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Morphological alterations of zooxanthellae in bleached cnidarian hosts

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Abstract: Studying the morphological changes of zooxanthellae in the host gastroderm is essential to understand the mechanisms of bleaching. Transmission electron microscopy was used to observe samples from four coral species – three collected from a barrier reef in Madagascar (*Acropora digitifera* (Dana, 1846), *Echinopora hirsutissima* Milne-Edwards & Haime, 1849 and *Porites (Synaraea) rus* Forskål, 1775)) and one cut from an aquarium-grown coral (*Pocillopora damicornis* (Linnaeus, 1758) – and from the hermatypic (zooxanthellae-containing) sea anemone *Aiptasia pulchella* (Carlgren, 1943). Zooxanthellae from bleached animals showed different stages of degradation or disorganization. Some were free, detached from the host gastroderm, associated or not with host-cell remains. Others were vacuolated, with abundant reserve material globules and angular holes probably created by the loss of crystalline material during cutting. Experimentally heat-shocked *P. damicornis* harboured, moreover, a greater number of dividing algae. Bleached individuals were found to vary as regards their response to stress, and zooxanthellae expelled from heat-shocked anemones showed a greater mitotic index and a higher survival rate than algae extracted or naturally externalized from healthy individuals. We propose a combination of morphological criteria for use in diagnosing the health state of algae-cnidarian symbiosis, so vulnerable in the case of bleaching.

Résumé : *Altérations morphologiques des zooxanthelles d'hôtes cnidaires atteints par le blanchissement.* L'étude des changements morphologiques s'opérant au sein des zooxanthelles présentes dans le gastroderme hôte est essentielle pour comprendre les mécanismes du blanchissement. Quatre espèces de coraux – trois provenant d'un récif barrière à Madagascar (*Acropora digitifera* (Dana, 1846), *Echinopora hirsutissima* Milne-Edwards & Haime, 1849 et *Porites (Syneraea) rus* Forskål, 1775)) et une produite par bouturage en aquarium (*Pocillopora damicornis* (Linnaeus, 1758)) – et l'anémone hermatypique *Aiptasia pulchella* (Carlgren, 1943) ont été échantillonnées et observées au microscope électronique à transmission. Les zooxanthelles des animaux blanchis présentent différents stades de dégradation ou de désorganisation. Certaines sont vacuolées, avec de nombreux globules de réserve et des trous anguleux créés par la perte de cristaux minéraux durant la coupe. Les échantillons de *P. damicornis* soumis à un stress thermique expérimental hébergent plus d'algues en division. Dans cette étude, nous avons montré une certaine variabilité de réponse au stress parmi

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les individus blanchis. De plus, les zooxanthelles expulsées par des anémones stressées thermiquement présentent un indice mitotique et un niveau de survie plus élevés que les algues extraites ou naturellement extériorisées chez des individus sains. Nous suggérons donc que divers critères morphologiques peuvent être utilisés pour diagnostiquer l'état de santé des symbioses algues-cnidaires, si vulnérables en cas de blanchissement.

Keywords: Zooxanthellae ● Ultrastructure ● Alterations ● Symbiosis ● Coral bleaching ● Cnidarians

Introduction

Mass bleaching appears as one of the greatest threats against reef ecosystems. Over the last two decades, a profusion of studies have shown an increasing impact of this phenomenon in all tropical oceans on a massive scale (Glynn et al., 1993; Hoegh-Guldberg & Salvat, 1995; McClanahan et al., 2001). Most reef-building corals, like some other cnidarians (e.g. some sea anemones), are hermatypic, meaning that they harbour endosymbiotic dinoflagellates called zooxanthellae (*Symbiodinium microadriaticum* Freudenthal, 1962, different clades described) within vacuoles of gastrodermal cells. Symbionts enhance skeleton precipitation (in corals) and allow better growth of the host by supplying sugars, glycerol, and amino acids. On the other hand, these algae benefit from metabolic products of the host, such as CO_2, phosphates, and nitrogenous compounds (Muscatine & Porter, 1977). Bleaching occurs as a disruption of the mutualistic association between hosts and their microalgal endosymbionts (Banin et al., 2000). Expulsion or degradation of zooxanthellae or loss of their photosynthetic pigments causes loss of colour, so that the white skeleton becomes visible through the transparent coral tissues (Gates et al., 1992; Jokiel, 2004). Different factors may induce this pathology, but the most often reported stress factor is increased water temperature (Glynn et al., 1993; Hoegh-Guldberg, 1999; Warner et al., 1996). Yet rather than being triggered by a single factor, bleaching seems to result from a combination of two or more factors such as low (Goreau, 1964) or high salinity (Nakano et al., 1997), low or high UV irradiation (Gleason & Wellington, 1993; Banaszak & Trench, 1995), particle sedimentation (Meehan & Ostrander, 1997), pollution (Jones & Steven, 1997), diseases due to microorganisms (Kushmaro et al., 1996; Rosenberg & Loya, 1999; Banin et al., 2000), high (Warner et al., 1996; Hoegh-Guldberg, 1999) or low temperature (Hoegh-Guldberg & Fine, 2004).

Despite the above definition of bleaching, there are no standards or criteria for qualifying the "bleached status". This should be taken into account in comparative studies. As a matter of fact, zooxanthellar densities and pigment concentrations are found to vary considerably among species, among colonies of a same species, from one region to another of a colony, and also according to various environmental factors (Drew, 1972; Falkowski et al., 1993; Rowan et al., 1997; Stimson, 1997; Fagoonee et al., 1999; Hoegh-Guldberg, 1999; Fitt et al., 2000; Kuffner, 2005; Pillay et al., 2005; Ladrière et al., work in prep.). Apprill et al. (2007) have shown that corals that appear healthy display variable pigment concentrations and symbiont phenotypes. Moreover, inside the host tissues and along the tentacles, the distribution and density of the zooxanthellae vary from one region of the body to another, according to a photic zonation pattern (Rowan et al., 1997; Ladrière, 2006). Bleaching is thus merely defined as an obvious discoloration of a single colony or of part of it through time.

A single host species can simultaneously harbour different clades of *S. microadriaticum* (or different species of *Symbiodinium*) inside the gastroderm, and some algal clades are more temperature-resistant than others (LaJeunesse, 2001). Hence, in the event of an environmental perturbation or stress, the host may be able to change its symbiont distribution and composition. This is called the adaptive hypothesis of bleaching (Buddemeier & Fautin, 1993). Symbiont loss can thus be very heterogeneous.

The actual mechanisms by which symbiosis is disrupted and which cause damage/injury to both host and symbionts during bleaching are not yet well understood. Various cellular mechanisms lead to a reduced number of endosymbionts in the host tissue. These are (1) detachment of zooxanthella-containing gastrodermal cells (Gates et al., 1992), (2) *in situ* degradation of zooxanthellae within the gastrodermal tissue of the host (apoptosis or necrosis), and (3) release of zooxanthellae from the gastroderm into the coelenteron (exocytosis or pinching off) (Gates et al., 1992; Brown et al., 1995). Dunn et al. (2002) have also demonstrated expulsion of healthy-looking zooxanthellae, when the host is exposed to acute high temperature stress, and degradation of zooxanthellae through necrotic or apoptotic death, when the host is exposed to chronic stress.

