Aquat. Living Resour. 2023, 36, 17

https://doi.org/10.1051/alr/2023010

Aquatic Living Resources
Available online at:
www.alr-journal.org

Special Issue - NORA 4 - Native Oyster Restoration Alliance 4th Conference

Research Article Open Access

Potential of calcein staining as growth monitoring marker in *Ostrea edulis*

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Received 30 September 2022 / Accepted 17 April 2023

Handling Editor: Tom C. Cameron

Abstract – Growth is an important biological trait and monitoring metric for the assessment of the success and progress of restoration projects with the European oyster (*Ostrea edulis*). However, sampling time and frequency are often limited, as well as the ability to survey individual growth. Chemical dyes, such as calcein, can be used to create incremental markings for measuring growth in shell cross-sections, especially in bivalves. However, potential negative effects and limits for successful staining have not been assessed for *O. edulis*, yet. In this study, three different calcein concentrations (100 mg l^{-1}, 150 mg l^{-1}, 200 mg l^{-1}) with three different immersion times (6 h, 12 h, 24 h) were tested for potential negative effects, the best incremental marking result and their potential to be used in growth measurements. Furthermore, the deposition of an annual growth line was investigated. Results showed that calcein is a reliable *in situ* fluorescence marker that produced sufficient growth lines in the cross-section of *O. edulis*. At a concentration of 100 mg l^{-1} or higher and immersion times of 6 h or more, no negative effects on growth and survival were observed after five months. Applications include the possibility for long-term, individual growth data for a large number of oysters for restoration monitoring as well as the option of marking restored oysters from aquaculture production to distinguish them from existing wild stock populations or natural offspring.

Keywords: European oyster / tagging / restoration / recapture / fluorochrome dye / *in situ* fluorescent marking

1 Introduction

In Europe, the history of harvesting and aquacultural usage of the native European oyster, *Ostrea edulis*, dates back as far as Roman times (Gerlach, 2001; Pogoda, 2019). Over the centuries, fishing pressure constantly increased due to drastic improvements of harvesting techniques. Ultimately, this led not just to the decline of the native European oyster in some areas, but even to the functional extinction in others, such as Belgium and the German Bight (Beck et al., 2009; Gercken and Schmidt, 2014). Considering the multitude of ecosystem functions and services provided by healthy oyster reef habitats, the ecological restoration of such ecosystem engineers is currently an important conservation measure (Northern Economics, 2009; Pogoda et al., 2017; Lown et al., 2020; zu Ermgassen et al., 2021).

To assess, evaluate and adapt the progress and success of restoration measures such as oyster habitat recovery it is of vital importance to develop and implement appropriate monitoring tools (zu Ermgassen et al., 2021). The European oyster habitat restoration monitoring handbook provides a comprehensive list of such metrics (zu Ermgassen et al., 2021). Depending on the exact project aims and the interests of the stakeholders, different parameters of the universal, supplementary, or restoration goal-based metrics can be chosen to evaluate the success of the restoration measure. Each of the metrics includes the assessment of growth as a monitoring parameter. Oyster growth rates are an important indicator of oyster productivity and an indicator that European oysters at restoration sites are experiencing suitable conditions

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(Rödström and Jonsson, 2000; zu Ermgassen et al., 2020). However, quantities (>20% of the population: Universal metric – oyster size-frequency) or sample frequency (every three months: Supplementary metric – growth rate) might be challenging to achieve for some study sites, such as those in offshore areas, where sampling time and frequency is limited (zu Ermgassen et al., 2021). In addition, growth rates are most accurately established by measuring the same individual more than once, as averaging over populations introduces a lot of variation (zu Ermgassen et al., 2021). Individual growth measurements often require an effective tagging or marking technique, preferably applicable to a large number of oysters with minimum effort.

Numerous techniques have been applied to tag individuals in marine environments. External and internal physical tagging is used to mark and recapture bivalves (integrated transponder tags: (Kurth et al., 2007; Evans et al., 2016); notching: (Sejr et al., 2002; Haag and Commens-Carson, 2008); paint (Nelson, 1981; Neves and Moyer, 1988), glued on tags or wire tags (Neves and Moyer, 1988; Lim and Sakurai, 1999; Bayne, 2002; Evans et al., 2016), or tethering of individuals (Lown et al., 2020). However, using physical tagging might cause issues, such as stressing of the animals due to extended air exposure or physical alteration of the shell; technical challenges such as loss or damage of tags by abrasion; or possible rejection of internal tags by animals (Riley et al., 2010). Additionally, time intense growth measurements have to be carried out periodically for each point in time.

