

SUITABILITY OF THREE STAINS TO MARK SHELLS OF *CONCHOLEPAS CONCHOLEPAS* (GASTROPODA) AND *MESODESMA DONACIUM* (BIVALVIA)

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ABSTRACT Different stains are used to internally mark calcified structures of mollusc shells in growth experiments. Because of interspecific variations in marking success, an assessment of suitability for each species is necessary. The potential of calcein, alizarin red, and strontium chloride hexahydrate (strontium chloride) was investigated for the Chilean abalone *Concholepas concholepas* and the surf clam *Mesodesma donacium*, two molluscs of commercial importance in Chile. Wild specimens from Northern Chile were marked using different concentrations and immersion periods of the three stains. Animals were reared for 20 days to allow growth, mortality, body condition index (BCI), and growth rate was measured to assess the effects of the treatments. To detect marks, individuals were culled and shell sections analyzed using scanning electron microscopy for strontium chloride and fluorescence microscopy for calcein and alizarin red, respectively. Strontium chloride produced narrow bright bands only at concentrations of 2,880 mg·l⁻¹ and 24 h exposure. Calcein markings produced fluorescent bands detectable in all treatments (50 and 100 mg·l⁻¹, 3 and 6 h) whereas alizarin red only yielded irregular bands with 50–100 mg·l⁻¹ and 6 h exposure. Our results show that growth rates of *C. concholepas* are significantly affected by the stains factor: Strontium chloride showed the lowest growth rates whereas that of alizarin red and calcein was similar to the control group. High concentrations of strontium chloride negatively affected ($P < 0.05$) the body condition of the gastropod. Although no statistical differences were found, BCI of *M. donacium* followed the same trend as observed for *C. concholepas*. In conclusion, calcein was the best growth marker for both species because it produced bright, long-lasting bands even at low concentrations and immersion times without detectable lethal or sublethal effects.

KEY WORDS: alizarin red, calcein, Chilean abalone, growth rate, strontium chloride, surf clam, *Mesodesma*, *Concholepas*

INTRODUCTION

Growth rate is one of the basic parameters to describe population dynamics. In fisheries growth as well as recruitment is used to estimate the sustainable stock yield, (Hilborn & Walters 1992, Vakily 1992, King 1995). The Chilean abalone, *Concholepas concholepas* (Bruguière, 1789), and the surf clam *Mesodesma donacium* (Lamarck, 1818) rank among the most important commercial species for Chilean and Peruvian shellfisheries (Bustamante & Castilla 1987, Rabí & Marabí 1997, Rubilar et al. 2001). Accordingly their growth rates have been analyzed using various methods, including length-frequency analysis, tagging-recapture experiments, and the interpretation of shell growth rings (Tobella 1975, Acuña & Stuardo 1979, Gallardo 1979, Castilla & Jerez 1986, Arntz et al. 1987, Stotz & Pérez 1992, Jerez et al. 1999). Estimations are, however, often contradictory (Wolff 1989), because current methods for growth and age determination of molluscs all have specific limitations. Length-frequency analyses require well-defined age cohorts and large sample sizes, invasive tagging-recapture methods promote physical disturbance and contingently uncharacteristic growth rates, whereas quantification of growth rings or internal growth marks are affected by surface erosion and disturbance events (Kaehler & McQuaid 1999, for revisions of growth methods see Griffiths & Griffiths 1987, Richardson 2001).

To overcome the shortcomings of current methods for growth and age estimations, a number of staining methods as

shell growth markers have been tested in a variety of marine animals. The organisms exposed to stains successfully incorporated the chemical into growing calcified structures in the form of an internal growth mark that could subsequently be used to estimate growth from time of exposure (e.g., Hernaman et al. 2000, Leips et al. 2001, Bashey 2004, Bernhard et al. 2004, Marschal et al. 2004, Heilmayer et al. 2005). Thus, this method provides a temporal frame for age validation of increment periodicity (Hales & Hurley 1991, Oliveira 1996) and enables the analysis of environmental controls on short-term growth rates (Schöne et al. 2003).