Mise & Hidaka (2003) have shown zooxanthellae of a naturally bleached coral, *Acropora nasuta* (Dana, 1846), to be swollen and vacuolated with loss of pigmentation,

suggesting necrotic death. This might be a way for symbiotic dinoflagellates to lose photosynthetic pigments without being expelled (Hoegh-Guldberg & Smith, 1989; Kleppel et al., 1989). To our knowledge, nothing is known about the physiological mechanisms that regulate the pigment concentration through bleaching. Ralf et al. (2001) suggest that zooxanthellae remain photosynthetically competent after being expelled from bleached corals at 33°C.

As very few studies have focused on morphological changes in zooxanthellae in naturally bleached corals and as given contradictions exist between different studies, the cellular mechanisms of stress-induced bleaching remain largely unknown. New tools are needed to study this phenomenon.

The present work highlights morphological observations as a diagnostic tool for determining the bleaching state of the host and for better understanding the mechanisms that lead to disruption of symbiosis and decreased symbiont density and/or pigment concentrations. We present a preliminary study of these morphological alterations and degradation states in cnidarian endosymbionts. In addition, we have investigated the survival and division rates of zooxanthellae outside their host (*Aiptasia pulchella* Carlgren, 1943) as a complementary approach used to determine the fate of zooxanthellae after their expulsion into the environment.

Materials and Methods

Specimen collection for morphological observations

Field samples of the corals *Acropora digitifera* (Dana, 1846), *Echinopora hirsutissima* Milne-Edwards & Haime, 1849, and *Porites (Synaraea) rus* (Forskål, 1775) were collected in December 2005 from the external reef slope of the barrier reef of Tuléar (Madagascar). Two colonies (healthy and bleached) were sampled in triplicates for each species.

Other coral samples were collected from our experimental tanks, i.e. from colonies of *Pocillopora damicornis* (Linnaeus, 1758) maintained at the Nausicaä aquarium (Boulogne-sur-Mer, France). Four previously adapted control colonies of *P. damicornis* were maintained under controlled conditions at 26°C ± 1°C, while four other colonies kept initially at 26°C were heat shocked by gradually raising the temperature by 1°C per day up to 30°C ± 1°C and then maintaining this temperature for five additional days. At the end of this heating experiment, two replicate fragments were sampled for each colony (four bleached and four unbleached).

Four hermatypic (zooxanthellate) sea anemones *Aiptasia pulchella* (Carlgren, 1943) were also sampled from a tank at the Aquarium Dubuisson (University of Liège, Belgium). Two of them were induced to undergo thermal bleaching by a heat shock of two weeks at 32°C.

Only samples (coral colonies and anemones) showing an obvious loss of colour were taken into account as bleached samples. Loss of colour was checked with the Coral Watch Card edited by the University of Queensland.

Microscopy procedures

For each species (corals and anemones), healthy and bleached organisms were compared according to ultrastructural morphological criteria. For all samples, two ultrathin slices were prepared for observation by transmission electron microscopy.

Observations were first made on healthy and "naturally" bleached corals (sampled in the field). Our findings were then compared with observations on *P. damicornis* coral kept in an aquarium and bleached under heat stress. As hermatypic anemones are emerging as a model for coral bleaching, easier than corals to maintain and to prepare for microscopy, we included this model in our study so as to test it in the present context and possibly to consolidate our results.

Samples were fixed in 2.5% glutaraldehyde (in 7/10 seawater at pH 7.2-7.4) and then rinsed and kept in seawater containing 20 mM NaN_3. Coral fragments were kept for several weeks at 4°C in 0.2 M EDTA pH 8.0 until complete dissolution of the calcified skeleton. They were then further fixed for 2 h in 2.5% glutaraldehyde solution (in 7/10 seawater at pH 7.2-7.4) and post-fixed for 1 h in 1% OsO_4 in distilled water. The tissues were then dehydrated by an ethanol/epoxypropane procedure (through a graded series of alcohol to absolute ethanol) before embedding in epoxy resin (SPI-CHEM, Spi-pon 812). The resin blocks were cured at 60°C for 72 h before the specimens were trimmed and prepared for sectioning. For final orientation, 500-µm-thick sections were cut with a diamond knife, stained in toluidine blue, and viewed by light microscopy. For EM, ultra-thin sections (~70 nm thick) were produced with a diamond knife on a Reichert-Jung ultra-microtome (Ultracut E), contrasted with uranyl acetate and lead citrate, and observed with a Jeol JEM 100-SX transmission electron microscope (TEM) at 80 kV accelerating voltage.

Testing the survival of zooxanthellae outside the host

The impact of expulsion on the division rate and mortality of zooxanthellae was assayed in the case of the hermatypic anemone *Aiptasia pulchella* under aquarium conditions. Three kinds of zooxanthellae suspensions were prepared and compared: 1) three replicate suspensions of intact

zooxanthellae extracted from five living, healthy anemones (each time) by the usual method (gently crushing the host tissues with a manual potter mortar), 2) three replicate suspensions of naturally externalized zooxanthellae collected from a mucus accumulation close to the pedal disk of at least ten non-bleached anemones each time, and 3) three replicate suspensions of zooxanthellae expelled (from 15 anemones each time) after induction of bleaching of 45 healthy anemones by increasing the temperature from 26 to 32°C over 72 h (2°C/24 h), collected as naturally externalized ones. The extraction method was routinely used to obtain intact algae. Collected algae were washed in 0.2 µm filtered seawater, resuspended in fresh aerated seawater under a 12-h light/dark cycle (60 µmol m^{-2} sec^{-1}), and incubated at 25 ± 1°C for 22 days.

Ten samples were taken periodically (from each of the three replicates for each condition) to evaluate the percentages of dividing and dead cells in the population. The algal mitotic index (MI), defined as the percentage of cells with a division plate (Wilkerson et al., 1983), and the mortality rate, defined as the percentage of dead cells, were determined in parallel by examination of each replicate under 40x magnification with an Olympus BX 50 microscope. Calculations were done on 30 microscopic fields (small squares) of a Bürker cell (haematocytometer) containing at least 15 cells per square. Two Bürker cell counts (one for the mitotic index, and one for the mortality rate) were performed five times for each replicate solution at each sampling time. The % MI equation has been used before *in vitro* as a method to determine stress (Wilkerson et al., 1988; Jones, 1997). Damaged cells, observed under phase contrast, were considered dead when permeable to a 1% solution of Trypan blue, according to Freshney (1987). The propidium iodide/fluorescein diacetate method, more sensitive, was found inadequate because it interferes with the fluorescence of the photosynthetic pigments. *t*-tests were done to compare the mitotic index and mortality rates of the zooxanthellae under the three conditions (extracted, naturally externalized, "bleaching-expelled").

Results

Morphology of zooxanthellae in bleached vs. unbleached organisms

In all studied coral and anemone species, quite similar differences appeared between healthy and bleached organisms. These differences concerned the ultrastructural morphology of the zooxanthellae rather than their number in the host gastroderm.

