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# A new approach for assessing cold-water coral growth *in situ* using fluorescent calcein staining

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**Abstract –** Research on the biology and ecology of cold-water corals (CWCs) is still in its infancy. The growth patterns of CWCs in their natural environments are poorly known. Growth rate investigations on these deep-sea reef builder species are needed to predict recovery times following damage to their ecosystems. This study investigates a new approach for analysing CWC growth rate, suitable for *in situ* application. *Lophelia pertusa* and *Madrepora oculata* (Scleractinian) were collected from the Lacaze-Duthiers canyon in the northwestern Mediterranean Sea (520 m depth), marked and then either redeployed *in situ* for 6 months, or maintained in aquaria for growth rate comparison at a constant temperature of 13 °C, corresponding to their habitat conditions. Two different types of staining (calcein and manganese) and three different exposure times (30, 60 and 240 min) were tested. The results show that calcein offers rapid incorporation and easy detection, making it particularly suitable for skeletal growth rate investigations compared with other chemical staining. *In situ* linear polyp growth rates of $7.5 \pm 1.2$ mm $y^{-1}$ and $3.5 \pm 2.1$ mm $y^{-1}$ were measured in new polyps of *L. pertusa* and *M. oculata*, respectively. Those values were significantly higher in young polyps than in older ones, where they decreased to $1.3 \pm 1.5$ mm $y^{-1}$ and $1.2 \pm 1.2$ mm $y^{-1}$. Beyond the study of coral reef growth processes, this approach offers a methodological basis for habitat quality assessment which could be used in the management of deep-sea marine protected areas (MPA).

**Keywords:** Deep-sea coral / Growth rate / Calcein and manganese labellings / Scleractinia / *Lophelia pertusa* / *Madrepora oculata* / Submarine canyon / Mediterranean Sea

## 1 Introduction

Scleractinian cold-water corals (CWC), which are widespread in the oceans, are key species for the associated deep-sea ecosystems (Freiwald et al. 2004). The hermatypic (or reef-building) CWC species *Lophelia pertusa* and *Madrepora oculata* excrete hard calcium carbonate skeletons as they grow, forming complex 3-dimensional reef structures that provide niches and nursery grounds for a variety of species, including commercial fish species like rockfish, orange roughy, and grenadiers (Costello et al. 2005; Roberts et al. 2006; Roberts et al. 2009). However, their slow growth compared with most shallow-water zooxanthellate species and their high sensitivity to disturbance render CWC reefs particularly vulnerable to anthropogenic activities like fisheries, waste discharges or ocean acidification (Hall-Spencer et al. 2002; Freiwald et al. 2004; Roberts et al. 2009; Clark and Tittensor 2010; Soffker et al. 2011; Form and Riebesell 2012; Maier et al. 2012). Huge damage (overlap and breaking of live colonies) are induced by fishing activities, particularly with abandoned fishing gear and trawl tracks (Purser et al. 2009). It is estimated that 30 to 50% of Norwegian coral reefs have been damaged by bottom trawling (Fossa et al. 2002). Consequently, in late 2006, the United Nations General Assembly called for responsible fisheries in the marine ecosystem (resolution 61/105, paragraph 80, subsection 10), including CWC environments, and marine protected areas (MPA) were established in Australia, Canada, Europe, New Zealand, the USA, and the Mediterranean Sea (Lophelia reef off Capo Santa Maria di Leuca, and the Gulf of Lion Marine Reserve).

Despite the importance of growth rate investigations on reef building CWC for predicting their resilience against

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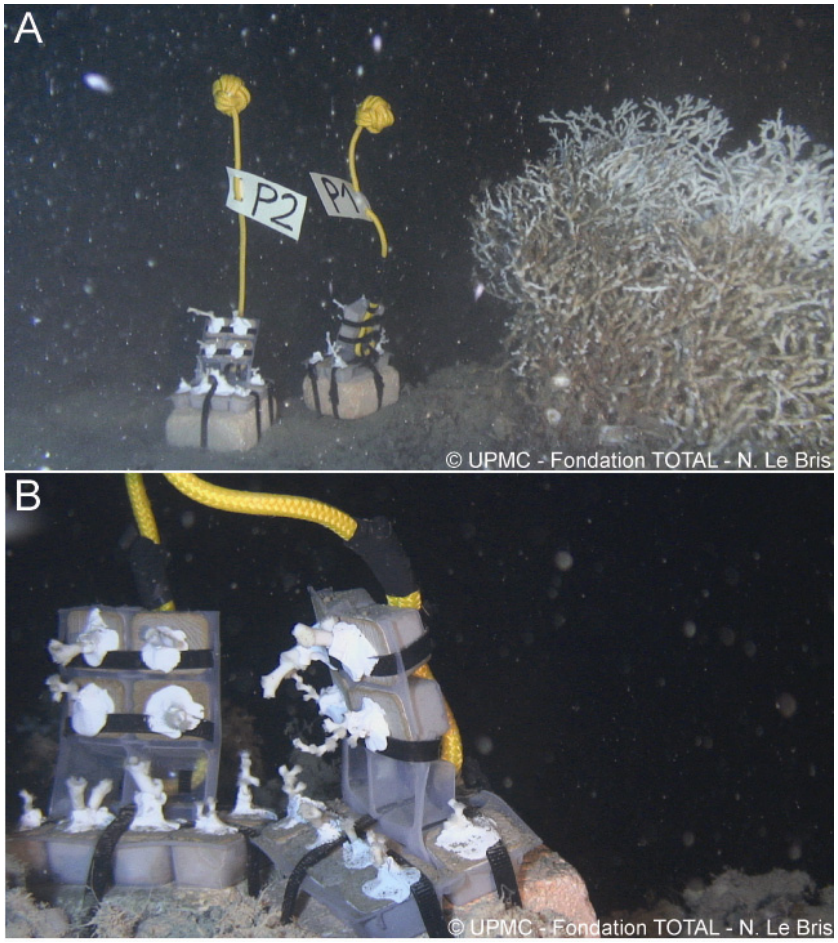