Alternatively, it is often possible to determine age or growth patterns by the biogenic depositions of calcifying organisms (Schöne and Fiebig, 2009). Relevant preconditions of this method are that the species grows in distinct increments which can be linked to a specific time period at a specific study site. Growth increment can be distinguishable e.g. by colour or shape and appears in bands or lines. Different environmental conditions form different increments when the conditions for the animal changes or are less favourable, such as under stress, during reproduction, or most prominent at low temperature, hence during low growth periods (Sejr et al., 2002; Haag and Commens-Carson, 2008; Schöne and Fiebig, 2009). The latter can potentially be used as growth lines to measure annual growth. In bivalves, incremental patterns can be viewed either on the shell surface or in a cross-section of a shell valve along the LSG (Line of strongest growth, umbo to longest edge), depending on the species (Herrmann et al., 2009; Schöne and Fiebig, 2009). However not in all cases is an incremental pattern legible enough to make precise growth measurements from it (Kesler and Downing, 1997).

Richardson, Collis et al. (1993) as well as Milner (2001) showed in cross-sections, that *O. edulis* is growing incrementally. The growth pattern is visible as increments of growth increments interrupted by growth lines of a different colour. Growth lines in British waters seem to form in March/April when environmental conditions, especially temperature, are less favourable for growth (Richardson et al., 1993). However, it was also found that disturbance lines in-between growth lines impair the readability significantly (Milner, 2001). Precise growth measurements cannot be obtained from the cross-sections since a reliable link between the formation of increments to a specific time period is not possible (Milner, 2001).

It is, however, possible to create distinct incremental markings within the growth patterns of incremental growing organisms using chemical dyes (Riascos et al., 2007; Gancel et al., 2019). These *in situ* fluorescent markings allow to mark and recapture e.g. reintroduced individuals as well as the marking of a specific point in time in the shell that can be used to measure sizes and calculate growth rates (Herrmann et al., 2009; Spires and North, 2022). Creating *in situ* fluorescent markings as part of fieldwork is a time-efficient way of building individual growth archives that can be applied to a large scale.

Numerous chemical dyes were used in the past, such as alizarin red (Day et al., 1995; Riascos et al., 2007), strontium (Fujikura et al., 2003), tetracycline (Day et al., 1995; Riascos et al., 2007) and calcein (Kaehler and McQuaid, 1999; Riascos et al., 2007; Herrmann et al., 2009; Fitzpatrick et al., 2013). Calcein has been shown to be the most reliant *in situ* chemical dye in the past (Herrmann et al., 2009; Fitzpatrick et al., 2013). Calcein is a potentially non-toxic, fluorochrome dye that binds to alkaline earth metals like calcium in suspension and gets incorporated into newly mineralized shells (Wilson et al., 1987; Day et al., 1995; Spires and North, 2022). The feasibility of such *in situ* fluorescent markings has been verified for several oyster species: *Ostrea chilensis* (Chaparro et al., 2018), *Crassostrea virginica* (Gancel et al., 2019; Spires and North, 2022), *Pinctada margaritifera* (Linard et al., 2011), and *Ostrea puelchana* (Doldan et al., 2018). Calcein has been proven to be non-toxic in the majority of usages, however, in some studies, a negative effect was reported (Bumguardner and King, 1996; Russell and Urbaniak, 2004). Equally, the efficiency, as well as toxicity of any chemical dye, can differ substantially between species. Hence, it is essential to test the technical and biological application to a new species prior to large-scale implementations. For *O. edulis*, this method has not yet been established.

This study provides the first data on the application of *in situ* fluorescent markings in *O. edulis* shells. *In situ* fluorescent markings were created using calcein with three different concentrations and immersion times to assess the best incremental marking result and their possible use in growth measurements. The results provide practical information for creating long-term, large-scale individual growth data for restoration monitoring as well as for the marking and recapturing of oysters from aquaculture production to distinguish them from existing wild stock.

2 Materials and methods

2.1 Origin of test animals

European oysters of this study were taken from a reintroduced *O. edulis* population (original 2 mm seed from France, MARINOVE), reared in oyster baskets (6 mm mesh size, 15 l baskets, 600 × 140 × 260 mm, SEAPA) at 26 m water depths within the marine protected area (MPA) 'Helgoländer Felssockel' (Merk et al., 2020) since 2017. In May 2021, 100 four-year-old oysters from the same size category were rapidly acclimated over 24 hours from ambient water temperature of 7–17 °C to activate filtration activity after winter for *in situ* fluorescence marking by implementing the stain to the shell (Buxton et al., 1981; Gancel et al., 2019).

2.2 Staining

Staining was conducted using calcein ($C_{30}H_{26}N_2O_{13}$, 1.02315.0005, MERCK). Previous studies used a wide range of different calcein concentrations (50–250 mg l^{-1}) and immersion times (1–48 h) in the staining process, that depended on the size and number of the specimens and the species itself (van der Geest et al., 2011; Fitzpatrick et al., 2013; Chaparro et al., 2018; Gancel et al., 2019). Based on these studies, ten different treatments were chosen based on similarity of species and sizes. Three different concentrations of calcein 100 mg l^{-1}, 150 mg l^{-1}, 200 mg l^{-1} and three immersion times 6 h, 12 h, and 24 h were tested and the treatments named accordingly (100/6, 100/12, 100/24, 150/6, 150/12, 150/24, 200/6, 200/12, 200/24). In addition, a control with no added calcein was run for 24 h. Each of the concentrations and the control were set up in a separate basin with a lid, including heating, temperature sensor, and aeration in filtered seawater (1 μm) at 17 °C. No change of water took place; food (*Chaetoceros muelleri*, 3 million cells ml^{-1}) was added at 0 h and 6 h to increase filter-feeding. Calcein was dissolved in 1 l of filtered seawater and added to the basins twice (at 0 h and 6 h), to keep the concentration of staining solution stable after feeding. After 6 h, 12 h, and 24 h, 10 oysters were removed from each treatment tank and cleaned with filtered seawater. Oysters from the ten different treatments were kept separate after staining.