In healthy corals, the gastroderm appeared as a consistent tissue with tightly disposed cells and tiny intercellular spaces (Fig. 1A). Regularly distributed zooxanthellae were located in vacuoles inside cytoplasmic extensions of the gastrodermal myoepithelial cells. They were enclosed within a succession of adjoining layers including the algal cell membrane, the algal cell wall, and the symbiosome (an apparently multilayered structure that closely adheres to the vacuolar membrane of the host cell). The zooxanthellae were generally surrounded by a very thin cytoplasmic flange of the myoepithelial host cell. Most of them (more than 80%) showed a regular, round section with a continuous cell wall (Fig. 1B). The organelles of the zooxanthellae were conspicuous, around a central nucleus characterized by condensed chromosomes. A peripheral electron-dense chloroplast was found to occupy more than 50% of the algal cell volume. The chloroplast generally showed regularly spaced thylakoids parallel to each other (Fig. 1B, C). A pyrenoid (chloroplast extension surrounded by a starch sheath) was also visible in most cases, depending on the section plane (Fig. 1B). Electron-lucent globules of reserve material (probably glucids) and angular holes (probably left by a crystalline material lost during cutting) were sometimes observed in the algal cytoplasm. Only a few algal cells (< 5%) were found to be dividing.

In healthy *Aiptasia pulchella* anemones, zooxanthellae were numerous in a consistent gastroderm with tight intercellular spaces. They displayed the same morphological characteristics as those observed in healthy corals: a regular shape with a continuous cell wall and a symbiosome adhering to the host vacuolar membrane, a central nucleus with condensed chromosomes, a peripheral chloroplast with parallel thylakoids, a visible pyrenoid, and some small reserve globules (Fig. 2).

In naturally bleached corals (*Acropora digitifera*, *Echinopora hirsutissima*, and *Porites rus*), the gastroderm appeared damaged. Especially the median and basal zones of the cells become inconsistent, showing only strands of clear cytoplasm and large intercellular spaces containing damaged cell remains (Fig. 3A). Free zooxanthellae (representing ~ 30% of the total count), apparently detached from their host cells, also appeared in these spaces or released into the gastrocoele. Those released into the gastrocoele, representing 40-50% of the released symbionts, were either nude or still surrounded by the thin cytoplasmic flange of the host cell. Moreover, especially in *E. hirsutissima*, areas of the gastroderm showed large, electron-lucent "empty" vacuoles about the size of a zooxanthella (Fig. 3B). Of the algae that were not released, some appeared severely degraded but many (30 to 60% of the total count) still remained in less altered parts of the gastroderm of these bleached corals. Like those released from the host tissue, these zooxanthellae showed external and internal signs of alteration. First, a space of variable size was apparent between the vacuolar membrane of the host cell and the

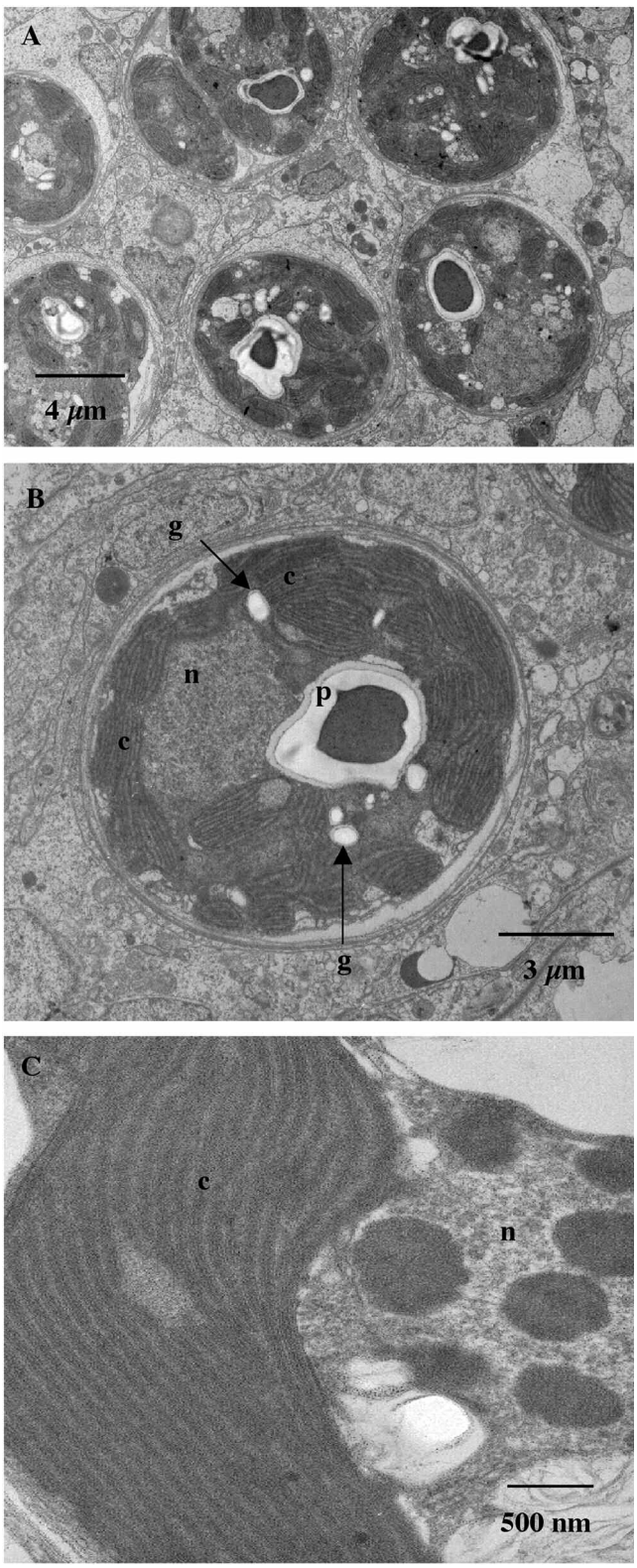

Figure 1. Healthy coral, *Pocillopora damicornis*. **A.** Consistent gastroderm with adjoining cells and well-structured zooxanthellae. **B.** Zooxanthella section showing different organelles: **n**: nucleus with condensed chromosomes, **c**: peripheric chloroplast with parallel thylakoids, **p**: pyrenoid, that appears as a globule of starch (electron-lucent) surrounding an extension of the chloroplast (electron-dense), **g**: reserve globules. **C.** The parallel position of the thylakoids in the chloroplast (**c**).

Figure 1. Corail sain, *Pocillopora damicornis*. **A.** Gastroderme cohérent, aux cellules jointives et zooxanthelles bien structurées. **B.** Coupe d'une zooxanthelle montrant différents organites : **n** : un noyau avec des chromosomes condensés, **c** : un chloroplaste périphérique avec des thylakoïdes parallèles, **p** : un pyrénoïde, qui apparaît comme une gaine d'amidon (claire aux électrons) entourant un prolongement du chloroplaste (dense aux électrons), **g** : globules de réserve. **C.** Arrangement parallèle des thylakoïdes dans le chloroplaste (**c**).

