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Evaluation of chemical markers for age validation of western Baltic cod (*Gadus morhua*) otoliths

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Abstract

Age determination of Baltic cod (*Gadus morhua*) is subject to inaccuracies caused by inconsistencies in the periodical pattern of the growth increments in the otoliths. The need of age validation has become a major concern to ensure adequate stock assessment of this ecological and economical important species.

This study investigated the performance of four commonly used chemical markers to time label the otoliths of Baltic cod. Alizarin red S, calcein, strontium chloride and tetracycline hydrochloride were tested regarding the effect on (1) mortality, (2) growth and (3) the formation of clear and distinct marks in the cod otoliths. Therefore, 746 Baltic cod were intraperitoneally injected with the different chemicals each at different concentrations and kept for 47 days in two netpens together with a control group (isotonic saline solution injections). The analysis of the observed mortalities, the calculated growth rates and the evaluation of the chemical–induced marks in the otoliths revealed that tetracycline hydrochloride in the concentrations of 50 mg/kg and 100 mg/kg fish had no negative effect either on mortality or growth of cod and induced high proportions of clear and distinct marks in the otoliths. Highest mortalities and lowest growth rates were found for cod injected with alizarin red S and calcein. Their successful application failed, because both chemicals were hardly soluble in their stock solutions. Strontium chloride did not negatively affect mortality and growth of cod. But marking success of the otoliths was 0 %. It was found that probably higher stock solution concentration of strontium chloride than this applied in the study are needed to successfully mark the otoliths.

The use of tetracycline hydrochloride in the concentrations of 50 mg/kg and 100 mg/kg fish were considered the most appropriate to mark Baltic cod for age validation in large-scale mark-recapture experiments.

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Acronyms

ALI	Alizarin red S
BLR	Binary logistic regression
CAL	Calcein
CTD	Conductivity, temperature, depth (hydrographic measurement equipment)
MNR	Multinomial logistic regression
NaCl	Sodium chloride
OTC	Oxytetracycline
RSD	Relative standard deviation
SD	Standard deviation
SEM	Scanning eletron microscope
Sr	Strontium
STR	Strontium chloride
TET	Tetracycline hydrochloride

1 Introduction

1.1 Age determination of Baltic cod

1.1.1 Age estimation of otoliths

Age is a fundamental and most important biological parameter, since from age key parameters are calculated which are crucial for fish stock assessment and management strategies, such as growth rates or mortality rates (Campana, 2001).

Age can be estimated from the hard tissues of fish, such as fin rays, scales, vertebrae, operculae and otoliths (Yamada, 1973), whereby otoliths are most commonly used for age determination (Campana, 2001, Gunn et al., 1992¸ Panfili & Ximenes, 1992). Otoliths are found in the inner ear of fish and contribute to the detection of sound and play a role for the equilibrioception and sense of direction (Campana, 1999). Whole or thin sectioned otoliths viewed under transmitted-light microscope, display periodic growth increments, which differ in their opacity (Hüssy et al., 2009; Stuby, 2007). This is due to differences in chemical composition of the increments. Opaque increments contain a higher amount of calcium carbonate and proteins and are referred to growth during summer time, whereas in translucent increments the content of calcium and proteins is reduced and represent periods of reduced growth, e.g. during winter time (Hüssy et al., 1992; Pannella, 1971).

These differentiable opaque and translucent increments are counted to estimate the age of fish on the annual basis or on the daily basis (Campana, 2001, Lang & Buxton, 1993). However using this method, the absolute age of fish cannot be determined and “age estimation” is the proper term to be applied (Campana, 2001), since the growth increments are often inconsistent and the assignment to the growth periods (summer, winter) is often difficult. This issue especially applies to Baltic cod (Gadus morhua Linnaeus, 1758), because of the estuarine character of the Baltic Sea.

1.1.2 Problems in age determination of Baltic cod

Baltic cod is exposed to highly variable environmental factors, altering the periodical increment formation in otoliths.

The Baltic Sea forms a highly variable environment for aquatic organisms due to its uneven hydrographic conditions, e.g. horizontal and/or vertical gradients in oxygen, temperature and salinity (Aro, 2000). To this heterogeneous hydrography are added a variety of internal and external factors, i.e. size, age and temperature and the availability of food, whereby in the case of Baltic cod temperature is assumed to have the highest impact on the opacity of the increments of the otoliths (Hüssy et al., 2009; Hüssy, 2010). Consequently, the formation of the growth increments in the otoliths of Baltic cod often does not follow a regular periodical pattern, leading to not clearly defined growth increments and a visually rather uniform increment pattern of the otoliths (Hüssy, 2010; Rehberg-Haas, 2012).

Therefore, Baltic cod otoliths are subject to possible errors in age reading and biased age estimates. This is further aggravated by the subjectivity and different levels of experience and expertise of the investigators. This is a problem to solve, since biased age determination can imply dramatic consequences for the stock assessment (e.g. overexploitation) (Campana, 2001, Reeves, 2003).

1.2 Chemical marking of otoliths

To overcome these inaccuracies in age determination for Baltic cod, validation of the age estimates is required. There are several methods dealing with age validation (reviewed in Campana, 2001; Geffen, 1992; Jones, 1992). One of the best methods for this purpose is the marking of otoliths by injecting chemical compounds. It presents a direct method for validating age in fish, since with the administration of a chemical (most often chemicals emitting fluorescence light are used) and its deposition in the otolith soon after marking (usually within one day), a visible reference is given of known date and valid age information is gained by counting the growth increments that have formed from beginning to the marking until the sacrifice of the fish and by comparing the counted number of increments to the calculated expected number from the known time between marking and sacrifice/recapture (Clear et al., 1999; Geffen, 1992).

In this study the chemical marking of otoliths of western Baltic cod was used to assess the suitability of four chemicals for possible use in future large scale mark-recapture experiments which are needed to gain valid age information of Baltic cod and to contribute to the improvement of the management of the cod stocks in the Baltic Sea.

The four chemicals were: alizarin red S, calcein, strontium chloride and tetracycline hydrochloride. Some of the main characteristics of the chemicals important for the purpose of this study are given in Table 1.

By now, few research studies have dealt with the investigation of an appropriate chemical to mark the otoliths of Baltic cod.

Hüssy et al. (2009) successfully used strontium chloride to mark the otoliths of Baltic cod. In the studies conducted by Nordeide et al. (1992) and Pedersen & Carlsen (1999), oxytetracycline (OTC) was administered to juvenile cod through the feed. Blom et al. (1994) investigated the suitability of two fluorescent chemicals, alizarin red S and alizarin complexone in immersion experiments of eggs, larvae and juveniles.

However the successful use, strontium chloride requires highly specialized equipment (scanning electron microscope) and is only detectable at considerable expense, it was estimated important to evaluate other potential chemical compounds which are more easily to detect and which did not been investigated for application on Baltic cod in large-scale mark-recapture experiments.

Table 1 Main characteristics of the chemicals used in the experiment.

	Chemical marker			
	Alizarin red S	**Calcein**	**Tetracycline hydrochloride**	**Strontium chloride**
Chemical group	Antraquinone[1]	Xanthene[1]	Natural[1], antibiotic agent	Chemical element, ionic compound
Marking applications	Bone structures[2]	Calcium ions, bone structures[2]	Bone structures[2]	Bone imaging[3]
Detection mode	Fluorescence microscopy	Fluorescence microscopy	Fluorescence microscopy	Scanning electron microscopy
Excitation/emission wavelength (nm)[4]	530-560/580	490/520	390/560	-
Excitation light[4]	green	blue	ultraviolet	-
Fluorescent colour	red	green	orange	-
Toxicity, lethal dose (LD_{50})	170 mg/l/48 h (Japanese medaka, *Oryzias latipes*)[5]	260 mg/kg (intrapritoneal injection of rats)[6]	220 mg/l/96 h (Lake trout, *Salvelinus namaycush*)[7]	405 mg/kg (intrapritoneal injection of rats)[8]

[1]Mason, 1999
[2]Sabnis, 2010
[3]The American Heritage® Medical Dictionary, 2004
[4]Olympus Microscopy Resource Center
[5]LIFELINE Cell Technology, 2008
[6]King Mongkut's University of Technology Thonburi, Energy Environment Safety and Health
[7]Carl Roth, 2013
[8]Hummel Croton Inc., 2009

1.3 Aim of this study

The aim of this study was to determine the suitability of four chemicals, alizarin red S, calcein, strontium chloride and tetracycline hydrochloride, to mark the otoliths of Baltic cod and their potential use for future large scale mark-recapture experiments with the objective to validate age of Baltic cod. The key assumptions made for a suitable marker in this study were that the chemical marker (1) may not affect mortality of the marked cod, (3) may not affect the growth of the cod and (2) provide good quality of the mark in the otoliths. Further, the chemical marker should be easy and fast to apply and require little specialized equipment for the detection and analysis.

2 Material and Methods

2.1 Experimental set-up

In total four marker groups were set corresponding to the four chemicals used (ALI, CAL, STR and TET) and one control group (isotonic saline solution) to assess the effect of intraperitoneal injection. Cod in the control group were administered 0,9 % of isotonic saline solution (µl/kg fish). Cod were equally distributed over the treatment groups the mean length of cod was ca. 25 cm. However, variability in the length distributions within and between the treatment groups occurred due to the fact that cod came from three different batches (Figure 1)

For each marker group and the control group single treatments with only the chemical and the isotonic saline solution, respectively were carried out and additionally for the marker groups ALI, CAL and TET double treatments with only the chemical marker plus STR in the concentration of 2 mg/kg fish were conducted (Table 2).

Further, for each marker group different degrees of concentration were injected (low, medium and high) to test which concentration of the chemical marker injected is most suitable to mark the otoliths of Baltic cod. The different degrees of concentration were chosen according to concentrations used in previous studies. The maximum concentrations of the chemical markers used here were determined according to concentrations successfully used in published experiments (Table 3). Decreasing concentrations were chosen to test, whether lower concentrations than the highest concentration would lead to an adequate internal mark in the otoliths. Table 3 gives concentrations for chemical markers used in previous studies.

Exclusively for ALI and CAL additional degrees below the lowest concentration were injected, because of high mortalities at the low, medium and high concentrations.

The concentrations were the same in the single and the double treatment, whereby for the double treatment always the highest STR concentration of 2 mg/kg fish was administered additionally to each concentration of the chemical markers. The maximum concentration of strontium chloride for the double marking experiment was chosen according to Hüssy et al. (2009). The double marking treatments were conducted to examine whether the combination of any of the three used fluorescent markers (ALI, CAL, TET) together with strontium chloride lead to clear marks in the otoliths. Furthermore, fluorescent marks as those produced by ALI, CAL and TET are known to disappear over time due to photobleaching, whereas strontium-um deposits permanently in the otoliths by substituting with calcium (Clear et al.,

2000). Combining STR with the fluorescent markers, age validation would be ensured even after potential loss of the light-sensitive fluorescent marks. In all, six mark groups were set, including the control group.

Table 2 shows the different concentrations for each marker group and the initial numbers of cod marked, such as the final numbers of cod marked after subtraction of cods, which have lost their external tag and thus could not be identified anymore.

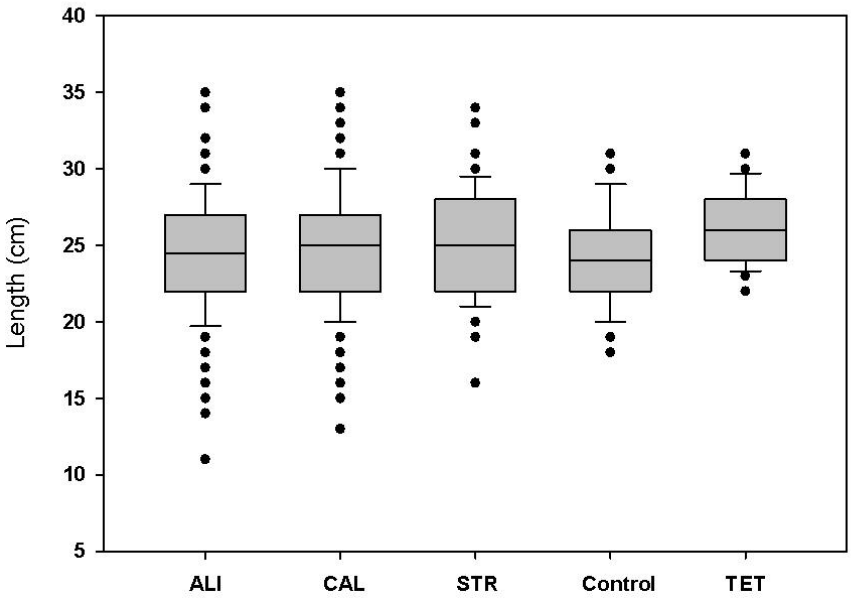


Figure 1 Length distribution of cod in the four marker groups (ALI, CAL, STR, TET) and the control group.

Table 2 Marker groups with chemical markers, injected concentrations (mg/kg fish) with initial numbers of cod marked and final numbers of cod marked after exclusion of cods losing external tag are given in parentheses.

	Chemical concentration (mg/kg fish [a])						
Chemical marker	Low/8 [b]	Low/2 [b]	Low/4 [b]	Low	Medium	High	Total N
Single treatment							
ALI	31,25	62,5	125	250	500	1000	
	20 (20)	20 (20)	20 (19)	25 (24)	25 (25)	25 (24)	135 (132)
CAL	1,25	2,5	5	10	25	50	
	20 (20)	20 (20)	21 (21)	25 (20)	25 (25)	25 (21)	136 (127)
STR	-	-	-	0,5	1	2	
				25 (24)	25 (25)	25 (25)	75 (74)
TET	-	-	-	25	50	100	
				25 (25)	25 (25)	25 (25)	75 (75)
Double treatment							
ALI&STR [c]	31,25	62,5	125	250	500	1000	
	21 (21)	20 (20)	20 (20)	25 (25)	25 (25)	3 (3)	114 (114)
CAL&STR	1,25	2,5	5	10	25	50	
	21 (21)	20 (20)	20 (20)	25 (24)	25 (23)	25 (25)	136 (133)
TET&STR	-	-	-	25	50	100	
				25 (23)	25 (22)	25 (22)	75 (67)
				Total N			746 (722)

ALI: alizarin red S, CAL: calcein, STR: strontium chloride, TET: tetracycline hydrochloride.

[a] kg fish = total fish wet weight. [b] Concentrations of Low/8, Low/4 and Low/2 correspond to an eighth, a quarter and a half of the concentration Low. [c] STR in the double treatment corresponds to 2 mg STR per kg fish.

Table 3 Examples of otolith staining studies using injection of chemical compounds.

Fish species	Chemical marker	Dose (mg/kg fish)	Reference
Gadus morhua	STR	2	Hüssy et al., 2009
Cyprinus carpio	CAL	4-25	Yamada, 1973
	TET	15-100	
	ALI	40-250	
Mice and rabbit	CAL	25-100	Suzuki & Mathews, 1966
	TET	25-100	
Young goldfish	TET	20-100	Kobayashi et al., 1964
Gadus morhua	TET	25, 50, 100	Jones & Bedford, 1968
Thunnus albacares and *Katsuwonus pelamis*	Oxytetracycline	125 (ml/4,54 kg fish)	Wild & Foreman, 1980
Sciaenops ocellatu	CAL	25, 50	Thomas et al., 1995
	Oxytetracycline	25, 50	
	Alizarin complexone	25, 50	
Diplodus sargos, *D. cervinus* and *Chrysoblephus laticeps*	Oxytetracycline	20-2000	Lang & Buxton, 1993
Oreochromis niloticus	Tetracycline	50	Massou et al., 2002
Hirundychthys affinis	Oxytetracycline	100	Oxenford, et al., 1994
Paralichthyus dentatus	CAL	25	Monaghan, 1993
	Tetracycline	50	
Esox lucius	Oxytetracycline	25-50	Babaluk & Craig, 1990
Oncorynchus spp.	Tetracycline	100	Jones, 1969
	Chlortetracycline		
	Oxytetracycline		
Sebastes auriculatus	STR	60	Kuroki et al., 2010
	STR	1,25 (g/l)	Moreno & Morales-Nin, 2003
Thunnus maccoyii	STR	76-270	Clear et al., 2000
Ginglymostoma cirratum	CAL	5-25	Gelsleichter et al., 1997

ALI: alizarin red S, CAL: calcein, STR: strontium chloride, TET: tetracycline hydrochloride.

2.2 Injection volumes

2.2.1 Calculation of injection volumes

First the weighed portion (mg) of the chemical marker to be dissolved in a given volume of the stock solution was calculated (500 ml and/or 250 ml stock solutions were mixed, according to the amounts needed). For this it was assumed that maximum weight of cod would be 500 g and maximum injection volume administered in cod would be 10 ml. Further, according to the maximum chemical marker concentration (High), a theoretical maximum chemical marker concentration of the stock solution was estimated. The theoretical maximum chemical marker concentration was always estimated higher than the actual maximum concentration for three reasons. First, to provide that the steps between the injection volumes from one smaller weight to a higher weight of cod were large enough to adjust the weight difference and second, to ensure that the calculated injection volumes fit best the volume steps of the Multipette Plus (Eppendorf) used for injections (see section 2.5.), and third, to provide that the calculated injection volumes did not exceed the maximum injection volume of 10 ml, to avoid the risk of physical harm due to inflation of the abdominal cavity of the test fish with the chemical compounds. In Table 4 the estimated theoretical maximum chemical marker concentration relative to the actual maximum chemical marker concentration are given with the calculated weighed proportion of the chemical marker that was dissolved in the stock solution.

Thus, the assumed weighed portion of the chemical marker w_0, dissolved in a stock solution of the theoretical maximum chemical marker concentration (*conc* 2), that would be injected into a 500 g fish was determined by solving equation (1):

$$\frac{conc\ 2\ (\mathrm{mg})}{1000\ \mathrm{g}} \triangleq \frac{w_0\ (\mathrm{mg})}{500\ \mathrm{g}}. \qquad (1)$$

Given the assumed weighed proportion w_0, the calculated weighed portion of the chemical marker w_1 (mg) per 500 ml stock solution was calculated in consideration of the limitation of 10 ml maximum injection volume:

$$\frac{w_0\ (\mathrm{mg})}{10\ \mathrm{ml}} \triangleq \frac{w_1(\mathrm{mg})}{500\ \mathrm{ml}} \qquad (2)$$

The injection volumes were calculated according to the measured wet weight of cod *w*(cod) (g). Given the degree of concentration of the chemical marker *conc* (mg/kg) that want to be injected into cod of given wet weight *w*(cod), the weighed portion of the chemical marker w_2 that would be dissolved in a stock solution of the given degree of concentration of the chemical marker *conc*, and injected into a cod of given wet weight *w*(cod) was calculated as follows:

$$\frac{conc\ (\mathrm{mg})}{1000\ \mathrm{g}} \triangleq \frac{w_2(\mathrm{mg})}{w(\mathrm{cod})(\mathrm{g})} \tag{3}$$

The injection volume *iV* (ml) for the weighed portion of the chemical marker w_2 that would be dissolved in a stock solution of the given degree of concentration of the chemical marker *conc*, and injected into a cod of given wet weight *w*(cod) for cod from the stock solution (calculated weighed portion of the chemical marker w_1 (mg) per 500 ml) was calculated by solving equation (4):

$$\frac{w_2(\mathrm{mg})}{iV\ (\mathrm{ml})} \triangleq \frac{w_1(\mathrm{mg})}{500\ \mathrm{ml}} \tag{4}$$

For the control group the wet weight of cod was multiplied by factor 2 to calculate the injection volumes to obtain injection volumes that were comparable to those injected in the marker groups.

Table 4 Estimated theoretical maximum chemical marker concentration (2) relative to the actual maximum chemical marker concentration (1) and calculated weighed proportion of the chemical marker w_1, dissolved in 500 ml stock solution.

	Max. concentration (mg/kg fish)		
Chemical marker	1	2	w_1
ALI	1000	1400	35000
ALI [a)]	125	350	4375 [b)]
CAL	50	76	1900
STR	2	20	500
TET	100	200	5000

[a)] Due to difficulties in dissolving ALI properly in the amount of 35000 mg, additional stock solution was prepared for the ALI concentrations 31,25, 62,5 and 125, where only 4375 mg of ALI were dissolved.
[b)] The volume of the stock solution was 250 ml instead of 500 ml.

2.3 Preparation of the stock solutions

The chemical markers were available in crystalline form and diluted either in distilled water or in 0,9 % of isotonic saline solution, depending on the preparation methods in published experiments followed. For the injections, 250 or 500 ml stock solutions of the chemical markers ALI and CAL were prepared. For STR, TET and the control group only 500 ml stock solutions were prepared. All chemicals were weighted to the nearest 0,001 g with a micro balance (Sartorius ME 235 P). The weighed portion of each chemical marker was calculated according to equations (1) and (2) (see 2.2.1. and Table 4).

2.3.1 Isotonic saline solution

For the control group, a 1 L stock solution of 0,9 % isotonic saline solution was prepared. The stock solution was mixed from crystalline sodium chloride (strontium hexyhydrate crist. pure, Köhler GMBH) and distilled water. A beaker was put on a top batching balance (Sartorius LA 5200 P, resolution ±0,01 g) and 9 g of sodium chloride were weighted and distilled water was added by stirring until the final weight of 1000 g (equals to 1000 ml) was reached.

2.3.2 Calcein

To prepare the 500 ml CAL stock solution, around 250 ml of distilled water was filled in a beaker. Then, 1900 mg of CAL (CAS No. 1461-15-0, Sigma) was added and the solution was vigorously stirred, first with a stirring rod, then the beaker was put on a magnetic stirrer to dissolve the CAL for 2 h, because CAL is poorly soluble in water of low pH (Yamada, 1973). The pH level of the initial solution was 2,25. To adjust the pH level of the stock solution to physiological pH level of fish (around 8 pH) and to entirely dissolve the CAL, 1 M potassium hydroxide (AppliChem, potassium hydroxide (KOH) standard volumetric solution 1M) was carefully added dropwise as described in Suzuki & Mathews (1966) and Tsukamoto (1988). By monitoring the pH level with a portable pH meter, the final pH level was approximately 7. Once the pH level was adjusted, the solution was poured in a 500 ml volumetric flask and distilled water was added until the final volume of 500 ml was reached. The solution was transferred in a 1 L amber glass bottle and stored in darkness.

The CAL stock solution was prepared a second time, because CAL did not fully dissolve in the first attempt. A 250 ml stock solution was prepared following the above procedure. To produce a 250 ml stock solution, 950 mg CAL were dissolved. The pH level was adjusted to 7. This time, CAL could be dissolved by intensively stirring the solution overnight. The 500 ml stock solution (first attempt) was used for the first single treatment injections of the concentrations of 10, 25 and 50 mg/kg. For double treatments and the single treatments with the lower concentrations 1,25, 2 and 5 mg/kg, the 250 ml stock solution (second attempt) was used.

2.3.3 Tetracycline hydrochloride

A 500 ml stock solution of TET (Tetracycline hydrochloride ≥ 95 %, CELLPURE®) was prepared following Suzuki & Mathews (1966). To prepare the 500 ml TET stock solution,around 250 ml of 0,9 % of NaCl in a beaker. Then, 5000 mg of TET was added and the solution was vigorously stirred, using a stirring rod and a magnetic stirrer. To adjust the pH level of the stock solution to physiological pH level of fish from initial pH values around 3, 1 M KOH was carefully added dropwise. By monitoring the pH level with a portable pH meter, the final pH level was around 7. The solution was transferred to a 500 mL volumetric flask and 0,9 % NaCl was added until the final volume of 500 ml was reached. The prepared TET stock solution was filled in a 1 L amber glass bottle and stored in darkness in a fridge at 8 °C.

2.3.4 Alizarin red S

ALI stock solution had to be prepared four times (ALI solution1, ALI solution2, ALI solution3 and ALI solution4), because difficulties were encountered in 1) dissolving the amount of ALI powder for the stock solution, 2) adjusting the pH and 3) exact weighing of the ALI powder. The latter problem was due to the fact that the powder probably absorbed atmospheric humidity and thus became gradually heavier.

ALI solution1, ALI solution2 and ALI solution3 were prepared in the same way as the CAL stock solution. For ALI solution1 a 500 ml and for ALI solution2 and ALI solution3, 250 ml stock solutions were prepared. For the 500 ml and 250 ml stock solution, 35000 mg and 17500 mg ALI was dissolved, respectively.

The amounts of 35000 mg and 17500 mg ALI were too high for successful dissolving in ALI solution1, ALI solution 2 and ALI solution3, respectively, although these amounts were under the referred maximum concentration of 77 g/l for ALI solubility in water (Ellis, 2003-2011).

The ALI solution2 was heated to approximately 50 °C on a magnetic stirrer and around 1 ml of 97 % ethanol was added to facilitate and accelerate the dissolving process. The powder dissolved nearly entirely. The adjustment of the pH level from 2,3 to around 7 was impossible, because the solution began to precipitate, by adding further 1 M KOH. The high amount of 1 M KOH added, caused the formation of insoluble salts, which formed precipitates.

For ALI solution3, a 1 L phosphate-buffered saline solution (PBS) was prepared. The solution had a final pH level of 7,4. The weighed ALI powder was directly added to the PBS to get the initial pH level of the stock solution near to 7. After nearly total dissolution of the ALI powder, the pH was approximately 5. To raise the pH, di-sodium hydrogen sulfate-12-hydrate (in crystalline form) was successively added. However, this attempt was abandoned, because the compounds of the solution did not entirely dissolve and the solution began to precipitate.

Only ALI solution1 was used for the single and double injections in the concentrations of 250, 500 and 1000 mg/kg of ALI.

For ALI solution4 was used for the single and double injections in the concentrations of 31,25; 62,5 and 125 mg/kg of ALI. Since ALI 125 mg/kg fish was the highest concentration, the estimated theoretical maximum chemical marker concentration of 350 mg/kg was assumed. As a result, 4375 mg ALI was dissolved for a 250 ml stock solution (Table 4).