2.3 Study site and environmental parameter

Oysters were placed into 10 oyster baskets (6 mm mesh size, 15 l baskets, 600 × 140 × 260 mm, SEAPA) sorted by treatment and redeployed at 10 m water depth (54°11.60'N 007°52.80'E). Oysters were kept in the field to grow for five months from May to November 2021. Environmental parameters at the study site and depth (~10 m) were extracted from the BSH circulation model for the German Bight (BSHcmod), provided by the German Federal Maritime and Hydrographic (Dick et al., 2001).

2.4 Growth and condition

Shell height (according to NORA monitoring guidelines (zu Ermgassen et al., 2021) was measured along the line of strongest growth (LSG, umbo hinge to longest edge) to the closest 0.1 mm at the beginning and the end of the experiment (DIGI-MET 1/100 150 × 40 mm IP 67 TM, Helios-Preisser). Individual shell size measurements were used to calculate mean shell sizes for each treatment to further calculate from these external growths of each treatments as the mean increase in mean shell size.

Wet weight of oysters was determined at the end of the experiment (Sartorius TE412, closest 0.01 g). Oysters were dissected, soft tissue was dry frozen (48 h, Alpha 1-4 LSC, Christ), and shells were dried at 60 °C for 48 h. To estimate potential negative effects of the *in situ* fluorescent marking, the dry weight of bodies (Sartorius LA230S, closest 0.1 mg) and shell (Sartorius TE412, closest 0.01 g) was determined to calculate the condition index for each individual according to Davenport and Chen (1987) and Walne and Mann (1975).

2.5 Cross-sectioning of shells

To determine *in situ* fluorescent marking, right valves were cut along the LSG and were examined in a cross-section under fluorescent light. For an even cut, a layer of two-compounded metal-epoxy resin (1:1 ratio; WIKO EPOXY METALL; GLUETEC, Greußheim, Germany) was applied as a coating to the inside and outside of the valve and set to for 24 h. Afterwards, each valve was cut using a circular saw bench (FKS/E; Proxxon, Föhren, Germany) and a diamond-coated cutting blade (blade: NO 28 735; Proxxon, Föhren, Germany). The cross-section surfaces were then sanded with abrasive paper (grinder: Phoenix Alpha; Buehler, Düsseldorf, Germany, grain sizes: 25 μm, 15 μm, and 8 μm) to create an even surface for visualisation.

2.6 Visualisation and measurement

In situ fluorescent markings were expected to be visible as a fluorescent green lines in the cross-sections. Distance measurements between the marginal endings of two *in situ* fluorescent markings represent the growth between the two marking events, alternatively the shell marginal end of the cross-section can be used as measurement point in case the time of death is known. The position of a growth line formed in winter as well as potential disturbance lines can be visualised under reflective light and compared to the *in situ* fluorescent marking.

In situ fluorescent marking of each individual cross-section was visualised under a microscope (Research Stereomicroscope System SZX12, Olympus, Hamburg, Germany) with an external fluorescence light source (Model U-ULS100HG, Olympus, Hamburg, Germany). Results were converted to images using a mounted camera (CCD-camera U-CMAD3 Colorview I; Olympus, Hamburg, Germany) and an image analysis software (AnalySIS 5.0 Copyright 1986–2004; Soft Imaging System GmbH, Olympus, Hamburg, Germany). Growth was measured in the cross-sections (AnalySIS 5.0 Copyright 1986–2004; Soft Imaging System GmbH, Olympus, Hamburg, Germany) as incremental growth represented by the distance from the *in situ* fluorescent marking to the marginal end of the cross-section in DOG (direction of growth) (see Fig. 1).

For a non-subjective measurement of brightness and therefore readability of the markings, the maximum brightness was recorded according to Fitzpatrick, Jeffs and Dunphy (2010) using the software ImageJ. Maximum brightness was measured for each individual and used to calculate the mean maximum brightness for each treatment.

2.7 Statistics

Mean values and standard derivations where calculated for the condition indices, external shell heights internal growth and wet weights over each treatment and compared.