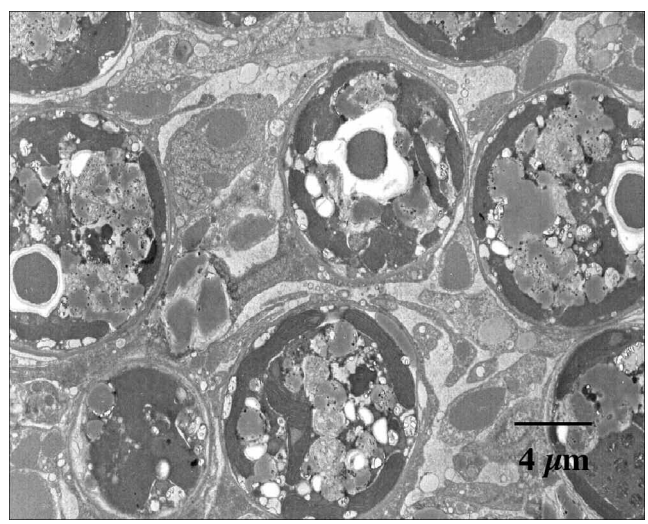

Figure 2. Gastoderm of a healthy anemone, *Aiptasia pulchella,* filled of well-structured zooxanthellae.

Figure 2. Gastoderme d'une anémone saine, *Aiptasia pulchella,* remplie de zooxanthelles bien structurées.

symbiosome, which were no longer tightly joined (Fig. 3C). This is interpreted as a retraction of the algal cell inside its vacuole. More severely altered algae were deformed, showing an irregular shape, rupture of the cell wall, and internal disorganization (Fig. 3C, D). Internal changes in the algal cells included disorganization of the thylakoids (notably loss of parallellism) (Fig. 3E), the presence, inside the algae, of large vesicles (Fig. 3C) containing angular holes probably created by loss of a hard "mineral" material during cutting, and the appearance of

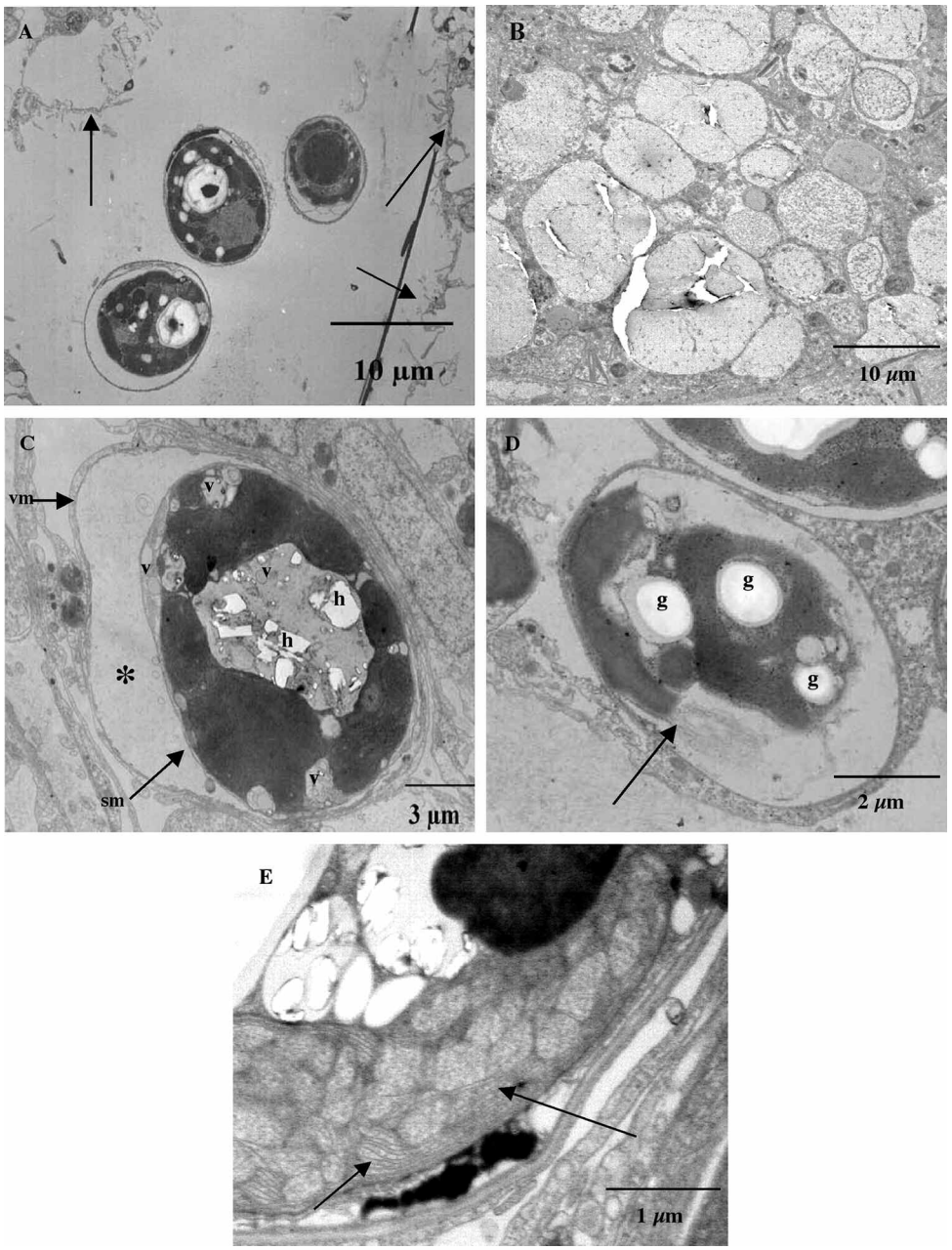

Figure 3. Naturally bleached corals. **A.** "Free" zooxanthellae still surrounded by the host cell cytoplasmic strip, after the lysis of the host gastroderm cells in *Acropora digitifera*. **B.** Empty rounded vacuoles probably left in the gastroderm of naturally bleached *Echinipora hirsutissima* by the loss of zooxanthellae. These "holes" are of the same size than zooxanthellae (about 8 µm in diameter). **C.** Retracted zooxanthella in *Echinopora hirsutissima*, with vesicles (**v**) containing white angular holes (**h**) probably created during cutting by the presence of a hard mineral material. The asterisk (*) shows the space between the vacuolar membrane (**vm**) and the symbiosome membrane (**sm**). **D.** Altered zooxanthella (necrosis) with reserve globules (**g**) in naturally bleached *Porites rus*. The arrow shows the rupture of a membrane. **E.** Thylakoids of the chloroplast loosing their organization and parallel position in *Acropora digitifera* (arrows).