The toxicity of some chemicals, even at low concentrations represents one serious potential drawback of these rapid marking methods (Bumguardner & King 1996, Gelsleichter et al. 1997). Consequently, a diversity of markers is needed to overcome species-specific variation in marking success (Bashey 2004). Suitable markers must be: (1) reliable and easy to detect in shell preparations; (2) not alter the viability of marked individuals; and (3) be retained for an appropriate period of time. Within the diversity of markers, calcein, alizarin red and strontium chloride have been proven to be suitable for molluscs (Day et al. 1995, Kaehler & McQuaid 1999, Moran 2000, Fujikura et al. 2003, Heilmayer et al. 2005). The aim of the current study is to test the potential of these growth markers for further studies concerning the environmental control on shell growth rates of the Chilean abalone *C. concholepas* and the surf clam *M. donacium*. The suitability of the marks produced at different concentrations and immersion periods and their effects on growth, body condition and survivorship are assessed.

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MATERIALS AND METHODS

Sampling Sites and Maintenance

In March 2005, 277 specimens of *C. concholepas* (anterior-posterior shell length, *SL*: 33.5–56.7 mm) were collected in southern San Jorge Bay (Antofagasta, Chile; 23°42.297'S; 70°25.482'W). Additionally 203 specimens of *M. donacium* (*SL*: 14.4–88.7 mm) were collected in Hornitos (22°54.998'S; 70°17.416'W), a sandy beach at the northern side of Mejillones Bay, Antofagasta. Specimens were maintained in 1,000 l-tanks with circulating seawater. The *SL* of all specimens was measured to the nearest millimeter using a vernier caliper. Sand collected from the study site was used to allow *M. donacium* to borrow. Water temperature of the rearing tanks was monitored every 30 min with a temperature data logger (StowAway Tidbit, ONSET). Because seawater temperature in the laboratory was slightly higher (1°C to 2°C) compared with the sampling sites, molluscs were acclimated for one month before the experiment started. During the maintenance period the carnivore *C. concholepas* was fed with the mussel *Perumytilus purpuratus* (8 kg per week and 277 specimens), whereas *M. donacium* was fed daily with a mixture of three micro algae in proportion 1:1:1 (*Isochrysis galbana*, *Chaetoceros gracilis* and *Pavlova lutheri*; 3×10^6 cell/L and 203 specimens). After the initial handling the first two days no mortality was observed during the acclimation time.

Staining Experiment

Three stains (calcein – Sigma, CAS 1461–15–0; strontium chloride hexahydrate [strontium chloride] – Sigma, CAS 10025–70–4, and alizarin red S – Sigma, CAS130–22–3) were used at different concentrations and immersion periods (Table 1). Concentrations and immersion periods were chosen in accordance with previous studies (Day et al. 1995, Kaehler &

McQuaid 1999, Moran 2000, Fujikura et al. 2003). Thirteen animals covering the whole size range available were randomly assigned for each treatment. The staining process was standardized as followed: (1) animals were placed in 4 l aquaria with aerated sea water containing the respective stain; (2) each aquarium was placed in the dark to prevent light degradation of the fluorescent chemicals during the immersion period; and (3) after immersion, molluscs were reared in the laboratory during 20 days to allow growth of the organisms. A control group was treated in exactly the same way without adding staining dye to the water.

Detection of Growth Marks

After the 20-day rearing period all animals were sacrificed. Empty shells were cleaned and oven-dried at 60°C for 24 h. A transverse shell section was cut across the longest growth axis. For the detection of incorporated marks produced by the immersion in strontium chloride, the transverse sections were embedded using Epoxycure resin (Buehler) before successively polishing on glass slides with 125–68–30–12 and 5- μ m SiC powder, and finally 1- μ m Al₂O₃ suspension (Brot). Finally, the embedded shell sections were gold-coated. The detection of Sr-enriched marks was performed using a scanning electron microscope (SEM) JEOL (JSM-6360LX). Once the Sr-marks were recognized as bright bands, strontium concentrations in those bands and outside the bands (background) were determined by an energy dispersive X-ray spectrometer (EDS) coupled to the SEM. The electron beam was irradiated at an accelerating voltage of 15 kV and a lifetime of around 150 s.

For detection of calcein and alizarin red marks, thin sections were prepared by successive polishing of transverse shell sections glued to a thin glass plate, as described earlier. Marks were detected under a fluorescence microscope (Olympus BX51) using blue (460–490 nm) and red (330–385 nm) light, respectively.