3 Results

3.1 Environmental parameter

Seawater temperatures increased from 7 °C at the beginning of the experiment in May 2021 (monthly mean), marking the beginning of the oyster growth period (Wilson and Simons,

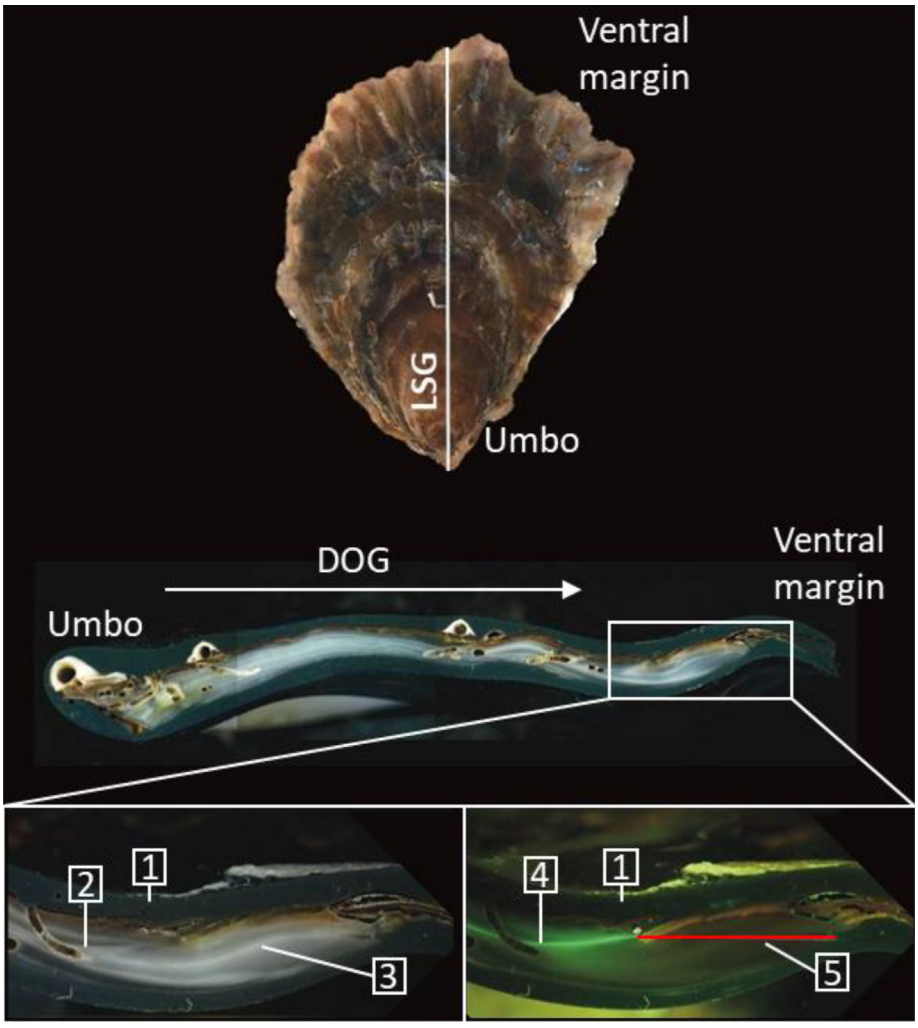

Fig. 1. Overview of the shell of *O. edulis*. Top: Outer view of the shell indicating the line of strongest growth (LSG) from the umbo to the margin, which runs perpendicular to the growth lines. Middle: Cross section following the LSG with the direction of growth (DOG) from left to right. Magnified is the measurement area under reflected (bottom left) and fluorescent light (bottom right) with 1–Coating, 2–Growth line, 3–Disturbance line, 4–*in situ* fluorescent marking, 5–measurement. Under florescent light, the shell does show natural fluorescence visible in yellow on the edge between shell and the coating.

1985) to 18 °C in August 2021, and decreased again to 12 °C in November 2021 at the end of the experiment. Salinity was constantly above 30. Oxygen concentration ranged from 7.6 to 10.0 mg l^{-1}.

3.2 Condition of oysters

Condition index of exposed oysters was 3.2 ± 0.7, with a minimum at 3.0 ± 0.6 (150/06 and 100/24) and a maximum at 3.5 ± 0.6 (200/24) (see Tab. 1). No difference in condition was observed between treatments with different calcein concentrations, immersion times or reference oysters (control). Survival over the experimental period was 100%. Total wet weight was 41.6 ± 17.6 g.

3.3. Visibility of *in situ* fluorescent marking

In situ fluorescent markings were visible in all animals across different staining treatments and not in reference oysters (control). According to the mean maximum brightness values, no significant differences were documented between the treatments (Fig. 2C). 11% of markings were hard to read (brightness < 45), but 24 % of markings were good (brightness: 45–75) and 64 % were very good to read (brightness > 75). The weakest *in situ* fluorescent marking across treatments was detected in 100/06, but was still applicable for detection and measurements. All *in situ* fluorescent markings can be viewed in Appendix I. *In situ* fluorescent markings were visible from the umbo along the cross-sections of each shell to the marginal end of the shell (Fig. 2A).