**Fig. 1.** (A) Cuttings of *Lophelia pertusa* and *Madrepora oculata* fixed on cement blocks and redeployed in Lacaze-Duthiers canyon close to a coral reef (520 m depth), after labelling on board. (B) Detail of the experimental structures showing the corals on the bottom.

various threats, their growth rates remains poorly defined, mainly due to the difficulties of accessing their habitat (Freiwald et al. 2004). Growth measurements of *L. pertusa* and *M. oculata* were performed in aquaria and revealed mean linear extension rates of 9 to 17 mm $y^{-1}$ and 3 to 18 mm $y^{-1}$, respectively (Mortensen 2001; Orejas et al. 2008, 2011), but there are possible artifacts introduced by extrapolating aquarium observations to field growth rates. *In situ* growth has been inferred from observations of colonies growing on man-made structures such as telegraph cables, oil platforms, long-lines or fishing nets, providing mean linear extension estimations of 6 to 27 mm $y^{-1}$ for *L. pertusa* (Bell and Smith 1999; Gass and Roberts 2006; Roberts et al. 2009; Gass and Roberts 2011) although no similar work has yet been done in *M. oculata*. Assuming the age of the largest colonies to be equal to the time that has passed since these artificial structures were deployed, probably overestimated age and thus underestimated growth rate (Brooke and Young 2009). Another method for growth rate estimations used skeletal stable isotopes of oxygen and carbon which provided a wide range of growth rate estimations for *L. pertusa* (6 to 25 mm $y^{-1}$) (Mikkelsen et al. 1982; Mortensen and Rapp 1998). However, the lack of an efficient sclerochronological profile to determine an adequate micro-analysis strategy inside the colony can lead to misinterpretation (Lartaud et al. 2010a). The development of radiogenic isotope analyses offered additional tools to determine the age and growth rate of CWC (Cheng et al. 2000; Pons-Branchu et al. 2005). U-series and $^{14}C$ dating are most successful on time scales from decades to thousands of years, making them unsuitable for the reconstruction of the individual growth rate of a single organism. $^{210}Pb$-$^{226}Ra$ are more adapted for recent corals (Andrews et al. 2002; Adkins et al. 2004). Using this method, Sabatier et al. (2012) assessed linear growth rates of *M. oculata* and *L. pertusa* colonies off Norway as 14.4 mm $y^{-1}$ and 8 mm $y^{-1}$, respectively. However, no independent *in situ* observations and growth rate measurements have been proposed to validate the radiometric dating technique.

Chemical marking techniques of biogenic carbonates are a good alternative for estimating skeletal growth rate, particularly for *in situ* experiments. Fluorochromes, strontium or manganese labelling provide bright artificial growth lines revealed by microscopy for many calcifying species (Kaehler and McQuaid 1999; Fujikura et al. 2003; Lartaud et al. 2010b,c) including corals (Dustan 1975; Cohen et al. 2004). Because incorporation of such chemical elements into carbonate is fast (a few hours), the particular methods provide a high-resolution chronologic framework, essential for growth