The amount of 4375 mg ALI was dissolved in a mixture of 125 ml of distilled water and 125 ml of ethanol, to make a stock solution of 250 ml. Even by dissolving in ethanol, the ALI powder could not be completely dissolved. Compared to ALI solution1, 2 and 3, the solubility of ALI in ALI solution4 was improved. By adding ethanol to the solution, it was not necessary to adjust the pH by adding 1 M KOH. Ethanol is a neutral molecule and does not dissociate when dissolved in water, thus the solution will have a pH level of 7 (pers. comm. Dr. Wolfgang Ruth, Institute of Chemistry of the University of Rostock, 2013).

2.3.5 Strontium chloride

The strontium chloride (STR) stock solution was prepared from 500 mg STR, dissolved in 500 ml destilled water. The solution was rigorously stirred on a magnetic stirrer, until full solution of STR. The initial pH level was of 5,5 and was raised to around 7, by dropwise adding 1 M KOH. The solution was filled in a 1 L amber glass bottle and stored in darkness.

2.4 Capture of cod

Three attempts were undertaken to collect Western Baltic cod for the present otolith marking experiment, one in March 2013 (trawl), July 2013 (trawl) and October 2013 (pound nets)

The first attempt was performed on 19th and 20th March 2013. Cod were caught with a bottom trawl (TV3-520, Steert 10 mm mesh size), with the research vessel "Solea" off Warnemünde (ICES Subdivision 22). About 450 cod were caught in four hauls at an average bottom depth of 17 m. The duration of bottom trawling was 20 minutes and the trawling speed was 3 knots. Right after capture, the fish were kept on board in two 1,2 m³ tanks, which were continually supplied with surface seawater. The fish were transferred to two swimming netpens, located at the mouth of River Warnow in Warnemünde harbour (Figure 3). At this site, water depth was around 6 m. The fish were transferred with dip nets from the holding tanks in three fish boxes, ("Hyttefade", Type 3, 100x60x40 cm, 240 l volume capacity, Midtlollands Glasfiber v/ Peter Jensen) and then transported over from the research vessel with the rubber dinghy "Belone" to the netpens. From the Hyttefade the fish were dip-netted into the netpens. Water surface temperature at site was 3 °C at that time. Cod showed pronounced skin lesions, mainly caused by trawling. Further, any attempts of feeding failed, probably due to the low water temperature at that time (3 °C) and hence, reduced metabolism of the fish. After 10 days the remaining fish were not considered suitable for an experiment and released.

The second attempt was performed during summer, on 26th July 2013. Cod were collected with a bottom trawl (TV3-520, Steert 10 mm mesh size), with the research vessel "Clupea". Stations were similar to those from the first attempt. About 500 kg cod were caught during five hauls at an average depth of 18 m. For the hauls 1 to 3, the duration of bottom trawling was 30 minutes. For the hauls 4 and 5, the trawling duration was reduced to 15 minutes. The trawling speed was 3 knots. Depth, temperature, salinity and oxygen saturation were recorded at each catching station, using a CTD. Average water temperature at the ground was 10 °C. Average water surface temperature was 21 °C. Right after catch, the fish were kept in two 1,2 m³ tanks on board, which were continuously supplied with surface sea-water. Due to 100 % cod mortality on board due to extreme high temperatures, no fish were available for the experiment.

The third attempt was performed during autumn, at the beginning of October 2013. Juvenile cod were collected from six stationary commercial pound nets (catch chamber at 3-5 m depth), installed south off Fehmarn. Fish were transferred from the pound net catch chambers

into holding tanks on board of commercial vessels. The tanks were aerated and continually supplied with surface seawater. The samples were brought to Burgstaaken harbour on Fehmarn, where the fish were transferred into Hyttefade with knotless dip nets. The Hyttefade were modified with a PVC-pipe of through which the fish were discharged, by lifting up the Hyttefade with a crane right into a 2 m^3 transport tank (provided by the Landesforschungsanstalt für Landwirtschaft und Fischerei Mecklenburg-Vorpommern). The fish slided gently through the PVC-pipe into the transport tank without any further handling. This reduced stress and the probability of skin damage as well as the abrasion of surface mucus of the fish. The transport tank was filled with local seawater and aerated during the whole transport to Rostock (ca. 4 h by car). The fish were transported di-rectly to the netpens in Warnemünde. No obvious mortality occurred during the three trans-ports. The dimensions of the net cages were 3x3x3 m and 20 mm mesh size. Nets of 100 mm mesh size were fixed above the cages to prevent predation. The construction allowed lifting the bottom of the netpens. Thus, the fish could be lifted carefully in the water column, which facilitated the handling of the fish (Mieske, 1998). The proximity of the netpens to the open sea ensured sufficient water exchange and adequate water quality. Water surface temperature and salinity were recorded, once at depature from Burgstaaken harbour and once at arrival in Warnemünde at the netpens, using a portable CTD (YSI Professional Plus) (Table 5), cod were transferred into the netpens. Cod were immediately removed from the transport tank with 10 L black buckets (ca. 6-8 fish/bucket) and carefully released into a netpen and counted. The buckets were held under water and fish swam into the netpen water without any further handling stress.

Table 5 Surface water temperature (°C) and salinity (PSU) for the three batches of cod at Burgstaaken and Warnemünde, measured using a portable CTD (YSI Professional Plus).

		Burgstaaken		Warnemünde	
Haul	Date	(°C)	(PSU)	(°C)	(PSU)
1	01.10.2013	14,20	12,29	13,80	13,46
2	08.10.2013	12,90	10,44	12,70	8,97
3	15.10.2013	11,70	10,77	12,40	13,14

2.5 Internal chemical marking and external tagging

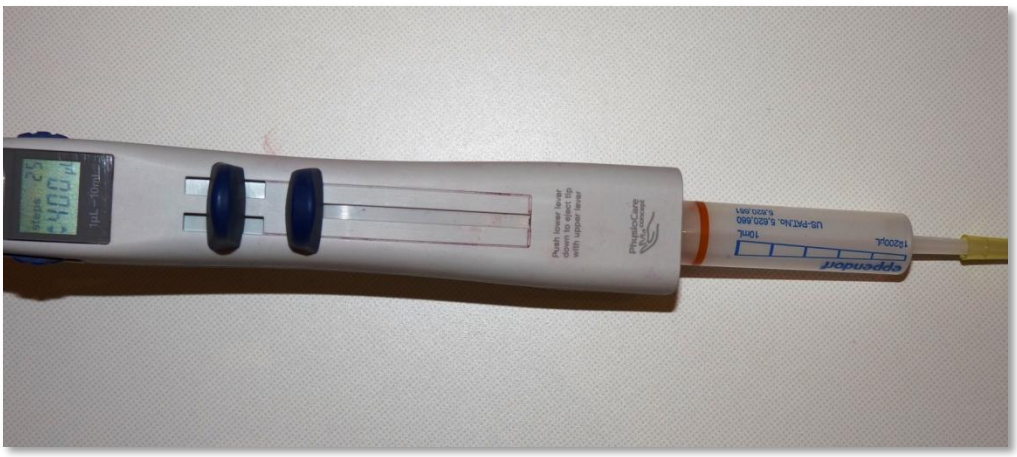
Only cod in very good external condition were taken for the experiment.

Fish were internally marked by intraperitoneal injection on the platform of a dalben in proximity of the netpens (Figure 3). A Multipette Plus with Combitips Plus (Eppendorf) was used to inject the chemicals. Eppendorf did not provide Combitips for hypodermic needles (Pers. Comm. Eppendorf). Therefore, on the top of the Combitips Plus a 200 µl pipette tip (PLASTIBRAND®) was mounted. The tip of the pipette was cut by third with a scalpel to get an even and proper edge. On that top of the pipette tip was fixed a disposable hypodermic needle (0,60 x 30 mm, Fine-Ject needles for single use, Henke-Sass, Wolf GmbH) (Figure 1).

For the internal chemical marking, five to ten fish were put from the netpens with a knotless dipnet and carefully put into 35 l white plastic boxes filled with local seawater. Each fish was processed individually. This involved gentle removal with two hands (gloved) and wet-weighting (Balance details), internally marked and external tagged.

According to the fish weight, the calculated injection volume was read from the injection volume table and the pipette was adjusted to the related volume. In the meantime, the fish was measured (total length) and the head region of the fish was covered with a wet tissue to provide humidity and to minimize stress. The fish was immobilized with one hand and with the other hand the needle was carefully inserted ventrally below the skin, anterior of the anus and the chemical was injected gently into the abdominal cavity (Figure 2).

A)



B)

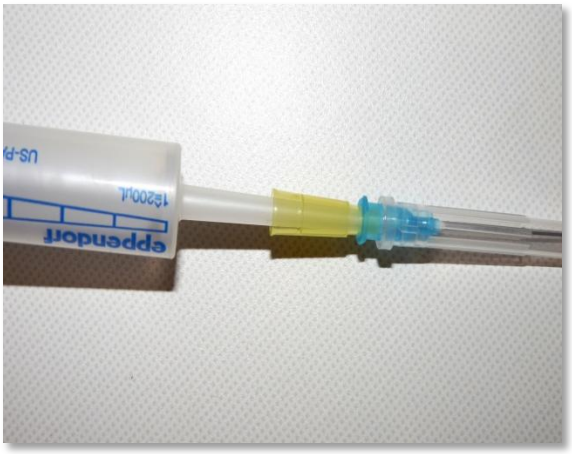


Figure 2 A) Multipette Plus (Eppendorf) for interperitoneal injection of cod, **B)** Combitip with pipette tip and fixed hypodermic needle. Photo by author

Figure 3 Performance of internal and external marking of cod on a dalben near the netpess

Figure 4 Interperitoneal injection of a chemical marker into a cod. Photo Thünen Institute of Baltic Sea Fisheries

Once marked internally, the fish was tagged externally with a T-bar anchor tag (type TBF-1, 20 mm, Hallprint) about 1 cm below the second dorsal fin, using a TBF tagging gun (No. 10312, Avery Dennison) as described in Nielsen (1992) (Figure 5), for individual identification. Handling time for one fish was ca. 1 minute.

After internal marking and external tagging, the fish was carefully transferred in a black bucket and then released again into the netpens.

A)

B)

C)

Figure 5 A) T-bar anchor tags with identification number (magnification below) and tagging gun and tagging needle. **B)** External tagging below second dorsal fin. **C)** Cod with external tag on measuring board. Photo by Thünen Institute of Baltic Sea Fisheries.

2.6 Holding of cod in netpens

Cod had similar size and divided over the two netpens. The total holding time was 7 weeks, i.e. from 1st October until 19th November 2013. The fish were fed on a daily basis with a mixed diet, consisting of shrimps, *Crangon crangon* (Linnaeus, 1758), deep-frozen herring, *Clupea harengus* Linnaeus, 1758, sandeel, *Hyperoplus lanceolatus* (Le Sauvage, 1824), and commercial fish pellets, fish were cut in pieces. Temperature, salinity and dissolved oxygen were recorded daily at the water surface, in 1 m and 3 m depth, with a portable CTD (YSI Professional Plus). A temperature logger (Onset, HOBO Data Logger) monitored local water temperature at 2 m beneath the surface. Whenever a fish was dead, it was removed from the netpens prior to each feeding event. Dead fish were weighted, measured and identified using the identification number on the external tag. Moreover, a post-mortem examination was conducted to determine possible reasons, which lead to the early death of the fish. Then were deep-frozen and stored in darkness until dissection of the otoliths.

After 7 weeks in the netpens, the fish were sacrificed on 19th November 2013. Total length, total weight (with and without inner organs), liver weight, gender and maturity, as well as stomach content and fullness were recorded before removal of the sagittal otoliths for each individual fish. The otoliths were cleaned in water and put on consecutively numbered moulds to dry. For the examination, only the right otoliths were taken. The left otoliths were archieved.

Figure 6 Two netpens used for the experiment in Warnemünde. Photo by author.

2.7 Otolith preparation and examination

The nuclei of the otoliths were marked with a pencil on the bottom side. The marks would serve later on as reference to embed the otoliths properly in succession. To embed the otoliths, 180 ml of synthetic resin (GTS polyester casting resin, Voss Chemie, 35-40 % styrene) was cautiously and air bubble-free mixed with 4 ml of a hardener (MEKP-hardener), which accelerated setting of the resin. Then, the resin mixture was poured half full in an aluminium mould. A waiting time of ca. 30 min followed for the resin to set, to prevent the otoliths from sinking into the resin. In a next step, the otoliths were laid onto the resin. The otoliths were arranged such that they laid on the resin with the marked bottom side pointing upwards. This adjustment ensured that the marks for the slicing position stayed discernible through the resin and that no air became trapped under the otoliths, thus ensuring a firm fixation in the resin. The marks served as reference, so that when sliced, the transversal cut was across the nuclei. The rest of the remaining resin was poured air bubble-free in the basin to fill it completely, so that the otoliths were entirely covered. The whole embedment procedure was carried out under a fume cupboard.

After a setting time of the resin of a minimum of 5 days in a drying cabinet, the otoliths were sliced. Thereto, the resins were mounted in a wet abrasive cut-off machine (Brilliant 250, ATM). The obtained thin sections had a thickness of 0,5 mm. At every step, it was required to keep and store the thin sections in the dark, to prevent the fluorescent marks to fade, when exposed to light (Geffen, 1992).

2.7.1 Preparing of otolith thin sections

The nuclei of the otoliths were marked with a pencil on the bottom side. The marks would serve later on as reference to embed the otoliths properly in succession. To embed the otoliths, 180 ml of synthetic resin (GTS polyester casting resin, Voss Chemie, 35-40 % styrene) was cautiously and air bubble-free mixed with 4 ml of a hardener (MEKP-hardener), which accelerated setting of the resin. Then, the resin mixture was poured half full in an aluminium mould. A waiting time of ca. 30 min followed for the resin to set, to prevent the otoliths from sinking into the resin. In a next step, the otoliths were laid onto the resin. The otoliths were arranged such that they laid on the resin with the marked bottom side pointing upwards. This adjustment ensured that the marks for the slicing position stayed discernible through the resin

and that no air became trapped under the otoliths, thus ensuring a firm fixation in the resin. The marks served as reference, so that when sliced, the transversal cut was across the nuclei. The rest of the remaining resin was poured air bubble-free in the basin to fill it completely, so that the otoliths were entirely covered. The whole embedment procedure was carried out under a fume cupboard. After a setting time of the resin of a minimum of 5 days in a drying cabinet, the otoliths were sliced. Thereto, the resins were mounted in a wet abrasive cut-off machine (Brilliant 250, ATM). The obtained thin sections had a thickness of 0,5 mm. At every step, it was required to keep and store the thin sections in the dark, to prevent the fluorescent marks to fade, when exposed to light (Geffen, 1992).

2.7.2 Categorization of otolith thin sections under fluorescence microscope

The analysis of otoliths marked with ALI, CAL and TET under the fluorescence microscope required further preparation of the thin sections.

The thin sections were put on conventional microscope slides (25x75 mm) and carefully fixed with adhesive tape at both ends on the slides.

An inverted fluorescence microscope (Nikon Diaphot 300 Phase Contrast Inverted Microscope) was used. The otoliths were viewed with a PLAN 2,5/0,08 160/- objective and a high pressure mercury lamp (Nikon) served as light source. To see the fluorescent marks of ALI, CAL and TET, different excitation and barrier filters were used (Table 7).

The filter combinations were determined according to the excitation and emission wavelengths of the chemicals and the filter combinations used in the literature (Bashey, 2004; Blom et al., 1994; Bumguardner & King, 1996; Leips et al., 2001; Monaghan, 1993).

Table 6 Filter combinations for the three fluorescence markers alizarin red S (ALI), calcein (CAL) and tetracycline hydrochloride (TET).

Chemical marker	Filter combination	Dichromatic mirror	Excitation filter	Barrier filter
ALI	G-2A[1]	565	510-560	590
CAL	B-2E[2]	505	450-490	520-560
TET	BV-2A[3]	455	400-440	470

G-2A[1], green filter combination including a dichromatic mirror at 565 nm, an excitation filter at 510-560 nm and a barrier filter at 590 nm. B-2E[2], blue filter combination including a dichromatic mirror at 505 nm, an excitation filter at 450-490 nm and a barrier filter at 520-560 nm. BV-2A[3], including a dichromatic mirror at 455 nm, an excitation filter at 400-440 nm and a barrier filter at 470 nm.

Each sliced otolith was photo-documented, using a digital camera (Nikon D7000), which was mounted on the microscope. For each chemical marker, the settings of the digital camera (length of exposure, ISO speed) were tested and the best settings were maintained for each chemical to ensure the comparison of the quality of the fluorescent marks within one marker group. The classification of the fluorescent marks in to more detailed categories than in to more superficial and comprising categories, e.g., "no mark, poor mark, fair mark, good mark" (Lorson & Mudrak, 1987) was found more appropriate. For statistical analysis it was more convenient to define comprised groups from multiple properties. In total, seven categories were evaluated with different subcategories for the description of the fluorescent marks:

1. Fluorescent mark (yes/no)
2. Clearness of mark (no mark, very clear, clear, faint)
3. Distribution (no mark, around whole otolith, partial)
4. Ventral edge (none, clear, diffuse)
5. Dorsal edge (none, clear, diffuse)
6. Position of mark (inner part, outer edge)
7. Autofluorescence (yes/no)

} Mark quality (no mark, poor, good)

The categories covered the features of the fluorescent mark of a given thin section, first in general with regard to fluorescent clearness and constancy of the mark around the otolith, then more precisely, qualitative features at the ventral and dorsal side. It was pointed out whether the fluorescent mark was clear, diffuse or partial. Furthermore, the position of the fluorescent

mark was specified, whether it was at the outer edge or further inside the section. After the evaluation of the specific categories, the final mark quality was determined (no mark, poor, good). The final mark qualities were then statistically assessed.

2.7.3 Detection of strontium chloride marks in SEM

Strontium chloride marks in the thin-sectioned otoliths were detected by energy dispersive X-ray spectroscopy (EDS) in a scanning electron microscope (SEM, type DSM 960A from Carl Zeiss). The EDS X-ray microanalysis is a method to determine qualitatively and quantitatively the elements present in a sample (Australian Microscopy & Microanalysis Research Facility, 2012). The electrons of the atoms of the sample are excited by an electron beam. When they return to their ground state photons are emitted, which are detected by the energy dispersive detector and constitute the characteristic X-rays with discrete energies. These characteristic X-rays are then displayed in a spectrum, where the number of X-rays is plotted against the energy (in thousands of electron-volts, keV). The discrete energies of the X-rays appear as peaks in the spectrum. Their positions and amplitudes are characteristic for one special element and therefore, the energies of the X-rays allow identifying the elements present in the sample as well as determining their concentrations (Gunn et al., 1992). For the present study, only strontium (Sr) and calcium (Ca) were determined qualitatively at the ventral edges of the thin sections. It is referred that preceding preparation of the thin sections prior to the detection of the strontium chloride marks are required (Gunn et al., 1992). Usually the thin sections have to be grounded and polished, to get a thickness of 0,2 mm (Hüssy et al., 2010) and an even as well as clean surface, to not alter the absorption of X-ray intensities (Gunn et al., 1992). For the purpose to only qualify Sr present in the strontium chloride marks of the otoliths, such preparations were not inevitably necessary (Australian Microscopy & Microanalysis Research Facility, 2012). For this reason and due to the short time window available to carry out the analyses, the thin sections of the otoliths analyzed were not treated any further.

Furthermore, preceding trials helped to set up the appropriate settings for the analyses. The trials showed that the thin section thickness of 0,5 mm was sufficient. The Sr signals were clearly detectable, so that it was proved that not any further treatment of grounding and polishing the thin sections was necessary.

For the EDS, the surface of the non-conductive thin-sectioned otoliths needed to be coated with a thin layer of carbon, to make the surface conductive and to prevent charge build-up during the EDS (Gunn et al., 1992). Thus, the thin sections were dried in a desiccator to evaporate any moisture from the probe and then coated with carbon under vacuum in a high vacuum sputter coater (Leica EM SCD500). Afterwards, the sections were stuck on SEM pin stubs and analyzed. To find the right settings for the qualitative analysis of Sr within the STR marks, in the first instance both EDS X-ray mappings and line scans were carried out. The EDS line scan provided the element detection and identification along a virtual line, set across the diameter of the ventral edge of the otolith sample. As a result, an image of the scanned area with the drawn line and graphics for each element identified were generated, where the number of X-rays was plotted against the position (in µm) on the virtual line.

Thus, each peak could be related to a discrete position on the line and therefore the elements detected could be located on the probe. In contrast, the EDS mapping generated two dimensional images for each element identified, where the intensity and distribution of the given element could be seen over the analyzed area of the probe. The images were greyscale images, where the difference in brightness was dependent on the number of characteristic X-rays of discrete energies, thus on the concentration of the given element (i.e., bright shades and dark shades were analogous to high and low concentrations, respectively). In a next step, the single images of the identified elements (i.e., Ca and Sr) were overlapped and different colours were attributed to each element, so that a combination map was obtained, which showed the distribution of all elements identified over the analyzed area of the probe. It was found that the EDS mappings gave the best results, thus for all probes EDS maps (single and combination maps) and the associated spectra were generated.

In total, six thin sections of otoliths from marked cod with STR were assessed: O1 STR1, marked with STR 1 mg/kg fish, O2 STR2 and O3 STR2, marked with STR 2 mg/kg fish and O4 TET50&STR2, double marked with TET 50 and STR 2 mg/kg fish. Additionally, two thin sections of otoliths from cod marked with STR 2 mg/ kg fish from a previous marking attempt in 2012 were analyzed (O5 STR2 and O6 STR2). Only a small number of samples could be analyzed in six sessions, because the access to the SEM was restricted, since the SEM was used by other researchers and the access to another SEM was not possible. The final settings for the qualitative Sr analysis were as follows. For each otolith analyzed the spatial distribution of Sr and Ca was detected across the ventral edge of the thin sections of the otoliths and the peak counts for Sr Kα, Sr Lα and Ca Kα lines were recorded (Kα and Lα refer to the electron shells of the elements). The maps were generated at magnifications of 100 µm

and consisted of 150x250 pixels. The maps were acquired within 180 minutes and the acceleration voltage of the electron beam was set at 25 keV

2.8 Data analysis

The statistical analyses were carried out with the statistic program IBM® SPSS® Statistics 20 and the statistic program R.

2.8.1 Mortality and growth

The non-parametric Kruskal-Wallis *H*-test was used to test for differences in mean mortalities between different concentrations within each marker group and between the concentrations of each marker group and the control group. To evaluate the differences in mean growths, the Kruskal-Wallis *H*-test was used to test for differences only between the treatment groups (marker groups and control group). The Kruskal-Wallis procedure in SPSS does not offer the opportunity to perform post hoc multiple comparison tests. In case of significant differences and when only small numbers of comparisons had to be tested, Mann-Whitney *U*-tests for comparison of two independent samples with additional Bonferroni-Holm correction of the significance level α were performed to find out the significantly different groups. The Bonferroni-Holm correction was calculated as:

$$\alpha * = \frac{\alpha}{m+(1-j)}, \tag{5}$$

where α* is the new calculated significance level, α is the given significance level (0,05), *m* the number of comparisons and *j* the number of the test. This correction was used to prevent α to inflate in multiple comparisons of two independent variables.

In case Mann-Whitney *U*-tests were not applicable, due to a too large number of comparisons, the Nemenyi-test for pairwise multiple comparisons was used. To compare the growth between the different treatment groups, only the Nemenyi-test was used. The Nemenyi-test is not included in SPSS and thus it was computed with R. The Nemenyi-test was computed after Siegel & Castellan (1988).

Binary logistic regression analyses were conducted to determine probable causes which lead to early death of cod. Logistic regressions were modelled for each marker group. The relationship between three independent predictor variables, likely to have an effect on mortality, the marker concentrations (single and double treatment), injection volumes and mean water temperatures, and the survival of cod, as the binary categorical dependent variable with the two outcomes, cod dead (cod did not survive) and cod alive (cod survived). In a further attempt, additionally to the marker concentrations, the chemical compound as such, without the concentration levels, was included. But it was excluded again, since problems of multicollinearity arise between the two categorical variables, marker compound and marker concentrations. All predictor variables were fitted to the survival of cod using the method backward stepwise selection, based on the likelihood ratio and the contrast method indicator for the categorical variables chemical marker concentrations. The overall logistic regression link function was:

$$Predicted\ logit\ (Y) = ln(\pi/(1-\pi)) =$$
$$\beta_0 + \beta_1(marker\ compound) + \beta_2(marker\ concentrations) +$$
$$\beta_3(injection\ volumes) + \beta_4\ (mean\ water\ temperatures)\ , \qquad (6)$$

where predicted logit (Y) is the link function, ln(π/(1-π) the natural logarithm of the odds with the probability π, β_0 is the constant (Y intercept) and β_1, β_2, β_3 and β_4 the estimated regression coefficients for the predictor variables chemical marker compound, the marker concentrations (single and double treatment), injection volumes and mean water temperatures, respectively (Peng et al., 2002).

The null hypothesis of the overall logistic model assumed that all the predictor variables equal 0 (Kopp & Lois, 2011), meaning that the predictor variables did not have any influence or effect on the survival of cod. This assumption was represented by the null model, which served as a baseline to predict the survival of cod. In contrast, the fitted logistic model included the independent predictor variables. The aim was to investigate whether or not the logistic model provided an improvement over the baseline and thus a better fit to the data than the null model (Peng et al., 2002). Statistics used, testing the goodness-of-fit of the logistic model were the Wald test and the negative double logistic likelihood (-2 log-likelihood or -2LL).