TABLE 1.

Stains, concentrations, immersion periods and results on quality of marks and mortality for treated Chilean abalone *Concholepas concholepas* and surfclams *Mesodesma donacium*.

Stain Concentration (mg·l ⁻¹)	Immersion Period (h)	Quality of Mark		Mortality (n)		
		<i>M. donacium</i>	<i>C. concholepas</i>	<i>M. donacium</i>	<i>C. concholepas</i>	
Strontium chloride						
Low: 225*	17	No mark	No mark	1	0	
	24	No mark	No mark	3	0	
	High: 900†	17	No mark	Faint mark	0	0
		24	No mark	Faint mark	2	0
Alizarin red						
Low: 50	3	Faint mark	Faint mark	2	0	
	6	Faint mark	Clear mark	2	0	
	High: 100	3	Faint mark	Clear mark	3	1
		6	Clear mark	Clear mark	1	0
Calcein						
Low: 50	3	Clear mark	Clear mark	1	1	
	6	Clear mark	Clear mark	2	2	
	High: 100	3	Clear mark	Clear mark	3	0
		6	Clear mark	Clear mark	4	0
Control						
—	—	—	—	1	1	

*, †: 30, 120 times, respectively, the concentration of strontium in coastal sea-water in Antofagasta (7.5 mg/l on average; J. Román personal communication).

Viability: Mortality, Growth, and Body Condition Index

Dead animals were registered daily and extracted from the tanks. Growth and *BCI* were calculated at the end of the rearing period.

Absolute growth rate was measured as shell growth along time:

$$\text{absolute growth rate} = \frac{SL_2 - SL_1}{t_2 - t_1} = \frac{\Delta SL}{\Delta t}$$

where, SL_1 is the shell length before staining (t_1) and SL_2 the shell length at the end of the rearing period (t_2). Because *M. donacium* growth was too slow to be detected reliably with a caliper, the shell length increase was determined on sectioned shells with a micrometer installed in a light microscope by measuring the distance (μm) between the staining mark and the growth tip. For growth comparisons we used the linear relationship between growth rate and \overline{SL} (Gulland & Holt 1959):

$$\frac{\Delta SL}{\Delta t} = a + b \times \overline{SL}_{(t)}$$

where \overline{SL} is the mean shell length between SL_1 and SL_2 , a the intercept and $-b$ the slope of regression, corresponding to K (the curvature parameter of the von Bertalanffy growth model).

For body condition comparisons, the *BCI* was estimated as:

$$BCI = \frac{DM}{SL} \times 100$$

where, *DM* is the soft tissue mass (g) dried at 65°C for 48 h and *SL* the shell length (mm).

Statistical Analysis

A log-linear analysis on a cross tabulation table ($4 \times 2 \times 2$) was performed to test effects of the different treatments on mortality (Zar 1984). An analysis of covariance (ANCOVA) was used to compare growth rates between treatments, with marker, concentration and time exposition as factors and *SL* as the covariate. Correspondingly, an ANCOVA was performed to compare *BCI* between treatments using the same factors and the covariate. Data were linearized to fulfill ANCOVA assumptions. Tukey honest significant difference (HSD) test for unequal sample sizes was used for multiple comparisons (Zar 1984). An isolated or "hanging" control group design was chosen to analyze the control treatment for analysis of covariance. All analyses were performed using the software STATISTICA for windows (StatSoft, Inc. 1998).

RESULTS

Quality of Marks

Results of the staining experiments are summarized in Table 1. Calcein produced clearly visible fluorescent growth bands in shells of both species at all concentrations and exposure times (e.g., Fig. 1a, d). Alizarin red markings were successful in a lesser extent: In *C. concholepas* a clear mark can be detected at concentrations of 50 mg·l⁻¹ after 6 h immersion and at 100 mg·l⁻¹