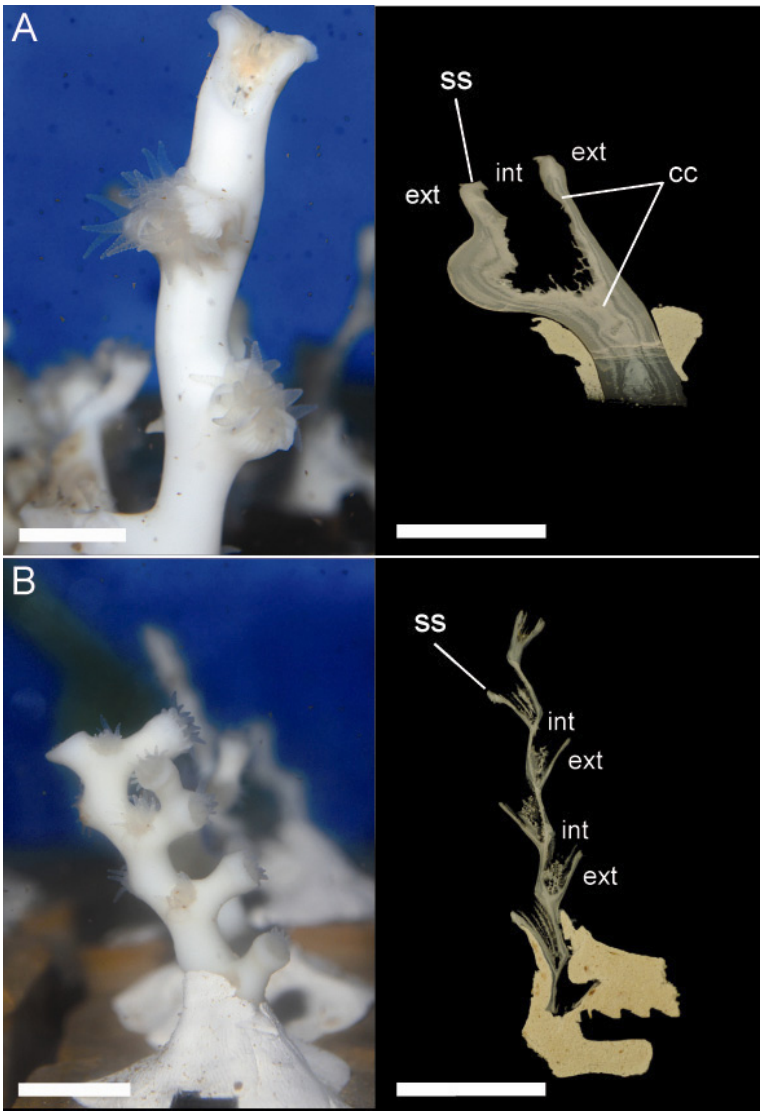


**Fig. 2.** (A) *L. pertusa* nubbin cultivated in aquaria and lengthwise section of the calyx along a major septum. (B) *M. oculata* nubbin in aquaria and lengthwise section along six polyps. cc = centre of calcification, ss = summit of the septum. Scale bar is 1 cm.

rate measurements. Using staining with alizarine red, a type of fluorochrome, Brooke and Young (2009) developed the first *in situ* measurement of growth of *L. pertusa*. New growth in coral fragments marked and deployed more than one year in the northern Gulf of Mexico was clearly visible, particularly on heavily-stained fragments. However, CWC incorporated alizarin red more slowly than shallow-water corals, which require a minimum staining period of 48 h (Brooke and Young 2009; Form and Riebesell 2012). This length of time impedes the use of alizarin red on board on a small research vessel that was not fully equipped with controlled aquarium facilities. Additionally, this stain was not taken up by the entire colony marked, as it was more visible in younger polyps than mature ones (Brooke and Young 2009).

Mark and recapture is a reliable technique for growth rate analysis of CWC but the chemical labelling chosen needs to be quickly incorporated into the coral skeleton to facilitate the operation (<1 day) at-sea, and easily detectable in both young and old polyps. In the present study, we investigated the use of two different markers (calcein and manganese) on the species *L. pertusa* and *M. oculata*, using different exposure times. The technique was developed for the first time on corals cultivated in aquaria and tested *in situ* experiment, using coral fragments marked and deployed in Lacaze-Duthiers canyon (northwestern Mediterranean Sea).

## 2 Materials and methods

### 2.1 Study site

The Lacaze-Duthiers (LD) submarine canyon runs in a northwest to southeast offshore of Banyuls-sur-mer in the Gulf of Lion. The canyon extends 23 km with depths from 150 to 1000 m at the Cap de Creus junction. Since the first dives in the 1960s using the submersible SP 300 of Commandant

**Table 1.** Mortality, budding and success rate of staining according to the rearing conditions, stain types and exposure times, Number (Nb) of colonies for *Lophelia pertusa* and *Madrepora oculata*.

| Type of culture | Species | Nb of colonies | Nb of nubbins | Total number of polyps | Marker | Concentr. (mg $L^{-1}$) | Exposure time (min) | Polyp mortality (%) | Nb of new polyps | Success rate of staining (%) |
|---|---|---|---|---|---|---|---|---|---|---|
| Aquaria | *L. pertusa* | 1 | 7 | 23 | Calcein | 150 | 30 | 0 | 0 | 75 |
| | | 1 | 7 | 20 | | | 60 | 0 | 0 | 40 |
| | | 1 | 5 | 15 | | | 240 | 6 | 1 | 100 |
| | *L. pertusa* | 1 | 6 | 24 | Manganese | 120 | 30 | 0 | 1 | 0 |
| | | 1 | 3 | 11 | | | 60 | 0 | 0 | 0 |
| | | 1 | 3 | 11 | | | 240 | 0 | 4 | 0 |
| Aquaria | *M. oculata* | 1 | 4 | 32 | Calcein | 150 | 30 | 0 | 1 | 75 |
| | | 1 | 4 | 34 | | | 240 | 0 | 2 | 45 |
| | *M. oculata* | 1 | 4 | 26 | Manganese | 120 | 30 | 0 | 1 | 0 |
| | | 1 | 4 | 29 | | | 240 | 0 | 3 | 0 |
| *in situ* | *L. pertusa* | 2 | 8 | 23 | Calcein | 150 | 60 | 26 | 4 | 100 |
| | *M. oculata* | 2 | 11 | 94 | | | 60 | 23 | 19 | 96 |

Cousteau, the LD canyon has been known to host CWC reefs (Petit and Laubier 1962; Reyss 1964; Reyss and Soyer 1965). Recent explorations (OOB-DIREN 2008-2009; AAMP MED-SEACAN 2009-2010) revealed an abundance of *L. pertusa* and *M. oculata* from 300 to 600 m depth (Fourt et al. 2012). A large part of the Lacaze-Duthiers canyon belongs to the recently created (10 November 2012) French Marine Reserve of the Gulf of Lion.

