Effects of acidification on a Mediterranean calcitic bryozoan

The first coastal transplant experiments designed to investigate the effects of naturally acidified seawater on the Mediterranean calcitic bryozoan *Myriapora truncata* are reported. Colonies were transplanted along a pH gradient (normal pH = 8.10, intermediate pH = 7.83 and low pH = 7.32) with increasing intervals of exposure in an area of natural volcanic CO_2 vents at Ischia Island (Tyrrhenian Sea). Multiple and integrated experiments have been performed to investigate organisms' responses to ocean acidification at several levels: skeletal structure, mineralogy, geochemistry, colony growth, soft tissue and protein profile. These transplanting experiments add themselves to a growing body of evidence showing that the acidifying effects of accelerating CO_2 emissions will be detrimental to important components of shallow water marine ecosystems

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Effetti dell'acidificazione su un briozoo calcitico mediterraneo

Vengono riportati i risultati dei primi esperimenti di trapianto realizzati per investigare gli effetti di acque naturalmente acidificate sul briozoo calcitico *Myriapora truncata*. Le colonie sono state trapiantate lungo un gradiente di pH (normale = 8.10, intermedio = 7.83, basso = 7.32) con intervalli crescenti di esposizione in un'area di emissioni vulcaniche di CO_2 all'Isola di Ischia (Mar Tirreno). Sono stati realizzati esperimenti multipli e integrati per analizzare come la specie reagisce a livello di struttura scheletrica, mineralogico, geochimico, crescita della colonia, tessuti e profilo proteico. Questi esperimenti di trapianto si aggiungono alla crescente evidenza che gli effetti dell'acidificazione prodotti dalle emissioni di CO_2 saranno nocivi per importanti componenti degli ecosistemi marini costieri superficiali

Ocean acidification (OA) is an undisputed fact [1]. Atmospheric CO_2 is the dominant greenhouse gas and its concentration, currently 380 ppm, is expected to rise to 790 ppm by 2100. This will cause a pH decrease of 0.3-0.5 units [2]. The ocean storage capacity takes up a quarter of the total atmospheric CO_2, with consequences on the geochemical balance of the oceans which are causing major changes in marine ecosystems [3]. Not only does the dissolution of carbon dioxide in sea water provoke an increase in hydrogen ions and a consequent decline in pH, but it also generates a decrease in a very important form of inorganic carbon: the carbonate ion (CO_3^{2-}). Numerous marine organisms such as corals, mollusks, crustaceans, bryozoans and sea urchins rely on carbonate ions in conjunction with organic material (proteins, carbohydrates, lipids) to form their calcareous shells or skeletons in a process known as calcification. The concentration of carbonate ions in the ocean largely deter-

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mines whether there is dissolution or precipitation of aragonite and calcite, the two natural polymorphs of calcium carbonate ($CaCO_3$), secreted in the form of shells or skeletons by these organisms [4]. Not only are calcifying organisms potentially affected by ocean acidification, but other main physiological processes such as reproduction, growth and photosynthesis are susceptible to be impacted, possibly resulting in an important loss in marine biodiversity.

Moreover, ocean acidification does not occur in isolation, but rather in concert with other changes, such as global warming, that may have synergistic, antagonistic, additive, or neutral effects. There are, however, few studies examining the interactive effects of acidification and other consequences of global change on marine organisms, and experimental data is limited. Recent reviews of OA studies show that marine biota, particularly coastal benthic organisms, are unlikely to respond uniformly to the changes expected during the 21st century, with some 'winners' (photosynthetic groups such as seagrasses and brown algae) as well as some 'losers' (most calcareous groups) [5, 6, 7, 8].

In the Mediterranean Sea, some marine calcifying taxa have the ability to create permanent carbonate structures that not only increase habit diversity but also exert a control over the distribution and abundance of associated species, promoting marine biodiversity [9]. The varied biomineralogy and proven potential of bryozoans as bioindicators [10] make them good candidates for exploring the effects of OA on skeletal morphology, biochemistry and physiology of bryozoan biomineralization.

In collaboration with the Laboratory of Benthic Ecology of the SZN, the first *in situ* biological transplantation experiments have been undertaken to investigate the effects of acidified seawater at a volcanic CO_2 vent area in Ischia, Italy [11], using the cheilostome bryozoan *Myriapora truncata* (Pallas, 1766) as study organism. Natural CO_2 venting sites are useful for assessing the long-term effects of ocean acidification on benthic biota because they can provide essential information about high-CO_2 effects on spatial and temporal scales which are otherwise difficult to address.