The Wald statistic tested the assumption of null hypothesis. The chi-square value was the deviance of the difference between the null model and the predicted model. The assigned *p*-value indicated whether or not the logistic model led to a significantly better prediction of the dependent variable as the null model, and thus, whether or not the null hypothesis could be rejected. The -2LL-value was the maximum likelihood estimate for the logistic model and represented the residuals of the deviance from the difference between the null and the logistic model. In consideration of the deviance, it could be stated, whether or not the logistic model showed a significantly improvement over the baseline of the null model (Quinn & Keough, 2002). This maximum likelihood estimation was computed iteratively, in 20 iterations and the iterations were stopped when the estimates varied less than 0,001.

Further, for each predictor variable in the equation the regression coefficients (logarithmized odds or logits β) with the assigned standard errors were determined, using maximum likelihood estimates (Quinn & Keough, 2002). With the Wald statistic, the significance of the regression coefficients was tested (β = 0, when $p > 0{,}05$). Positive or negative β-values indicated the direction of the relationship between a predictor variable and the dependent variable (Kopp & Lois, 2011).

Along with the each regression coefficient, the natural logarithm base e, raised to the exponent of the slope β ($e^{(\beta)}$) was calculated. It represents the conversion of the logarithmized odds (β) back to the odds ($e^{(\beta)}$). Odds are ratios of probabilities of the dependent variable happening (i.e., cod alive) to probabilities of the dependent variable not happening (i.e., cod dead) (Peng et al., 2002).In contrast to β, the odds gave the degree of the relationship between the odds of the outcome of the dependent variable (i.e., cod alive) and the predictor (Kopp & Lois, 2011).

The conversion from the odds to the probability was calculated as follows:

$$\pi = \frac{e^{(\beta)}}{(1+e^{(\beta)})}, \tag{7}$$

where π is the probability of the outcome of interest, $e^{(\beta)}$ the natural logarithm base e, raised to the exponent of the slope β and β is the regression coefficient (Peng et al., 2002).

2.8.2 Mark quality

The concentrations (single and double treatment) were opposed to the mark quality, with the three categories no mark, poor mark and good mark, in contingency tables for each marker group and the proportions of the mark qualities for each concentration was computed. For each marker group two contingency tables were computed, one including the total number of cod marked and one, only including cod, which survived until the end of the experiment (cod surviving). To test for significant differences in the proportions of mark qualities between the concentrations within one marker group, the chi-square test of independence was conducted. The null hypothesis of this test stated that the opposed categorical variables marker concentration (single and double) and mark quality are independent and not related, thus the observed proportions would be similar to the expected proportions and not any significant different proportions would be detected between the concentrations and the mark quality. The probability p of significance was assumed be significant, given $p \leq 0{,}05$. If the chi-square test was significant the computed standardized residues were used to detect significantly different proportions within the sample. Provided the standardized residues had a value of 2 or more, than the observed proportions were significantly different from the expected proportions (Bühl & Zöfel, 2005). In case of significant different proportions the effect size Cramér V was used to qualify the strength of the proved relationship. Contingency tables for ALI and CAL, which included only the surviving cod could not be statistically assess, because sample sizes were too small to obtain valid results, due to high mortalities recorded for these groups. For TET the assessment was valid, since the sample size was large enough, due to a high survival rate for this marker group.

Multinomial logistic regressions (MLR) were conducted for each marker group to assess the relationship between the fluorescent mark quality with three categories, no mark, poor and good, and the concentrations of the marker (single and double treatment). Additionally the days of survival were included as covariate to investigate whether longer survival increased the probability of good mark quality. The analysis of the MLR model was analogous to that of the binary logistic regression, with the only difference that the dependent variable comprised three, instead of two outcome categories and thus, two logistic models were calculated. The model fit was assessed by the likelihood ratio chi-square test, testing the null hypothesis, that all predictors in the logistic model equal 0 and by the -2LL. The multinomial regression coefficients (β) were estimated using maximum likelihood estimates and tested for

significance using the Wald-statistic and the odds ratios ($e^{(\beta)}$) were calculated. For the multinomial logistic regression two logistic models were predicted:

$$Predicted\ logit\ (Y = poor\ mark/Y = no\ mark) = \beta_0 + \beta_1(marker\ cocentrations) + \beta_2(days\ of\ survival) \quad (8)$$

$$Predicted\ logit\ (Y = good\ mark/Y = no\ mark) = \beta_0 + \beta_1(marker\ concentrations) + \beta_2(days\ of\ survival), \quad (9)$$

where predicted logit (Y = poor mark/Y = no mark) and (Y = good mark/Y = no mark) are the link functions for poor and good mark quality with no mark as reference group, β_0 is the constant that refers to the Y intercept and β_1 and β_2 the estimated regression coefficients for the predictor variables the marker concentrations (single and double treatment) and the days of survival.

3 Results

3.1 Mortality

3.1.1 Control group

The control group displayed significantly lower mortalities compared to the marker groups ALI and CAL (single and double treatment), while the mortalities were significantly higher than the mortalities of the TET concentrations from the single treatment.

To exclude the possibility that the isotonic saline solution, injected in cod of the control group could have had an effect on the survival of cod, a binary logistic regression was also computed for the control group. The results of the analysis are given in Table 8. As it was expected, the logistic model did not fit well to the survival of cod (-2LL = 29,767, chi-square = 0,495 and p = 0,482) and the maximum likelihood estimates of the model parameters were all not different from zero ($p > 0,05$).

Table 7 Results from logistic model fitted to the control group and the binary dependent variable survival of cod (dead/alive). Regression coefficient β (in log-odds unit), standard error (SE) of β, chi-square test of the null hypothesis, degrees of freedom *df*, probability *p* of significance and the natural logarithm base *e*, raised to the exponent of the slope β that equals to the odds ratio (conversion of β).

Predictors		β	SE β	Wald Chi-square	*df*	*p*	$e^{(\beta)}$
	Constant[a)]	0,891	5,703	0,024	1	0,876	2,438
	Injection volumes	-0,003	0,004	0,488	1	0,485	0,997
	Mean water temperature	0,051	0,496	0,011	1	0,917	1,053
Goodness-of-fit statistics			coefficient	Chi-square	*df*	*p*	
	-2 log-likelihood		29,767				
	Wald test			0,495	1	0,482	

[a)] The constant represents the control group. Nagelkerke $R^2 = 0$

3.1.2 Mean water temperatures and injection volumes

To assess whether or not the mean water temperatures and the injection volumes had significant impacts on the survival of cod and probably contributed to mortality, a binary regression model only with these two factors as predictor variables for the survival of cod was computed (Table 8). The results indicated that the logistic model fit was poor and thus a significant improvement over the null model was not demonstrated (-2LL = 29,272, chi-square = 0,495 and p = 0,482). Both the mean water temperatures and the injection volumes were not significant and did not differ from zero. Accordingly, they did not have significant impacts on the survival of cod.

Table 8 Results from the logistic regression model between the mean water temperatures, the injection volumes, and the binary dependent variable survival of cod (dead/alive). Regression coefficient β (in log-odds unit), standard error (SE) of β, chi-square test of the null hypothesis, degrees of freedom *df*, value of significance *p* and the natural logarithm base *e*, raised to the exponent of the slope β that equals to the odds ratio (conversion of β).

Predictors		β	SE β	Wald Chi-square	*df*	*p*	$e^{(\beta)}$
	Constant	0,891	5,703	0,024	1	0,876	2,438
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	Mean water temperatures	0,051	0,496	0,011	1	0,917	1,053
Goodness-of-fit statistics			coefficient	Chi-square	*df*	*p*	
	-2 log-likelihood		29,272				
	Wald test			0,495	1	0,482	

Nagelkerke $R^2 = 0$

3.1.3 Tetracycline hydrochloride

Total mortality rates for TET were found highest for the double treatment and lowest for the single treatment. The total mortality rates of the concentrations from the double treatment were close to each other and to the mortality rate of the control group (27,3 (±7,4) % for the control group, compared to 30 (±6,6), 18 (±9,6) and 14 (±11,4) % for TET25&STR, TET50&STR and TET100&STR, respectively). The total mortality rates of the TET concentrations from the single treatment were extremely low (8 (±13,6), 12 (±10,8) and 4 (±19,6) for 25, 50 and 100 mg TET/kg fish respectively) and even below the mortality rate of the control group (cf. Table 14).

Not any significant differences in absolute mortalities could be detected among the concentrations of the single treatment and double treatment (Kruskal-Wallis *H*-test; $p = 0{,}107$). But when compared to the control group, significant differences in absolute mortalities were proved (Kruskal-Wallis *H*-test; $p = 0{,}007$). Multiple Mann-Whitney *U*-tests revealed that mortality of the control group was significantly higher from mortalities of the TET concentrations from the single treatment (Mann-Whitney *U*-tests; $p < \alpha^*$, Bonferroni-Holm adjusted *p*-values to new calculated significance levels α^*). Figure [number] shows the different mortality rates for the tested TET concentrations from the single (A) and the double treatment (B). The significant difference of mortality of the control group from the mortalities of the concentrations from the single treatment is indicated with an asterisk.

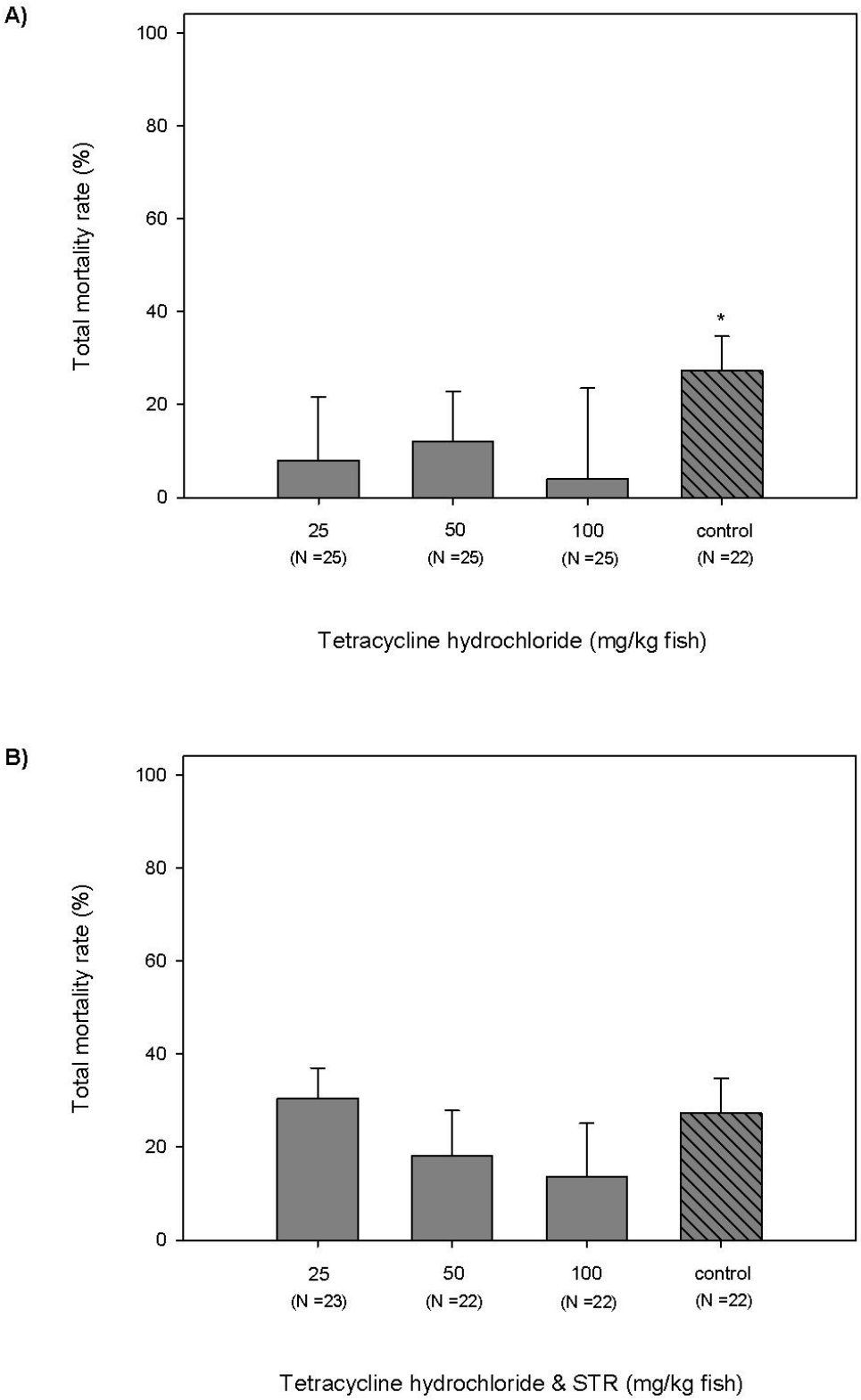


Figure 7 Total mortality rates (%) with error bars (±RSD%) for the control group as reference (striped bar) and the tested TET concentrations, A) TET single treatment and B) TET double treatment with 2 mg/kg fish strontium chloride. Sample sizes are given in parentheses. Significantly differences in mortality rates are indicated with asterisks (*) ($p \leq 0{,}05$).

The results of the binary logistic regression for TET are given in table [number]. The overall null hypothesis, that there was no relationship between the survival of cod and the predictor variables TET concentrations (single and double treatment), injection volumes and mean water temperatures, could not be rejected. The Wald-test of the goodness-of-fit of the logistic model was not significant (-2LL = 111,769, chi-square = 8,554 and p = 0,128). Therefore, the logistic model did not improve upon the null model. Not any of the regression coefficients of the predictor variables were found significantly different from zero in estimating the survival of cod (p > 0,05). Thus, no relationship between the survival of cod and the predictor variables was proved. These results were consistent with the findings from the assessment of the mortality rates, where significant differences in mortalities were not found among the concentrations (single and double treatment). The absolute mortalities were even significantly lower than those of the control group (cf. Table 13 and Figure 7).

Table 9 Results from logistic model fitted to the concentrations of alizarin red S from single treatment and the binary dependent variable survival of cod (dead/alive). Regression coefficient β (in log-odds unit), standard error (SE) of β, chi-square test of the null hypothesis, degrees of freedom *df*, value of significance *p* and the natural logarithm base *e*, raised to the exponent of the slope β that equals to the odds ratio (conversion of β). The concentrations (mg/kg fish) of TET were 25; 50 and 100, respectively (single treatment and double treatment). STR refers to strontium chloride, which concentration was 2 mg/ kg fish.

Predictors		β	SE β	Wald Chi-square	*df*	*p*	$e^{(\beta)}$
	Constant	13,878	7,938	3,056	1	0,08	1064679,09
Marker conc.	TET25	-0,386	1,41	0,075	1	0,784	0,68
Single treatment	TET50	0,069	1,354	0,003	1	0,96	1,071
	TET100	0,83	1,393	0,355	1	0,551	2,293
Marker conc.	TET25&STR	-1,536	1,147	1,793	1	0,181	0,215
Double treatment	TET50&STR	-0,831	1,066	0,609	1	0,435	0,435
	Injection volumes	0,055	0,35	0,024	1	0,876	1,056
	Mean water temperature	1,791	4,111	0,19	1	0,663	5,994
Goodness-of-fit statistics			coefficient	Chi-square	*df*	*p*	
	-2 log-likelihood		111,769				
	Wald test			8,554	5	0,128	

Note: reference group for TET concentrations was TET100&STR. Nagelkerke R^2 = 0

3.1.4 Strontium chloride

For STR, highest total mortality rates were recorded for the concentration STR1 (60 (±3,3) %) and lowest total mortality rates at the concentrations STR0,5 and STR2 (33 (±5,9) and 28 (±6,4) %, respectively). For the concentration STR2 mg/kg the total mortality rate was close to the total mortality rate of the control group (27,3 (±7,4) %).

Significant differences in absolute mortalities were not found between the concentrations (only single treatment) (Kruskal-Wallis *H*-test; $p = 0{,}092$) and neither when compared to the control group (Kruskal-Wallis *H*-test; $p = 0{,}187$).

Figure [number] shows the different total mortality rates for the tested concentrations. It can be seen that total mortality rates did not differ significantly between the concentrations and were close to the mortality rate of the control group.

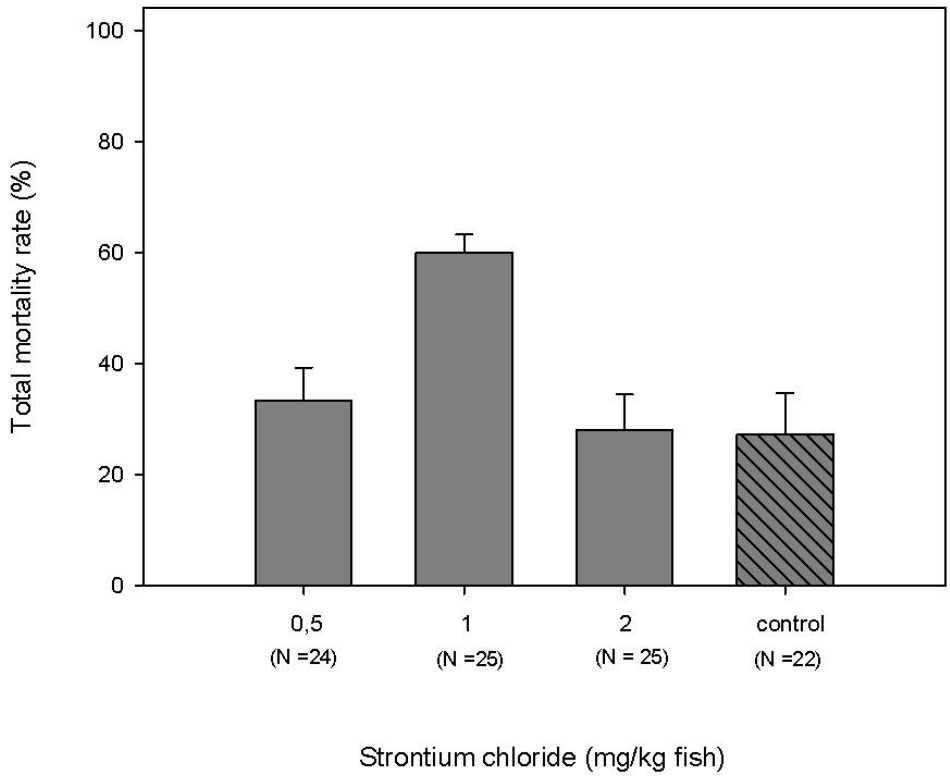


Figure 8 Total mortality rates (%) with error bars (±RSD%) for the control group as reference (striped bar) and the tested STR concentrations. Sample sizes are given in parentheses.

The results from the binary logistic regression are given in Table 11. The logistic model improved significantly upon the null model and the null hypothesis that the predictor variables had not any effect on the survival of cod could be rejected (-2LL = 87,462, Wald-test, chi-square = 12,623 and p = 0,006). With regard to the parameter estimates, it can be noticed that the regression coefficients of STR, STR0,5, STR1 and the injection volumes were significant and thus were assumed to have an effect on the survival of cod ($p \leq 0{,}05$). The estimated logarithmized odds for the survival of cod were negatively related to STR0,5, STR1 and the injection volumes (β = -2,129, β = -2,691 and β = -0.,008, respectively).

In consideration of the estimated odds ratio ($e^{(\beta)}$) for the concentrations STR0,5, STR1 relative to STR2, the odds for cod to survive rather than to die were expected to decrease by the factor of 0,119 and 0,068, respectively. Therefore cod marked with STR2 were more likely to survive rather than to die, than cod marked with STR0,5 and STR1. Accordingly, cod marked with STR0,5 and STR1 were by 10,63 % and 6,37 %, respectively less likely to survive than cod marked with STR2. Normally it would have been assumed that the probability that cod survived rather than died would be higher at the lower concentrations STR0,5 and STR1 than at the highest concentration STR2. The interpretation of these findings had to be made with care, since the low probabilities of 10,63 % and 6,37 % suggested that not a clear result could have been obtained from the logistic model.

For the injections volumes, the odds for cod to survive rather than to die decreased by 0,008 times with each unit increase of the injection volumes. Therefore, with each unit increase of the injection volumes, the probability that cod survived was by 49,80 % lower than the probability that cod died. The high probability of ca. 50 % suggested that the injection volumes had a noticeably negative effect on the survival of cod.

Table 10 Results from logistic model fitted to the concentrations of strontium chloride and the binary dependent variable survival of cod (dead/alive). Regression coefficient β (in log-odds unit), standard error (SE) of β, chi-square test of the null hypothesis, degrees of freedom *df*, probability *p* of significance and the natural logarithm base *e*, raised to the exponent of the slope β that equals to the odds ratio (conversion of β). The concentrations (mg/kg fish) of STR were 0,5; 1 and 2, respectively.

Predictors		β	SE β	Wald Chi-square	*df*	*p*	$e^{(\beta)}$
	Constant	2,62	3,593	0,532	1	0,466	13,732
Marker conc.	STR0,5	-2,129	1,078	3,9	1	**0,048***	0,119
	STR1	-2,691	0,946	8,088	1	**0,004***	0,068
	Injection volumes	-0,008	0,004	5,18	1	**0,023***	0,992
	Mean water temperature	0,086	0,305	0,08	1	0,777	1,09
Goodness-of-fit statistics			coefficient	Chi-square	*df*	*p*	
	-2 log-likelihood		87,462				
	Wald test			12,623	3	**0,006****	

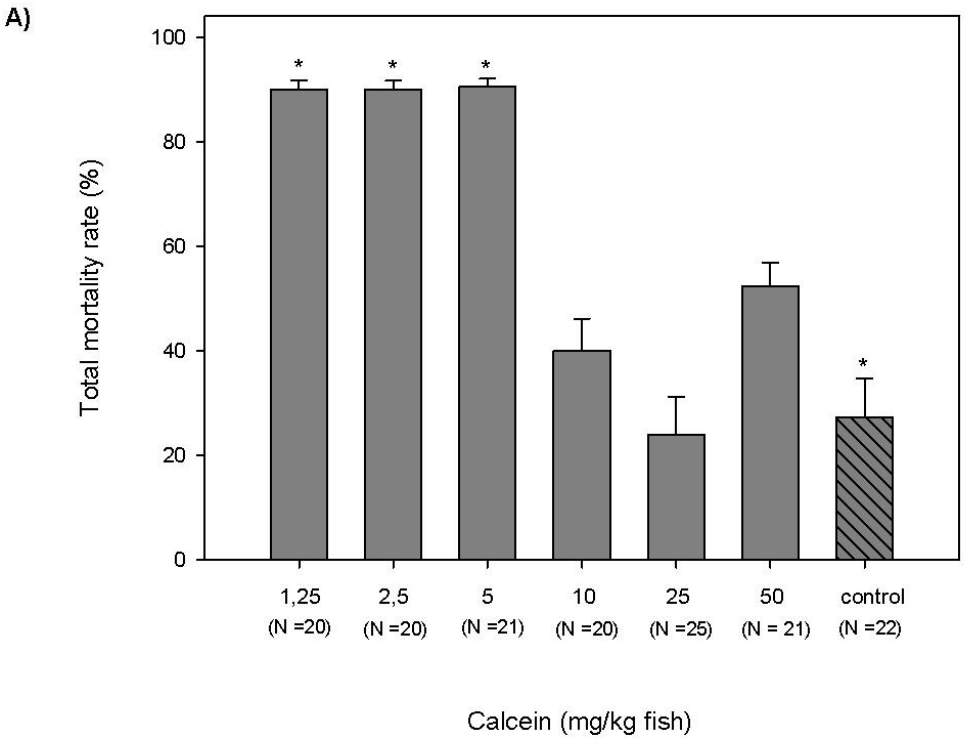
Note: reference group for STR concentrations was STR2. Nagelkerke $R^2 = 0,21$
*$p \leq 0,05$, **$p \leq 0,01$

3.1.5 Calcein

Considering Table 13 the total mortality rates for CAL single treatment were near 100 % for the concentrations CAL1,25, CAL2,5 and CAL5 (ca. 90 (±1,7) %). The higher concentrations CAL10, CAL25 and CAL50 showed lower total mortality rates, whereby at the concentration CAL25 the lowest mortality rate was recorded (40 (±6,1), 24 (±7,1) and 52 (±4,4) %, respectively). For the double treatment all cod died at the concentrations CAL2,5 and CAL5 (100 (±0,0) %) and reached near 100 % for the concentrations CAL1,25, CAL10, CAL25 and CAL50, where latter concentration had the highest mortality rate (76 (±2,7), 75 (±2,4), 74 (±2,6) and 92 (±1,2) %, respectively). Compared to the total mortality rate of the control group (27,3 (±7,4) %) the mortality rates for the concentrations of the single and double treatment were markedly higher.

Kruskal-Wallis *H*-tests revealed significant differences in absolute mortalities among the concentrations ($p \leq 0,001$) and compared to the control group ($p \leq 0,001$). For multiple comparisons, the Nemenyi-test was used. The Nemenyi-test revealed that the mortalities for the concentrations of the single treatment CAL1,25, CAL2,5 and CAL5 were significantly higher than the mortalities for CAL10, CAL25 and CAL50 (chi-square = 12,59, $p \leq 0,05$).

Concentrations of the double treatment did not show any significant differences in mortalities (chi-square = 12,59, $p > 0,05$), neither did the comparisons between concentrations of the single and double treatment yield in significant differences in mortalities (chi-square = 19,68, $p > 0,05$). Whereas, significant differences in mortalities were indicated between the control group and the concentrations of the single treatment CAL1,25, CAL2,5 and CAL5, as well as between the control group and all concentrations of the double treatment (CAL1,25&STR, CAL2,5&STR, CAL5&STR, CAL10&STR, CAL25&STR and CAL50&STR), where the control group showed significant lower mortality (chi-square = 12,59, $p \leq 0,05$). In Figure 9 the total mortality rates for each concentration of the single and double treatment are displayed with indication of the significant different concentration groups (asterisks).