### 2.2 Staining experiments

Samples of *L. pertusa* and *M. oculata* were collected in November 2010 from six live colonies in the Lacaze-Duthiers canyon (42° 32′ 44″N, 3° 25′ 15″E) at 520 m depth, using the Super Achille ROV operated by COMEX (Fig. 1). On board, the coral fragments were transferred into aerated tanks maintained at 13 °C using a chiller, and were later placed in aquaria in a cold room (13 °C) at the laboratory. The corals were maintained in a 80 L tank with continuous flow of Mediterranean seawater pumped from 4 m depth at a rate of 18.5 L $min^{-1}$. The seawater was not filtered and thus contained low amounts of organic and inorganic nutrients. Corals were kept in the dark and fed three times a week with *Artemia salina* nauplii (~$10^3$ nauplii $L^{-1}$).

Coral fragments from six colonies (three for *L. pertusa* and three for *M. oculata*) were cut into small nubbins, each containing 3 ± 1 polyps for *L. pertusa* and 8 ± 4 polyps for *M. oculata*, before marking with calcein ($C_{30}H_{26}N_2O_{13}$) or manganese ($MnCl_2$, $4H_2O$) and then either cultivated in aquaria or deployed in the canyon. The fragments were photographed to create a pre-deployment baseline data for branch extension and addition of new polyps.

Thirty one nubbins from one colony of *L. pertusa* (=104 polyps) and 16 nubbins from one colony of *M. oculata* (= 121 polyps) were used for the staining experiments in aquaria. In order to test the feasibility of *in situ* daily operations combining collect, marking and redeployment, they were first marked for 60 min with calcein fluorescein at 150 mg $L^{-1}$ (Mahe et al. 2010), immediately after collection. One month later, the nubbins were divided into ten groups to study the effects of different exposure times (30, 60 and 240 min) of calcein or manganese labelling for both species (Table 1). Twelve nubbins (= 41 polyps) of *L. pertusa* and 8 nubbins (= 62 polyps) of *M. oculata* were collected after two and six months for sclerochronological analyses. The eight nubbins from two colonies of *L. pertusa* (= 23 polyps) and 11 nubbins from two colonies of *M. oculata* (= 94 polyps) to be used for the *in situ* experiment were fixed on cement blocks, stored for 12 hours in tanks filled with cold-water (13 °C) for acclimation, and marked with 150 mg $L^{-1}$ of calcein for 60 min prior to the deployment in their site of collection in the LD canyon for 6 months (Fig. 2, Table 1).

### 2.3 Detection of labelling and growth rate measurements

Upon collection, the nubbins were photographed in the same position as the pre-deployment images, and live, dead and broken polyps were recounted to compare with the pre-deployment data, according to Brooke and Young (2009). The percent survival and number of new polyps were assessed for each nubbins.

Removed from the concrete bases, the polyps were cleaned for 12 hours in a hydrogen peroxide solution ($H_2O_2$ 3.4%) at 60 °C to remove the tissues, and then rinsed in demineralized water. Polyp skeletons were embedded into epoxy resin for 24 hours, cut into slices with a Buehler Isomet low-speed saw, mounted on slides, and then polished manually to ~100 $\mu$m thickness using $Al_2O_3$ powders (Fig. 2). Sections of corals marked with calcein were observed under an epi-fluorescence microscope (Olympus IX51) with excitation at 495 nm (blue light). Sections of corals marked with manganese were observed under cathodoluminescence using an optical microscope coupled to a cold cathode (Cathodyne-OPEA, 12–20 keV and 200 to 400 $\mu$A $mm^{-2}$ under a pressure of 0.06 Torr) (Lartaud et al. 2006; Mahe et al. 2010). Images were captured using a digital camera.

Image analyses were carried out using ImageJ software (rsbweb.nih.gov/ij/) in order to measure the distance from the stain bands to the outer edges of the calices. Polyp linear