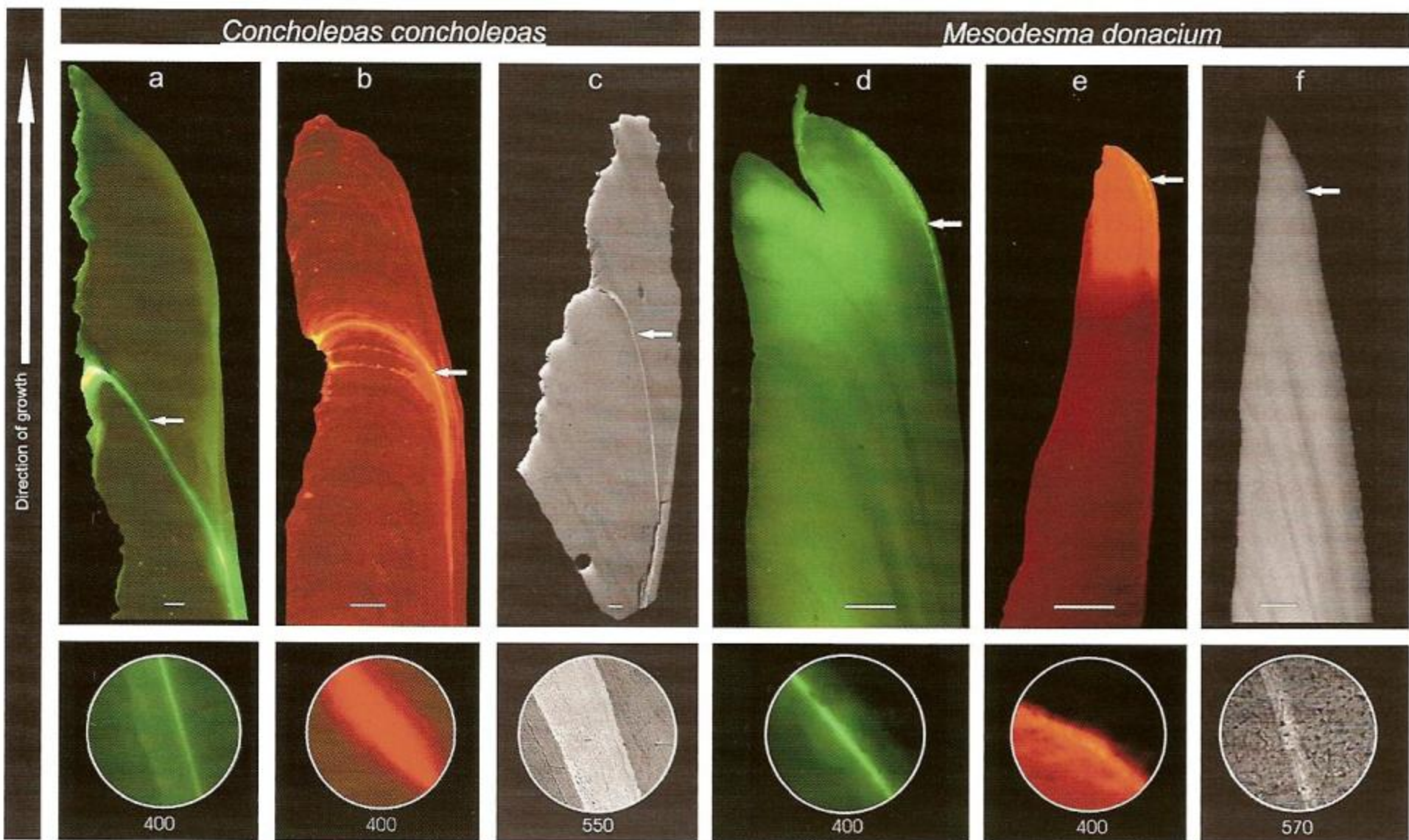


Figure 1. Photomicrographs of shell sections (*Concholepas concholepas*: a, b, c; *Mesodesma donacium*: d, e, f) after staining. The left side of each shell section corresponds to the outside part of the shell, whereas the right side corresponds to the mantle cavity side. Arrows indicate the place where the bright band below was magnified. Calcein treatment (a, d): 100 mg·l⁻¹, 6 h; Alizarin red treatment (b, e): 100 mg·l⁻¹, 6 h; Strontium chloride (c, f): 2880 mg·l⁻¹, 6 h. Scalebars: 20 μm .

after 3 h and 6 h immersion (e.g., Fig. 1b). In *M. donacium* alizarine red produced distinct fluorescent bands only after 6 h immersion and at higher concentration (e.g., Fig. 1c).