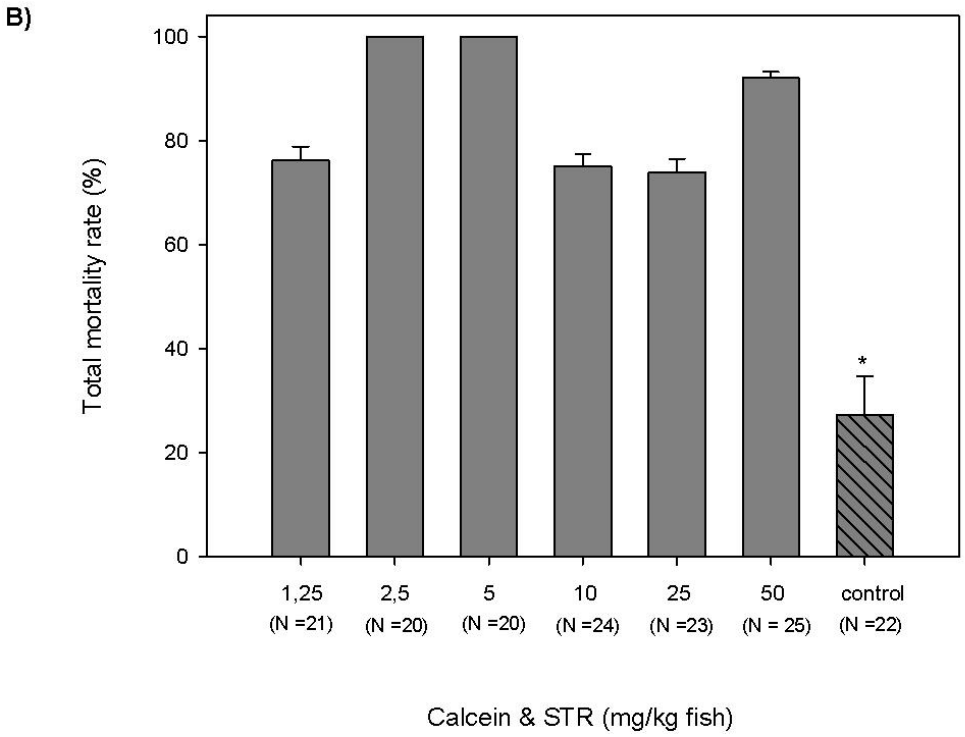


Figure 9 Total mortality rates (%) with error bars (±RSD%) for the control group as reference (striped bar) and the tested CAL concentrations, A) CAL single treatment and B) CAL double treatment with 2 mg/kg fish of strontium chloride. Sample sizes are given in parentheses. Significantly differences in absolute mortalities are indicated with asterisks (*) ($p \leq 0{,}05$).

The results from the binary logistic regression (Table 11) indicated that the logistic model fitted well to the survival of cod. The -2LL likelihood ratio for the logistic model was 226,855 and by 68,321 (chi-square = 68,321) times lower than the null model The Wald-statistic of the goodness-of-fit showed a highly significant improvement of the logistic model over the null model ($p \leq 0{,}001$), therefore a significant effect of the predictor variables CAL concentrations (single and double treatment), injection volumes and mean water temperatures, on the survival of cod was demonstrated.

In consideration of the β-values with the assigned *p*-values from the Wald chi-square tests, the concentrations CAL10, CAL25 and CAL50 were significantly different from zero and had a significant effect on the survival of cod ($p \leq 0{,}001$, $p \leq 0{,}01$ and $p \leq 0{,}05$, respectively).

The logarithmized odds for the survival of cod were positively related to the concentrations CAL10, CAL25 and CAL50 ($\beta = 2{,}661$, $\beta = 2{,}857$ and $\beta = 2{,}138$, respectively).

In consideration of the estimated odds ratio ($e^{(\beta)}$) for the concentrations CAL10, CAL25 and CAL50 relative to CAL50&STR, the odds for cod to survive rather than to die were expected to increase by the factors of 14,311, 17,403 and 8,486, respectively. Therefore, cod marked with these concentrations were more likely to survive rather than to die, than cod marked with CAL50&STR. Speaking in terms of probability, cod marked with CAL10, CAL25 and CAL50 were by 93,47 %, 94,57 % and 89,46 %, respectively more likely to survive than cod marked with CAL50&STR. From the probabilities it can be seen that cod injected with CAL10 and CAL25 had higher probability to survive, compared to cod injected with CAL50 (relative to cod marked with CAL50&STR).

These findings from the logistic model applied to the results from the evaluation of the mortality rates, where CAL10, CAL25 and CAL50 showed the lowest mortality rates (cf. Table 14 and Figure 9).

Table 11 Results from logistic model fitted to the concentrations of calcein from single and double treatment and the binary dependent variable survival of cod (dead/alive). Regression coefficient β (in log-odds unit), standard error (SE) of β, chi-square test of the null hypothesis, degrees of freedom *df*, probability *p* of significance and the natural logarithm base *e*, raised to the exponent of the slope β that equals to the odds ratio (conversion of β). The concentrations (mg/kg fish) of CAL were 1,25; 2,5; 5; 10; 25 and 50 respectively (single treatment and double treatment). STR refers to 2 mg/kg fish strontium chloride.

Predictors		β	SE β	Wald Chi-square	*df*	*p*	$e^{(\beta)}$
	Constant	-3,192	2,278	1,963	1	0,161	0,041
	CAL1,25	-0,347	1,455	0,057	1	0,811	0,707
	CAL2,5	0,115	1,385	0,007	1	0,934	1,122
Marker conc.	CAL5	-0,337	1,413	0,057	1	0,811	0,714
Single treatment	CAL10	2,661	1,292	4,239	1	**0,04****	14,311
	CAL25	2,857	1,178	5,876	1	**0,015****	17,403
	CAL50	2,138	0,964	4,917	1	**0,027***	8,486
	CAL1,25&STR	0,206	1,311	0,025	1	0,875	1,229
Marker conc.	CAL2,5&STR	-19,292	8987,18	0	1	0,998	0
Double treatment	CAL5&STR	-0,286	1,304	0,048	1	0,827	0,751
	CAL10&STR	0,584	1,114	0,275	1	0,6	1,794
	CAL25&STR	0,687	0,944	0,53	1	0,467	1,987
	Injection volumes	0	0	0,018	1	0,892	1
	Mean water temperature	0,108	0,141	0,588	1	0,443	1,114

Goodness-of-fit statistics		coefficient	Chi-square	*df*	*p*
	-2 log-likelihood	226,855			
	Wald test		68,321	11	***

Note: reference group for CAL concentrations was CAL50&STR. Nagelkerke $R^2 = 0,34$

*$p \leq 0,05$, **$p \leq 0,01$ and ***$p \leq 0,001$

3.1.6 Alizarin red S

Total mortality rates for the single (only ALI) and the double treatment (ALI&STR) varied between 88 (±1,5) % and 100 (±0,0) % for the single treatment and between 66,7 (±23,6) % and 100 (±0,0) %.for the double treatment. The maximum total mortality rates of 100 % occurred at the concentrations ALI62,5 and ALI125 both for the single and double treatment. Compared to the total mortality rate of the control group (27,3 (±7,4) %), the mortality rates of the single and double treatment were markedly high. The lowest total mortality rate of 66,7 (±23,6) % of ALI1000&STR is misleading, since the marking had to be aborted, because cod died right after injections. The small number of total cod internally marked (N = 3) is not representative for this concentration group and has not any statistical validity and thus this concentration group was excluded from the statistical tests. The mean days of survival varied between 4 and 9 days and did not exceed 9 (±14,3) days for both single and double treatments (disregarding ALI1000&STR). A total number of 123 out of 132 (single treatment) and 107 out of 114 (double treatment) died after intraperitoneal injections with ALI and ALI&STR, respectively (Table 13).

The absolute mortalities did not differ significantly between concentrations (single and double treatment), (Kruskal-Wallis *H*-test; $p = 0{,}368$). Whereas, including the control group in the test, the result of the Kruskal-Wallis *H*-test showed significant differences in mortalities (Kruskal-Wallis *H*-test; $p < 0{,}001$). Multiple comparisons with Bonferroni-Holm adjusted *p*-values, revealed that the control group had a significantly lower mortality as the concentrations of ALI of the single and the double treatment (Mann-Whitney *U*-tests; $p < \alpha^*$, where α^* was the new calculated significance level). Figure 10 shows the total mortality rates of the ALI concentrations from single and double treatment. The significant difference in mortality of the control group is indicated with an asterisk.

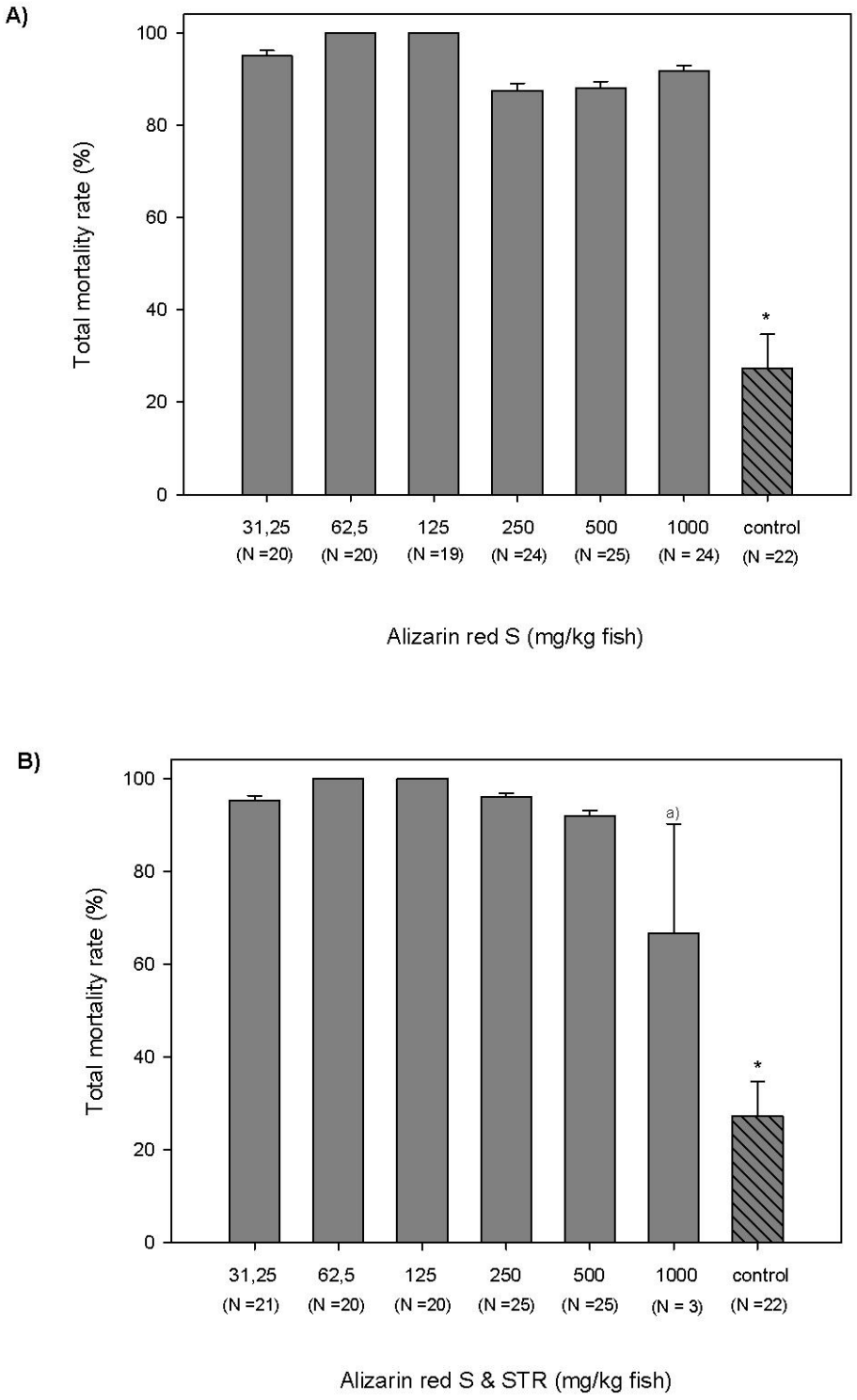


Figure 10 Total mortality rates (%) with error bars (±RSD%) for the control group as reference (striped bar) and the tested ALI concentrations, A) ALI single treatment and B) ALI double treatment with 2 mg/kg fish of strontium chloride. Sample sizes are given in parentheses. Significantly differences in absolute mortalities are indicated with asterisks (*) ($p \leq 0{,}05$). [a)] The concentration group ALI1000&STR from the double treatment was excluded from statistical tests, due to high mortality of cod right after injections.

The results from Kruskal-Wallis *H*-test indicated that significant differences did not exist between the concentrations of ALI (single and double treatment). This suggested that concentration did not have an effect on the absolute mortality of cod. To test, whether the ALI concentrations from the single and double treatment and other possible factors, as the injection volumes and the mean water temperatures were related to the survival of cod; a binary logistic regression analysis was conducted. The results of the logistic model are summarized in Table 13.

The Wald test of goodness-of-fit was highly significant (-2LL = 64,849, chi-square = 42,241 and $p \leq 0{,}001$) thus, the logistic model led to a significantly better prediction of the survival of cod as the null model.

Regarding the maximum likelihood estimates of the predictors, the null hypothesis could be rejected, it was expected that at least one predictor did not equal zero and was expected to have a significant effect on the survival of cod. Considering the *p*-values from the Wald chi-square tests, it can be noticed that among the predictor variables, the only regression coefficient, which was highly significant was that of the mean water temperature ($\beta = -1{,}549$, chi-square = 26,971, $p \leq 0{,}001$). The predictor variables ALI concentrations (single and double treatment) and the injection volumes were not significant ($p > 0{,}05$). Consequently, the mean water temperature was assumed to significantly effecting the survival of cod from ALI treatments. Further, it could be stated that the logarithmized odds for cod to survive were negatively related to the mean water temperatures ($\beta = -1{,}549$). In consideration of the odds ratio ($e^{(\beta)} = 0{,}213$), it could be concluded that with each unit increase in mean water temperature, the odds that cod survived were 0,213 times lower than those that cod died. In other terms, with each unit increase of the mean water temperature the probability that cod survived was by 18 % (0,213/(1+0,213)*100) higher than the probability that cod died.

With regard to the β-values of ALI62,5, ALI125, ALI62,5&STR and ALI125& STR, it is striking that they had very low negative β-values with high standard errors. One probable reason might be that the mortality rates for these concentrations were 100 % (see table [number]) and that therefore the two outcomes of the dependent variable survival of cod, dead and alive, were completely separated, since these concentrations only predicted perfectly the outcome cod dead and not any case of cod alive was observed. Consequently the iterative maximum likelihood estimation could not compute a final solution for these four concentrations and the parameter estimates were infinite. Due to this explained circumstance and in addition to the non-significant Wald-test and the assigned high *p*-values ($p > 0{,}05$), these ALI concentrations were not assessed any further for the logistic model.

Table 12 Results from logistic model fitted to the concentrations of alizarin red S from single and double treatment and the binary dependent variable survival of cod (dead/alive). Regression coefficient β (in log-odds unit), standard error (SE) of β, chi-square test of the null hypothesis, degrees of freedom *df*, probability of significance *p* and the natural logarithm base *e*, raised to the exponent of the slope β (odds ratio). The concentrations (mg/kg fish) of ALI were 31,25; 62,5; 125; 250; 500 and 1000 respectively (single and double treatment). STR refers to 2 mg/kg fish strontium chloride.

Predictors		β	SE β	Wald Chi-square	*df*	*p*	$e^{(\beta)}$
	Constant	17,671	4,147	18,156	1	***	47241313,33
	ALI31,25	-2,004	1,646	1,483	1	0,222	0,135
	ALI62,5	-19,755	8948,267	0	1	0,998	0
Marker conc.	ALI125	-19,471	9174,618	0	1	0,998	0
Single treatment	ALI250	-1,039	1,585	0,43	1	0,512	0,354
	ALI500	-0,823	1,412	0,34	1	0,56	0,439
	ALI1000	-0,462	1,439	0,103	1	0,748	0,63
	ALI31,25&STR	-2,119	1,519	1,945	1	0,163	0,12
Marker conc.	ALI62,5&STR	-20,012	8556,77	0	1	0,998	0
Double treatment	ALI125&STR	-19,274	8820,01	0	1	0,998	0
	ALI250&STR	-0,958	1,406	0,464	1	0,496	0,384
	Injection volumes	-0,001	0,001	0,394	1	0,53	0,999
	Mean water temperatures	-1,549	0,298	26,971	1	***	0,213

Goodness-of-fit statistics		coefficient	Chi-square	*df*	*p*
	-2 log-likelihood	64,849			
	Wald test		42,241	10	***

Note: ALI1000&STR of the double treatment was excluded from the statistical tests and reference group for ALI concentrations was ALI500&STR. Nagelkerke $R^2 = 0,448$

*** $p \leq 0,001$

Table 13 Total mortality rates (%) with relative standard deviations (± %RSD), mean days of survival with standard deviations (± SD) and total holding time in days of the control group and the different concentrations of the four marker groups, alizarin red S (ALI), calcein (CAL), strontium chloride (STR) and tetracycline hydrochloride (TET), from single and double treatments. The numbers after the acronyms of the chemicals indicate the tested concentrations (mg/kg fish). For the double treatments, STR refers to 2 mg/kg fish of STR.

	Number of cod (N)				
Marker group	marked	dead	Total mortality rate (%)	Mean days of survival	Total holding time (days)[a)]
Single treatment					
ALI31,25	20	19	95,0 ±1,1	6 ±6,5	34
ALI62,5	20	20	100,0 ±0,0	5 ±0,9	34
ALI125	19	19	100,0 ±0,0	4 ±1,1	34
ALI250	24	21	87,5 ±1,6	9 ±14,3	46
ALI500	25	22	88,0 ±1,5	7 ±14,4	46
ALI1000	24	22	91,7 ±1,3	4 ±12,5	46
CAL1,25	20	18	90,0 ±1,7	7 ±8,6	33
CAL2,5	20	18	90,0 ±1,7	7 ±8,7	33
CAL5	21	19	90,5 ±1,5	7 ±8,5	33
CAL10	20	8	40,0 ±6,1	30 ±19,3	46
CAL25	25	6	24,0 ±7,1	35 ±16,8	46
CAL50	21	11	52,4 ±4,4	27 ±18,8	46
STR0,5	24	8	33,3 ±5,9	29 ±17,6	47
STR1	25	15	60,0 ±3,3	24 ±19,2	47
STR2	25	7	28,0 ±6,4	34 ±17,0	47
TET25	25	2	8,0 ±13.6	44 ±10,9	47
TET50	25	3	12,0 ±10,8	42 ±12,9	47
TET100	25	1	4,0 ±19,6	46 ±4,1	47
Control	22	6	27,3 ±7,4	31 ±13,7	39
Double treatment					
ALI31,25&STR	21	20	95,2 ±1,1	6 ±6,0	34
ALI62,5&STR	20	20	100,0 ±0,0	5 ±1,3	34
ALI125&STR	20	20	100,0 ±0,0	4 ±0,7	34
ALI250&STR	25	24	96,0 ±0,8	4 ±7,1	39
ALI500&STR	25	23	92,0 ±1,2	5 ±10,3	39
ALI1000&STR[b)]	3	2	66,7 ±23,6	18 ±15,8	39
CAL1,25&STR	21	16	76,2 ±2,7	11 ±12,2	33
CAL2,5&STR	20	20	100,0 ±0,0	5 ±2,7	33
CAL5&STR	20	20	100,0 ±0,0	5 ±1,9	33
CAL10&STR	24	18	75,0 ±2,4	13 ±15,6	40
CAL25&STR	23	17	73,9 ±2,6	14 ±15,5	40
CAL50&STR	25	23	92,0 ±1,2	8 ±9,7	40
TET25&STR	23	7	30,4 ±6,6	32 ±12,8	40
TET50& STR	22	4	18,2 ±9,6	35 ±11,0	40
TET100&STR	22	3	13,6 ±11,4	35 ±11,8	40

[a)]Maximum holding time was 47 days.

[b)]ALI1000&STR: marking of cod was aborted, because cod died right after injections. This concentration group was excluded from statistical tests.

3.2 Growth

Figure 11 shows the spread and the differences in growth between the treatment groups ALI, CAL, STR, TET and control group (NACL). For the marker groups ALI and CAL it can be seen that the median equals zero and that the growth rates show an extreme left-skewed distribution, due to no growth of the fish. The variances of the growth rates of STR, TET and the control group are similar, whereas the variance in growth for TET marker group seemed to be smaller.

The assessment of significant differences in growth between the treatment groups revealed that a growth did differ significantly between the treatment groups ALI, CAL, STR, TET and control group (Kruskal-Wallis *H*-test; $p \leq 0{,}05$). The multiple comparison test after Nemenyi revealed further that growth in the marker groups TET and STR as well as in the control group significantly differed from the marker groups ALI and CAL (chi-square = 9,49; $p \leq 0{,}05$). No significant differences in growth were indicated between the marker groups ALI and CAL and between the control group and the marker groups STR and TET (chi-square = 9,49; $p > 0{,}05$). But the marker group TET did significantly differ in growth from the STR treatment group (chi-square = 9,49; $p \leq 0{,}05$).

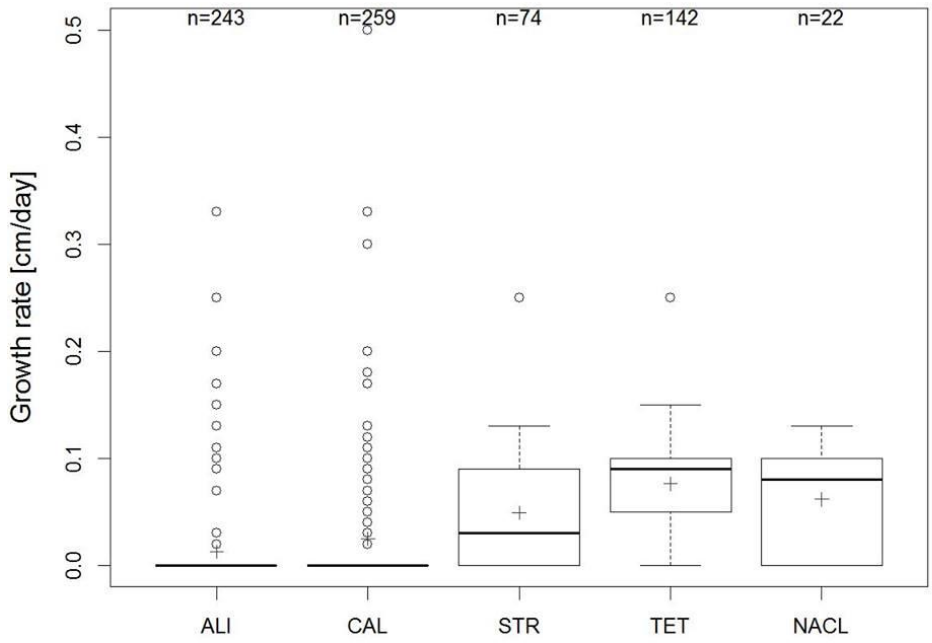


Figure 11 Differences in growth rates between the treatment groups ALI, CAL, STR, TET and the control group (NACL).

3.3 Mark quality

3.3.1 Tetracycline hydrochloride

The frequencies of the mark qualities no mark, poor and good for the TET concentrations from the single and the double treatment are given in Table 14.

The majority of the otoliths examined from the single treatment displayed good marks. The concentrations TET50 and TET100 recorded the highest proportion of otoliths with good mark and the lowest proportions of otoliths with no marks (60 % and 68 % relative to 8 % and 4 %). Otoliths marked with TET25 showed most often poor marks (40%), this proportion was in the same range as that of otoliths with good marks (36 %).

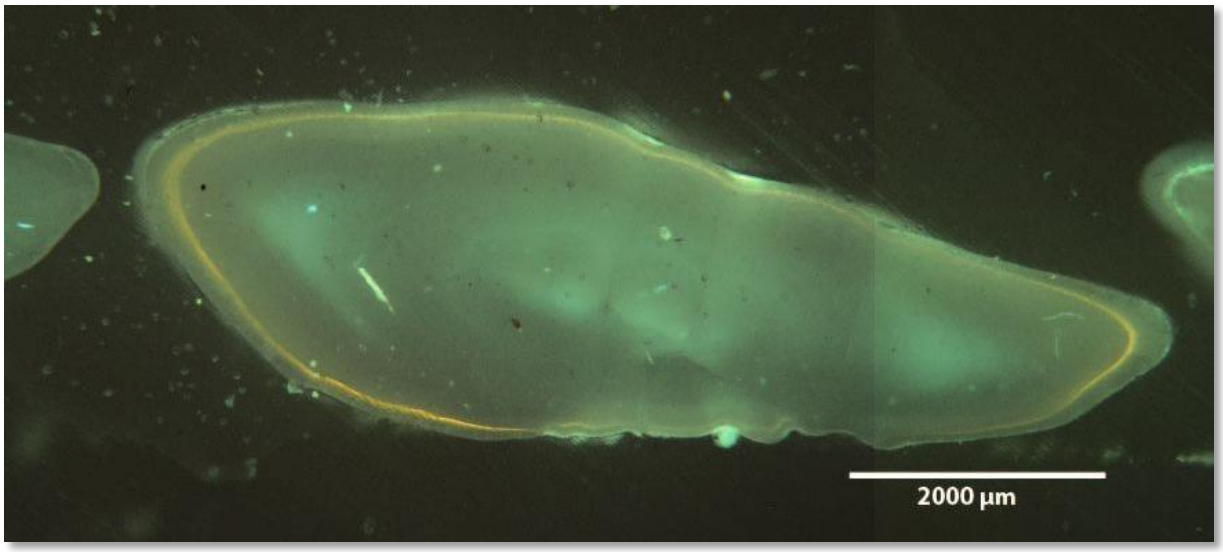
The opposite was found for the mark qualities from the double treatment. The majority of otoliths appeared to have no fluorescent marks. This was observed especially for otoliths marked with TET25&STR and TET50&STR (74 % and 64 %, respectively).