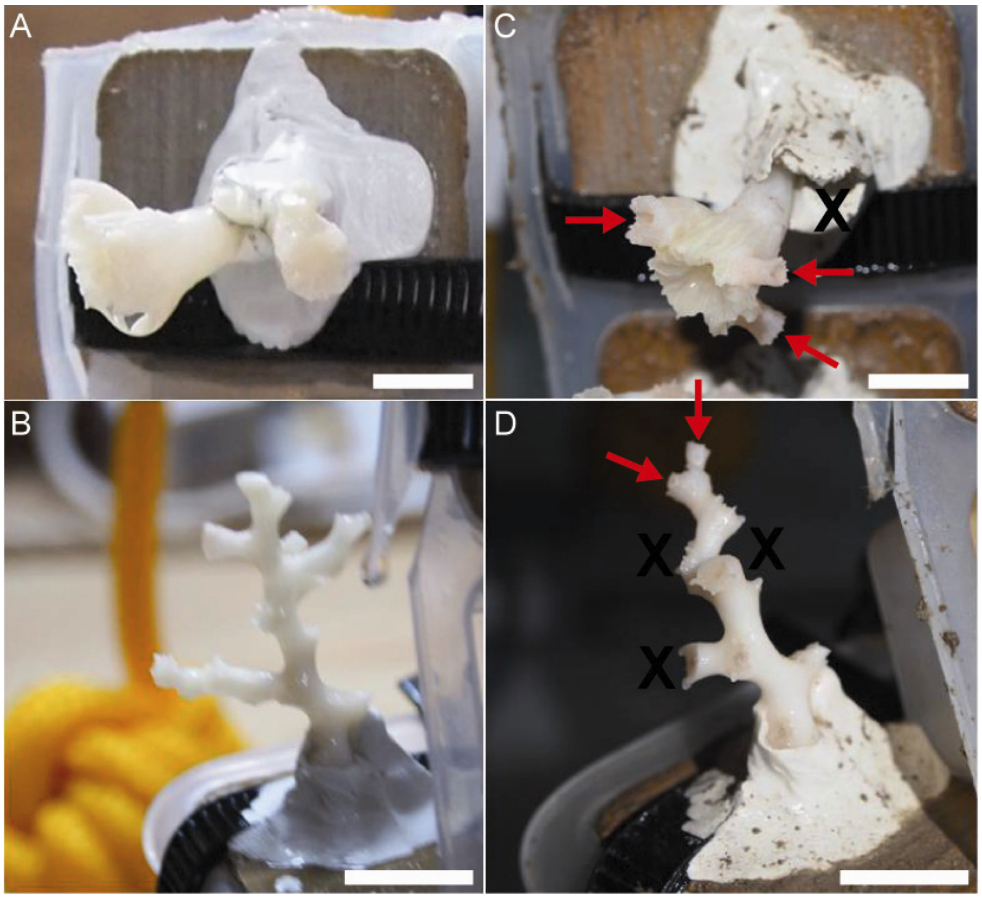


**Fig. 3.** Nubbins of *L. pertusa* and *M. oculata* before the deployment *in situ* in November 2010 (A-B) and after the recapture in May 2011 (B). Red arrows show the addition of new polyps and black crosses show the broken fragments. Scale bar is 1 cm.

growth rate was inferred from the mean linear extension (repeated 10 times on each septum of each polyp) from the stain line to the summit of the septum (= date of death). Average values are presented ± SD. Overpolish can alter the summit of the septae and thus decrease the growth rate estimations. Only polyps providing well-defined and well-preserved observations of both the labelling line and the end of the septum were chosen for growth rate measurements. The data were statistically evaluated using non-parametric Mann and Whitney U-tests to assess the growth differences between species, type of culture (*in situ* or in aquaria), and young and old polyps.

## 3 Results

Calcein and manganese had no lethal effect on CWC (Table 1). Only one *L. pertusa* polyp died in the aquaria and all *M. oculata* survived throughout the experiment. About 25% of both *L. pertusa* and *M. oculata* polyps redeployed *in situ* died. This was mainly related to fragment breakage (Fig. 3), likely caused by damage during transfer from the surface to the experiment site or natural breaking up caused by hydrological conditions.

Addition of new polyps occurred primarily at the terminal ends of the branches. In the aquaria, 8 new polyps of *L. pertusa* and 7 of *M. oculata* formed whatever the staining, which corresponded to a rate of new polyp addition of 6% for the both species. *In situ*, 4 new polyps of *L. pertusa* and 19 of *M. oculata* were formed, which corresponded to polyp addition rates of 17% and 20%, respectively (Table 1).

Results of the suitability test of the staining techniques are summarized in Table 1. Among the two chemical markers tested on the corals (calcein and manganese), only calcein was efficient. It produced a clearly visible fluorescent growth band in the coral skeleton of both *L. pertusa* and *M. oculata* at all exposure durations (Fig. 4). There was no variation in the intensity of fluorescence among exposure times. Success rate of the staining was variable (from 40 to 100%) but again unrelated to the exposure time (Table 1). The marks of the first calcein treatment conducted on board immediately after collection were undetectable for *L. pertusa* and occurred rarely for *M. oculata* (success rate of 5%). Calcein staining of corals deployed *in situ* appeared to have the same intensity as in aquaria but the overall success rate in these groups was higher (>96%) (Table 1, Fig. 4).