Strontium chloride markings showed poor results to produce detectable bands (Table 1). Only faint marks were observed after staining at $900 \text{ mg}\cdot\text{l}^{-1}$ for 24 h immersion in shells of *C. concholepas*, whereas no marks were observed for *M. donacium*. The concentrations used here were based on the study of Fujikura et al. (2003) (30 and 120 times the strontium concentration of natural seawater). However, because it is important to know the concentration needed to produce visible marks an additional staining was performed ($2880 \text{ mg}\cdot\text{l}^{-1}$ for 24 h). This treatment produced clear bright bands in *C. concholepas* and *M. donacium* (e.g., Fig. 1c, f). Energy-dispersive X-ray spectrometry confirmed that bright shell bands in animals treated with strontium chloride at $2880 \text{ mg}\cdot\text{l}^{-1}$ for 24 h correspond to enhanced levels of strontium. The background strontium concentration was close to the detection limit (below $2 \text{ counts}\cdot\text{s}^{-1}$), whereas the strontium concentration in the bands was notably higher (Fig. 2), indicating that strontium was accumulated because of the staining process during shell formation. The boundary between strontium-enriched and nonenriched areas appeared more distinct compared with fluorescent and nonfluorescent areas. No differences in stability of marks were observed between the stains; all the marks were readily detected in stored samples at least seven months after the experiment.

On the other hand, interspecific differences in staining success were observed. Shells of *C. concholepas* showed wider and clearer bright bands than shells of *M. donacium*, particularly for strontium chloride and alizarin red markings (Fig. 1, Table 1). Moreover, because bright bands in shells of *M. donacium* were close to the shell margin, they were often difficult to identify.

Effects of Staining on Viability

Analysis of covariance showed differences in growth rates of *C. concholepas* between markers ($F_{3,108} = 4.1651$, $P = 0.018$) whereas no differences were found for concentrations, immersion periods or interactions among these factors. The HSD test, however, did not reveal the individual differences between markers because the P value was too close to the critical value and *post hoc* test are more conservative. Based on these results, mean growth rates of the studied length classes were plotted for each marker (Fig. 3). Animals tagged with strontium chloride showed a lower mean growth rate in almost all length classes, whereas faster mean growth rates were observed in specimens marked with calcein, alizarin, and nontreated individuals. Growth of *M. donacium* was slow (around $0.002 \mu\text{m}\cdot\text{day}^{-1}$) and only small specimens formed bright bands, allowing measures of growth increments. Moreover, some treatments failed to produce a detectable mark on the shells (Table 1). Consequently, comparisons of short-term growth between treatments of this species were not possible.

