Finding of 132, 173-cyclopheophorbide a enol as a degradation product of chlorophyll in shrunk zooxanthellae of the coralMontipora digitata

FINDING OF 13^2, 17^3–CYCLOPHEOPHORBIDE *a* ENOL AS DEGRADATION PRODUCT OF CHLOROPHYLL IN SHRUNK ZOOXANTHELLAE OF THE CORAL *Montipora digitata*[1]

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Abstract

In this study, we examined the morphology and pigment composition of zooxanthellae in corals under normal temperature (27°C) and thermal stress (32°C) conditions. Morphologically several types of zooxanthellae cells with normal and abnormal shapes were observed. Normal zooxanthellae were intact with unbroken chloroplasts (healthy), while abnormal ones were shrunk with partially degraded or broken chloroplasts, and bleached without chloroplasts. Under normal temperature, most of zooxanthellae cells were healthy and were retained in coral tissue, whereas shrunk zooxanthellae were released from coral tissue. During thermal stress, abundance of healthy zooxanthellae decreased and that of shrunk/abnormal zooxanthellae increased in coral tissue, and abundance of expelled zooxanthellae during experiment were less than normal temperature. Pigment analysis of shrunk cells showed the presence of a chlorophyll-like pigment, which is ordinarily not seen in healthy cell. From the analysis of absorption spectrum, absorption maxima and retention time during HPLC, the pigment was identified as 13^2, 17^3–cyclopheophorbide *a* enol (cPPB-*a*E) which is frequently found in marine and lacustrine sediments and protozoans that graze on phytoplankton. This is the first report of cPPB-*a*E found in symbiotic zooxanthellae on coral tissue. The production of cPPB-*a*E in shrunk zooxanthellae suggests that shrunk cells are partially

degraded cells in which chlorophylls are converted to cPPB-*a*E, a compound that is no fluorescent and has no reactivity to oxygen. Our results indicate that coral bleaching is a self-produced physiological mechanism in which zooxanthellae are degraded to avoid injury from reactive oxygen species (ROS) which are mainly generated by zooxanthellae with damaged chloroplast under thermal stress.

Key index words: coral bleaching, '13^2, 17^3–cyclopheophorbide *a* enol', degraded zooxanthellae, thermal stress, reactive oxygen species

List of Abbreviations

cPPB-*a*E, 13^2, 17^3–cyclopheophorbide *a* enol

ROS, reactive oxygen species

Introduction

Reef-building corals have symbiotic algae (zooxanthellae) within their endodermal cells. Zooxanthellae produce organic matter which is translocated to host corals and is used by them as source of organic matter. Therefore the presence of zooxanthellae is essential for the life of coral. In the last two decades world climate has changed rapidly, as a

result serious damages have occurred to reef-building corals all over the world. Coral bleaching is one of them, and it is well known that bleaching results from the loss of symbiotic zooxanthellae from the host (Hoegh-Guldberg and Smith 1989, Gates 1990, Brown et al. 1995, Jones 1997) or the degradation of photosynthetic pigments in zooxanthellae cells (Fitt and Warner 1995, Fitt et al. 2001). The apparent loss of zooxanthellae occurs with changes in environmental conditions, including high light intensity and UV radiation (Dustan 1979), elevated seawater temperature (Hoegh-Guldberg and Smith 1989), cold stress (Saxby et al. 2003, Hernández et al. 2010, Lirman et al. 2011, Paz-García et al. 2012), low salinity (Coles and Jokiel 1978, van Woesik et al. 1995), low availability of food (plankton) availability (Titlyanov et al. 1996), and bacterial infection (Kushmaro et al. 1996). High water temperature particularly affects zooxanthellae cells. It has also been shown that bacteria accelerate bleaching (Higuchi et al. 2013). During the massive bleaching in 1998 at Okinawan waters, morphological changes with loss of pigments were observed in zooxanthellae cells retained in coral tissue (Kuroki and van Woesik 1999). Different types of zooxanthellae were observed in tissue of naturally bleached coral in summer (Mise and Hidaka 2003, Reimer et al. 2007). Thermal stress causes damages in the thylakoid membrane of the chloroplast due to changes in lipid composition (Tchernov et al. 2004), inducing the production of reactive

oxygen species (ROS) (Smith et al. 2005), and this ultimately breaks down the chloroplasts (Salih et al. 1998). Thermal stress affects the coral not only under high light stress, but also under dark condition producing damages to photosynthetic system of zooxanthellae (Suwa and Hidaka 2006). However the mechanism of bleaching is not yet well understood. In particular it is unclear how the loss of zooxanthellae occurs and how is the state of zooxanthellae and coral response under stress.