Further, for these two concentrations not any otolith was observed with good mark quality. For the highest concentration TET100&STR, over half of the otoliths displayed poor marks (55 %) and the proportion of otoliths with no marks was as much as that of otoliths with good marks (23 %). Figure 12 shows three examples of marked otoliths viewed under UV-light in a fluorescence microscope that displayed different mark qualities.

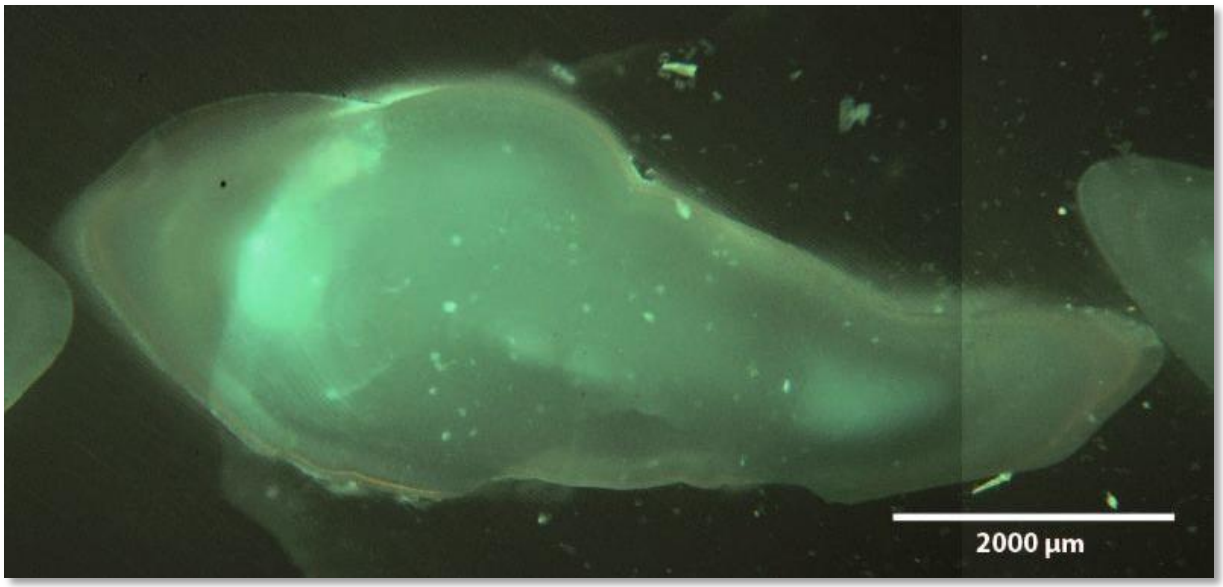
Table 14 The proportions (%) of fluorescent mark qualities (no mark, poor and good) after intraperitoneal injections with TET (single and double treatment). Proportions given in parentheses refer to mark qualities of surviving cod. Total number of cod marked, the numbers of surviving cod until the end of the experiment are given in parentheses.

		Mark quality (% of N)		
Marker	Number of cod (N)	No mark	Poor	Good
Single treatment				
TET25	25 (23)	24,0 (21,7)	40,0 (39,1)	36,0 (39,1)
TET50	25 (21)	8,0 (9,5)	32,0 (19,0)	60,0 (71,4)
TET100	25 (24)	4,0 (4,2)	28,0 (29,2)	68,0 (66,7)
Double treatment				
TET25&STR	23 (16)	73,9 (68,8)	26,1 (31,2)	0,0 (0,0)
TET50&STR	22 (18)	63,6 (55,6)	36,4 (44,4)	0,0 (0,0)
TET100&STR	22 (19)	22,7 (15,8)	54,5 (57,9)	22,7 (26,3)

A)



B)



C)

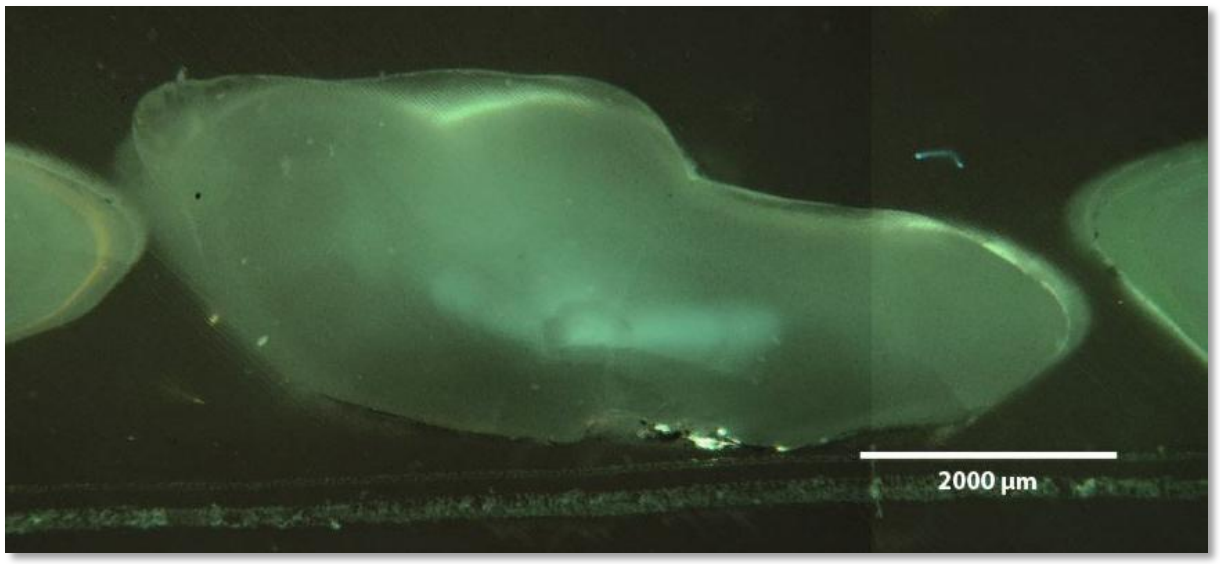


Figure 12 Examples of otoliths viewed under UV-light in a fluorescence microscope displaying different mark qualities A) good mark quality (TET100), B) poor mark quality (TET25), C) no mark (TET100&STR). Photo by author.

Two chi-square tests of independence were conducted, including the total number of cod marked and including just the number of surviving cod. The chi-square test, including the total number of cod was significant (chi-square = 65,907, *df* = 10, $p \leq 0{,}001$). Significant residuals were found for TET50 and TET100. At both concentrations, the proportions of good marks were significantly highest (60 % and 68 %, respectively) in contrast to TET25, TET25&STR, TET50&STR and TET100&STR. Further, at TET100 absent marks were significantly lowest than at the other concentrations (4 %). For TET25&STR and TET50&STR, significantly highest proportions of absent marks were found (74 % and 64 %, respectively), and both concentrations showed the lowest proportions of good marks (0 %). The relationship between these significant concentrations and the mark qualities was moderate (Cramér V = 0,482, $p \leq 0{,}001$).

The test results for the surviving cod were corresponding, except that the proportions were slightly different, due to smaller sample sizes. The chi-square test was highly significant (chi-square = 63,727, *df* = 10, $p \leq 0{,}001$). TET50 and TET100 proved significantly highest proportions of good marks (71 % and 67 %, respectively), additionally TET100 showed the lowest proportions of no marks (4 %). At TET25&STR and TET50&STR significantly highest proportions of absent marks were demonstrated (69 % and 56 %, respectively), and both concentrations showed the lowest proportions of good marks (0 %). The relationship between the significant concentrations and the mark qualities was proved moderate (Cramér V = 0,476, $p \leq 0{,}001$).

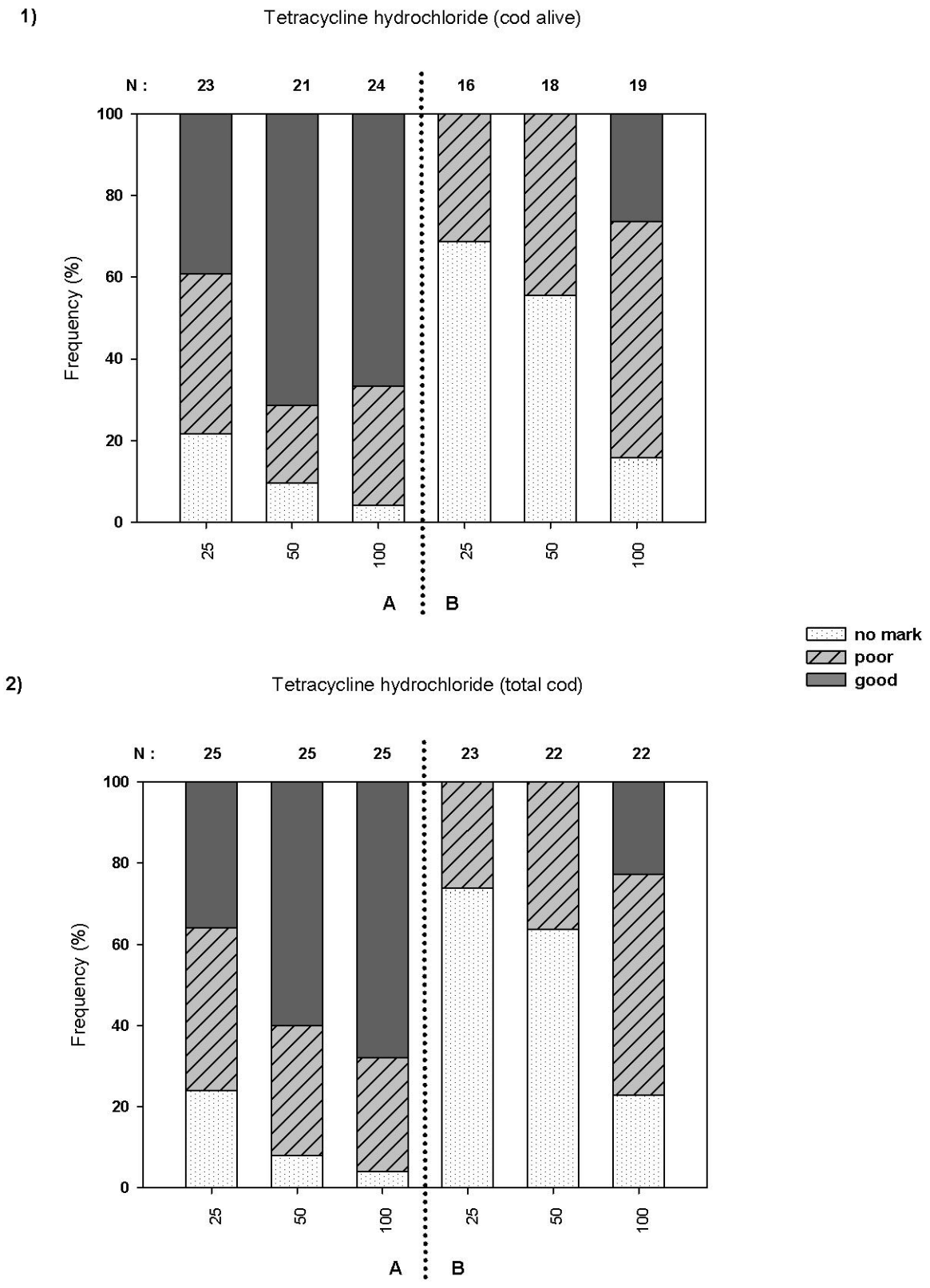


Figure 13 The proportions (%) of fluorescent mark qualities (no mark, poor and good) in thin sectioned otoliths marked with TET, 1) mark quality of otoliths o surviving cod, 2) mark quality of otoliths of total cod.

The results of the multinomial logistic regression for TET to investigate the relationship between the fluorescent mark quality and the TET concentrations (single and double treatment), including the days of survival as covariate, are given in Table 15.

The fit of the multinomial logistic model was highly significantly better upon the null model ($-2LL_{null}$ = 143,214 compared to $-2LL_{log}$ = 53,661). The likelihood ratio test yielded in a highly significant difference in deviance between the null model and the logistic model (chi-square = 89,553, $p \leq 0{,}001$). Regarding the maximum likelihood estimates for the predictor variables, it was found that for poor mark quality in contrast to no mark, the Wald-tests for TET25&STR and TET50&STR were significant, suggesting that these concentrations differed significantly from zero and had a significant effect on the categorization of otoliths in these two mark quality categories poor and good relative to the category no mark (chi-square = 6,962, p = 0,008 and chi-square = 4,516, p = 0,034, respectively). For good mark quality relative to no mark, the days of survival and the constant were proved to be high significant (chi-square = 5,029, p = 0,025 and chi-square = 6,363, p = 0,012, respectively).

Considering only the parameter estimates for poor mark quality relative to no mark, it was found that poor quality was negatively related to both significant predictor variables TET25&STR and TET50&STR (β = -1,911 and β = -1,499, respectively). As a consequence and provided that all the other predictor variables were held constant, the odds ($e^{(\beta)}$) of otoliths to display poor marks rather than no marks would be expected to decrease by 0,148 times with TET25&STR and by 0,223 times with TET50&STR. In terms of probability this means that in contrast to TET100&STR, TET25&STR decreased the probability of otoliths to have poor marks rather than no marks by 12,89 % (0,148/(1+0,148)*100), and TET50&STR relative to TET100&STR decreased the probability of otoliths to have poor marks rather than no marks by 18,23 %. The low predicted probabilities indicated that, although the predictor variables TET25&STR and TET50&STR proved to be significant, they had a low effect on the categorization of poor mark quality relative to no mark. Consequently, the interpretation of these relationships had to be made with care.

For good mark quality relative to no mark, it could be concluded that with each unit increase in the days of survival, the logarithmized odds of otoliths to display good mark quality rather than no marks increased by 0,11 times, given the other predictor variables were held constant. This means that with each unit increase in days of survival, the probability of otoliths to display good marks rather than no marks increased by 53,74 %, provided the other predictor variables were evaluated at zero.

This relative high portion of the survival time to raise the probabilities of good marks rather than no marks with further increase of the survival time, suggested that the relationship between both variables was strong.

Table 15 Results from the multinomial logistic regression describing the relationship between the concentrations of tetracycline hydrochloride from single and double treatment and the categorical dependent variable mark quality (no mark, poor, good). Regression coefficient β (in log-odds unit), standard error (SE) of β, chi-square test of the null hypothesis, degrees of freedom *df*, probability *p* of significance and the natural logarithm base *e*, raised to the exponent of the slope β that equals to the (odds ratio). The concentrations (mg/kg fish) of TET were 25; 50 and 100 respectively (single treatment and double treatment). STR refers to 2 mg/kg fish of strontium chloride.

						Mark quality good					
β	SE β	Wald Chi-square	*df*	*p*	$e^{(\beta)}$	Predictor	β	SE β	Wald Chi-square	*df*	*p*
-0,075	0,805	0,009	1	0,926	0,928	Constant	-4,083	1,821	5,029	1	**0,025**
0,029	0,018	2,452	1	0,117	1,029	Days of survival	0,11	0,044	6,363	1	**0,012**
-0,611	0,774	0,623	1	0,43	0,543	TET25	-0,465	0,917	0,257	1	0,612
0,642	0,982	0,427	1	0,514	1,9	TET50	1,875	1,079	3,019	1	0,082
0,742	1,219	0,37	1	0,543	2,1	TET100	1,876	1,265	2,198	1	0,138
-1,911	0,724	6,962	1	**0,008****	0,148	TET25&STR	-22,175	0	-	1	-
-1,499	0,705	4,516	1	**0,034***	0,223	TET50&STR	-22,055	0	-	1	-
0	-	-	0	-	-	TET100&STR	0	-	-	0	-

s

Days of survival			
coefficient	Chi-square	*df*	*p*
53,661			
	89,553	12	*******

Note: reference category for mark quality was “no mark” and reference group for TET concentrations was TET100&STR.

Significant *p*-values: **p* ≤ 0,05, ***p* ≤ 0,01, ****p* ≤ 0,001

3.3.2 Calcein

The frequencies of the mark qualities no mark, poor and good for the CAL concentrations from the single and the double treatment are given in Table 15. The majority of the otoliths examined displayed no marks or poor mark quality for the CAL concentrations of the single and the double treatment.

The results for CAL concentrations from the single treatment showed that most of the otoliths without fluorescent marks were found at the concentrations CAL1,25, CAL2,5 and CAL5. Equal observations were also made for the same CAL concentrations in the double treatment. High portion of otoliths displaying good marks were recorded for CAL10 and CAL25 (40 % and 36 %), whereby CAL50 showed the highest portion of otoliths with good marks (71 %). No good mark quality was found for CAL31,25.
Further, otoliths from the double treatment displayed most often no fluorescent marks, where the highest concentration CAL50&STR showed the highest proportion of otoliths with no marks (80 %), followed by CAL1,25&STR, CAL2,5&STR and CAL5&STR (70 %, 75 % and 70 %, respectively). Accordingly, at these concentrations the lowest portion of otoliths with good marks was recorded (4 % and 5 %, respectively). For CAL10&STR and CAL25&STR, 54 % and 57 %, respectively, of otoliths showed no marks. The proportions of otoliths displaying poor and good marks were similar for CAL10 &STR (21 % poor marks and 25 % good) and identical for CAL25&STR (poor and good marks each at 22 %) and at these concentrations the highest portion of otoliths with good marks was recorded.

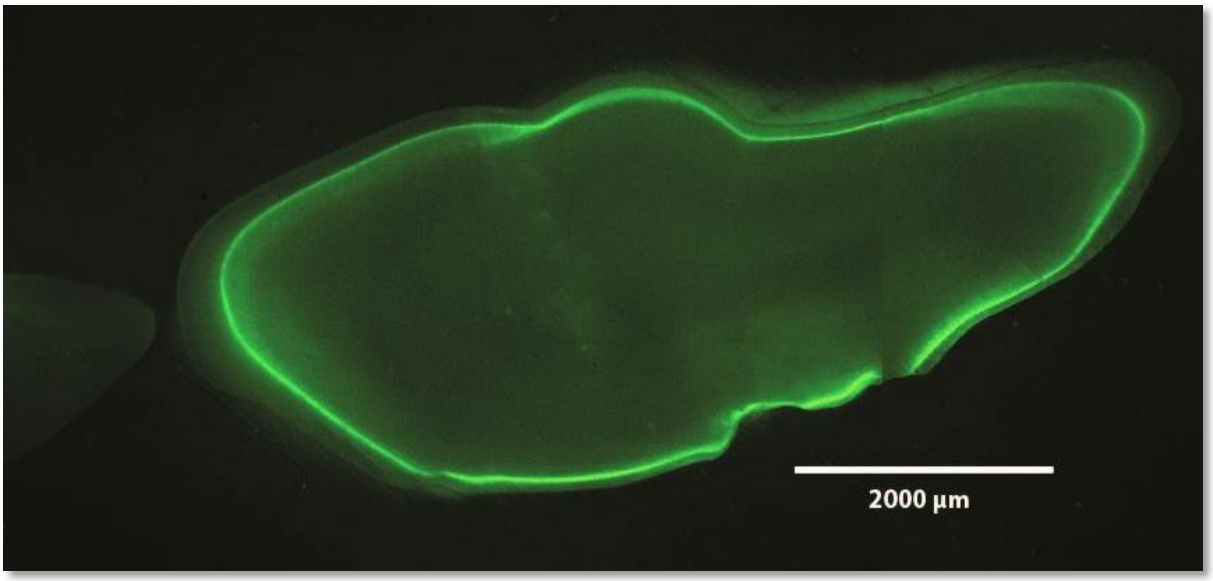
The chi-square test of independence to assess, whether the proportions of no mark, poor mark quality and good mark quality were the same for the CAL concentrations was highly significant for the single treatment (chi-square = 71,910, $df = 22$, $p \leq 0{,}001$). In consideration of the standardized residues, CAL31,25 proved significantly lowest observed proportions of otoliths with marks of good quality (0 %), whereas, at concentration CAL50, significantly highest proportions of good marks were indicated (71 %) and the lowest proportions of no marks (24 %). The effect size Cramér V showed that the association between these CAL concentrations and the mark qualities was moderate (Cramér V = 0,371, $p \leq 0{,}001$). For all remaining concentrations, the observed proportions in the three mark quality categories did not differ significantly and thus, mark quality was and concentrations were independent.

The chi-square test of independence was not computed for the contingency table with only surviving cod, because sample sizes for the concentrations were too small, due to high mortalities at each concentration and valid results would not have been obtained.

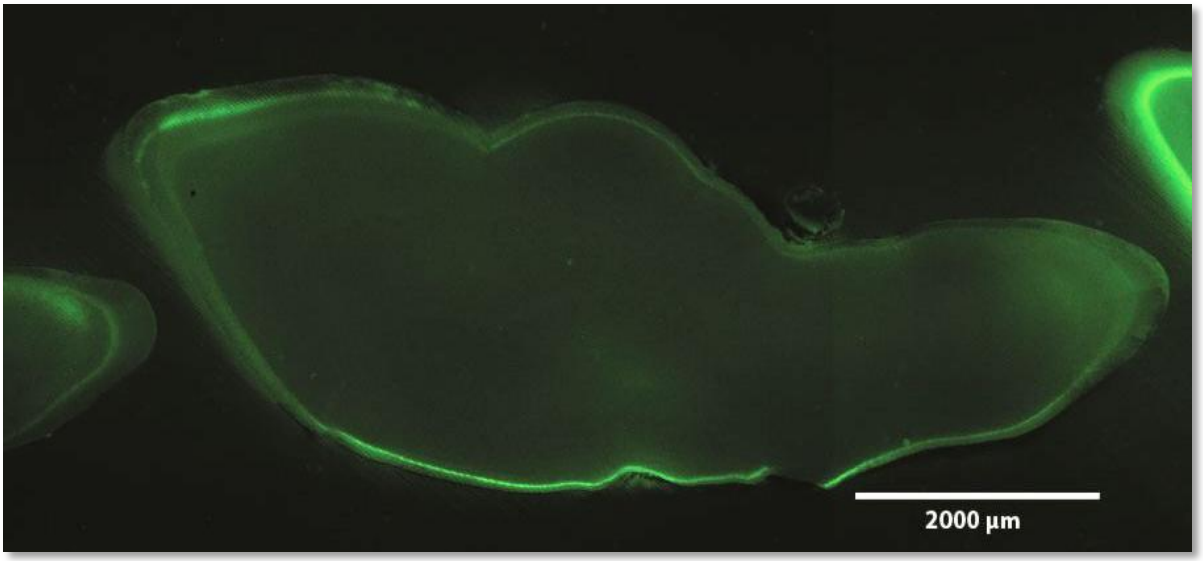
Table 15 The proportions (%) of fluorescent mark qualities (no mark, poor and good) after intraperitoneal injections with CAL (single and double treatment). Proportions given in parentheses refer to mark qualities of surviving cod. Total number of cod marked, the numbers of surviving cod until the end of the experiment are given in parentheses.

		Mark quality (% of N)		
Marker	Number of cod (N)	No mark	Poor	Good
Single treatment				
CAL1,25	21 (3)	66,7 (0,0)	33,3 (100,0)	0,0 (0,0)
CAL2,5	21 (2)	66,7 (0,0)	28,6 (100,0)	4,8 (0,0)
CAL5	21 (2)	57,1 (0,0)	23,8 (0,0)	19,0 (100,0)
CAL10	20 (12)	35,0 (8,3)	25,0 (25,0)	40,0 (66,7)
CAL25	25 (19)	32,0 (31,6)	32,0 (21,1)	36,0 (47,7)
CAL50	21 (10)	23,8 (0,0)	4,8 (0,0)	71,4 (100,0)
Double treatment				
CAL1,25&STR	20 (4)	70,0 (0,0)	25,0 (75,0)	5,0 (25,0)
CAL2,5&STR	20	75,0	20,0	5,0
CAL5&STR	20	70,0	25,0	5,0
CAL10&STR	24 (6)	54,2 (16,7)	20,8 (0,0)	25,0 (83,3)
CAL25&STR	23 (6)	56,5 (16,7)	21,7 (16,7)	21,7 (66,7)
CAL50&STR	25 (2)	80,0 (0,0)	16,0 (100,0)	4,0 (0,0)

A)



B)



C)

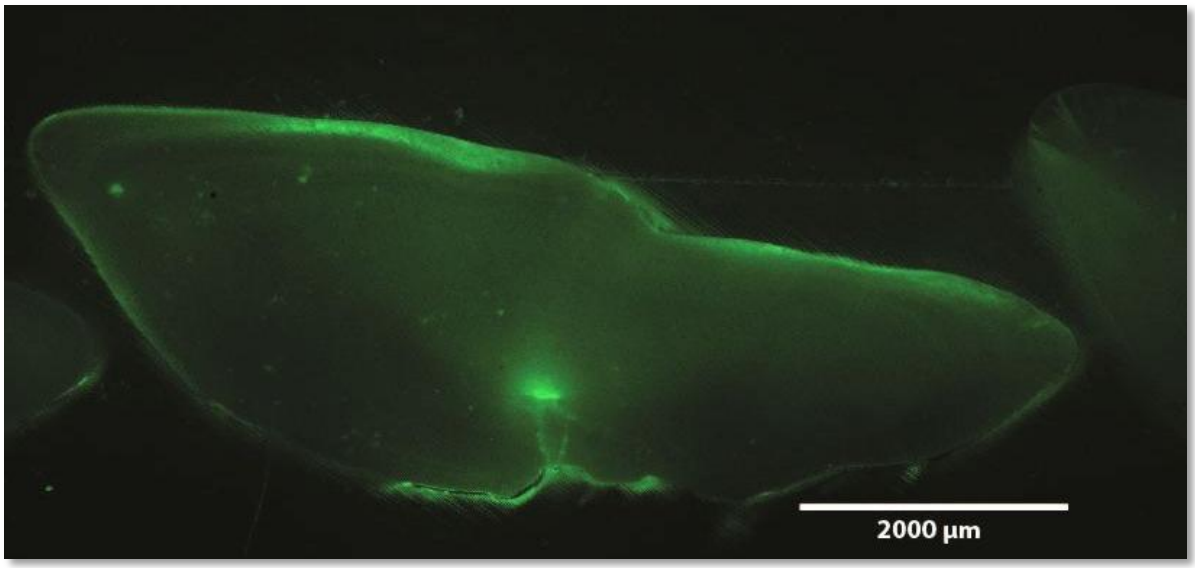


Figure 14 Examples of otoliths viewed under UV-light in a fluorescence microscope displaying different mark qualities A) good mark quality (CAL50), B) poor mark quality (CAL10), C) no mark (CAL25). Photo by author.