Here, we provide results of multiple and integrated experiments on colonies of a calcitic bryozoan transplanted at increasing duration time and at various distances from CO_2 vents, creating a gradient of different pH conditions (from 7.2 to 7.9), included that expected for 2100. The aims of these experiments were i) to analyse the effects of acidification on skeletal structure and ultrastructure on the calcitic skeleton of living bryozoan colonies, ii) to describe and quantify the mineralogical and geochemical effects; iii) to extend our knowledge in terms of how the organic constituents involved in biomineralization react to decreased pH conditions, the effects on growth, organic tissue and protein composition have also been analysed.

Materials and methods

Target species

Myriapora truncata (Figure 1 a) is a widespread Mediterranean bryozoan species forming erect tree-like colonies with a robust skeleton composed of magnesium-rich calcite (>8 wt% $MgCO_3$)[12]. Growing in dim light infralittoral and circumlittoral rocky environments, *M. truncata* occurs from the surface down to 60 m [13] on hard substrates such as rocks, crustose red algae and shells. Colonies begin growing from an encrusting base that quickly develops erect branches composed of radiating, undifferentiated autozooids (feeding units). Autozooids have calcified basal, vertical (lateral, proximal and distal) and frontal walls. The main bodies of the zooids – the polypides – are housed within the box-shaped skeletal walls of the zooids and protrude their tentacle crowns through the orifice in the frontal wall for feeding [14].

Field procedure

Colonies of *Myriapora truncata* were collected from a rocky bank far from the volcanic area and transported to the laboratory where they were maintained in 20 l aquaria with a turnover rate of seawater of 50% h^{-1}. Fragments (up to 3 branches each) were attached to tagged plastic plates using epoxy glue (HoldFast®), mounted on PVC plates and distributed in 6 cages (30 × 50 cm) (Figure 1b) with 12 fragments $cage^{-1}$. The cages were transplanted to the south side of Castello Aragonese (40° 43.84' N, 13° 57.08' E) where gas comprises 90–95% CO_2, 3–6% N_2, 0.6–0.8% O_2, and 0.2–0.8% CH_4 and is emitted at a rate of about 1.4 X 10^6 l $days^{-1}$ at room temperature and atmospheric pressure [11]. This vented gas lowers seawater pH from the normal value of about 8.17 to as low value as 6.57. Cages were fixed at 3 to 4 m depth along a 200 m transect at 3 sites (2 cages $site^{-1}$) where different mean pH conditions (normal, intermediate and low) due to CO_2 vents have been documented. Each experiment had a different number of replicates and variable duration (45 and 128 days for analyses of skeletal corrosion, mineralogical and geochemical effects, 16, 34, 57, 87 days

FIGURE 1

a) Red and orange, branched colonies of the bryozoan *Myriapora truncata* attached to plastic plates for the transplanting experiments; b) cage positioned at 4 m depth in the low pH site. Volcanic CO_2 vent bubbles are visible

for analyses of effects on growth, organic tissue and protein composition).

Laboratory analyses

Dead and live colonies were used. Colonies were soaked into a 20% sodium hypochlorite solution to remove organic material, washed in water, dried and examined uncoated in a LEO 1455VP low-vacuum scanning electron microscope. Differences in skeletal thickness were determined from cross-sections of branches. Skeletal area, branch diameter and thickness of zooidal frontal walls were measured using IMAGE.NET (Hesp Technology Software).

Mineralogical and geochemical analysis: quantitative X-ray diffraction (XRD) was carried out using an INEL Curved Position Sensitive Detector (PSD) powder diffractometer for mineralogical analysis to determine differences in Mg content of the calcite. Elemental compositions (spot analysis) and distributions were evaluated in polished sections using CAMECA SX50 Electron Probe Microanalysis.

Zooidal growth: the newly formed zooids were counted at the growing tips of colony fragments, individually tagged with plastic plates, photographed before being transplanted. A binocular microscope (Leica Z16 APO) with a digital camera (Leica DFC 300 FX) connected to a computer with dedicated software (Leica LAS©) was used.