We used the coral *Montipora digitata* as a model for our experiments. We applied temperature stress to examine the morphology and abundance of zooxanthellae remained inside the coral tissue and also those expelled to the water column. Furthermore, expelled zooxanthellae from the coral during experimental period were collected to classify, enumerate and analyze their pigments by using HPLC. We wanted to know what morphological and physiological differences are found between zooxanthellae released out and retained inside the coral tissue. We also examined pigment composition to know the fate of their pigments under thermal stress. We also wanted to know how corals are affected and is their response to zooxanthellae changes during thermal stress.

Materials and Methods

Coral Samples, aquarium and incubation

Branches of *M. digitata* were collected from a single colony at Bise, Motobu, Okinawa, Japan (26°42'N and 127°52'E) on May 2011. Collected corals were transported to the laboratory of the Tropical Biosphere Research Center (University of the Ryukyus) at Sesoko Island and kept in aquarium with natural seawater for adaptation during 10 days. Each 3 branches about 5 cm long were placed in 2 glass bottles filled with 800 ml of filtered seawater using cartridge filter with a pore size of 0.2 μm (ADVANTEC MFS, Inc., California, USA). Incubation vessels were maintained in a water bath at 27°C (control) and 32°C, respectively. Filtered seawater was continuously supplied to each incubation vessel with a flow rate of 10 ml min^{-1} and mixed by stirrer. In order to observe expelled zooxanthellae, outlet water was collected into 10 L polycarbonate bottles. These bottles were changed each 12 h. Half of the collected water was gently filtrated using 2.0 μm Nucleopore polycarbonate membrane (Whatman, GE Healthcare, Springfield Mill, UK) for the observation and counting of zooxanthellae and the other half was filtered using GF/F filter (Whatman) for pigment analysis. Light was provided with metal halide lamps with a photon flux density of 400 μmol m^{-2} s^{-1} with dark:light period of 12 h.

Zooxanthellae collection and pigment measurement

All coral branches were washed with 3.5% NaCl solution to remove loosely attached plankton and other organisms, and zooxanthellae in coral tissue were collected by removing the coral tissue from skeleton using a Water Pik (Johannes and Wiebe 1970) with 3.5% NaCl solution and homogenized with glass homogenizer. Coral tissue solution was centrifuged at 3,000 rpm during 15 min, and the supernatant was removed. Pelet of zooxanthellae was resuspended into new NaCl solution. This treatment was repeated twice to remove remaining of coral tissue. From final zooxanthellae solution, 5 ml were used for pigment analysis and 1 ml for zooxanthellae count. Mixtures for pigment analysis were filtrated using GF/F filter (Whatman) using plastic syringe and filter holder. Three replicates were prepared. Data were normalized by surface area of coral branch (cm^2) as described below. Expelled zooxanthellae were collected from the seawater around the incubated coral: during incubation, all outlet water was collected every 12 h from 6-18 and 18-6 h. Outlet water was filtrated with vacuum filtration using GF/F filter (Whatman) for pigment analysis and 2.0 μm Nucleopore polycarbonate membrane (Whatman) for cell observation and counting. Two L of outlet water was used for cell observation and count and the rest was used for pigment analysis. The number of expelled zooxanthellae during 12 h were normalized to surface area of coral branch (cm^2) (see next section).

All filters for pigment analysis were stored at -30°C and the pigment analysis was done within 1 week after collection. Cell observation and counting were done within 1 h after collection.

Surface area

The surface areas of respective coral branches were determined using the aluminum foil method (Marsh 1970); whereby coral skeletons were carefully wrapped with pieces of aluminum foil as uniform single layer, which was then weighed to establish the surface area of the foil. A calibration curve of the surface area was constructed based on pieces of aluminum foil with known area (27 mg cm^{-2}, $R^2 = 0.9885$, $N = 12$), which was then used to back-calculate the surface area of aluminum pieces wrapped around each coral sample.

Zooxanthellae observation and counting

We defined 3 types of zooxanthellae: i) healthy aspect cell with normal expanded chloroplast (healthy); ii) shrunk cell with reduced cell size, partially broken chloroplast and dark color (shrunk); iii) bleached cell with pale and uncolored chloroplast (bleached) (Fig. 1). Cell number of zooxanthellae was counted using microscope ECLIPSE 80i

(Nikon, Tokyo, Japan). Zooxanthellae in coral tissue were counted using Neubauer-line hemacytometer (Erma Inc., Tokyo, Japan). Expelled zooxanthellae were counted onto the filter after mounting it on a slide glass for microscopic observation. Expelled cell number in 10 visual fields was counted and normalized to surface area as cells cm^{-2} of coral surface during 12 h. Zooxanthellae and their fluorescence were photographed using a fluorescence microscope Olympus IX-72 (Olympus corp., Tokyo, Japan).