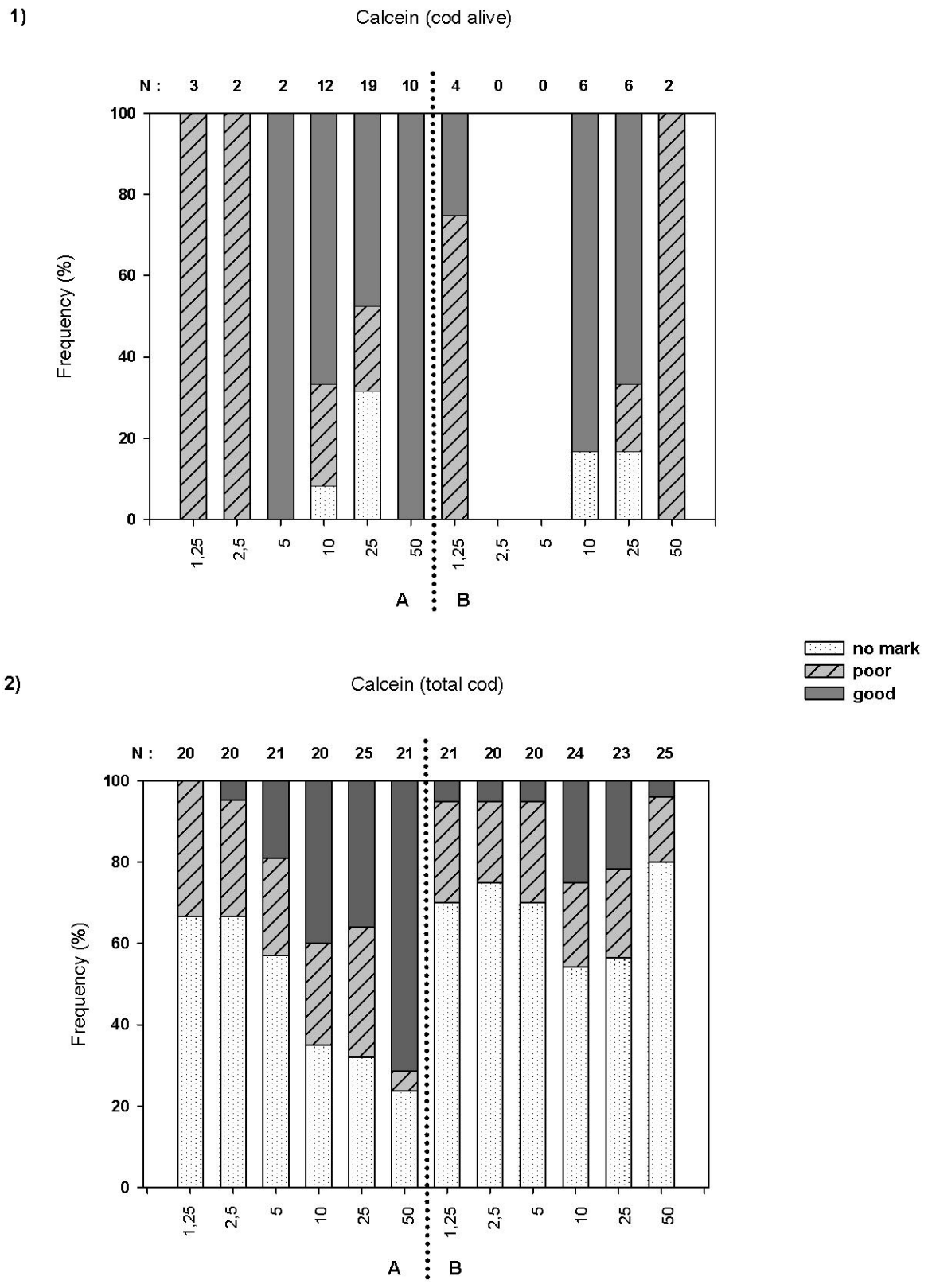


Figure 15 The proportions (%) of fluorescent mark qualities (no mark, poor and good) in thin sectioned otoliths marked with CAL, 1) mark quality of otoliths of surviving cod, 2) mark quality of otoliths of total cod.

The likelihood ratio test was highly significant (chi square = 154,716, df =24, $p \leq 0{,}001$), thus the logistic model, including the predictor variables fitted better than the null model ($-2LL_{null}$ = 402,593 compared to $-2LL_{log}$ = 247,877). Considering the maximum likelihood estimates for the predictors, it can be seen that the predictor variable days of survival was highly significant for categorizing both poor and good mark quality in contrast to no mark (chi-square = 16,234, $p \leq 0{,}001$ and chi-square = 39,512, $p \leq 0{,}001$, respectively). Both, poor and good mark quality were positively related to days of survival (β = 0,066 and β = 0,155, respectively). When the CAL concentrations were assumed to be constant, then for each unit increase in the days of survival, the probability of otoliths to display poor marks rather than no marks increased by 51,64 % (1,068/(1+1,068)*100; $e^{(\beta)}$ = 1,068) and the probability of otoliths to display good marks rather than no marks increased by 53,97 % ($e^{(\beta)}$ = 1,168). None of the CAL concentration had a significant influence on poor mark quality. Whereas CAL5 and CAL50 had a high significant effect on good mark quality (chi-square = 4,653, p = 0,031 and chi-square = 8,102, p = 0,004, respectively). Good mark quality was related positively to both concentrations (β =3,281 and β = 4,263, respectively). Assuming that all the other CAL concentrations and the days of survival were constant, then CAL5 increased the probability of otoliths to have good marks rather than no marks by 96,38 % ($e^{(\beta)}$ = 26,614), in contrast to CAL50&STR, and CAL50 relative to CAL50&STR increased the probability of otoliths to have good marks rather than no marks by 98,61 % ($e^{(\beta)}$ = 70,996).

Table 16 Results from the multinomial logistic regression describing the relationship between the concentrations of calcein from single and double treatment and the categorical dependent variable mark quality (no mark, poor, good). Regression coefficient β (in log-odds unit), standard error (SE) of β, chi-square test of the null hypothesis, degrees of freedom *df*, probability *p* of significance and the natural logarithm base *e*, raised to the exponent of the slope β (odds ratio). The concentrations (mg/kg fish) of CAL were 1,25; 2,5; 5; 10; 25 and 50 respectively (single treatment and double treatment). STR refers to 2 mg/kg fish of strontium chloride.

						Mark quality good					
β	SE β	Wald Chi-square	*df*	*p*	$e^{(\beta)}$	Predictor	β	SE β	Wald Chi-square	*df*	*p*
-2,143	0,585	13,394	1	***	0,117	Constant	-5,596	1,439	15,128	1	***
0,066	0,016	16,234	1	***	1,068	Days of survival	0,155	0,025	39,512	1	***
0,85	0,75	1,285	1	0,257	2,34	CAL1,25	-18,002	0	-	1	-
0,863	0,758	1,297	1	0,255	2,371	CAL2,5	1,081	1,743	0,385	1	0,535
0,933	0,782	1,423	1	0,233	2,541	CAL5	3,281	1,521	4,653	1	**0,031**
0,422	0,918	0,211	1	0,646	1,525	CAL10	0,642	1,518	0,179	1	0,672
0,11	0,91	0,015	1	0,904	1,116	CAL25	-0,19	1,458	0,017	1	0,896
-0,056	1,237	0,002	1	0,964	0,945	CAL50	4,263	1,498	8,102	1	**0,004**
0,411	0,795	0,267	1	0,605	1,509	CAL1,25&STR	0,148	1,712	0,007	1	0,931
0,492	0,803	0,376	1	0,54	1,636	CAL2,5&STR	2,048	1,726	1,408	1	0,235
0,774	0,774	1	1	0,317	2,168	CAL5&STR	2,125	1,727	1,513	1	0,219
0,747	0,785	0,907	1	0,341	2,111	CAL10&STR	2,417	1,504	2,581	1	0,108
0,581	0,792	0,54	1	0,463	1,789	CAL25&STR	1,726	1,501	1,322	1	0,25
0	-	-	0	-	-	CAL50&STR	0	-	-	0	-

s

Days of survival			
coefficient	Chi-square	*df*	*p*
247,877			
	154,716	24	***

Note: reference category for mark quality was “no mark” and reference group for CAL concentrations was CAL50&STR.

*$p \leq 0,05$, **$p \leq 0,01$, ***$p \leq 0,001$

3.3.3 Alizarin red S

Table 18 shows the frequencies of the mark qualities no mark, poor and good for the ALI concentrations from the single and the double treatment as well as those of cod, which survived until the end of the experiment. Considering the total sample size, it was found that the majority of the otoliths examined displayed no marks or poor mark quality and the frequencies were in the same range for both mark qualities for the ALI concentrations in the single and the double treatment. Otoliths displaying good mark quality were less frequent for ALI concentrations from both single and double treatment. No good mark quality was recorded for otoliths marked with ALI62,5, ALI1000, ALI31,25&STR, ALI250&STR and ALI500&STR. Furthermore, otoliths marked with ALI31,25 and ALI31,25&STR most often did not showed no marks (80 % and 86 %, respectively), followed by otoliths marked with ALI62,5 and ALI62,5&STR (65 %). To assess, whether the proportions of no mark, poor mark quality and good mark quality were the same for the ALI concentrations (null hypothesis), the Chi-square test of independence was conducted for all observations in the single and double treatment. The chi-square test was high significant (chi-square = 41,256, *df* = 22, *p* = 0,008) and therefore, the null hypothesis could be rejected. In consideration of the standardized residues, only ALI250 proved significant high proportion of otoliths with marks of good quality (17 %). At ALI1000&STR also significant high proportion of good mark quality was indicated (33 %). But it has to be kept in mind that the sample size only consisted of three cod. Therefore, this result needs to be interpreted with care.

In addition, to evaluate the strength of association between the ALI concentrations and the mark quality, the effect size Cramér V was computed. Cramér V was high significant and indicated a moderate association between ALI250 and ALI1000&STR concentrations and the mark qualities (Cramér V = 0,290, *p* = 0,008). For all remaining concentrations significant different proportions for the mark qualities were not proved.

The chi-square test of independence was not computed for the contingency table with only surviving cod, because sample sizes for the concentrations were too small, due to high mortalities at each concentration and valid results would not have been obtained.

Table 17 The proportions (%) of fluorescent mark qualities (no mark, poor and good) after intraperitoneal injections with ALI (single and double treatment). Proportions given in parentheses refer to mark qualities of surviving cod. Total number of cod marked, the numbers of surviving cod until the end of the experiment are given in parentheses.

		Mark quality (% of N)		
Marker	Number of cod (N)	No mark	Poor	Good
Single treatment				
ALI31,25	20 (1)	80,0 (0,0)	15,0 (0,0)	5,0 (100,0)
ALI62,5	20	65,0	35,0	0,0
ALI125	19	47,4	42,1	10,5
ALI250	24 (3)	37,5 (0,0)	45,8 (0,0)	16,7 (100,0)
ALI500	25 (3)	48,0 (0,0)	44,0 (33,3)	8,0 (66,7)
ALI1000	24 (2)	45,8 (0,0)	54,2 (100,0)	0,0 (0,0)
Double treatment				
ALI31,25&STR	21 (1)	85,7 (0,0)	14,3 (100,0)	0,0 (0,0)
ALI62,5&STR	20	65,0	25,0	10,0
ALI125&STR	20	50,0	45,0	5,0
ALI250&STR	25 (1)	40,0 (0,0)	60,0 (100,0)	0,0 (0,0)
ALI500&STR	25 (2)	52,0 (50,0)	48,0 (50,0)	0,0 (0,0)
ALI1000&STR	3 (1)	33,3 (0,0)	33,3 (0,0)	33,3 (100,0)

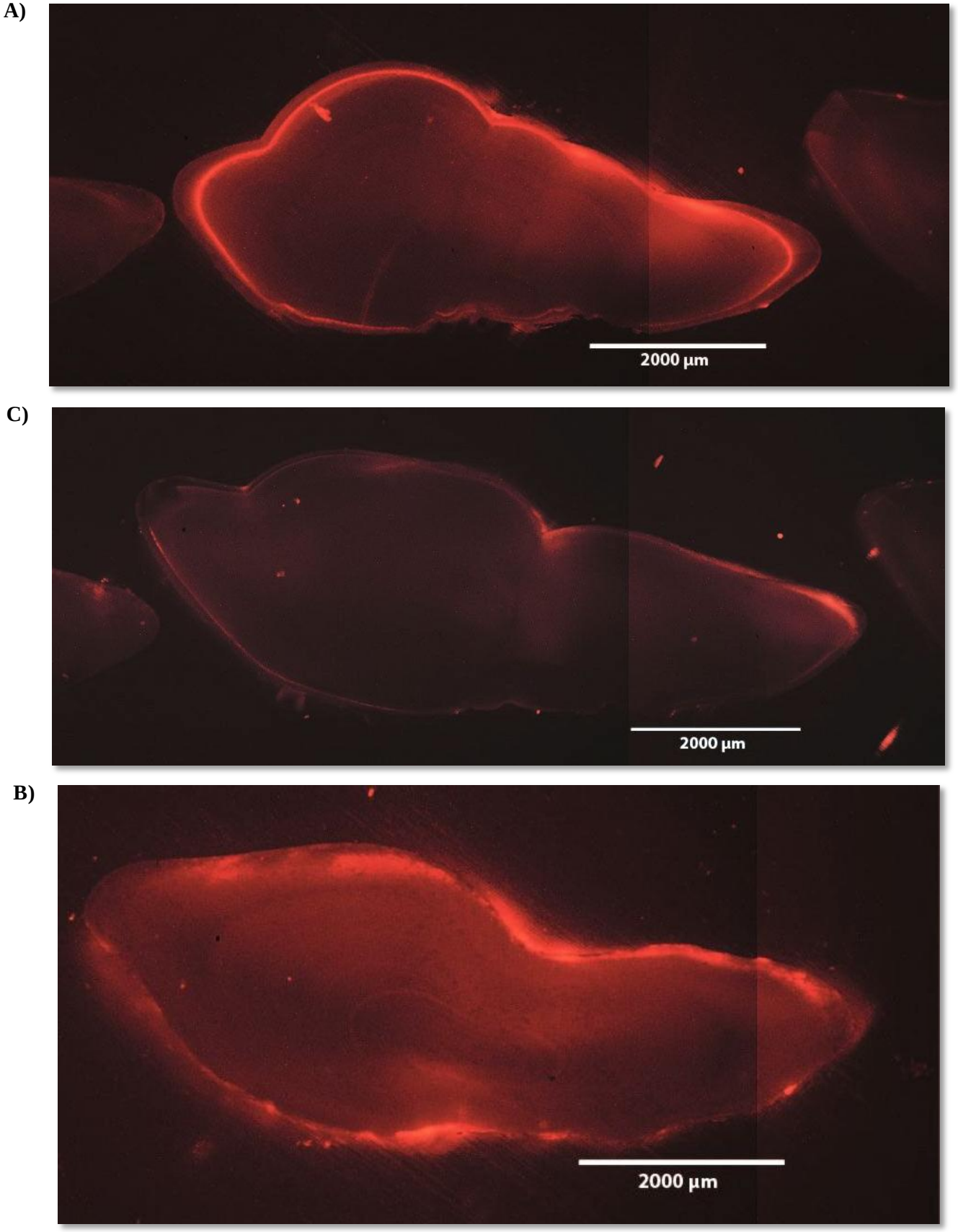


Figure 16 : Examples of otoliths viewed under green light in a fluorescence microscope displaying different mark qualities, A) good mark quality (ALI250), B) poor mark quality (ALI500), C) no mark and autofluorescence (ALI62,5&STR). Photo by author

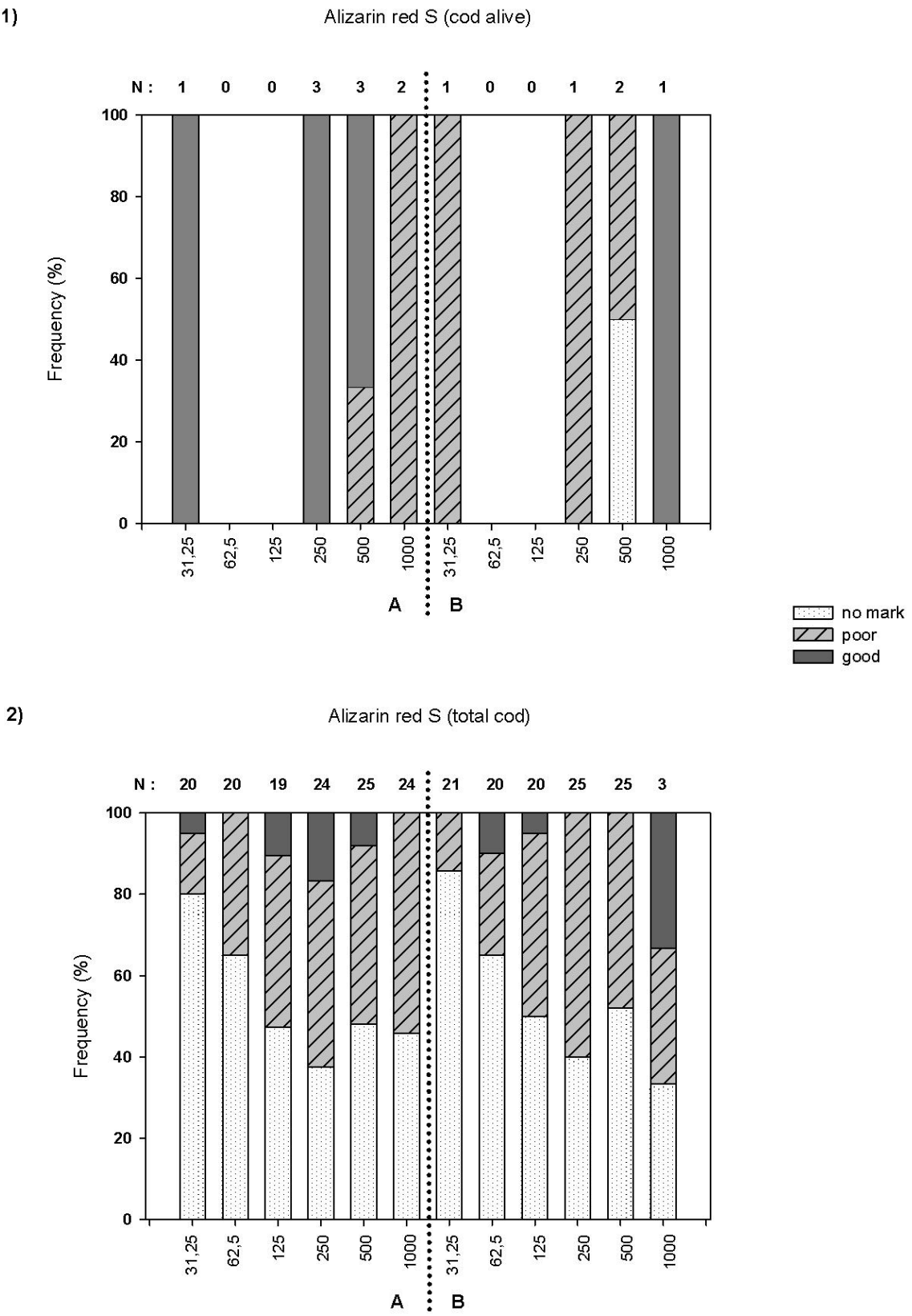


Figure 17 The proportions (%) of fluorescent mark qualities (no mark, poor and good) in thin sectioned otoliths marked with ALI, 1) mark quality of otoliths of surviving cod, 2) mark quality of otoliths of total cod.

To assess the relationship between mark quality and the ALI concentrations (single and double treatment), a multinomial logistic regression analysis was conducted with mark quality as the categorical dependent variable with the three categories no mark, poor and good, where good was chosen as reference group, and the ALI concentrations (single and double treatment) as categorical predictor variables. Additionally, as a continuous predictor variable the days of survival were added as a covariate. The results are shown in Table 19.

The interpretation of the results is analogous to the logistic regression model. The likelihood ratio test of the overall goodness-of-fit of the logistic model proved significantly better compared to the null model (chi-square = 89,800, $p \leq 0{,}001$). The rejection of the null hypothesis, stating that there is no difference between the null model and the logistic model, including the predictors was supported.

The likelihood-ratio comparison tests for effects of the predictors on the dependent variable, yielded for both ALI concentrations and days of survival in a significant result. Both predictors were assumed to have a highly significant effect on mark quality (Wald chi-square = 55,702, $p \leq 0{,}001$ and Wald chi-square = 206,307, $p \leq 0{,}001$, respectively). Thus, the null hypothesis that the predictor variables had any significant effect on the dependent variable could be rejected. These test results were consistent with the result of the model fit test.

Thus, it could be assumed that the predictor regression coefficients did not equal zero. Considering the maximum likelihood estimations of the regression coefficients (β) and their assigned *p*-values obtained by the Wald test, only the predictor variable days of survival was significant for categorizing poor mark in contrast to no mark (chi square = 4,390, $p = 0{,}036$). Poor mark quality was positively related to days of survival ($\beta = 0{,}077$) and the odds ratio was $e^{(\beta)} = 1{,}080$. Therefore it could be concluded that for a one unit increase in days of survival, the odds of otoliths to display poor marks rather than no marks would be expected to increase by 1,080 times, while all the other predictor variables were held constant. In terms of probability this means that when the ALI concentrations were assumed to be constant, then for each unit increase in the days of survival, the probability of otoliths to display poor marks rather than no mark increased by 51,92 % (1,080/(1+1,080)*100). None of an effect was found for the days of survival for good mark quality, although the *p*-value was just over the significance level of 0,05 (chi square = 3,592, $p = 0{,}058$). The regression coefficients β were not proved to be significantly different from zero, neither for ALI concentrations of the single treatment, nor for ALI concentrations of the double treatment ($p > 0{,}05$).

Therefore, the ALI concentrations were assumed to have any effect on the classification of poor and good mark qualities in contrast to no mark. In addition, considering the maximum likelihood estimates for good mark quality of ALI62,5, ALI1000, ALI31,25&STR, ALI250&STR and ALI500&STR, it can be noticed that the values display a great variance. Considering more precisely the standard errors for the β-values (SE β) it is striking that they were far higher than the estimated regression coefficients. These high standard errors were obtained due to complete separation for category good, since no good mark was achieved at these concentrations (Table 18).

Table 18 Results from the multinomial logistic regression describing the relationship between the concentrations of alizarin red S from single and double treatment and the categorical dependent variable mark quality (no mark, poor, good). Regression coefficient β (in log-odds unit), standard error (SE) of β, chi-square test of the null hypothesis, degrees of freedom *df*, probability *p* of significance and the natural logarithm base *e*, raised to the exponent of the slope β (odds ratio). The concentrations (mg/kg fish) of ALI 31,25; 62,5; 125; 250; 500 and 1000 respectively (single treatment and double treatment). STR refers to 2 mg/kg fish of strontium chloride.

						Mark quality good					
β	SE β	Wald Chi-square	*df*	*p*	$e^{(\beta)}$	Predictor	β	SE β	Wald Chi-square	*df*	*p*
-0,578	1,485	0,152	1	0,697	0,561	Constant	-13,310	18,603	0,512	1	0,474
0,077	0,037	4,390	1	**0,036***	1,080	Days of survival	0,568	0,300	3,592	1	0,058
-1,486	1,591	0,871	1	0,351	0,226	ALI31,25	1,174	22,937	0,003	1	0,959
-0,411	1,536	0,072	1	0,789	0,663	ALI62,5	-10,180	8375,932	0,000	1	0,999
0,136	1,543	0,008	1	0,930	1,146	ALI125	9,276	18,072	0,263	1	0,608
0,548	1,536	0,127	1	0,721	1,729	ALI250	8,712	18,019	0,234	1	0,629
0,241	1,532	0,025	1	0,875	1,273	ALI500	-8,879	17,871	0,247	1	0,619
0,536	1,537	0,122	1	0,727	1,710	ALI1000	-28,454	0,000	-	1	-
-1,746	1,595	1,198	1	0,274	0,175	ALI31,25&STR	-21,146	5031,196	0,000	1	0,997
-0,754	1,554	0,235	1	0,628	0,470	ALI62,5&STR	8,376	17,950	0,218	1	0,641
0,193	1,536	0,016	1	0,900	1,213	ALI125&STR	8,865	18,166	0,238	1	0,626
0,693	1,525	0,206	1	0,650	1,999	ALI250&STR	-22,973	5688,959	0,000	1	0,997
0,145	1,525	0,009	1	0,924	1,156	ALI500&STR	-24,117	5502,643	0,000	1	0,997
0	-	-	0	-	-	ALI1000&STR	0	-	-	0	-

s

Days of survival			
coefficient	Chi-square	*df*	*p*
202,902			
	89,800	24	***

Note: reference category for mark quality was “no mark” and reference group for ALI concentrations was ALI1000&STR.

