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In situ fast marking study of manila clams (*Ruditapes philippinarum*)

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Abstract
Arcachon Bay, in the southern Bay of Biscay, is the first French production area of Manila clam (*Ruditapes philippinarum*) with an annual commercial fisheries production around 400 tons in recent years. This bivalve mollusc was introduced into the bay in the 1980s for aquaculture purpose and quickly succeeded in natural settlement so that its high market value led to the progressive establishment of a perennial fishery from the mid-1990s. Local management plans applied to this species are based on a licensing system for the commercial, protected fishery areas and days of prohibited fishing for both commercial and recreational fishermen. A minimum catch size is also prescribed by European regulation, and so growth rates are an important matter in this context. This work focuses on studying shell growth from the recapture of shells, previously marked using a chemical marker (calcein) and then grown in natural conditions, and was performed in order to determine shell growth patterns. Manila clams were marked by immersion *in situ* in seawater containing calcein. In order to study the limitations of calcein on the mark readability *in situ* and manila clam mortality, various exposure times, from 30 minutes to 1 hour, and concentrations, from 50 $mg.L^{-1}$ to 200 $mg.L^{-1}$ were tested. After a period of 35 days, 69 growth micro-increments were observed, and, it was found that increment deposition in manila clams occurs with a tidal periodicity. Moreover, this study showed that a method for fast-marking of manila clams, and potentially other species, is feasible *in situ.*

Keywords: *Calcein, In-situ* marking, ruditapes philippinarum, bivalve mollusks, fisheries

1. Introduction
Growth information is necessary for population structure assessment of economically important and exploited bivalve species. The Manila clam *Ruditapes philippinarum* (Adams and Reeve 1850), endemic to Indo-Pacific waters, is one of the most commercially exploited bivalve molluscs in the world. Introduced in different geographical areas, the Manila clam is now widely distributed along the Pacific coast of the United States, the European Atlantic coast, the Adriatic and Aegean seas and the Indo-Pacific region [1]. This species was introduced into Arcachon Bay at the beginning of the 1980s for aquaculture purposes [2] and it rapidly colonised all intertidal flats of the lagoon. Arcachon Bay has the highest catch levels in French waters at around 400 tonnes per year [3, 4]. Current methods for age estimation of bivalves rely on quantification of growth rings on the shell surface. However, for this species, it is not possible to distinguish external growth increments due to typically slow growth in winter and also from occasionally unfavourable summer conditions (*e.g* hypoxia). Therefore, establishing a fixed, internal shell mark and could potentially be used to investigate growth. Several chemical markers have been tested to validate animal age through the deposition of an internal growth increment, as the organisms exposed to chemical markers incorporate it into the growing calcified structures. An efficient marker must present certain characteristics: being harmless to the organisms, detectable, easy-to-use, long-lasting etc. Several markers have been used and experience has demonstrated that marker suitability is species-specific [5, 6]. Among fluorochromes, calcein has presented little toxicity and reliable marking quality [6, 7]. In the present study, the potential of *in-situ* marking with very short exposure time to the fluorochrome calcein has been investigated in *R. philippinarum*, specifically in relation to mark quality and mortality rate, depending on concentration and exposure time. Determination of the periodicity of shell growth ring production typically is accomplished by marking shells in the laboratory and then the animals return to the wild. This long manipulation of the shellfish increases the stress level and consequently the mortality level of the organisms.

Moreover, in the specific context of Arcachon Bay characterized by a lot of small areas production, it is very difficult to realize one 'traditional' marking study in the laboratory across this entire of these areas without to introduce the bias in the methodology between different locations. Consequently, this *In-situ* fast marking study of Manila Clams wants to answer at two questions:

- Is it possible to realize *In-situ* fast marking study in thirty minutes to follow the differential growth of Manila Clams in many locations during the same environmental conditions and to limit the potential effects of the marking step on the growth of this shellfish?
- The tidal deposition of growth ring: is it clearly present from the cold shock as observed by Richardson [8] in Wales or from calcein-marked individuals during 3 hours used the benthic chamber in the Gulf of Morbihan [9]?