Figure 3. Coraux blanchis naturellement. **A.** Zooxanthelles libres, encore entourées d'une bande cytoplasmique de la cellule hôte, après la lyse des cellules du gastroderme hôte chez *Acropora digitifera*. **B.** Vacuoles arrondies vides probablement laissées dans le gastroderme d'*Echinipora hirsutissima* naturellement blanchi par la perte de zooxanthelles. Ces "trous" sont de taille identique aux zooxanthelles (environ 8 µm de diamètre). **C.** Zooxanthelle rétractée chez *Echinopora hirsutissima*, avec des vésicules (**v**) contenant des trous anguleux blancs probablement créés durant la coupe par la présence d'un matériel minéral dur (**h**). L'astérisque (*) indique l'espace entre la membrane vacuolaire (**vm**) et la membrane du symbiosome (**sm**). **D.** Zooxanthelle altérée (nécrose) avec des globules de réserve (**g**) chez *Porites rus,* naturellement blanchi. La flèche indique la rupture d'une membrane. **E.** Thylakoïdes du chloroplaste perdant leur organisation et leur position parallèle, chez *Acropora digitifera* (flèches).

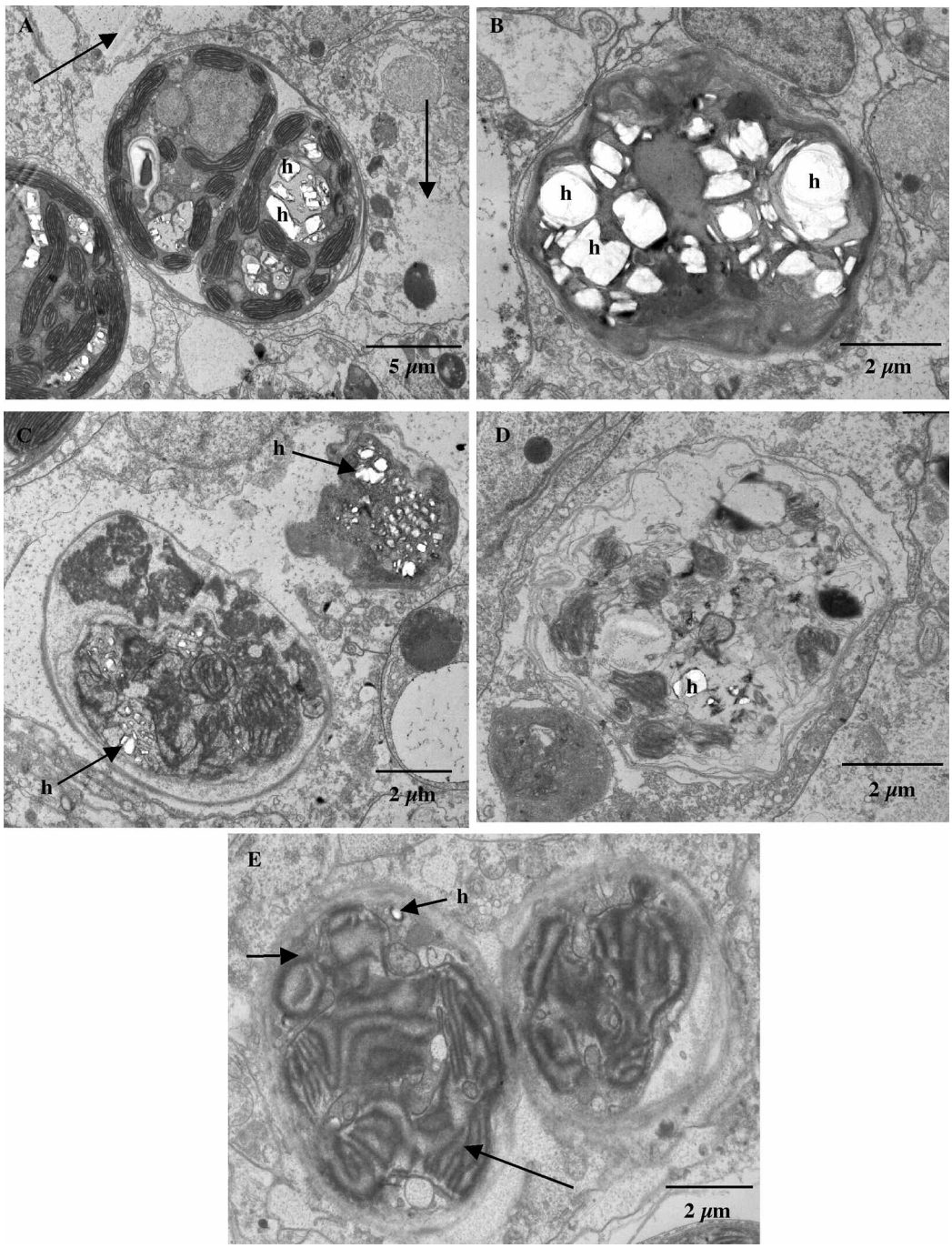

Figure 4. Experimentally bleached *Pocillopora damicornis.* **A.** Zooxanthella in division with lysis of the host cell (arrows). **B.** Degraded zooxanthella in a lysed host cell all around. **C.** Deeply altered alga showing internal disorganisation and the lysis of the different organelles. **D.** Advanced step of zooxanthella lysis with remains of organelles. **E.** Necrotic zooxanthella where thylakoids appear dilated in condensed chloroplasts (disruption of thylakoid membranes?) (arrows). For all pictures, white angular holes in vesicles of the zooxanthella were created, during cutting, probably by the presence of a hard mineral material (**h**).

Figure 4. *Pocillopora damicornis* expérimentalement blanchi. **A.** Zooxanthelle en division avec lyse de la cellule hôte (flèches). **B.** Zooxanthelle dégradée dans une cellule hôte lysée tout autour. **C.** Algue profondément altérée montrant une désorganisation interne et la lyse de différents organites. **D.** Etape avancée de la lyse d'une zooxanthelle, avec des restes d'organites. **E.** Zooxanthelle en nécrose où les thylakoïdes paraissent dilatés dans un chloroplaste condensé (rupture des membranes thylakoïdiennes ?) (flèches). Pour toutes les photos, des trous blancs anguleux dans des vésicules de la zooxanthelle ont été créés pendant la coupe, probablement par la présence d'un matériel minéral dur (**h**).

bigger electron-lucent globules (probably reserve materials such as glucids) than in healthy individuals (2 to 3 times more, Fig. 3D). As in healthy corals, only a few zooxanthellae were in division.