*$p \leq 0,05$, ***$p \leq 0,001$

3.3.4 Strontium chloride

No strontium chloride (STR) marks could be detected in the thin sections of the marked otoliths from the present experiment (O1 STR1, O2 STR2, O3 STR2 and O4 TET50&STR), except of the two otoliths O5 STR2 and O6 STR2 from a previous marking attempt in 2012, where the analyses yielded in positive results. Figure 18 shows the distribution for O5 STR2 of calcium and strontium in a combined X-ray map (A) and the distribution of calcium in a single X-ray map (B) as well as the EDS spectrum of calcium and strontium identified in the probe (C) at the ventral edge of the sample. Considering the combined X-ray map (A), a clear mark of strontium (in green) can be seen near the ventral edge of the thin section. The map of the calcium distribution (B) shows a clearly discernible dark band. Its position is congruent with the strontium mark seen in the combined map (A). The darker shade in the calcium map proved that at this position calcium concentrations were reduced. The reduced calcium concentration at the position of the strontium mark, gave the best evidence for strontium incorporation, since calcium were substituted by strontium. The EDS spectrum (C) clearly displayed a Kα X-ray peak for strontium. This confirmed the detection of enhanced strontium in the otolith. Strontium Lα line was barely detected, because it was below detection limit. The same results were obtained for O6 STR2. The strontium mark was equally detected at the ventral edge of the otolith and at this position the calcium concentration was reduced, which was displayed by a dark band on the X-ray single map for calcium Kα line. For O1 STR1, O2 STR2, O3 STR2 and O4 TET50&STR, no clear characteristic X-ray peak of either strontium Kα or Lα line was indicated. Further, the single X-ray maps of calcium did not display a dark band, which would have proved the presence of enhanced strontium at this position.

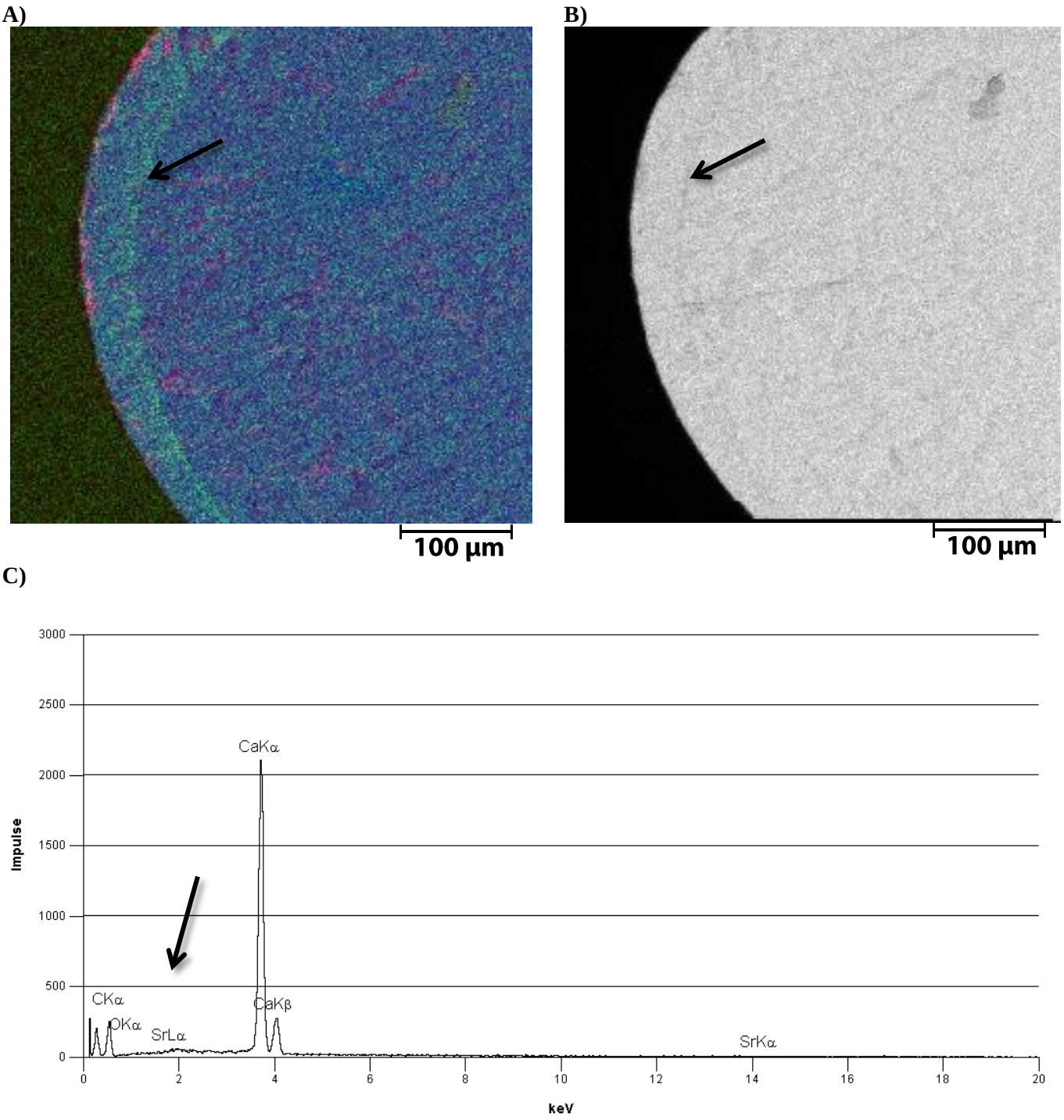


Figure 18 Distribution maps of calcium and strontium at the ventral edge of the test otolith O5 STR2, detected by X-ray mapping. A) Combination map with calcium distribution (blue) and the strontium mark (green), B) Single map of calcium distribution and C) Spectrum of detected elements, number of X-rays, plotted against the energies (keV).

4 Discussion

4.1 Mortality

Among the marker groups, single injections of TET showed the lowest mortalities at the concentrations of 25 mg/kg, 50 mg/kg and 100 mg/kg fish compared to the TET double treatment and the marker groups ALI, CAL and STR. The mortality at these concentrations was even significantly lower than the mortality in the control group. The total mortality rate of cod injected with isotonic saline solution was by 27 ±7,4 % (N = 22). Lang & Buxton (1993) reported that the control fish injected with isotonic saline solution showed stress reactions after the injections, but recovered more rapidly than fish injected with the chemical marker, but the study did not include the assessment of the mortality of the treated fish. Further, the binary logistic regression for the control group revealed that the injection of the saline solution did not affect the survival of the fish. It cannot be excluded that handling stress may have contributed to the early death of the fish in the control group, although fish injected with TET were handled in the same way. Further, the sample size of the control group was reduced by three cod, which have lost their tags, whereas the sample sizes in TET single treatment were not reduced due to loss of tags and therefore higher.
The mortality in the STR marker group was lower than the mortality recorded for the ALI marker group and the CAL marker group, and in the same range of that for the control group, whereas, the mortality was higher than the mortality in the TET treatments.

The marker group ALI together with the CAL marker group showed the highest mortalities in both the single and double treatments relative to the control group and the marker groups TET and STR, whereby the mortalities recorded for ALI even exceeded those of CAL in both single and double treatment. At CAL10, CAL25 and CAL50 the mortalities were exceptionally lower.

The assessed factors in the binary logistic regression analyses, which were considered likely to affect the survival of cod (the chemical marker concentrations, the injection volumes and the mean water temperatures) were proved not significant predictors of the survival of cod. This was in good agreement with the real conditions at the time of the experiment, since the mean water temperatures in the netpens ranged from 12 °C to 8 °C during the period of the study. Presumably, the decreasing water temperatures during the period of the experiment even promoted survival of cod towards the end of the experiment, since cod are known to prefer water temperatures below 10 °C (Fischer, 2003). The injections volumes were in the range of 10 to 2500 µl/kg fish for the single treatments and between 70 and 4900 µl/kg fish for the double treatments. The maximum injection volume was set at 10 ml. The volumes injected were without an exception far below that upper limit.

The mortality analyses further indicated no consistent tendency, whether or not lower or higher concentrations and single or double injections were more or less detrimental for the survival of cod. Moreover, the results obtained from the logistic models did not adequately explain the large variations within the survival of cod and led to results that were not consistent with the real conditions of the experiment. This suggested that other than these factors evaluated, affected the survival of cod in this experiment. One factor, which was most crucial for the high mortalities within the marker groups, was the quality of the stock solutions and the degree of complete solution of the chemicals, this was primarily important in the case of the marker group ALI and CAL. While the mixing of the TET and STR stock solutions presented any problem, the preparation of ALI and CAL stock solutions was difficult and complete solution of the chemicals was not reached. The majority of published work dealing with internal chemical marking of fish gave poor or no information about how the stock solutions of the chemicals used were prepared (e.g. Babaluk & Craig, 1990; Clear et al., 2000; Gelsleichter, 1997; Thomas et al., 1995). Furthermore, most of these studies mainly focus on the assessment of the mark qualities induced by the administered chemicals and did not evaluate the mortality of the fish injected with the chemicals tested (e.g. Jones & Bedford, 1968; Kuroki et al. 2010; Yamada, 1973). This crucial lack of detailed protocols of the mixing procedure for the chemicals made the preparation of the stock solutions in the case of ALI and CAL difficult. But this also demonstrated the inapplicability of these two chemicals for large-scale mark-recapture experiments with Baltic cod, since fast and easy application of the chemical(s) used is required, without causing acute mortality.

Other factors, which affected to a lesser degree the survival of cod, were probably the experience level of the person executing the injections and the critical insertion depth of the injection needle (Hack et al., 2010), as well as the handling stress in general (transport, injections and tagging). Adult cod is referred to support high densities up to 1000 kg/m^3, given sufficient oxygen is provided (Staurnes et al., 1994). The conditions of transport of cod were adequate, since sufficient oxygen supply was given. Moreover, the number of cod transported never exceeded the loading capacity of the transport tank, which had a volume of 2 m^3. For the three loads, the number of cod transported was 245, 338 and 417 cod. The handling of cod included collecting cod out of the netpens. Chase and capture as well as the transfer out of water are known to induce fight-or-flight stress reactions in fish (Staurnes et al., 1994). Stress was tried to be kept at a minimum, as cod was calmly and carefully caught by netting and handling procedure of cod (weighting, injecting, measuring and tagging) only last ca. one minute per fish, and marked fish were released right after treatment back into the netpens. The cod released back into the netpens were observed to swim lively towards the bottom of the netpens and did not remain at the water surface. Probably more stress was caused while injecting the chemicals and tagging of cod. But, this was considered suitable for cod, since cod of the control group recovered rapidly and very well after injection and tagging. So that high mortalities recorded in the ALI and CAL treatments and observed abnormal behavioural patterns, e.g. longer cessation of swimming and apathetic drifting under the water surface, probably were related to the intolerance towards the chemical injected as consequence of the poor solubility of the chemicals.

Cod from three different batches were used for the experiment. However, the fact that cod from the batches 2 and 3, displayed more superficial excoriations and deeper lacerations from attacks by cormorans, while cod were caught in the pound nets, differences in survival fit could not be proven, as no control group for each batch was considered. Moreover, only fish in good condition were injected and tagged.

The holding of cod in the netpens was considered optimal for cod. Maximum holding capacities of the netpens were not covered (540 kg fish per 27 m^3) since the total number of cod marked was 746 cod and the cod were spread equally over the two netpens. Thus, the cod had enough space to swim and collisions of cod were reduced.

Moreover, the environmental conditions were adequate as the mean surface water temperatures ranged from 12 °C to 8°C at the end of the experiment and no important fluctuations of the water temperature were recorded, and due to the proximity of the netpens to the open sea, sufficient water exchange and adequate water quality was ensured. Furthermore, it was observed that cod adapted very quickly to confinement conditions. After transferring cod from the transport tank into the netpens, cod swam calmly and began feeding after two days of acclimatization. Feeding of cod was very good during the whole time of the experiment.

Kock (1974) reported high mortalities of cod while held in netpens during spring and summer due to infections caused by pathogens (e.g. fin rot). Cod displaying fin rot died within 3-4 days. Moreover it was found that necrotic wounds formed around the external tags, especially during summer, which also led to the rapid death of cod. High water temperatures were assumed of having promoted the development of lethal wounds and the proliferation of pathogens. However, the observations in the current study do not support the findings of Kock (1974). In the present study not any infections emerged during the confinement time of cod. Good water exchange and good water quality and low surface water temperatures gave adequate conditions for cod to survive without lethal impairments of health during the whole period of the experiment (in autumn). Therefore, to conduct the experiment during autumn when surface water temperatures are no more extreme and tend to cool down, seemed to perfectly fit to the demands of the cod and was favorable for the experiment.

Only several individuals displayed superficial excoriations and deeper lacerations. Latter were caused by cormorans (*Phalacrocorax* sp.) attacking on cod while cod were caught in the pound nets. But these wounds did not noticeably deteriorate health of cod and for the majority of cod wounds healed during the period of the experiment. Suzuki & Mathews (1966) reported that after intraperitoneal injections of mice with both TET and DCAF (related chemical compound of CAL), neither skin lesions or alopecia or peritoneal lesions were found. This was in good agreement with this study however some individuals displayed irritations around the puncture wound of both injection and tag. In rare cases, wounds around the injection and the tag puncture developed to inflammatory wounds.

4.1.1 Tetracycline hydrochloride

The lowest total mortality rate was recorded at TET100 (4 ±19,6 %, N = 25), although the mortality rates at TET25 and TET50 were only insignificantly higher (8 ±13,6%, N =25 and 12 ±10,8%, N = 25). The mortalities of the double treatment were in the range of the mortality of the control group. At the concentrations of the double treatment relative to the single treatment, a low increase in the mortality was recorded, but this increase was not significant and therefore not any direct link to a possible negative effect on the survival of cod by combining TET with STR was indicated. Unfortunately, no studies were available, who conducted similar double treatments so that comparisons with other results were not possible.

These results from the single treatment were consistent with findings by Babaluk & Craig (1990), who intraperitoneally injected pikes (*Esox lucius* L.) with oxytetrcycline (OTC) and found low and insignificant mortality at concentrations of 50 mg OTC per kg fish, and considered injection concentrations in a range of 25-50 mg/kg fish of OTC as suitable for marking pike. Abdel-Hadi et al. (2011) assessed OTC-induced mortality in talipia (*Oreochromis niloticus*) by intraperitoneally injecting different concentrations of OTC (12,5, 25 and 50 mg/kg fish) and reported that at the concentration of 25 mg OTC/kg fish the lowest mortality was found and no significant mortalities were proved among the concentrations of OTC. In a feeding study by Weber & Ridgway (1967), no mortality right after feeding Pacific salmon (*Oncorynchus* spp.) with diets containing different concentrations of tetracycline occurred and 30 % of mortality of stockeye salmon fingerlings, which were fed with 2 g of oxytetracycline per kg body weight over 2 months (N = 382) was recorded. Since, the binary logistic regression analysis revealed not any effect of the factors tested, the extremely high survival rate for cod marked with TET might be mainly attributable to its bacteriostatic effect. Tetracyclines in general are broad-spectrum antibiotics and extensively used in human and veterinary medicine in the treatment of a wide range of infections caused by pathogens. In fish aquaculture tetracyclines have been used until the late 1970s (Babaluk & Craig, 1990; Celik & Eke, 2011). Tetracyclines have a low toxicity and minimal side effects when taken by animals (Todar, 2009). Therefore, TET has probably promoted the health of the fish and thus might have contributed to the high survival of cod in this marker group.

4.1.2 Strontium chloride

The mortalities did not differ significantly between the concentrations (0,5 mg/kg, 1 mg/kg and 2 mg/kg fish) and the total mortality rates at these concentrations ranged from 28 ±6,4 % to 60 ±3,3 %, while at 2 mg/kg fish the lowest total mortality rate was recorded. No studies were available, which evaluated the effect of different STR concentrations on the mortality of either cod or other fish species, which were marked with STR by injection. Comparisons could only be made for single concentrations of STR used in published marking studies. For instance, Hüssy et al. (2009) successfully intraperitoneally injected Baltic cod (*Gadus morhua*) in the concentration of 2 mg/kg fish of STR in a mark-recapture study. The cod marked and tagged were recaptured during the 50-100 days after release. Unfortunately, no mortality of the cod marked was evaluated, but the relatively high percentage of cod recaptured (51 %) proved that STR injections in the concentration of 2 mg/kg fish did not cause any mortality of 51 % of the cod marked after more than 100 days. This indicates that STR in the concentration of 2 mg/kg fish might be suitable for intraperitoneal injections of Baltic cod. However, in this experiment, no significant differences in the mortalities between the concentrations were proved, although at 2 mg STR/kg fish mortality of cod was lowest among the concentrations (28 ±6,4 %, N = 25). In another large-scale mark-recapture study, where southern Bluefin tuna (*Thunnus maccoyii*) were intramuscularly injected with STR in the concentrations of 100 mg STR/kg body weight showed that STR injected in even higher concentrations did not adversely affect the survival of fish marked, since the initial number of fish marked and tagged was recaptured. STR is a salt naturally occurring in sea water and non-toxic (Clear et al. 2000). The LD_{50} (lethal dose at which 50 % of the population is killed) of intraperitoneally injected rats was reported to be at 405 mg/kg body weight (Hummel Croton Inc., 2009). The concentrations injected were far below this limit, thus, a toxic effect on the fish injected was unlikely. Further, the mean water temperatures and the injection volumes were also considered unlikely to cause the early death of cod. However, the injection volumes were proved significant in the binary logistic regression analysis, they probably did not contribute to the death of the fish injected with STR, since the lowest injection volumes were administered, compared to those injected in the single treatments of the other marker groups. The mean injection volume applied with STR was 172 µl and hence below the mean injection volumes of CAL (456 µl/kg fish), TET (641 µl/kg fish) and ALI (771 µl/kg fish). The injection volumes were even higher (on total average 1130 µl/kg fish) in the double treatments, since injection volumes of STR 2 mg/kg fish were added to the injection volumes

of the chemical markers. Moreover, contrary to the assumption that lower concentrations would affect less the survival of cod, the logistic model revealed that cod marked with STR0,5 and STR1 were by 11 % and 6 %, respectively less likely to survive than cod marked with STR2. This result indicated that the concentrations were probably not responsible for the different survival probabilities of cod in this marker group. These findings demonstrated that the logistic model could not prove a consistent relationship between the factors tested and the survival of cod. It was more probable that the higher mortalities at the lower concentrations STR0,5 and STR1 were caused by accident during the injections, since these injections were executed the first of all injections. Injecting live cod was not intuitively done and required some experience. While injecting the chemical, the injection needle could have been accidentally inserted too deep into the abdominal lumen and as a consequence, inner organs might have been penetrated (e.g. intestine, liver, urinary bladder, spleen). Such wrongly administered injections are considered a high risk associated with intraperitoneal injections (Hack et al., 2010).

4.1.3 Calcein

The mortalities at the CAL concentrations of the double treatment did not differ between each other and were significantly higher relative to the mortality in the control group (total mortality rates 74 ±2,6 % - 100 ±0,0 %). Regarding the single treatment, at the lowest concentrations CAL1,25, CAL2,5 and CAL5, significantly higher total mortality rates in the range of 90 ±1,7 % were proved, compared to the higher concentrations CAL10, CAL25 and CAL50 and the control group. This was surprising, since by further reducing the concentration CAL10 by a half, a quarter and an eighth (i.e. CAL5, CAL2,5 and CAL1,25 mg/kg fish), mortality was expected to decrease, but the opposite was proved, regarding the high total mortality rates. This finding was in contrast to those of Gelsleichter (1997), who intramuscularly injected nurse shark (*Ginglymostoma cirratum*) with CAL in the concentrations of 5 mg/kg, 10 mg/kg and 25 mg/kg body weight. Gelsleichter (1997) recorded high and rapid mortality of 80 % (N = 5) at 25 mg/kg body weight, whereas in this study the lowest total mortality rate was found at the same concentration (24 %, N = 25). Latter finding was in good agreement with that of Monaghan, Jr. (1993), who intramuscularly injected summer flounders (*Paralychthys dentatus*) with CAL in the concentrations of 25mg/kg and 50 mg/kg body weight and who stated that fish after injections were active and vital. Further, Thomas et al. (1995) reported for intraperitoneal injections of red drums (*Sciaenops ocellatus*)

with CAL in the concentrations of 25 mg/kg and 50 mg/kg body weight even no mortality at both concentrations after injections (100 % survival, N = 10). Gelsleichter (1997) reported further that no nurse shark died at CAL concentrations of 5 mg/kg and 10 mg/kg body weight (N = 1 at each concentration). This result underlines the assumption stated above that lower injection concentrations may increase the probability of fish to survive and implies that the contradictory finding in this study was clearly other than concentration- or treatment-linked (single treatment/double treatment), since at CAL10 a low total mortality rate was recorded (40 %, N = 20), which was even about three times lower than at CAL5 (91 %, N = 21). Further, the results of the logistic regression analysis, indicated that cod marked with the concentrations CAL10, CAL25 and CAL50 were by 93 %, 95 % and 89 %, respectively more likely to survive than to die, whereas no significant effects on the survival of cod of the lower concentrations CAL1,25, CAL2,5 and CAL5 were proved, although the mortalities were proved significantly highest at these concentrations. The results of the logistic model for the concentrations of the double treatment were not proved significant either. Thus, possible adverse effects on the survival of cod by combining calcein with or without strontium chloride could not be demonstrated.

The discrepancies were caused, because the main cause affecting the survival of cod in this marker group was assumed to be the CAL solution injected, since the CAL powder was hardly soluble or did not completely dissolve in the two attempts to prepare homogenous stock solutions (see section 2.3.2). Despite the fact that no residues were visible in the stock solution from the second attempt, schlieren were observed that had formed, indicating that CAL did not dissolve completely. CAL is classified as being non-hazardous (Sigma-Aldrich, SDS Calcein, 2013), but forms acidic solutions and is poorly soluble in water at low pH (Yamada, 1973). Although the pH of the solution was raised near pH 7 to reach homogeneity of the solutions (Suzuki & Mathews, 1966; Tsukamoto, 1988), the undissolved CAL residues in the solution might have decreased the pH again, after the last pH recordings. Acid stress in fish causes a decrease of the blood pH, which decreases further the oxygen transport in the blood and leads to the death of the fish due to hypoxia (Fromm, 1980).

4.1.4 Alizarin red S

ALI was found to be the marker group with markedly highest mortality rates, which were in the range of 88 ±1,75 % to 100 ±0,0 % for each concentration from the single and the double treatment. Contrary to the expectation that mortalities were more likely to increase with increase of the marker concentration, no significant differences in mortalities among and between the concentrations from single and double treatment were found. This finding was in contrast to that by Thomas et al. (1995), who recorded only very low mortality of red drum (Sciaenops ocellatus) after intraperitoneal injections of alizarin complexone in the concentrations of 25 mg/kg and 50 mg/kg fish. Other published experiments were not available for further comparisons. ALI or related alizarin compounds were more commonly used in immersion experiments (e.g. Bashey, 2004; Beckman & Schulz 1996; Blom et al., 1994, Day et al., 1995; Morales-Nin et al., 2010). Regarding the high mortalities at each concentration, obviously other than a concentration-epffect might have caused these extremely high mortalities. As in the case of CAL, the stock solutions and the extremely poor solubility of ALI very likely caused the death of the fish. In contrast to Yamada (1973), ALI was found to be hardly soluble in water and very difficult to. The adjustment of the pH close to that of the living fish (pH 7) with potassium hydroxide (KOH) (Tsukamoto, 1988) was inefficient, since the solution began to precipitate while further increase of the pH level close to 7. This fact was supported by Day et al. (1995), who noted that ALI in the amounts of 50-60 mg/ L sea water was relatively insoluble and that ALI frequently began to precipitate. The authors suggested increasing the volume of the stock solution to solve this problem. Despite all the efforts made to increase the solubility of ALI (see section 2.3.4), ALI did not completely dissolve in any of the attempts.

the mean water temperatures for the ALI marker group were proved to significantly affect the survival of cod, these results were misleading and inconsistent with the real conditions. The mean water temperature at 12 °C at the time of injecting cod with ALI was in the tolerable range for cod. Moreover, cod from the STR and TET group were injected at the same time and did not show such high mortalities.

4.2 Growth

Besides the use in treatment of various infections in human and veterinary medicine, Tetracyclines are extensively used in live-stock productions to enhance the growth of the live-stocks (Pils & Laird, 2007; Visek, 1978). The results of the multiple comparisons confirmed probable growth-promoting by TET, since cod injected with TET grew significantly faster than cod in the control group or injected with STR. This suggests that TET might have enhanced growth of cod. This finding was contrary to results from other studies, where the effect on growth of fish treated with TET related tetracyclines was tested. Both studies by Nordeide et al. (1992) and by Weber & Ridgway (1967) did not demonstrate enhanced growth of the fish treated with tetracyclines. And Suzuki & Mathews (1966) even reported that bone formation was inhibited at high administration levels of tetracycline. Nevertheless, a high positive effect of TET injected cod was demonstrated in this study, as the lowest mortalities were observed

4.3 Mark quality

Within the marker groups, the best marking results (i.e. the highest proportions of good marks displayed in the otoliths) were obtained by injecting cod with TET in the concentrations of 50 mg/kg and 100 mg/kg fish (71 % and 67 %, respectively), with CAL in the concentration of 50 mg/kg fish (71 %) and with ALI in the concentration of 250 mg/kg fish (17 %). For all the remaining concentrations within the marker groups, the majority of fluorescent marks were more often absent than faint and diffuse and they were less frequently clear and distinct Further, no concentration related effects were found, since the proportions of a mark quality were similar at each concentration, despite some exceptions, were the proportions of absent or good marks were significantly higher or lower. None of the otoliths marked with STR in this experiment displayed a strontium mark, probably because the concentration of the STR stock solution was too low. However the marking success with STR was proved by evaluating otoliths from a former marking attempt where the injection solution was higher concentrated.

Poor mark quality was esteemed to be not sufficient for successful marking the otoliths, because the marks were often inconsistent, faint or diffuse and would be subject to possible misinterpretations and thus they would not be recommended for time labelling the otoliths of Baltic cod. The marking success at ALI250 was markedly lowest, compared to TET50,

TET100 and CAL50, so that this concentration would not be recommended to mark the otoliths of Baltic cod.

The fluorescent colours varied depending on the chemical injected and the filter combinations used. ALI induced red marks under green light excitation, CAL marks emitted green light when irradiated with blue light and otoliths injected with TET displayed orange marks when irradiated with UV-light.