2. Materials and Methods

2.1 Study site

Arcachon Bay is a 156 km² semi-sheltered lagoon on the south-west coast of France. Mostly composed of tidal flats (110 km long within the inner lagoon); this mesotidal system is characterized by a sediment composition ranging from mud to muddy sands and colonized by vast *Zostera noltii* seagrass meadows. The bay is influenced by external neritic waters and by continental inputs [10], and presents a semidiurnal macrotidal rhythm. The manila clam is, in terms of biomass, the dominant species of these intertidal flats [10, 11]. In February 2011, 80 living specimens were collected in the same geographical area. The samples represented all *R. philippinarum* length classes which were between 10.4 and 45.0 mm (length defined as the longest distance from front edge to back edge).

2.2 Staining experiment

The manila clams were divided into 8 groups of 10 specimens, each with a similar size distribution (from 10 mm to 45 mm in length). The clams were marked by immersion *in situ* in seawater containing calcein (CAS 1461-15-0) (Fig. 1). In order to study the limits of calcein on the mark readability *in situ* and the manila clam mortality, various calcein exposure times, from 30 minutes to 1 hour, and concentrations, from 50 $mg.L^{-1}$ to 200 $mg.L^{-1}$ have been tested. 80 individuals were marked during 30 minutes with the calcein concentrations of 50 $mg.L^{-1}$, 100 $mg.L^{-1}$ and 200 $mg.L^{-1}$, and during 1 hour for only the calcein concentrations of 100 $mg.L^{-1}$ with 2 replicates for each tagging condition (i.e. exposure time-calcein concentration). After the marking procedure, all manila clam groups were replaced in an experimental structure. These cages were built as a cube with a side length of 50 cm to obtain a density equal to that observed and a depth covering the life depth of this species, living mostly in the first ten centimetres of the substratum [12] (Fig. 1).

2.3. Detection and periodicity of growth mark

After a period of 35 days, all manila clams from each group were recaptured and sacrificed. Empty shells were cleaned and oven-dried at 30°C for 48h to enable the shell inclusion into the resin. To analyse internal micro-growth increments, shells were embedded in thermoplastic resin then transversely cut along the maximum growing axis (from the umbo to the ventral margin) using a precision saw with a blade thickness of 0.25 mm (Fig. 2). The thin sections (thickness of 0.2 mm) were first mounted in slides, where were then ground on a lapidary wheel using 600-grade grit followed by 1000-grade grit waterproof abrasive paper, and polished with wrapping film sheets (3M, St. Paul, MN, USA) using grits of 2000, 4000, 8000, 10 000, 15 000 and 40 000 grades consecutively on both sides. Polished surfaces were examined by stereomicroscope for scratches using reflecting light, and polishing was repeated until all visible scratches were removed.

Manila clams marked with calcein were observed through Zeiss motorized microscope combined to motorized stage, capable rebuild one mosaic image from many calibrated images and capable of fluorescent imaging with wavelengths of 460-490 nm. Image analyses were carried out by means of numerical camera (Hamamatsu) dedicated to the fluorescent light and piloted by the TNPC software (Calcified Structure Digital Processing, www.tnpc.fr) in order to determine the number of increments mineralized during the 35 days of the experiment. To identify the growth increments, two experts analysed the shell area between the fluorescent band along the growth axis parallel to the growth axis. Moreover, grey levels have been extracted by image analysis measuring the luminous intensity of each pixel along this growth axis. These measurements and its location along the growth axis were used to identify the limits between each opaque band and each hyaline band, reflecting each one growth increment.

3. Results

No mortality occurred during the experiment in any of the 8 groups. Thus, calcein marker treatments proved not to be lethal at maximal concentrations of 200 $mg.L^{-1}$ and for the longest exposure time of 1 hour. Calcein produced a clearly visible fluorescent growth band in shells at all concentrations and exposure time (Fig. 2). Calcein fluorescent bands were easily identified in all recovered manila clams from 30 minutes exposure with a concentration of 100 $mg.L^{-1}$ (Fig. 2). The mark intensity of fluorescence essentially depends on the calcein concentration; the influence of exposure time appears limited (Tab. 1).