For *L. pertusa* and *M. oculata* from the experiments in aquaria and *in situ*, the stain bands viewed under lengthwise and transverse sections along a polyp showed that growth occurred both on the outer and inner edge of the calyx, but was less in the latter (Fig. 4). According to the observation of

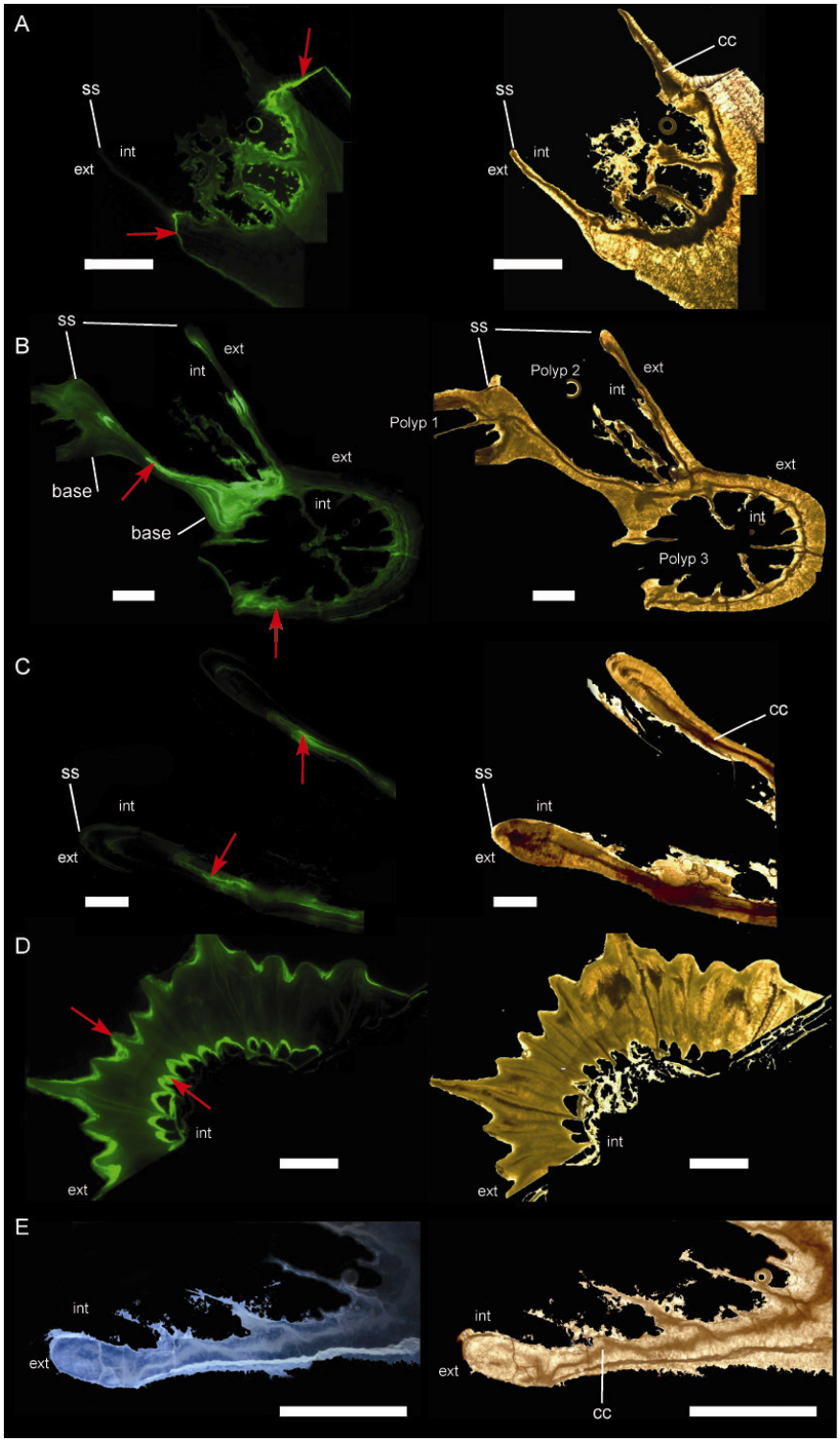


**Fig. 4.** Plate showing calcein and manganese staining revealed under fluorescence or cathodoluminescence (left) and section of polyps under transmitted light (right). Lengthwise section of a *M. oculata* polyp stained for 4 h with calcein and cultured in aquaria (A). Lengthwise and transverse sections of three *M. oculata* polyps stained for 1 h with calcein and redeployed *in situ* (B). Lengthwise section of a *L. pertusa* polyp stained for 1 h with calcein and redeployed *in situ* (C). Transverse section of a *L. pertusa* polyp stained for 4 h with calcein and cultured in aquaria (D). Lengthwise section of a *M. oculata* polyp stained for 4 h with manganese and cultured in aquaria, (E). Red arrows show the calcein stain bands. cc = centre of calcification, ss = summit of the septum. Scale bar is 1 mm.