Organic tissue: Cuticle and polypides were separated from the skeleton of the zooids using forceps under a binocular microscope. Cuticles were fixed with a solution of 2.5% glutaraldehyde and 0.25% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) for 2 h, rinsed in PBS and fixed in 2% OsO4 for 30 min. Specimens were dehydrated in acetone and embedded in EPON resin. Cuticle and polypide sections were analysed using SEM at 1000× magnification, and thickness (m) was measured on SEM pictures with Image J®.

Proteins: colony fragments were rinsed extensively with deionised water and dried overnight in an oven at 37°C. 1 g of each sample was crushed to a fine powder and dissolved with 23 ml of an aqueous solution of EDTA (0.2 M, pH 8.0) and 0.1 ml of dithiothreatol (DTT). Protein concentration was determined using a Nanodrop spectrophotometer. The samples were separated alongside protein standards of known molecular weight.

Results

During the experiment periods, at the 3 sites along the gradient pH was 8.10 ± 0.07 ('normal'), 7.83 ± 0.41 ('intermediate') and 7.32 ± 0.47 ('low').

Skeletons of live colonies in the low pH site after 45 days of exposure were less corroded than those of dead colonies, suggesting that the organic tissues enveloping the skeleton play a protective role. Colonies remained alive at the intermediate and low pH sites during the 45-day experiment, but corrosion was very striking after 128 days, with colonies from the low pH site, showing significant loss of skeleton (Figure 2). Compared to the control, these colonies also had lower levels of Mg (mean 8 versus 9.5 wt% Mg) within their skeletons. Electron microprobe mapping showed Mg to be higher in the outer layers of the skeletal walls in colonies from the normal pH site. Corrosion of outer layers of the walls probably explains the lower Mg level found in colonies exposed to acidic conditions.

M. truncata formed new and complete zooids at the normal site, whereas at the intermediate and low pH sites, neither partial nor complete zooids were pro-

duced. After 34 d at intermediate and low pH conditions, the organic cuticle which envelops the skeleton increased in thickness when compared to normal colonies, suggesting an initial protective role against dissolution of the high-Mg calcite skeleton. However, after 57 and 87 d, samples from the intermediate pH site showed a progressive decrease in cuticle thickness (57 d: 8.43 ± 0.52 m; 87 d: 5.95 ± 0.43 m). Samples from the low pH site showed a substantial decrease in cuticle thickness from 10.73 to 7.12 m between 34 and 57 d. Comparing ultrathin sections of the lophophore tentacles of samples from all 3 sites, there were no observable differences in tissue organization after 34, 57 and 87 d of exposure, whereas differences related to cellular functioning (i.e., heterochromatin densely clumped at the nuclear margins) were clearly detected in samples exposed to low pH conditions even after 34 d of exposure.

Protein analyses showed that samples from the normal pH site showed the most distinctive bands, which remained almost identical over time (34, 57 and 87 d) (Figure 3). Samples from intermediate and low pH sites showed an increase in protein production during the initial 34 d. However, after 57 and 87 d, there was a marked decrease in protein production, with samples from the low pH site showing almost no protein or very diffuse bands, particularly after the longest exposure.

Discussion

There is increasing awareness that biological systems will respond to ocean acidification at several levels, including gross morphological changes in individuals, physiological and biochemical shifts, and ecological shifts in species distribution. Our results show different effects on skeletal structures, soft tissue, growth and protein profile in the carbonate-secreting bryozoan *M. truncata* transplanted into a site along pH gradient in a natural CO_2 vent area. Samples in low pH conditions showed heavy and irregular corrosion across branches [14]. The geochemical composition may be important considering that the greatest loss is from the zooidal frontal wall, where the highest levels of Mg were found, as the solubility of calcite increases with higher levels of Mg [14]. Susceptibility to skeletal corrosion can be influenced by carbonate mineralogy and geochemistry. Corrosion could result in a weakening of the skeleton, potentially increasing vulnerability to predators and to hydrodynamic breakage. The survival of live samples of *M. truncata* in extreme acidic conditions after a short period of exposure can be interpreted as due to the presence of soft tissues (i.e., the cuticle and epithelium plus the coelom beneath) that completely envelop the skeleton of *M. truncata*, thus exerting a protective role against dissolution of the high Mg calcite skeleton of colony growing tips.