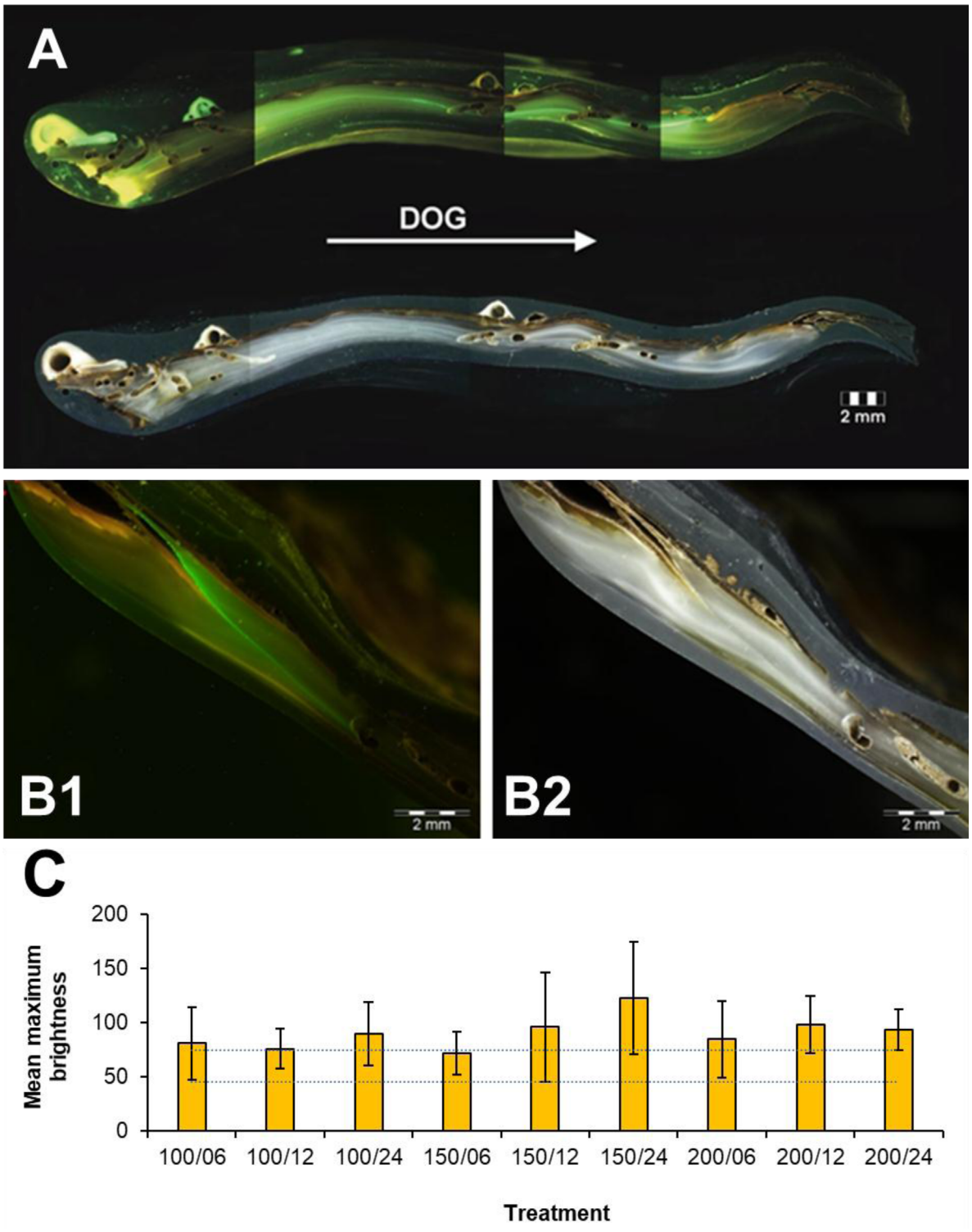

Fig. 2. A – Exemplary overview of a cross-section under fluorescent (top) and reflective (down) light (Treatment 200/12). *In situ* fluorescence markings are visible and congruent with the growth line. Identification of growth lines without *in situ* fluorescence marking is challenging due to disturbance lines (Treatment 200/12). B – Cross-section along the line of strongest growth viewed under fluorescent lights (B1) and reflected light (B2). *In situ* fluorescent marking is visible as a green line under fluorescent lights. C – Mean maximum brightness of *in situ* fluorescent markings in the cross-sections of *O. edulis* shells. Staining was performed for nine treatments using three different calcein concentrations (100 mg l^{-1}, 150 mg l^{-1}, 200 mg l^{-1}) and three immersion times (6 h, 12 h, 24 h). The higher the brightness the higher the readability and the applicability for measurements. Markings were hard to read at a brightness $\leq$ 45 (lower dotted line), good to read at a brightness of 45–75, and very good to read at $\geq$75 (upper dotted line). The mean maximum brightness showed no significant difference between the treatments. Abbreviation: DOG, Direction of growth.

3.4 Verification of growth line

Shells were stained at the beginning of the growth period to obtain an *in situ* fluorescent marking at the outer rim of the growth line (Richardson et al., 1993; Milner, 2001). In all shells, the *in situ* fluorescent marking corresponded with the end of the growth line formed in low growth periods. Identification of the growth line without an *in situ* fluorescent marking was significantly challenging since all cross-sections revealed significant disturbance lines in addition to the growth line (Fig. 2A-B and appendix).