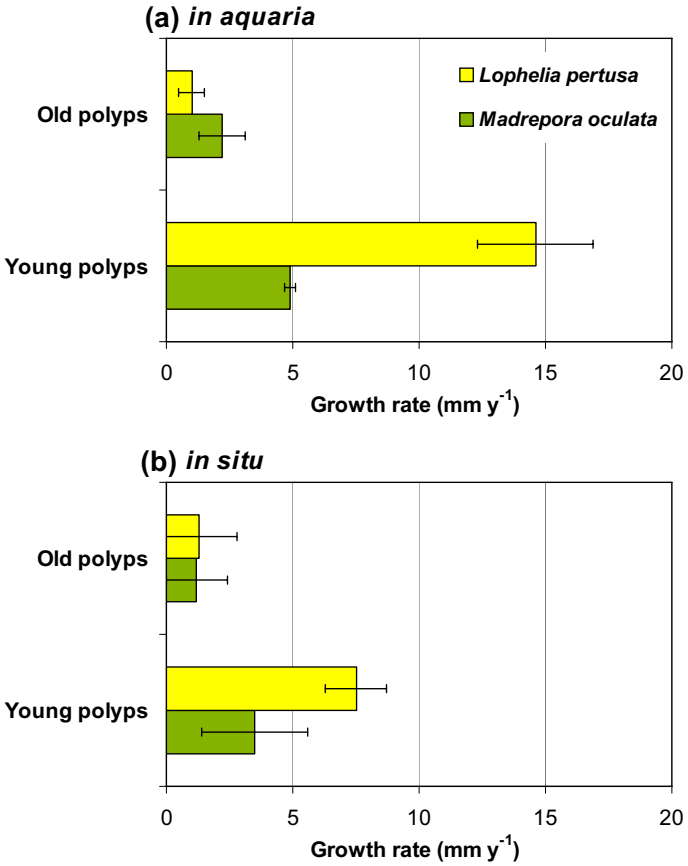


**Fig. 5.** *M. oculata* and *L. pertusa* mean linear polyp growth rates in aquaria and *in situ*. Error bars represent SD.

the stain band, the new deposition of skeleton around the calyx was not constant, including at the summit of the septum. For instance, growth repair of broken fragments (e.g., Fig. 4 top right corner) started with thin carbonate biomineralization in the inner edge of the calyx and extended to the outer edge later, to produce a well-defined septum. Additionally, as growth was lower in transverse section, a lengthwise section in major septae seemed more appropriate for growth rate measurements, because this area corresponded to the maximum growth axis and allowed linear polyp growth analysis, which reflected colony extension.

In the aquaria, new polyps of *M oculata* had a mean growth rate of $4.9 \pm 0.2$ mm $y^{-1}$ but this was only $2.2 \pm 0.9$ mm $y^{-1}$ for old polyps. The difference in growth rate between new ($14.6 \pm 2.3$ mm $y^{-1}$, mean ± SD) and old polyps ($1 \pm 0.5$ mm $y^{-1}$) was greater for *L. pertusa* than for *M. oculata* (Fig. 5).

In the submarine canyon, there was also a significant difference in the mean growth rate for both species between new ($3.5 \pm 2.1$ mm $y^{-1}$ for *M. oculata* and $7.5 \pm 1.2$ mm $y^{-1}$ for *L. pertusa*) and old polyps ($1.2 \pm 1.2$ mm $y^{-1}$ for *M. oculata* and $1.3 \pm 1.5$ mm $y^{-1}$ for *L. pertusa*) (Mann and Whitney U-test, $n = 35$, $p < 0.01$). This large range in growth rate values explained there was no significant difference between aquaria and *in situ* conditions for either *M. oculata* (Mann and Whitney U-test, $n = 31$, $p = 0.072$) or *L. pertusa* (Mann and Whitney U-test, $n = 15$, $p = 0.12$) considering all polyps together (new and old), but new *L. pertusa* had a higher growth rate in aquaria than *in situ* (Fig. 5). Furthermore, in their natural habitat, new polyps of *M. oculata* grew significantly less than new polyps of *L. pertusa* (Mann and Whitney U-test, $n = 13$, $p < 0.01$). However, there was no significant difference between the species for old polyps (Mann and Whitney U-test, $n = 22$, $p = 0.403$).

## 4 Discussion

A calcein staining could produce a fluorescent band in the skeleton at all exposure times, it appears to be a suitable technique for mark and recapture experiments on framebuilding cold-water corals. As previously observed in bivalve shells (Kaehler and McQuaid 1999; Mahe et al. 2010), the calcein labelling is rapidly incorporated into the carbonate skeleton in less than 3 h, which is faster than other types of markings tested on CWC (2 to 8 days with alizarin red in Brooke and Young 2009; Form and Riebesell 2012). However, calcein labelling requires observations of slides by microscopy, in contrast by alizarin red which can be visually identified on the skeleton. Surprisingly, staining of freshly collected corals did not give the desired results, contradicts previous on-board observations made on CWC calcification rates by Maier et al. (2012). Stressful conditions with direct exposition to calcein are suspected as samples marked after 12 hours acclimation displayed particularly elevated success (Table 1). Additionally,

**Table 2.** Comparison of in situ growth rates measured for *L. pertusa* and *M. oculata*.

| Species | Localization | Growth rates (mm $y^{-1}$) | Method | References |
|---|---|---|---|---|
| *L. pertusa* | Arctic circle, Norway | 8 | Pb-Ra dating | Sabatier et al. 2012 |
| *L. pertusa* | North Sea | 26<br>19–34<br>27.4 ± 5.0 | Observations of colonies from man-made structure | Bell and Smith 1999<br>Gass and Roberts 2006<br>Gass and Roberts 2011 |
| *L. pertusa* | Gulf of Mexico | 2.4 to 3.8 | Mark and recapture | Brooke and Young 2009 |
| *L. pertusa* | Mediterranean Sea | 7.5 ± 1.2 (new polyps)<br>1.3 ± 1.5 (old polyps) | Mark and recapture | this study |
| *M. oculata* | Arctic circle, Norway | 14.4 ± 1.1 | Pb-Ra dating | Sabatier et al. 2012 |
| *M. oculata* | Mediterranean Sea | 3.5 ± 2.1 (new polyps)<br>1.2 ± 1.2 (old polyps) | Mark and recapture | this study |

calcein is clearly observed both in young and old polyps contrary to Alizarin red (Brooke and Young 2009), making this technique particularly well adapted for individual growth pattern studies.