Ultrathin sections of tentacles from zooids at the colony growing tips exposed to low pH conditions for 34 d revealed cells with heterochromatin-rich nuclei especially associated with the nuclear envelope [15]. Abundance of heterochromatin may imply reduced nuclear and cell activity induced by stress factors [16].

The protein analyses, along with the physical changes, suggest that *M. truncata* may initially attempt to overcome the decrease in pH by up-regulating protein production, perhaps as a response to hypercalcification, but eventually, especially in the lowest pH condition, it appears to exhaust the biochemical energy needed to maintain this rate of hypercalcification. Seemingly the organism responses to com-

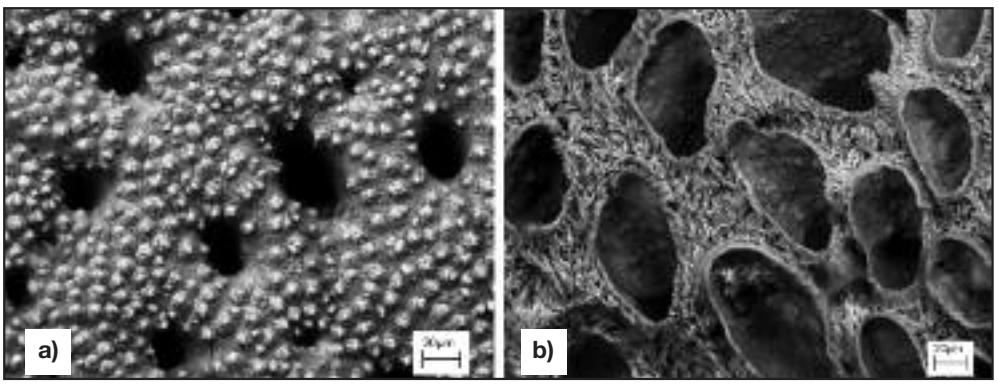

FIGURE 2

Appearance of zooidal wall pores of live colonies of *Myriapora truncata* after 128 days of exposure at normal (a) and low pH (b) sites. The skeleton is very heavily corroded at the low pH site, with extreme enlargement of the orifice. Scale bars: 20 µm (modified from [14])

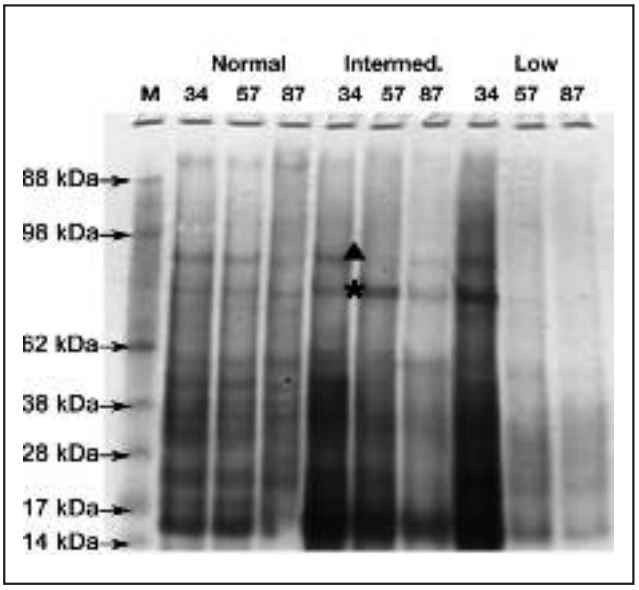

FIGURE 3 Silver stained SDS PAGE gel of *Myriapora truncata* fragments for protein analysis. Molecular weight marker (M), samples exposed to normal, intermediate and low pH conditions for 34, 57 and 87 d periods (from [14])

bat the effects of ocean acidification may be dependent on intrinsic and extrinsic factors such as metabolism or gene/protein expression and vary with life stages (e.g., developmental stages) and body size or age and, in addition, will be highly influenced by environmental conditions [17, 18].

This study highlights that biogenic minerals — since they are made up of organic and inorganic components [19]— should be considered when assessing the vulnerability of organisms and their ability to respond to ocean acidification. To date, we have a sufficient basic understanding of the biological and ecological processes involved with OA to claim that if we fail to reduce CO_2 emissions and subsequent OA, many marine species and ecosystems will experience profound modifications because effects on individuals accumulate into effects on whole ecosystems.

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