3.5 Growth

Shell height ranged from 58.2 ± 10.3 mm (100/06) to 65.3 ± 7.6 mm (control) at the beginning of the experiment in May and from 64.9 ± 10.1 mm (100/24) to 76.1 ± 6.0 mm (control) at the end of the experiment in November. The largest growth measured externally was 13.1 ± 15.7 mm in 100/06, smallest growth was 5.9 ± 15.8 mm in 100/24 (see Tab. 1). No significant difference in external growth was found between oysters exposed to calcein and reference oysters (control). Differences between external and incremental growth were not significant. Growth did not differ notably between oysters exposed to calcein and the control treatment.

Incremental growth was measured from the cross-section of each shell as the distance between the *in situ* fluorescent marking and the end of the shell as an approximately parallel line to the outer shape of the shell. The largest incremental growth was measured in 150/06 (11.0 ± 3.6 mm), the smallest in 100/24 (6.8 ± 3.3 mm). Differences between external and incremental growth were not substantial.

4 Discussion

This study examined the potential of incremental measurement in *O. edulis* as a tool for collecting data on individual growth rates. The focus was on the possibility to create *in situ* fluorescent markings using three different calcein concentrations and three different immersion times.

Applied calcein concentrations showed no toxic or negative effect on *O. edulis* after 5 months regarding survival, growth and condition. Calcein has been shown to be potentially non-toxic for other bivalves (Kaehler and McQuaid, 1999; Tada et al., 2010; van der Geest et al., 2011; Gancel et al., 2019). Staining of bivalve species at Calcein concentrations of 100–800 mg l^{-1} did not show measurably effect on shell growth rate, body condition and size (van Geest, 2011). Staining of *Crassostrea virginica* with concentration of 250 mg l^{-1} at three different size classes (juvenil and adult oysters) showed also a similar or better survival rate compared to the control (Spires and North, 2022).

However, two studies have reported a negative influence. Juvenile striped bass *Morone saxatilis* exposed to calcein levels of 250 mg l^{-1} showed a greater exhibited stressed behaviour and therefore concentrations above 125 mg l^{-1} should be used with caution (Bumguardner and King, 1996). Green sea urchins, *Strongylocentrotus droebachiensis* showed, after staining a temporary reduction in growth rate compared to the control group in the first week following the application of

Table 1. Measurements and calculations of *O. edulis* ($N = 100$) over all 10 treatments (Control; calcein concentrations: 100 mg l^{-1}, 150 mg l^{-1}, 200 mg l^{-1}, immersion times: 6 h, 12 h, 24 h). Condition index was calculated from the dry weight of bodies and shell according to Davenport and Chen (1987) and Walne and Mann (1975). Shell height (umbo hinge to longest edge) to the closest 0.1 mm, was measured in May 2021 and in November 2021. Both shell heights were used to calculate external growth [mm]. Internal growth was calculated from measurements in valve cross-sections using *in situ* fluorescent markings.

Treatment	Shell height [mm] May 2021	Shell height [mm] Nov 2021	Dry weight body [g] Nov 2021	Dry weight shell [g] Nov 2021	Wet weight [g] Nov 2021	Condition index Nov 2021	External growth [mm]	Internal growth [mm]
Control	65.3 ± 7.6	76.1 ± 6.0	1.0 ± 0.2	30.0 ± 8.1	48.1 ± 12.6	3.5 ± 0.7	10.9 ± 9.6	
100/06	58.2 ± 10.3	71.4 ± 11.9	0.8 ± 0.4	26.7 ± 8.7	42.2 ± 13.1	3.1 ± 0.7	13.1 ± 15.7	10.8 ± 4.1
100/12	61.6 ± 10.4	68.8 ± 9.1	0.8 ± 0.3	25.6 ± 7.8	41.8 ± 12.2	3.2 ± 0.7	7.2 ± 13.8	8.7 ± 2.4
100/24	59.1 ± 12.9	64.9 ± 10.1	0.7 ± 0.3	21.9 ± 9.1	33.4 ± 13.0	3.0 ± 0.6	5.9 ± 16.4	6.8 ± 3.3
150/06	61.5 ± 12.7	74.4 ± 11.1	0.9 ± 0.4	32.1 ± 12.0	48.8 ± 18.4	3.0 ± 0.6	12.8 ± 16.9	11.0 ± 3.6
150/12	59.9 ± 14.7	68.1 ± 12.6	0.7 ± 0.4	25.3 ± 13.6	39.0 ± 21.2	3.0 ± 0.8	8.2 ± 19.4	9.3 ± 3.6
150/24	60.7 ± 15.9	72.0 ± 13.6	0.8 ± 0.4	25.4 ± 10.2	40.7 ± 17.2	3.0 ± 0.8	11.3 ± 20.9	9.0 ± 3.0
200/06	62.1 ± 10.5	74.6 ± 12.0	1.0 ± 0.4	31.6 ± 13.3	50.6 ± 21.0	3.3 ± 0.6	12.6 ± 15.9	9.4 ± 3.5
200/12	61.4 ± 11.0	67.3 ± 11.3	0.7 ± 0.4	22.7 ± 8.3	36.6 ± 15.2	3.1 ± 0.7	5.9 ± 15.8	7.8 ± 3.5
200/24	59.1 ± 17.2	68.0 ± 15.6	0.8 ± 0.4	22.0 ± 11.9	351 ± 19.1	3.5 ± 0.6	8.9 ± 23.2	9.0 ± 3.6
Total	60.9 ± 12.8	70.6 ± 12.1	0.8 ± 0.4	26.3 ± 11.1	41.6 ± 17.6	3.2 ± 0,7	9.7 ± 17.6	9.0 ± 3.9