Manganese marking in contrast was not incorporated into the coral skeleton, at any exposure time. This technique is known to rapidly produce fine stained growth lines in calcite mollusc shells (Barbin et al. 2008; Lartaud et al. 2010c), but the marking efficiency seems to be reduced in the aragonite part of biogenic carbonates (e.g., *Crassostrea gigas*, *Cerastoderma edule*), with lower intensity of luminescence (Barbin et al. 2008; Mahe et al. 2010). Aragonite cold-water corals may not incorporate enough $Mn^{2+}$ because they do not substitute it for $Ca^{2+}$ in the crystal lattice.

Internal growth organization revealed by staining and microscopy observation indicates that engineer CWC have different growth processes to scleractinian shallow-water reef-building corals. In contrast to their shallower-growing counterparts, where small polyps are linked together by the coenosarcal tissue producing growth bands for the entire structure (Allemand et al. 2004), reef-builder CWC have individual large polyps (5 mm in diameter for *M. oculata* and 15 mm for *L. pertusa*) which grow on top of each other. In this study, the labelling highlights that a single polyp produces growth increments both inside and outside the calyx, leading to complex bi-directional growth structures (Gass and Roberts 2011) that mix at polyp junctions (Fig. 4), making the time-calibrated sampling difficult for geochemical analyses.

Even when considering new polyps, which grow faster than old polyps, the mean annual growth rate of *L. pertusa* and *M. oculata* cultivated in aquaria were lower in the present study than according to previous estimated by Orejas et al. (2008, 2011) on corals collected in the western Mediterranean Sea (9 to 17 mm $y^{-1}$ for *L. pertusa* and 3 to 18 mm $y^{-1}$ for *M. oculata*). In these previous studies, which concern growth rate estimations after 1 year (Orejas et al. 2008) and 2 years (Orejas et al. 2011) on the same experiment, growth decreased with time Orejas et al. (2008, 2011) fed the corals 5 times a week with a mixed diet composed of Mysidacea, frozen Cyclops and *A. salina* nauplii. Because these species can achieve higher capture rates when exposed to high food densities (Purser et al. 2010), the difference between these previous studies and our own suggests that high feeding rates in captivity can sustain higher growth rates of CWC compared with the natural conditions. Interestingly, compared with the experiments of Orejas et al. (2008, 2011), the mean *in situ* polyp growth rate in our study is still lower than in aquaria, which suggests that CWC corals are limited in food supply (qualitatively or quantitatively) in the Lacaze-Duthiers canyon. However they should benefit from falling organic matter during winter cascading events or severe coastal storms (Heussner et al. 2006; Sanchez-Vidal et al. 2012). Temperature drop and increase in current velocity are additional stress factors associated with dense shelf water cascading that might affect CWC coral growth (Company et al. 2008). Effects of local oceanographic conditions are supported by the wide range of growth rates measured *in situ* for both species (Table 2). With growth rates up to 34 mm $y^{-1}$, *L. pertusa* from North Sea seems to benefit from better conditions for growth compared with *L. pertusa* from the Gulf of Mexico (<4 mm $y^{-1}$) (Gass and Roberts 2006; Brooke and Young 2009). A variety of biotic and abiotic factors such as food supply, turbidity, temperature, hydrography and ocean chemistry are suggested to influence growth (Cairns and Parker 1992; Guinotte et al. 2006; Thiem et al. 2006; White et al. 2007; Roberts et al. 2009), but further studies are needed to better quantify their role.

Previous studies have shown that young polyps grow faster than old ones (Mortensen 2001; Maier et al. 2009), but our results indicate that old polyps show a strong decrease in skeletal growth, suggesting that linear extension of the colony is primarily driven by the addition of new polyps (Gass and Roberts 2011). The biomineralization of old polyps thus appears as a cementation process between polyps and branches of the colony, allowing reinforcement of the reef structure.

## 5 Conclusion

Calcein marking is easily incorporated into the skeleton of *M. oculata* and *L. pertusa*, demonstrating the suitability of this method for polyp growth process studies. The method will allow the determination of growth rates from macroscopic methods (e.g., counting of polyps, weight measurement) to be related to microscopic accretion rate and related processes. Using this approach, growth rhythms can be studied in order to investigate the impact of environmental variability.

Our staining approach can be conducted with short term (<3 h) exposure times, which are particularly suitable for *in situ* experiments. Moreover, the methodology developed here including collection, staining and redeployment could

provide a useful approach for the survey of deep ecosystems, particularly the characterization of habitat quality for CWC growth in different deep-sea habitats, including fisheries and marine protected areas.

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