In contrast to ALI, TET and CAL, otoliths from the control group only emitted a greenish light throughout the entire otolith. This observation was also reported by Lang & Buxton (1993), who interpreted the greenish light as to be autofluorescence of the otolith. Indeed, bone tissues are known to emit green fluorescence when irradiated with UV light. This autofluorescence is caused by the collagen present in bone tissues (Prentice, 1967). Autofluorescence was also observed for otoliths marked with ALI, CAL or TET, but which did not display any marks or very faint and indistinct marks. A probable cause for no marks or poor marks might be the leakage of the solution out of the puncture wound, but these losses observed for injections of each chemical marker, only were minimal and were also estimated to have no apparent effect on marking quality (Thomas et al., 1995). Moreover, when greater losses of the chemical injected occurred mostly due to leakage out of the anus of the fish, the fish was not considered for the experiment.

Despite, the multinomial logistic regression (MLR) analyses proved the survival time of cod to be the main cause for otoliths to display poor or good fluorescent marks relative to no marks. The MLR analyses revealed a significant positive relationship between the survival time of cod and the probability of otoliths to display poor or good marks relative to no marks. The results indicated that with each increase of the survival time by one day, the probability of otoliths to display poor marks rather than no marks would be expected to increase by 52 % and the probability to show good marks rather than no marks increase in the range of 52-64 %, meaning that the longer cod lived, the more likely otoliths would be expected to display poor or good marks rather than no marks. This finding concurred with the actual observations made. It was found that mark quality appeared to depend on whether the mark was located in the inner part of the otolith or on the outer edge of the otolith. The marks in the inner part of the otolith appeared to display more often good quality, whereas the marks on the outer edge of the otoliths appeared most frequently diffuse and not clearly visible. This finding matched also with those observed by Riascos et al. (2007), who found that ALI marks in shell of *Mesodesma donacium* (Bivalvia), close to the edge of the shell were most often difficult to

determine. Furthermore, in consideration of the proportions of the fluorescent mark qualities of the otoliths from the different marker groups, such a tendency was obvious, since comparing the proportions of good mark quality of the otoliths between the marker groups, it appeared that the majority of otoliths from cod that survived throughout the period of the experiment, tended to display more often good marks rather than poor or no marks. This was especially pronounced for the TET concentrations, because the surviving cod could be analyzed separately from the dead cod. For the marker groups ALI and CAL, such general tendencies were difficult to state, since in these marker groups the mortality was highest and the sample sizes between dead and surviving cod were highly unequal. This relationship was confirmed by Riascos et al. (2007), who noted a relationship between the immersion time and the mark quality of incorporated marks in the shells of Chilean abalone (*Concholepas concholepas*) and the surf clam (*Mesodesma donacium*).

Further, the mark quality and the position of the mark were directly linked to the growth of the fish. Further Day et al. (1995) stated that the marking success depended on the growth rates of the abalone (*Halioris rubra*) immersed, since in active forming calcifying structures, the chemical stain is more likely incorporated.

4.3.1 Tetracycline hydrochloride

The results showed that at both concentrations TET50 and TET100, the proportions of otoliths with good marks were significantly highest in contrast to those at TET25 and all the concentrations of the double treatment (71 % and 67 %, respectively). The marking success at the concentrations TET25, TET25&STR, TET50&STR and TET100&STR was poor, demonstrating that those concentrations were not appropriate for marking the otoliths of cod, whereas This finding was consistent with that of Kobayashi et al. (1964), who injected juvenile goldfish intraperitoneally with different concentrations of TET and who found that 100 mg/kg body weight of TET gave strong intense fluorescent marks in the otoliths, whereas the intensities at 20 mg/kg body weight resulted in weaker marks. The authors concluded that the concentration of 50 mg/kg body weight of TET might be appropriate to mark the otoliths of juvenile goldfish. And intraperitoneal injections of OTC in the concentrations of 25 mg/kg and 50 mg/kg fish showed equally faint marks at 25 mg/kg fish and clear distinct marks at 50 mg/kg fish in the otoliths of juvenile red drum (*Sciaenops ocellatus*) (Thomas et al., 1995). Several other studies confirmed high marking success at the concentration of 50-100 mg/kg fish, using besides TET also OTC, tetracycline and oxytetracycline hydrochloride for marking

the otoliths of fish (Campana and Neilson, 1982; Fargo & Chilton, 1987; Jones & Bedford, 1968; Panfili & Ximenes, 1992).

On the contrary, Babaluk & Craig (1990), who marked pike (*Esox lucius* L.) with oxytetracycline (OTC) and found 100 % fluorescent marks in the otoliths and other calcified structures at concentrations of 25-50 mg OTC per kg fish and also contrary to McFarlane & Beamish (1987), who found the appropriate dosage for injections of stablefish (*Anoplopoma fumbria*) with OTC to be 25-35 mg/kg fish; in this study TET concentrations of 25 kg/mg fish did not show successful marking in Baltic cod.

Further Yamada (1971) found very distinct marks in otoliths of six carp (*Cyprinus carpio* L.), which were intramuscularly injected with TET in the concentrations of 15-20 mg/kg body weight, but not any mark was found in otoliths of carp injected intraperitoneally. This finding was contrary to that in this study, since intraperitoneal injections lead to observable marks in the otoliths of cod. However, at the concentration of 25 mg TET per kg fish (single treatment), only very low marking success was found (9 % of good mark) and at the same concentration of the double treatment, respectively, not any good mark was observed in the otoliths of cod. Contrasting findings were also made in other studies, where faint marks were detected at single concentrations of 100 mg/kg fish and double concentrations of 30 mg/kg fish in the otoliths of fish after recapture (fish were intraperitoneally injected with oxytetracycline hydrochloride) (Oxenford et al., 1994).

The MLR indicated no significant relationship between TET50 and TET100 and the probabilities of otoliths to display good marks relative to no marks. But although the results for TET50 and TET100 were not proved significant, the model showed accordance with the findings from the contingency table analysis. At TET50 and TET100 the estimated regression coefficients were highest, which suggested that both concentrations had the strongest effect on the categorization of good marks relative to no marks, by increasing the probabilities of otoliths displaying good marks rather than no marks by 87 %. Further, it was found that for poor mark quality in contrast to no mark TET25&STR and TET50&STR were expected to have a significant negative effect. TET25&STR decreased the probability of otoliths to have poor marks rather than no marks by 13 % and TET50&STR decreased the probability of otoliths to have poor marks rather than no marks by 18 %. This was in good agreement with the observations that at TET25&STR and TET50&STR significantly highest proportions of absent marks (69 % and 56 %, respectively) and the lowest proportions of good marks (0 % both) were found.

4.3.2 Calcein

In the Calcein marker group otoliths displaying good marks were more frequent than for ALI. Nonetheless, the majority of otoliths from the single and double treatment showed rather no marks than poor marks and less often good marks.

At the highest concentration of 50 mg/kg fish of the single treatment, significantly highest proportions of good marks were recorded (71 %) and consequently the lowest proportions of no marks (24 %). Whereas, at the lowest concentration 1,25 mg/kg fish of the single treatment significantly lowest proportions of otoliths with marks of good quality were observed (0 %). The proportions of no mark, poor mark quality and good mark quality were in the same range and were not significantly different for the CAL concentrations (single and double treatment) and not any clear relationship between the mark qualities and the CAL concentrations was proved, except for CAL1,25 and CAL50.

Nonetheless, the significant strong influence of CAL50 on good marks relative to no marks was in perfect agreement with the real observations and was confirmed by Thomas et al., (1995), who reported that intraperitoneal injections of CAL in the concentrations of 25 mg/kg and 50 mg/kg fish showed clear distinct marks in the otoliths of juvenile red drum (*Sciaenops ocellatus*). Yamada (1971) concluded that intraperitoneal injections of CAL in the concentration of 4-5 mg/kg body weight were appropriate to produce very distinct marks in the otoliths of six carps (*Cyprinus carpio* L.). This was contrary to the findings in this study, where CAL in the comparable concentrations of 1,25-5 mg/kg fish did not lead to successful marking of the otoliths of cod (0-19 % good marks).

The MLR indicated that CAL5 and CAL50 were found to increase the probability of otoliths to have good marks rather than no marks by 96 % and by 99 %, respectively. Latter result was expected, since CAL50 had significantly highest proportions of otoliths displaying good marks. But the significant high impact of CAL5 on good mark quality relative to no mark was surprising, since not any evidence was given that at this concentration the proportion of good marks was significantly high or as high as at CAL50. Actually, the proportion was only of 19 % and therefore four times lower than that of CAL50 (71 %). This striking result was very likely to be computed by error. As a consequence, it was assumed that the logistic model probably was biased as it obviously over-estimated the maximum likelihood for CAL5. In fact, it was found evidence for such an assumption, since 57 % of the observed frequencies for the mark qualities were zero percent. This led probably to a quasi-complete separation of the data. Therefore it was not possible to compute maximum likelihood estimates for some of

the concentrations (i.e. CAL1,25, where 0 % of good marks were found) or the estimates for the regression coefficients probably were infinite, as it was likely the case for CAL5.

4.3.3 Alizarin red S

At the concentration ALI250 of the single treatment a significant high proportion of otoliths with marks of good quality (17 %) was proved. At ALI1000&STR, the significant high proportion of 33 % of good mark quality was not interpreted, because this result was not valid, since the sample size consisted only of three cod. The double marking of cod with ALI1000&STR had to be aborted due to high mortalities right after the injections. All the other concentrations did not show significant different proportions for the mark qualities and marking success was very low (0-10 %), which was expected, since nearly all cod at these concentrations died soon after the injections.

Only for ALI and TET the MLR showed some inconsistencies regarding the significance of the survival time of cod on the mark qualities. The MLR for the ALI marker group indicated that the regression coefficient for days of survival for good mark quality was just above the critical z-value at $\alpha = 0{,}05$ ($p = 0{,}058$). But the estimate was very likely to be significant, since the logistic model analysis for the maximum likelihood estimates of good mark quality relative to no mark, was probably biased due to frequent proportions of zero percent of observed good marks for five concentrations, due to high mortality of cod (ALI62,5, ALI100, ALI31,25&STR, ALI250&STR and ALI500&STR). The MLR for TET revealed that the survival time of cod did not significantly influence poor mark quality relative to no mark, whereas it was significant for good mark quality. The MLR was likely to be biased, because the data contained a high number of frequencies for the mark quality that yielded in zero percent (49 %), leading probably to a quasi-complete separation of the data so that the maximum likelihood estimates for the concentrations could not be computed correctly and thus were incorrect. Yamada (1971) demonstrated that intraperitoneal injections of ALI in the concentrations of 40-250 mg/kg body weight did not produce any observable mark in the otoliths of six carps (*Cyprinus carpio* L.). This was in contrast to the findings of this study. Although a high number of otoliths with absent fluorescent marks was recorded (55 %), few otoliths were found displaying good marks (5 %) and, a relative high proportion of otoliths showed poor marks (40 %). And at ALI250 significantly highest marking success was proved. Intraperitoneal injections of alizarin complexone in the concentrations of 25 mg/kg and 50

mg/kg fish showed no marks in the otoliths of juvenile red drums (*Sciaenops ocellatus*) and alizarin complexone was not recommended as suitable marker for injections by the authors (Thomas et al., 1995). This finding was also confirmed in this study, where the marking at the comparable concentrations ALI31,25 and ALI62,5 led only to very low marking success (0-5 %), since the mortality at these concentrations reached 100 %.

Moreover, caution has to be applied, as the chi-square test of independence, used to test for significant associations between the mark qualities and the concentrations might be erroneous, because the sample size required for the test was not satisfied (39 % of the expected frequencies were less than 5, the upper limit is set by 20 % for this test), due to the fact that frequently not any good mark quality was observed for ALI concentrations, resulting in proportions of zero percent, which probably lowered the statistical validity of the test.

No good mark quality was recorded for otoliths marked with ALI62,5, ALI1000, ALI31,25&STR, ALI250&STR and ALI500&STR. Only at ALI250 the highest proportion of good marks was observed (17 %). Accordingly, the proportions of the mark qualities were assumed independent from the ALI concentrations and not any significant differences in mark qualities between the concentrations could be clearly demonstrated. This finding was confirmed by the results from the multinomial logistic regression modelling, where all ALI concentrations were not proved significant and thus were not expected to have an impact on the survival of cod. Instead it was found that the days of survival were significantly affecting mark quality, especially poor quality. Provided that the ALI concentrations had not any effect on the survival, then for each unit increase in the days of survival, the probability of otoliths to display poor marks rather than no marks would be expected to increase by 51,92 %. The regression coefficient for days of survival for good mark quality was just above the critical z-value at $\alpha = 0{,}05$ ($p = 0{,}058$). Presumably, the time of survival of cod was also very likely to have an effect on otoliths displaying good marks, in the way that with each unit increase in the days of survival, the probability of good marks in otoliths rather than no marks, would increase by even 64 %. Latter probability estimate was very likely to be significant, since the logistic model analysis for the maximum likelihood estimates of good mark quality relative to no mark, was probably biased due to frequent proportions of zero percent of observed good marks for five concentrations (ALI62,5, ALI100, ALI31,25&STR, ALI250&STR and ALI500&STR).

Furthermore, these high probabilities were adequate, since they were consistent with the observations made during evaluating mark qualities of the thin-sectioned otoliths for all the

marker groups under the fluorescence microscope. It was found that the marks in the inner part of the otolith displayed more often good quality, whereas the marks on the outer edge of the otoliths most frequently were diffuse and not clearly visible. These findings matched also with those observed by Riascos et al. (2007), who found that alizarin red S marks in shell of *Mesodesma donacium* (Bivalvia), close to the edge of the shell were most often difficult to determine.

Better results seemed to be obtained in studies where fish was immersed in solutions containing ALI or related chemical compounds. Immersions of larval and juvenile red drum (*Sciaenops ocellatus*) in 100 mg alizarin complexone per l solution for 2 h were more successful than intraperitoneal injections of the same chemical (25 mg/kg and 50 mg/kg fish) and produced clear marks (Thomas et al., 1995). Lang & Buxton (1993) immersed juvenile backtail (*Diplodus sargus capensis* (Smith)) and zebra (*D. cervinus hottentotus* (Smith)) in alizarin complexone solutions in the concentrations of 100-200 mg/l for 24 h and found that these concentrations lead to distinct marks in the otoliths of the fish.

Beckman & Schulz (1996) found highest mortality (93%, N = 150) of juvenile central stonerollers (*Campostoma anomalum*) and southern redbelly dace (*Phoxinus erythrogaster*) when immersed in ALI solution at a concentration of 400 ml/l and no marks were displayed in the otoliths.

The relative high proportion of immersing studies involving ALI or related chemical compounds as chemical marker for fish, suggest that ALI might be successfully administered in fish by immersion, but led to only low marking success when injected, since no solubility problems was reported. Whether this is also true for Baltic cod needs further investigations as in this study only intraperitoneal injections were carried out.

4.3.4 Strontium chloride

Marking otoliths of cod with strontium chloride (STR) was not successful. In any of the thin-sectioned otoliths of this study STR marks were shown. However, the evaluation of two otoliths from a previous attempt to mark otoliths of cod with STR (O5 STR2 and O6 STR2) yielded in successful detections of STR marks in the otoliths. The presence of a STR mark in the otolith was proved, when the characteristic X-ray peaks of strontium (Sr) were distinguishable from background concentrations of Sr and above the detection limit. Further, high concentrations of Sr were displayed in the single X-ray map of calcium. Darker shades in

the calcium map proved that at this position calcium concentrations were reduced, because high concentration of Sr deposited at this position. The reduced calcium concentrations at the position of the strontium mark, gave the best evidence for strontium incorporation, since calcium were substituted by strontium. The negative results of otoliths displaying any STR mark from this study was probably obtained, because the STR concentration of the stock solution was not high enough to provide detectable Sr above the detection limit of the EDS X-ray microanalysis. O5 STR2 and O6 STR2 were marked with STR from another stock solution provided by Dr. Karin Hüssy that was the same stock solution, which was injected in cod during the mark-recapture study Hüssy et al. (2009). This stock solution was prepared from 30,4 g STR per 1 L ringer solution, thus 15, 2 g per 500 ml ringer solution (30,4*500/1000 = 15,2 g/500 ml) (pers. comm. Hüssy, 2011). In this study the amount of STR dissolved in 500 ml isotonic saline solution was 5 g of STR. The concentration of the stock solution of this study was by factor 3 lower than that of Hüssy et al. (2009). Therefore, the concentration of the STR stock solution prepared in this study was assumed too low to yield in detectable Sr concentrations in the otoliths. No other comparisons with published works can be made, since the concentrations of the STR stock solutions for injections were not mentioned or immersion experiments were conducted and the STR amounts used in immersion solutions are not directly comparable with those for injection solutions.

Nonetheless the absence of STR marks in otoliths of this study, there are numerous examples of studies, where STR was successfully applied to fish to mark calcified structures within the fish. Hüssy et al. (2009) demonstrated that intraperitoneally injected cod (*Gadus morhua*) with STR in the concentration of 2 mg/kg body weight led to detectable mark in the otoliths.

Whereas Clear et al. (2000) obtained less distinct STR marks in otoliths by intramuscular injections of STR in concentrations of 100 mg/kg body weight of large southern blue fin tuna (*Thunnus maccoyii*). The authors hypothesized that a probable explanation might be the loss of STR from the muscle tissues after injections. Leakages out of the injection sites were also observed for STR and the other chemicals, but these losses were negligible, since only a very small drop of the chemical solution leaked out of the injection site. Moreover, the assessment of the otolith O4 TET50&STR, double marked with TET50 and STR2, showed a visible mark under UV-light, induced by TET, thus it can be assumed that when TET was deposited into the otolith, STR was also esteemed likely to be incorporated.

This finding was not confirmed by Moreno & Morales-Nin (2003), who immersed sand-smelt (*Atherina presbyter*) in 1,25 g STR/l for 24 h and found that the majority of otoliths with clear Sr marks was relatively low. Further they noted high background Sr that might be due to

contamination of Sr during polishing of the otoliths. Although in this study the otoliths were not polished, background Sr was also found, but this was due to the fact that Sr is naturally present in otoliths and other bone structures.

4.4 Limitations of the study

The main limitation of this study was the unsuccessful preparation of the solutions, especially those of ALI and CAL. The crucial lack of detailed description in published work dealing with chemical marking experiments, regarding the preparation steps for mixing the chemical solutions and solution approaches to solve probable difficulties encountered during the preparation, is fundamental to provide the reproducibility of the experiments conducted and further to ensure the comparability between experiments using the same types of chemicals and the same mode of chemical administration. The problems encountered in this experiments by preparing the injection solutions from ALI and CAL, were not described elsewhere in the published works studied, and despite several attempts involving different strategies to obtain homogenous solutions from both chemicals, a final solution to this problem could not be found. This implies further research and the provision of more detailed information about the methods in published work.

Regarding the statistical analyses, the high mortalities led to extremely unbalanced sample sizes for the mortality and mark quality assessments. This fact was further enhanced due to tag losses that occurred during the time of the experiment and it was not possible to allocate retrospectively the otoliths of the cod that lost their tags to the marker groups. The high mortalities within the concentrations led to frequent proportions of zero percent observations, which tend to bias the logistic regression models. To this is added, particularly in the case of ALI and CAL that the poor stock solution qualities above all other possible factors affected the survival of cod and caused the high mortality of cod. Therefore, possible concentration related effects on either the mortality or mark quality could not be determined. Furthermore, due to the high mortalities the separate evaluation of surviving cod from dead cod could not be conducted for the marker groups ALI and CAL, solely for the TET treatment, because the sample sizes were too small to compute statistically valid results. The information obtained from the assessment of the surviving fish was considered most important.

Further one limitation of this study was that only one control group was considered for this experiment. In further investigations the inclusion of more control groups should be

considered. Particularly with respect to multiple treatment group comparisons more than one control group or larger sample sizes might be preferable, to compensate possible random variations in the mortality within the control group and to provide better discrimination between possible effects of the different treatments on the examination object.
Another constraint was the evaluation of the otoliths marked with STR, because the access to the SEM was limited. Appointments had to be fixed in advance and the SEM was nearly booked out by other researchers. Thus, only a small sample size was evaluated. Moreover, the analysis is time consuming, if the application of STR is considered in the future, these restrictions have to be included.

4.5 Conclusion

This experiment was a first step to assess the efficacy of four commonly used chemical markers to time label the otoliths of Baltic cod for age validation in envisaged large-scale mark-recapture experiments. The aim of this study was to determine among the four chemicals tested, namely alizarin red S, calcein, strontium chloride and tetracycline hydrochloride, in single and double treatments at different concentrations, the chemical which (1) caused lowest mortalities, (2) which had no effect on growth and (3) which showed best mark quality in the otoliths of Baltic cod.

Among the four chemicals tested in this study, intraperitoneal single injections with TET in the concentrations of 50 mg/kg fish and 100 mg/kg fish proved ideal to mark the otoliths of juvenile Baltic cod, based on a high survival rate of the cod, significantly highest growth rates and highest proportions of clear and distinct fluorescent marks in the otoliths of Baltic cod. Intraperitoneal injections at these concentrations would be recommended for future large-scale mark-recapture studies for the purpose to validate the periodicity of growth increments in the otoliths of Baltic cod. The retention time is reported to be sufficiently long and ensures detectable marks in the otoliths even after 20 years (Beamish & McFarlane, 2000). The usefulness of this chemical was further emphasized by its uncomplicated and easy preparation and relative low detection costs. The detection of the TET-induced fluorescent marks requires a fluorescence microscope and a filter combination for UV-light. This little specific equipment enables the rapid evaluation of high numbers of otoliths and makes it applicable for standardized age validation of Baltic cod. Moreover, the investment costs of TET are relatively low (e.g. 50 g, 64 EUR).

In contrast to TET, ALI and CAL were not found suitable for chemical marking Baltic cod, Both chemicals caused high mortalities at the different concentrations tested in the single and double treatments, primarily due to great difficulties in obtaining adequate injection solutions, since the chemicals were poorly soluble. STR might to be preferable to the fluorescent markers ALI, CAL and TET with regard to the retention time, as STR deposits permanently into the otolith structure (Clear et al., 2000). Nonetheless, due to the long and expensive detection of Sr marks in the otoliths, STR is not considered suitable for large-scale mark-recapture experiments either. The potential benefit of double marking cod with TET and STR is not disregarded, since TET is susceptible of possible fading and degradation due to its light-sensitivity (Fargo & Chilton, 1987; Geffen, 1999). This impairment would be compensated by the Sr mark, which would be ideally detectable in the marked otoliths throughout the lifetime of the fish. However, in this study it was found that the double treatment of cod with both TET and STR is not suitable for marking Baltic cod either, since the marking success was noticeably poor compared to single injections of TET, despite relatively low mortality of cod.

The toxicity of tetracyclines is reported to be low and their use as antibiotics is approved in human and veterinary medicine as well as in fish aquaculture (Chopra & Roberts, 2001; Celik & Eke, 2011). In the European Union, the maximum residue limit for tetracyclines is set at 100 ng/g in muscle for all species (Wen et al., 2006). Whether or not, the injected concentrations of 50-100 mg/kg fish would respect this limit needs further investigation. However, the high tendency of the tetracyclines to deposit in calcifying structures by forming stable chelate complexes with calcium and the serum half-life times are reported to range between 6-11 h, suggest that the concentration of TET in the body might rather reduce with the time after injection (Agwuh & MacGowan, 2006; Lhafi et al., 2008).

Therefore, in consideration of the extensive use of tetracyclines in Baltic fish farms, where large quantities of tetracyclines end up in the sediments (Tamminen et al., 2011) and the common apply in human medicine as approved antibiotic (Chopra & Roberts, 2001; Weber & Ridgway, 1967), the potential environmental impact and human health risk of TET injected into cod might be low.

Nonetheless, the concern of a probable hazardous impact on human health by consuming cod marked with TET must be reconsidered further and prior to the application of TET in large-scale experiments, the official permission from the competent authorities must be granted.

This study demonstrated that the use of tetracycline hydrochloride in the concentrations of 50 mg/kg and 100 mg/kg fish as chemical marker is recommended over the use of the chemicals alizarin red S, calcein and strontium chloride to time label the otoliths of Baltic cod. TET at these concentrations proved not any effect on the mortality of cod and even seemed to promote the growth of the fish. The marks induced by TET at these concentrations were clear and distinct. This chemical, administered in the concentrations of 50-100 mg/kg fish is recommended to be used in future large scale-recapture studies to contribute to validate age of Baltic cod.

4.6 Further research

Since the unsuccessful application of ALI and CAL, because both chemicals were nearly insoluble in their stock solutions, further research should focus on whether or not these chemicals when applied completely dissolved would be more efficient in marking the otoliths of cod.

STR showed besides TET low mortalities and positive results in the mark detection, the application together with other commonly used chemicals for age validation studies should be considered further, since contrary to fluorescent markers, STR marks are detectable throughout the lifetime of the fish. Double marking treatments similar to these carried out in this study would ensure age validation in case the fluorescent mark is no more detectable.

Since the purpose of this study was to investigate among different chemicals, the one which produce non-lethal marks in the otoliths of Baltic cod, in a second step further studies should prove the application of TET in age validation studies.

And processes should be designed which includes TET in a standardized age validation. But prior potential mark-recapture experiments in the Baltic Sea, possible human health risks by

In conclusion, the results presented in this study contribute to first designs of large-scale mark-recapture experiments to validate age in Baltic cod. This first recommendation of the use of TET at the concentrations of 50 mg/kg and 100 mg/kg fish (intraperitoneal injections), implies further research to prove the suitability of this chemical

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Declaration of Authorship

I, Aisha Karim Degen-Smyrek, hereby declare that this thesis entitled

“Evaluation of chemical markers for age validation of western Baltic cod (*Gadus morhua*) otoliths“

and the work presented in it is entirely my own. Where I have consulted the work of others, this is always clearly stated.

Rostock, 18/03/2014 **Aisha Degen-Smyrek**