Although "bleaching" was macroscopically obvious in *Pocillopora damicornis* corals subjected to heat stress at 30°C in an aquarium, the zooxanthellar density was not diminished, in contrast to what occurred in naturally bleached corals. Yet we did observe disorganization of the gastroderm with increased intercellular spaces and fragmentation of host cells. No "free" zooxanthellae were observed, but the gastrodermal cells displayed large electron-lucent vacuoles having about the same diameter as a zooxanthella. As compared to healthy and naturally bleached organisms, the proportion of dividing zooxanthellae was 4 times greater (15-20%) (Fig. 4A). All zooxanthellae in division appeared as two daughter cells, each with its own membrane and cell wall, in the same symbiosome within a host vacuole. Regarding their ultrastructure, up to 40% of the zooxanthellae showed signs of alteration that can be interpreted as necrosis (Fig. 4B-E). Many zooxanthellae had an irregular shape, with a sinuous cell wall (Fig. 4B, D). Lysis and disorganization of organelles were often conspicuous inside algal cells, their main manifestation being disruption of organellar membranes to the point that they were no longer distin-

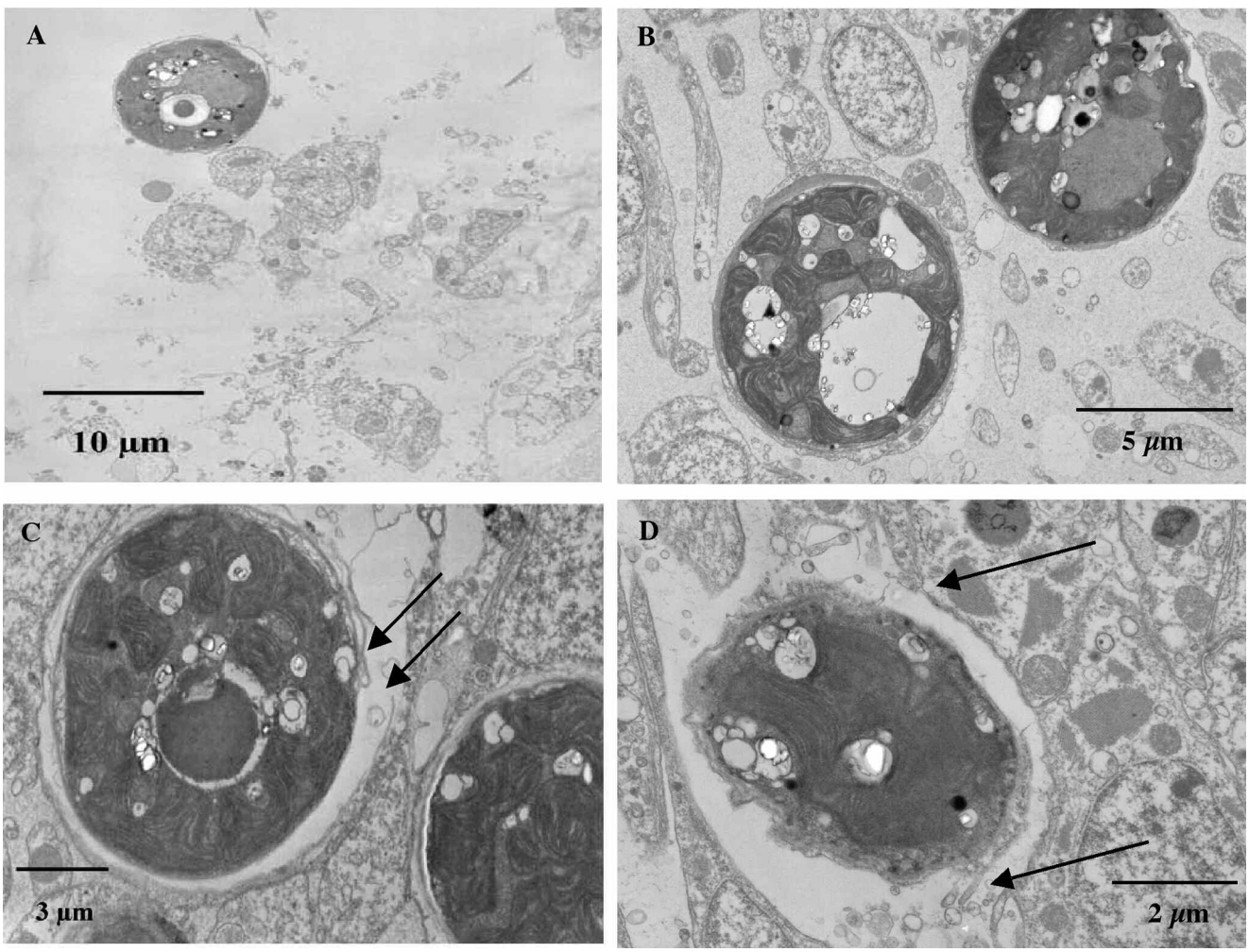

Figure 5. Gastroderm of a bleached anemone, *Aiptasia pulchella*. **A.** Freed zooxanthella and lysed gastroderm cells. **B.** Vacuolated zooxanthellae in lysed host cells. **C.** Zooxanthella with few globules of reserve material. Arrows show the lysis of the symbiosome membrane. **D.** Zooxanthella of irregular shape with a sinous periphery. The arrows show the rupture of the vacuolar membrane.

Figure 5. Gastroderme d'une anémone blanchie, *Aiptasia pulchella*. **A.** Zooxanthelle libérée et cellules gastrodermiques lysées. **B.** Zooxanthelles vacuolées dans des cellules hôtes lysées. **C.** Zooxanthelle avec peu de globules de matériel de réserve. Les flèches indiquent la lyse de la membrane du symbiosome. **D.** Zooxanthelle de forme irrégulière à périphérie sinueuse. Les flèches indiquent la rupture de la membrane vacuolaire.

guishable (Fig. 4C, D). In chloroplasts, the thylakoids appeared dilated, loosing their parallelism and showing some disruption of membranes (Fig. 4E). Most zooxanthellae (~ 75%) appeared vacuolated and exhibited vesicles containing angular holes, probably left by mineral crystals as in naturally bleached organisms (Fig. 4A-E).

Naturally bleached anemones showed alterations very similar to those observed in corals. Freed algae were again present in the gastrocoele or the spaces between inconsistent and lysed gastrodermal cells (Fig. 5A). The algal cell wall was shaped irregularly and the symbiosome membrane was disrupted in many cases (Fig. 5B-D). As in experimentally bleached corals, the zooxanthellae appeared vacuolated and exhibited angular holes. This alteration correlated with host cell lysis in 70% of the cases (Fig. 5B). There were greater spaces between organelles (dispersed or lysed). In addition, the majority of symbionts contained 2 to 3 times fewer electron-lucent reserve globules than those of healthy anemones (Fig. 5C).

Zooxanthellae survival

Potter-extracted algal cells appeared more damaged than those naturally externalized or those expelled after induced thermal bleaching, the mortality rates in these respective populations being 21.4 ± 5.2%, 18.5 ± 3.4%, and 13.6 ± 4.2% on the first day. Extracted zooxanthellae showed a further mortality increase over the following days (to 34.9% on day 3 and 36.5% on day 5 after extraction), whereas naturally externalized zooxanthellae and ones expelled after induced bleaching displayed a stable mortality rate over this period (18.2-19.5% and 12.4-13.8% respectively) (Fig. 6A). After day 5, the mortality decreased and remained stable for the next 15 days, at around 9.8 to 13.4% dead cells in all three populations.

Zooxanthellae externalized or "bleaching-expelled" from sea anemones were further examined by phase contrast and fluorescence microscopy. The former revealed alterations (shape changes, disorganization of the internal structure) similar to those observed by TEM in zooxanthellae within tissues from bleached anemones. Moreover, damaged cells displayed a loss of natural fluorescence, suggesting an alteration of the photosynthetic apparatus, in keeping with the changes in thylakoids observed by TEM.