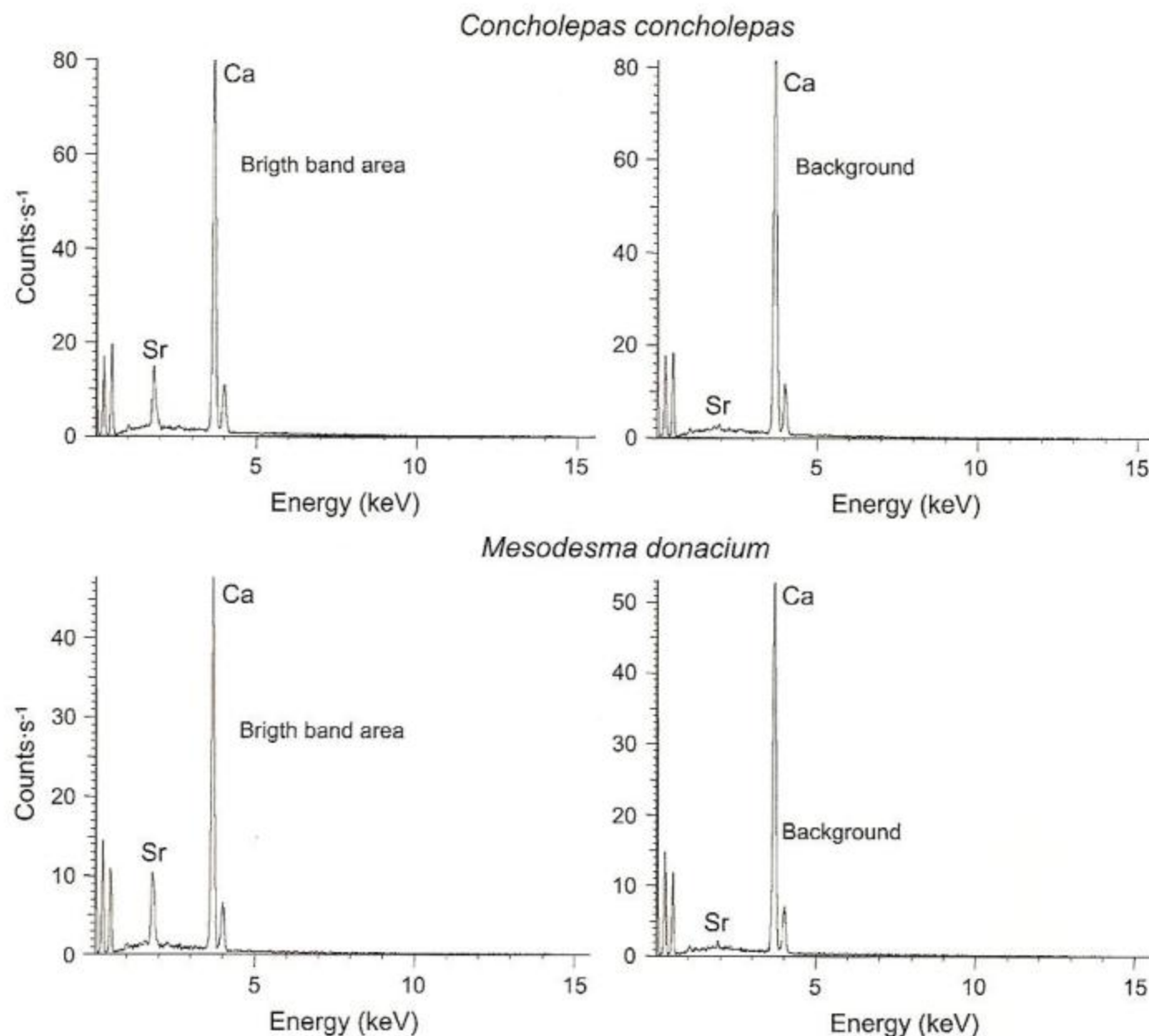


Figure 2. Strontium concentrations detected by energy dispersive X-ray of bright bands and background areas in shell sections of *Concholepas concholepas* and *Mesodesma donacium* stained with strontium chloride ($2880 \text{ mg}\cdot\text{l}^{-1}$, 6 h).