Single marked increments have been identified by fluorescence each time the experiment was successful and the mark was distinguishable almost along the whole shell, from the ventral margin to the hinge, suggesting uptake along the whole mantle edge. Additionally, distinct growth lines are recognized under fluorescence microscopy (Fig. 3). The experts, with image analysis, identified 69 growth micro-increments on the shell from the calcein band to the edge during the experiment relevant to the period of 35 days and 69 tides (Fig. 3). Consequently, it is established that increment deposition in manila clams occurs with a tidal periodicity.

Fig 1: *In situ* fast marking study of Manila Clam with the calcein fluorochrome dye.

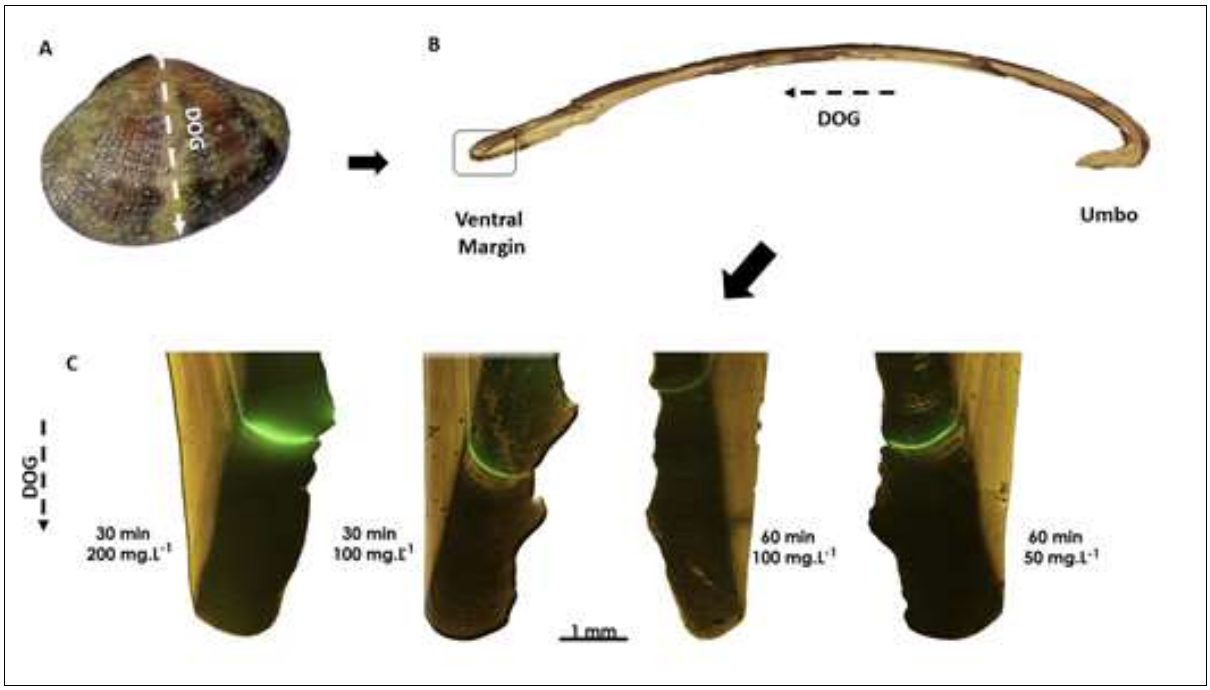

Fig 2: A Valve of Manila Clam with the line which represents the direction of growth (DOG); B) Thick section of shell (thickness of 0.2 mm); C) Ventral margin of a calcein-marked shell with a fluorescent mark (Calcein exposure time and concentration are indicated for each staining experiment)