We then estimated the MI in populations of zooxanthellae released or extracted from anemones. There appeared significant differences (*t*-test, $P < 0.001$) between "bleaching-expelled" (2.9 ± 0.8%) (Fig. 6B), naturally externalized (2.0 ± 0.4%), and extracted zooxanthellae (1.6 ± 0.4%). The "bleaching-expelled" zooxanthellae seemed

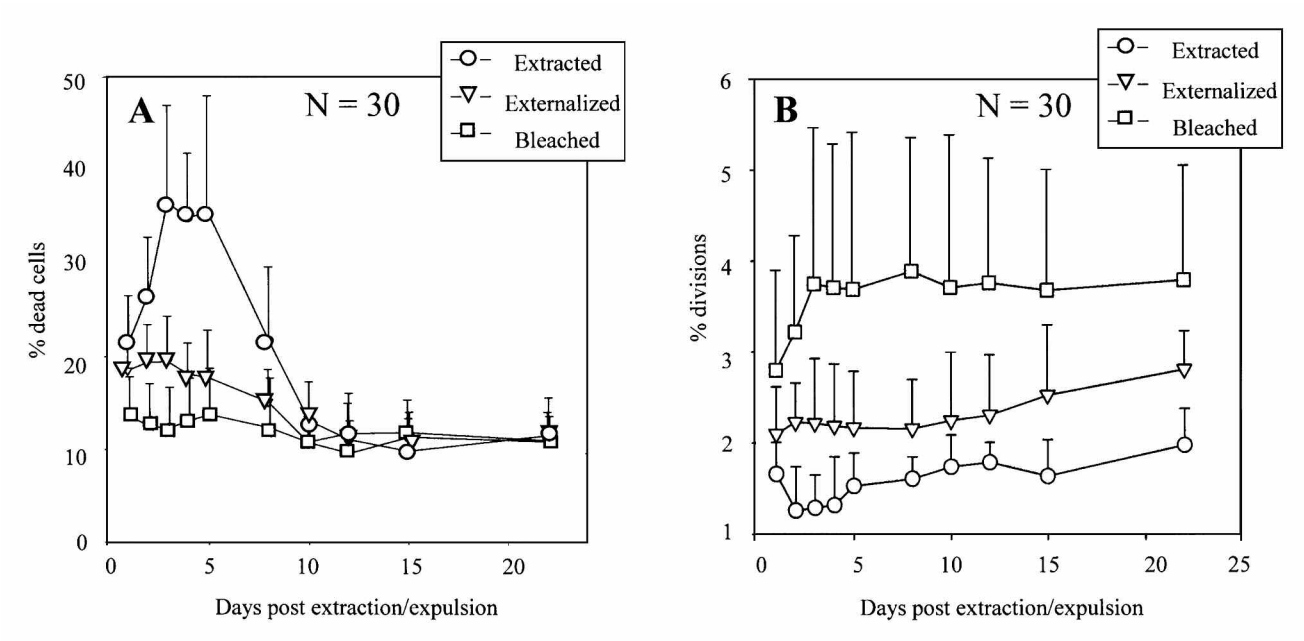

Figure 6. Percentage of dead (**A**) and dividing zooxanthellae (**B**) at different time after extraction/expulsion from intact extracted algae (O), naturally externalized algae (Δ) and "bleaching-expelled" algae ( ) from anemones *Aiptasia pulchella*. The counts were realised on 30 microscopic fields, for each sample condition, at each sample time.

Figure 6. Pourcentage de zooxanthelles mortes (**A**) et en division (**B**) à différents temps après l'extraction/expulsion d'algues extraites intactes (O), d'algues externalisées naturellement (Δ) et d'algues expulsées par blanchissement ( ) d'anémones *Aiptasia pulchella*. Les comptages ont été réalisés sur 30 champs microscopiques, pour chaque condition d'échantillonnage, à chaque temps de prélèvement.

to divide approximately 2 times more rapidly than those extracted from healthy anemones. This is in keeping with the TEM data showing enhanced division of algal cells in experimentally bleached corals. For two to five days after expulsion, the MI of "bleaching-expelled" zooxanthellae was found to increase further (up to 3.9 ± 1.5%) and then to remain stable for the next 15 days (3.7 to 3.9% of the population). The MI increase was much lesser in naturally externalized (max of 2.8 ± 0.4%) and extracted algae (max of 1.9 ± 0.4%) (significant differences, *t*-test, $P < 0.05$).

Discussion

We here provide details of the morphological alterations caused by bleaching in cnidarian hosts, at the level of the host gastroderm and its endosymbiotic zooxanthellae. Our TEM data reveal differences between healthy, naturally bleached, and experimentally bleached corals on the one hand, and between healthy and bleached anemones on the other. Slight differences also appear between corals and anemones subjected to heat-induced bleaching.

As described in the literature (Brown et al., 1995; Salih et al., 1997; Mise & Hidaka, 2003), healthy coral samples show a consistent and "well-structured" gastroderm filled with "healthy-looking" algae. Each constituent organelle is distinguishable and limited by a membrane, so that the internal organization appears structured. In healthy organisms, the chromosomes of algal cells are condensed and almost always visible in the nucleus, as is characteristic of dinoflagellates. Thylakoids are conspicuous in the chloroplasts of the symbionts and their parallelism is suggestive of active photosynthesis in the algae.

Healthy anemones have a gastroderm full of zooxanthellae sticking almost perfectly to the vacuolar membrane of the host cells, as in healthy corals. The organelles of the algae are clearly distinguishable, with intact membranes, as described above (for the zooxanthellae of healthy corals) and in the literature (Dunn et al., 2002).

In naturally bleached corals, both the zooxanthellae and the gastrodermal host cells show various alterations, in addition to a decreased zooxanthellar density (usually a 40 to 75% decrease). First, the space between the vacuolar membrane of the host cell and the symbiosome increases, seemingly as a result of retraction of the algal cell. Various stages of zooxanthella degradation or disorganization are also observed, with or without host cell lysis, preceding expulsion or necrosis. Necrosis is suggested by the rupture of organellar membranes, an increased number of reserve material globules (glucids), the presence of fewer electron-dense chloroplasts, the appearance of electron-lucent vesicles at the edges of or inside many algae, and an increased number of angular holes. The loss of chloroplast contrast might be due to loss of pigmentation, but this possibility should be investigated further. The potential loss of pigments and the observed increase in the number of vacuoles within the zooxanthellae are in agreement with the results of Mise & Hidaka (2003), showing that the zooxanthellae of a bleached coral (*Acropora nasuta* (Dana, 1846)) were vacuolated, with in some cases loss of pigmentation, suggesting necrotic death. Our data show that loss of photosynthetic pigments appears in endosymbionts that have not been expelled, as described by Hoegh-Guldberg & Smith (1989) and Kleppel et al. (1989), and that zooxanthella degradation appears within the gastrodermal tissue, as observed by Brown et al. (1995). Some zooxanthellae deteriorate but remain in the host cell despite rupture of the cell membrane, as described by Fang et al. (1998). When expulsion of zooxanthellae does occur, it is suggested by disorganization of the host gastroderm (becoming inconsistent) where detached zooxanthellae are observed. Unlike Gates et al. (1992), we saw no detachment of alga-containing gastrodermal cells but rather host-cell lysis with release of algae.