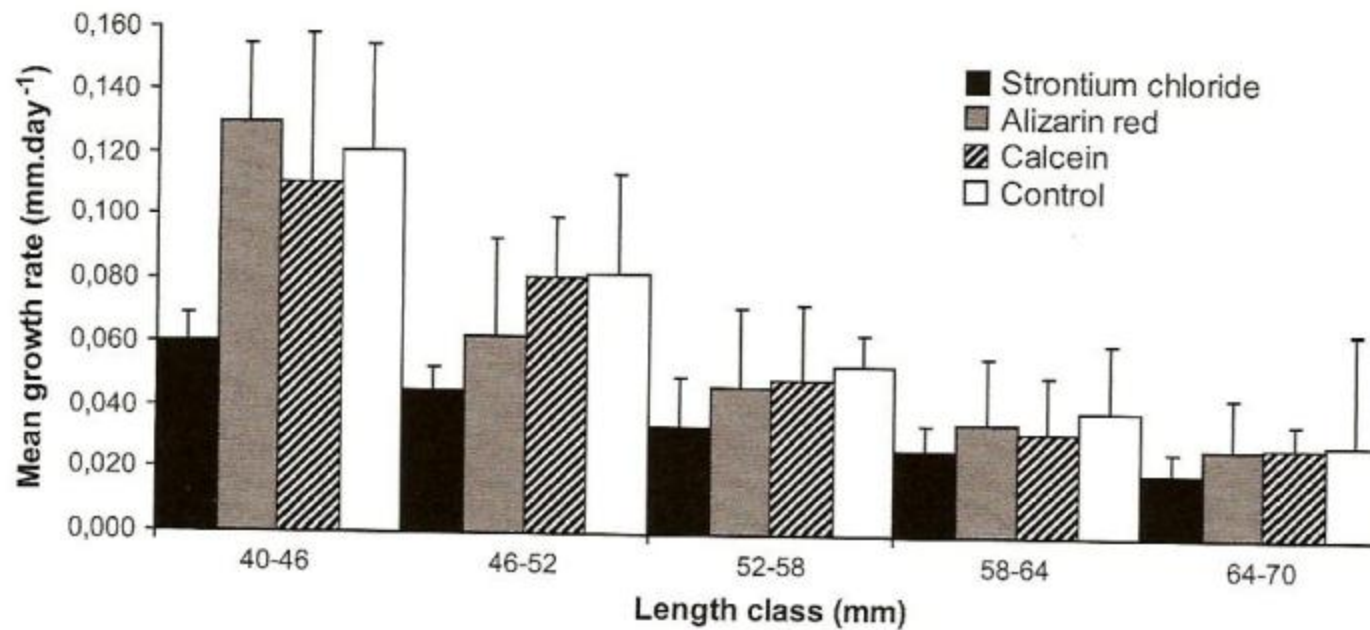


Figure 3. Comparison of growth rates ($\text{mm}\cdot\text{day}^{-1}$) of *C. concholepas* stained with strontium chloride, calcein and alizarin red among length classes. Standard deviations are included.

Analysis of covariance showed significant different of *BCI* between concentrations of stains for *C. concholepas* ($F_{3,29} = 8.208$, $P = 0.007$); the HSD test for multiple comparisons showed that observed differences in *BCI* are explained by low and high concentrations of strontium chloride. No significant differences in *BCI* between factors were found for *M. donacium*. Mean *BCI* corresponding to the high and low concentrations of strontium chloride and each stain and control for *C. concholepas* and *M. donacium* are shown in Table 2. Although no statistical differences in *BCI* of *M. donacium* were found between stains ($F = 0.106$, $P > 0.1$) mean *BCI* agrees well with the observed pattern of mean growth rates between treatments in *C. concholepas* (Fig. 3): animals marked with strontium chloride showed the lowest mean *BCI*, those marked with alizarin red and calcein showed intermediate values and non-marked animals showed the highest mean *BCI*.

The number of dead animals for each treatment and the control are summarized in Table 1. For both species mortality was relatively low, only four individuals (2.36%) of *C. concholepas* and 24 (14.20%) of *M. donacium* died after staining whereas only one individual of each species from the control group did not survive. The number of dead animals was independent from treatments for *C. concholepas* ($\chi^2 = 0.120$, $df = 10$, $P = 0.99$) and *M. donacium* ($\chi^2 = 0.985$, $df = 10$, $P = 0.999$).

TABLE 2.

Comparison of mean *BCI* between concentrations and stains for *Concholepas concholepas* and *Mesodesma donacium*.

	Mean <i>BCI</i>	
	<i>M. donacium</i>	<i>C. concholepas</i>
Low concentration (strontium chloride)	1.951	10.268
High concentration (strontium chloride)	1.647	6.282
Strontium chloride	1.773	8.428
Alizarin red	2.039	8.732
Calcein	2.083	8.835
Control	1.977	10.748

DISCUSSION

Quality of Marks

The incorporated marks in shells of *C. concholepas* and *M. donacium* showed a different quality, depending on (1) the stain type, concentration and immersion time and (2) interspecific differences in growth rate. Calcein produced clearly visible fluorescent bands in shells of both species, even at low concentrations and immersion times, whereas alizarin red often showed imprecise bands only at higher concentrations and immersion periods (Fig. 1, Table 1). Strontium chloride produced clear and narrow bright bands; however, it needed a high concentration (around 30 times that of calcein) and a long immersion time, thus making this staining method expensive and time-consuming for this species. Our results agree with several studies showing that calcein produces clear marks, which enables short-term, high-resolution growth studies (e.g., Rowley & Mackinnon 1995, Day et al. 1995, Kaehler & McQuaid 1999, Hernaman et al. 2000, Heilmayer et al. 2005, Thébault et al. 2005).