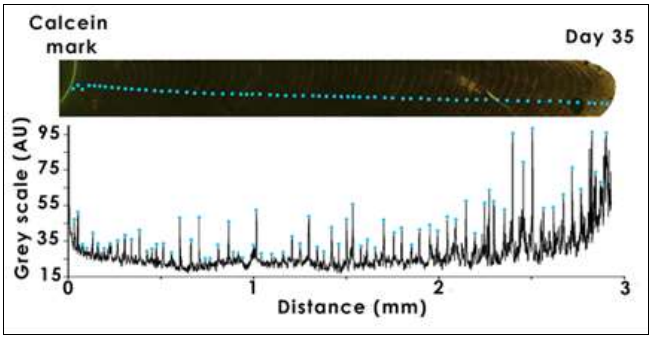

Fig 3: Magnification of a ventral margin in Manila clam: green fluorescent mark produced following 30 minutes calcein solution exposure (100 mg L−1) and observed growth increments before and after marking. Along a growth axis, grey levels have been measured (AU: arbitrary unit) and each grey peak is identified (blue circle) as the limit between each opaque band and each hyaline band, reflecting each one growth increment.

Table 1: Quality marking (Mark intensity in arbitrary units, AU) and mortality according to calcein concentration and exposure time.

Groups	Concentration (mg L−1)	Exposure time (min)	Mortality (%)	Mark intensity (AU)
1	200	30	0	122
2	100	30	0	87
3	100	60	0	83
4	50	30	0	68

4. Discussion

In recent studies on other bivalve growth, calcein exposure time and concentration used varied from 3 to 72 hours and from 10 to 500 mg.L^{-1} [6, 13, 14, 15, 16, 17]. Several studies on bivalve growth have suggested that calcein fluorochrome exhibits little toxicity [6, 13, 14, 16, 18, 19]. Similarly, in this work, the range of calcein concentrations used (from 50 to 200 mg.L^{-1}) had no lethal effect for all clam length classes during the experiment. In the same way, to limit the effects of stress to the clams during calcein marking, *In situ* experiments have been performed. A marking study of subtidal populations of manila clams in the north of the Bay of Biscay was realised in 3 hours with the calcein concentration of 150 mg.L^{-1} using benthic chambers [9]. Our study showed in 30 minutes, *in situ* exposure is enough to realise an effective shell mark on the species. Consequently, *in situ* calcein marking is efficient and has a time benefit allowing work during a single tidal period and also limits the stress to the manila clams caused by manipulation.

Manila clam's shell is composed of two aragonitic layers, an inner homogeneous layer and an outer prismatic layer. Our images analysis showed 69 growth micro-increments within the 35 days. Consequently, this periodicity was considered to be tidal. The result corroborated other studies on this species [8, 9, 20] and confirms the tidal regime as the main environmental factor dictating shell growth in intertidal habitats [21]. As shell growth of bivalves is limited to high tides [22, 23], during aerial exposure at low tide, the animals are forced to keep their valves tightly closed, and retract the mantle into the shell, leading to shell growth cessation and the formation of a growth-check line [9, 20, 24, 25]. Other intertidal bivalve species have presented a similar tidal periodicity of growth micro-increments in *Clinocardium nuttalli* [22], *Cerastoderma edule* [26, 27], *Tapes philippinarum* [8], *Chione fluctifraga* and *C. cortezi* [28], *Phacosoma japonicum* [29], *Mesodesma donacium* [30], *Phacosoma japonicum* [31] and *Saxidomus gigantea* [32], *Anadara granosa* [33]. However, it was not the case for all bivalves, as daily [34, 35, 36, 37, 38] or fortnightly periodicities have also been shown [20, 28, 29, 30, 31, 32]. These periodicity growth increment mainly could be explained by the hydric stress differences due to the location in the neritic zone (subtidal *versus* intertidal) and the emersion time in the intertidal area.

5. Conclusions

This preliminary study showed that it was possible to realise the relatively simple *in situ* marking of bivalves during a

single tide with no observed mortality. This type of approach could be applied to the other bivalve species.

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7. Authors' contributions
FA and NCM supervised the study design and the *in situ* experiment. EB analysed data. KM interpreted the data and wrote the manuscript. All authors read and approved the final manuscript.

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