calcein. This difference disappeared in the subsequent weeks of the experiment. It was concluded that the application of calcein stains has no long-term effect on growth and survival (Russell and Urbaniak, 2004). In this study, the survival across all treatments, including the control, was 100%. The condition index of all oysters was good and within the range of previous data from this population and season (Pogoda et al., 2011; Merk et al., 2020). As no significant difference was detected for oysters exposed to calcein and reference oysters, we assume that applied calcein concentrations did not influence growth or condition. Calcein is therefore a suitable non-hazardous *in situ* fluorescent marker for *O. edulis growth*.

Across all treatments, the markings were visible in the cross-section of the shell. No significant difference was observed between the different concentrations and immersion times. The variability was not affected by treatment although the lowest values were in the 6 hours treatments. Only 11% of markings were hard to read, however, exceeded the readability of the natural growth line and were sufficient for growth measurement. Concentrations and immersion time differ significantly between studies, ranging from 50 mg l^{-1} to 250 mg l^{-1} depending on species, size of the animals, and the number of specimens (van der Geest et al., 2011; Fitzpatrick et al., 2013; Chaparro et al., 2018; Gancel et al., 2019). This study shows that staining of *O. edulis* with 100 mg l^{-1} for 6 h is creating reliable *in situ* fluorescent markings. It is therefore also possible to successively generate multiple *in situ* fluorescent markings in the shells of living *O. edulis* with little time and cost effort in the field that can later be used to calculate reliable and individual growth rates.

The staining success is potentially linked not just to the concentration of calcein, but also the submersion volume and therefore the total quantity of staining pigment. Since the staining mechanism depends on the infiltration of pigments by the oyster, the amount of pigment filtered in total is relevant. Considering a filtration rate of 1.7 l h^{-1} (comparable age/size class with the oysters in this study) (16 °C, (Sytnik and Zolotnitskiy, 2014)), one single oyster in this experiment may have filtered half of the staining solution volume after ~6 h (10.2 l), the complete volume of staining solution after ~12 h (20.4 l) and the complete volume of staining solution twice after ~24 h (40.8 l). Hence, the oyster was exposed to half (6 h: 1020 mg, 1530 mg, 2040 mg), the total (12 h: 2000 mg, 3000 mg, 4000 mg) or twice the total amount (24 h: 2 × 2000 mg, 2 × 3000 mg, 2 × 4000 mg) of the pigment diluted in the staining solution, depending on the immersion time and concentration. If the volume of the submersion solution would have been 2 l, as applied by other studies (Herrmann et al., 2009) the oyster would have been exposed to all the pigment at around 1 h. It is unknown how much of the pigment is implemented during the immersion time. Therefore, this theory neglects the decrease of pigment quantity due to the intended binding process. However, the volume of water is important to consider when applying this method.

O. edulis in the German Bight forms a winter line until May. The deposition time of growth lines is not transferable between sites, as seen in Richardson et al. (1993), Richardson (2001), and needs verification for each new area. For all oysters in the present study, the *in situ* fluorescent marking was congruent with the marginal edge of the growth line. After winter, and assuming a resting period of no growth at temperatures < 7 °C (Wilson and Simons, 1985), a growth line had formed. Growth lines, under reflective light, showed up as white-translucent compared to the grey tone of the regular increments under reflective light. Identification of growth lines, however, was challenging due to the high abundance of disturbance lines in all cross-sections similar to previous studies (Richardson et al., 1993; Milner, 2001). The visibility of growth lines could eventually be improved by etching, even though this would not eliminate disturbance lines (Milano et al., 2017). Disturbance lines can be caused by a disruption of growth, although the exact reasons are not always clear. Disturbance lines in *O. edulis* may be caused by reproduction and the related hermaphroditic change (Milner, 2001). Linking disturbance lines to spawning events would be an interesting monitoring approach, however, this was not examined in the present study. *In situ* fluorescent marking is therefore a suitable method to create measurement points that are more reliable to calculate growth and can be adapted to sampling time and frequencies.