In general better marks were observed in *C. concholepas* than in *M. donacium* (Fig. 1, Table 1). This agrees with Day et al. (1995) who showed that the success of staining depends on the growth rate of each species because the transfer of ions or the stain through the mantle epithelium is quicker in rapidly growing organisms. As can be seen in Figure 1 *C. concholepas* grew faster than *M. donacium*. Hence, the observed differences in staining success can be attributed to these differences in growth rate. Additionally, as the rate of shell accretion decreases throughout the life span of many organisms, an age-dependent effect on the success of staining can be expected (Day et al. 1995, Thébault et al. 2005), which explains the higher staining success in small individuals compared with large ones.

Viability of Individuals After Staining

Assessing the suitability of a stain for short-term growth studies includes that marked individuals have unaltered viability after treatment. Traditionally mortality rate was the only parameter used to evaluate the effects of staining (e.g., Monaghan 1993, Brooks et al. 1994, Day et al. 1995, Bumgardner & King 1996, Gelsleichter et al. 1997, Kaehler & McQuaid 1999, Fujikura

et al. 2003, Bernhard et al. 2004). However, parameters evaluating sub lethal effects on treated animals (activity rates, filtration rates, body condition, and growth rate) have been rarely addressed (Moran 2000, Leips et al. 2001, Frenkel et al. 2002, Bashey 2004, Thébault et al. 2005). According to our results, none of the treatments affected the mortality of the stained animals, but showed significant effects on growth rate and body condition index.

Variations in growth rates of molluscs are controlled by environmental factors and physiological constraints (Wilbur & Owen 1964, Schöne et al. 2003). The underlying goal of a study looking for a suitable growth marker is the accurate measurement of growth after the mark produced by the stain and the identification of the environmental and/or physiological controls. Consequently, identifying the effects of staining on growth rate should be an obvious requirement.

Figure 3 shows the effect of the stains on the growth rate of *C. concholepas*: Animals immersed in strontium chloride showed the lowest mean growth rate along all length classes, whereas those immersed in alizarin red and calcein showed similar values to the nonmarked control individuals (Fig. 3). Calcein and alizarin red remain between the most commonly tested stains for growth studies; despite the fact that calcein seems to affect the survival of juvenile fishes (Brooks et al. 1994, Bumguardner & King 1996, Gelsleichter et al. 1997) several studies demonstrate no effects of these stains on growth in a variety of animals (Rowley & Mackinnon 1995, Moran 2000, Leips et al. 2001, Frenkel et al. 2002, Bashey 2004). Effects of strontium chloride, in contrast, have been scarcely tested. Fujikura et al. (2003) did not show conclusively results on growth effects because they did not include any control treatment and they used only three individuals in each treatment. Peck et al. (1996) found no significant differences on growth of treated and control individuals of the patellid gastropod *Nacella concinna* reared in seawater enriched with strontium (between 20–80 times the natural concentrations of strontium in seawater). However, because the shell material secreted during the experiments always contained less strontium than expected from the levels used in treatments, they concluded that *N. concinna* discriminate against strontium during shell deposition, which seems a common feature of gastropods and bivalves (Dodd 1967). A physiological mechanism, and therefore an energy investment would be needed to discriminate against strontium in a strontium-enriched environment. Because higher concentrations of strontium were used in our

experiments, consequences of this energy investment on growth rate could be expected.

The body condition index of *C. concholepas* differed between high and low concentrations of strontium chloride (Table 2), which could be related to the mechanism stated before. No significant effects of the treatments on *BCI* of *M. donacium* were detected; this may be because of the fact that the specimens closed the valves and stopped filtering water several times during the experiment. Consequently, the success of staining was lower for *M. donacium*. It is nonetheless interesting that the mean *BCI* for each stain follows a similar trend in both species (Table 2) and this resembles the trend observed for the effects on growth of *C. concholepas*, in which strontium chloride showed stronger departures from the control (Fig. 3). Because these results could hardly be obtained by chance, they may be interpreted as an approximation of the effects of the stains on *M. donacium*.

In conclusion and taking into account the results on quality of marks and effects of the stains, calcein is recommended as shell growth marker for both species for the following reasons: (1) it produces bright, long-lasting fluorescent bands with an appropriated magnification level for observation, even when the animals are stained at low concentrations (2) non detectable lethal or sublethal effects were found for this stain, (3) simpler methods are required for detection of marks (i.e., light microscopy) thus enabling the analysis of large numbers of samples, and (4) short immersion time is required for staining, which enables the tagging of the animals in the field thus reducing the manipulation effects.

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