Experimentally bleached corals, like naturally bleached ones but more conspicuously, displayed different stages of zooxanthella degradation. The zooxanthellar density did not decrease in aquarium-bleached corals, but the proportion of dividing zooxanthellae was four times greater than in healthy or naturally bleached corals. This suggests that the stability of the zooxanthellar density results from two processes that compensate for each other: expulsion of zooxanthellae and algal multiplication (an additional factor to be taken into account being the heterogeneous distribution of zooxanthellae within their host). Baghdasarian & Muscatine (2000) have evidenced a significant linear correlation between the rate of algal expulsion and the rate of algal division. Factors that increase the division rate (e.g., elevated temperature) also increase the expulsion rate. With such an increase of dividing cells, we can therefore assume that expulsion of algae occurs or that colonies are entering a recovery or adaptation stage. Morphologically, experimentally heat-shocked corals were more severely affected than "naturally" bleached individuals. Algal retraction might be an objective sign of a post-stress reaction appearing before and/or during degradation or expulsion of algae. Large reserve globules are also observed within altered symbionts, in keeping with the results of Salih et al. (1997), who observed lipid globules (another reserve material) in altered zooxanthellae. We can thus hypothesize that zooxanthellar symbionts receive a signal that "prepares" them for expulsion, leading them to make reserves (after the signal from the host and before expulsion or before they are subjected to a greater stress in the host tissue). If this view is accurate, then the signal preceding expulsion or degradation of the algae might induce symbionts in all

bleached corals to divide and increase their reserve material globules. This should be confirmed by cytophysiological observations. Furthermore, vacuoles containing crystals might be a forewarning of cell degeneration. Such crystals can be interpreted as signs of exocytosis dysfunction in stressed algae, leading to precipitation of some compounds in vacuoles. Gates et al. (1992) propose that isolated algae can result from various mechanisms (exocytosis, apoptosis, necrosis, pinching off, host cell detachment), but in our material, we have observed only necrosis of the alga or necrosis of the host cell, resulting in the release of zooxanthellae associated or not with host-cell remains (as observed by Searle et al., 1982).

Actually, in healthy and naturally bleached corals, we observed only two mechanisms leading to a decreased zooxanthellar density: host-cell lysis with release of zooxanthellae (intact or not) and *in situ* degradation of the algae. Yet no decrease in zooxanthellar density was observed in experimentally bleached corals. The loss of colour should thus reflect a loss of pigments due to thylakoid alteration. Still, we must bear in mind that the distribution and expulsion of zooxanthellae can be very heterogeneous and not always be observed in microscopy because of the small area observed. Empty round spaces like "holes" the size of a zooxanthella are present in abundance in some regions of bleached corals, suggesting that these holes have been left by expelled algae. Such spaces cannot be sites of the zooxanthella degradation since no content or algal remains were observed.

Bleached anemones harbour approximately threefold fewer zooxanthellae than healthy individuals, and the algae seem to retract, with a tendency to detach from the gastroderm. Degradation of the algae can be characterized as necrotic death (different stages) according to Dunn et al. (2002), but no sign of apoptosis (fragmentation of the cell with the appearance of electron-dense bodies) was observed. As in bleached corals, many symbionts of bleached anemones show rupture of the algal cell wall, a sinuous algal membrane, rupture of organellar membranes, vacuolization, and angular holes.

In nature, to our knowledge, *Aiptasia pulchella* anemones have never been observed without endosymbiotic zooxanthellae. Loss of algae thus appears as a real stress associated with morphological alterations and damage. In keeping with the alterations observed by microscopy, experimentally produced aposymbiotic or partially bleached anemones appear considerably fragilized and mechanically much less resistant than healthy hermatypic ones (personal observations).

The zooxanthellae of bleached anemones show less reserve material than those of healthy ones, in contrast to the zooxanthellae of bleached corals. In the case of anemones, the stress applied thus results in zooxanthellae with less reserve material (due either to reduced accumulation or to increased consumption of reserves, to be assessed).

By testing the survival of zooxanthellae outside their host sea anemone (*Aiptasia pulchella*), we aimed to assess the survival potential of zooxanthellae in the external environment, since bleaching causes some zooxanthellae to be expelled from the host into the surrounding water. It is interesting to know how they respond to this new situation after the bleaching stress. We show here that "bleaching-expelled" zooxanthellae have a lower mortality and a higher mitotic index than extracted or naturally externalized algae. The high mortality observed among extracted zooxanthellae during the first days after extraction might be an artefact due to the extraction technique (potter mortar, classically used to extract zooxanthellae), but most of the damaged cells were eliminated during the washing steps and the long-term survival of these algae (from day 5 to day 22) did not differ significantly from that of the other studied algae populations (naturally externalized or bleach-expelled). Light microscopy applied to "bleaching-expelled" zooxanthellae revealed the same features as in bleached corals and anemones: vacuolization, internal disorganization, and particularly disorganization of the photosynthetic apparatus, leading to alteration of the fluorescence signal. In these algae, the MI continued to increase after expulsion. According to the adaptive bleaching hypothesis of Buddemeier & Fautin (1993), expelled zooxanthellae might not be sufficiently suited to the host. This expulsion would thus prepare the cnidaria for further recolonization by more resistant clades of algae (a high MI leading to quick multiplication of the remaining, better-suited population or to colonization by external free-living algae). The fact that algae expelled after "stress-induced" bleaching continue to divide outside the host confirms the findings of Baghdasarian & Muscatine (2000, preferential expulsion of dividing cells). These authors have shown that algae naturally expelled from *Aiptasia pulchella* and *Pocillopora damicornis* have a higher mitotic index than the algae remaining in the hosts, and that the host preferentially expels algal cells that have entered the S-phase of the cell cycle. Their results on *Aiptasia pulchella* are comparable to ours. Our results further suggest that experimentally heat-induced bleaching enhances the natural phenomenon of preferential loss of dividing cells, since temperature increases resulting in higher division rates also result in enhanced expulsion of algae (Baghdasarian & Muscatine, 2000) and hence in more severe bleaching.

Conclusion

This study highlights criteria that can be used to diagnose the health state of alga-cnidarian symbiosis: zooxanthellar

density in the gastroderm, the internal organization of the algae, chloroplast contrast, and thylakoid parallelism, organelle lysis, the abundance of vacuoles and angular holes, the abundance of dividing algae (an increased mitotic index), the presence of freed zooxanthellae. Yet laboratory experimentation with systematic observations during stress-induced bleaching is absolutely necessary in order to know the sequence of degradation phases and to define the precise beginning of morphological bleaching. Correlatively, as these induced morphological changes are coupled with a physiological response of the algae (mitotic index), it would be interesting to unravel the physiological mechanism involved, and notably to determine where, when, and how the signal(s) given to the symbiont appear(s).

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