Incremental measurements in the cross-section of *O. edulis* using *in situ* fluorescent marking can be used to calculate growth rates. No significant difference was present between external and incremental growth measurements. External measurements of incremental growth are not easy to perform with the marginal edge a measurement point, since this area is fragile and could have been damaged during handling. Indeed, we find a higher variation in the external measurements than in the internal ones. Similar the incremental measurement from one *in situ* fluorescent marking to a second *in situ* fluorescent marking, stained at a different, time will further improve the accuracy of the data. Furthermore, measurements of older oysters with shorter increments will be more accurate and propose the possibility to define a van Bertalanffy curve for the study area (Von Bertalanffy, 1957; Richardson et al., 1993; Liddel, 2008; Herrmann et al., 2009). *In situ* fluorescent marking as a tool for growth rate measurements has already been established for different bivalves (Herrmann et al., 2009; van der Geest et al., 2011; Merk, 2015; Spires and North, 2022) and can be recommended for *O. edulis* as well.

Results indicate that *in situ* fluorescent marking in *O. edulis* can be successfully applied at moderate environmental temperatures. Marking was successful for 100% of oysters, despite the environmental temperature just reaching 7 °C and therefore marking the start of the growing period of *O. edulis* (Wilson and Simons, 1985). In the presented study, oysters were rapidly acclimated to a higher temperature before staining, which, according to Buxton et al. (1981), should increase the scope for growth momentarily, however, be still significantly below the growth of oysters that are long-term acclimated to warmer temperatures. Despite the increased temperature stress, survival was 100% and filtration rates were sufficient to create *in situ* fluorescence markings for all oysters over the respective immersion times. Hence, *in situ* fluorescent marking is possible for *O. edulis* throughout the year with a reasonable short period of acclimatization to the warmer temperature of the staining solution.

Calcein as an *in situ* fluorescence marking dye has an explicit application potential for *O. edulis* restoration: It allows for the generation of continuous individual growth data over longer periods with a potential high resolution. Furthermore, practical efforts to conduct staining of large numbers of oysters

are moderate, creating long-term and representative data sets. *In situ* fluorescence marking has a minimum or no impact and high reliability, especially for smaller animals, compared to external tagging methods for individual re-capture and external measurement such as etching, painting, or tags (Lim and Sakurai, 1999; Bayne, 2002; Evans et al., 2016). A high quantity of shell height measurement can furthermore be facilitated as one of the key parameters for modelling or evaluation of other monitoring metrics (zu Ermgassen et al., 2021; Pineda-Metz et al., submitted).

In addition, calcein markings can be used to differentiate between natural recruitment and relocated oysters at restoration sites. In areas with a wild population, *in situ* fluorescence marking can improve growth and survival monitoring of introduced oysters as well as the assessment of natural spat fall (Spires and North, 2022). Being able to distinguish between wild stock and introduced oysters optimises the practicability and accuracy of these essential monitoring metrics. Furthermore, for some bivalves, calcein-marking was already produced in larvae, which, once confirmed for *O. edulis*, would e.g. allow for detailed assessments of connectivity between restoration sites (Fitzpatrick et al., 2010; Chaparro et al., 2018; Gancel et al., 2019; Pogoda et al., 2020: Pogoda et al., *in prep*).

In conclusion, calcein as an *in situ* fluorescence marking produces reliable growth lines in the cross-section of *O. edulis*, at a concentration of 100 mg l^{-1} or higher at an immersion time of 6 h or more without negative effects on survival, growth and condition. It can be used to create long-term, individual growth data for a large number of oysters and facilitate monitoring of restoration success. It can also be used as a marker for aquacultural produced or relocated oysters within wild stock populations.

Acknowledgements. We thank the Centre for Scientific Diving at AWI, namely Prof. Dr. Philipp Fischer and Dr. Markus Brand for their constant and valuable support to implement and investigate the fieldwork. We thank the Helgoland Oyster Hatchery, namely Dr. Aldi Nel and Lennard Klingforth, as well as Anna Feuring for their support in conducting the staining and Kerstin Beyer for their support in visualising the calcein markings. Furthermore, we would like to thank the Federal Maritime and Hydrographic Agency (BSH) for providing environmental modelling data. This research is part of the testing and development project RESTORE, funded and supported by the Federal Agency for Nature Conservation (BfN) (grant numbers FKZ 3516892001, FKZ 3519892016).

Supplementary Material

The Supplementary Material is available at https://www.alr-journal.org/10.1051/alr/2023010.

Appendix. Ostrea edulis shell cross-section under fluorescent (left) and reflective (right) light. Staining was conducted for nine treatments with three different Calcein concentrations (100 mg l-1, 150 mg l-1, 200 mg l-1) and three immersion times (6 h, 12 h, 24 h). Additionally a Control treatment (0 mg l-1,24 h) was conducted. In-situ fluorescence markings (green line) are visible under fluorescent light and corresponding to the growth line underreflective light. Additional disturbance lines interfere with the identification of growth lines.

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Cite this article as: Merk V, Hausen T, Ameis T, Colsoul B, Boersma M, Pogoda B. 2023. Potential of calcein staining as growth monitoring marker in *Ostrea edulis*. *Aquat. Living Resour.* 36: 17