Photosynthetic activity of symbiotic zooxanthellae

As an index of the photosynthetic activity, the maximum fluorescence of the symbiotic zooxanthellae was measured using a portable pulse amplitude modulated fluorometer (PAM) (DIVING-PAM, Walz, Effeltrich, Germany) according to the method of Schreiber et al. (1998). The optimal quantum yield was calculated according to Krause and Weis (1991) as F_v/F_m, where $F_v = F_m - F_o$, where F_o is the initial fluorescence in the dark adapted state and F_m is the maximal fluorescence in the dark adapted state, therefore coral branches were placed in darkness for 15 to 30 min before measurements. Fluorescence data were taken more than 3 times from different parts of each coral branch.

Pigment analysis

Pigment analysis was performed with an HPLC according to the method reported by Zapata et al. (2000). Filters containing coral tissue were cut into small pieces and homogenized with 3 ml of cold 95% (v/v) methanol in a mill. Pigments were extracted using sonic treatment for 5 min. Extracts were filtered through a syringe filter (0.2 μm, Millex-LG, Millipore, Bedford, MA, USA) to remove cell debris. To avoid shape distortion by earlier eluting peaks, methanol extract (1.0 ml) was mixed with 0.2 ml of deionized water (Milli-Q water) just prior to injection, according to the protocol described by Zapata et al. (2000). These extracted samples (200 μl) were immediately injected into the HPLC system (LC-10A, Shimadzu, Kyoto, Japan). All samples were prepared under subdued light and were subjected to HPLC analysis within 5 min after extraction to avoid pigment deterioration. The HPLC system was equipped with a Waters (Milford, MA, USA) Symmetry C8 column (4.6 x 150 mm). Pigments were eluted at a flow rate of 1.0 ml per min at 25°C with a programmed binary gradient elution system. Solvents used were A: methanol:acetonitrile:0.25 M aqueous pyridine solution (50:25:25, volume to volume) and B: methanol:acetonitrile:acetone (20:60:20, volume to volume). Separated pigments were detected spectrophotometrically with a photodiode array detector (SPD-M10A, Shimadzu) with an optical resolution of 1.2 nm, measuring from 320 to 720 nm and monitoring five channels of representative wavelengths. Each peak

was identified by comparing HPLC retention times with the absorption spectra of standards, and data from photodiode array detection. Absorption spectra of chlorophyll *d* and *f* are referred from Larkum and Kühl (2005) and Chen et al. (2010) respectively. Photosynthetic pigments of zooxanthellae in coral tissue were normalized to surface area of coral and those of expelled zooxanthellae to surface area of coral and time (12 h). Dr. Y. Kashiyama (Fukui University of Technology, Fukui, Japan) kindly provided us with the standard of cPPB-*a*E.

Results

Color of coral surface and maximum quantum yield (F_v/F_m)

After 4 days of incubation, surface colors of coral branches were still brown at both 27°C and 32°C, but the colors were more brownish at 27°C (Fig. 2).

Maximum quantum yield (F_v/F_m) is shown in Figure 3. The values of F_v/F_m did not differ at two incubation conditions (27°C and 32°C) with respect to initial.

Zooxanthellae count

Three types of zooxanthellae were observed in coral tissue. (Fig. 1). However, bleached cells were rare (0.39% at 27°C and 1.97% at 32°C of the total cells in tissue). As shown

in Figure 4, the density of zooxanthellae in coral branches after 4 days of incubation at 27°C was similar to initial, as well as the proportion between shrunk and healthy zooxanthellae. At 32°C incubation, zooxanthellae density significantly (t-test, $p = 0.002$) decreased to 42% of the initial. Moreover, number of shrunk cells increased from 3.78×10^4 to 4.25×10^5 cells cm^{-2} comprising near ~18% of the total zooxanthellae.

Expulsion rates of zooxanthellae under each condition are shown in Table 1 and Figure 5. At 27°C, expelled cell number ranged from 3.78×10^2 to 1.82×10^4 cells cm^{-2} during 12 h (Fig. 5). Relatively more numbers of zooxanthellae were expelled during daytime and most of them were of shrunk type. On the other hand, at 32°C, expelled cell number ranged from 2.27×10^2 to 1.41×10^3 cells cm^{-2} during 12 h. Total numbers of expelled cells during 4 days were 4.39×10^4 at 27°C and 6.00×10^3 at 32°C representing ~1% of the total zooxanthellae contained in coral tissue at initial condition.

Table 1 shows the expulsion rate of zooxanthellae at 27°C and 32°C during day and nighttime: at 27°C it was 22 times higher than at 32°C. Also at 27°C, expulsion rate was higher during daytime than nighttime as 13 in case of shrunk zooxanthellae. However at 32°C, expulsion rate at daytime and nighttime was similar.

Pigment analysis

For pigment characterization, shrunk zooxanthellae were collected from outlet seawater of the incubation at 27°C and their pigments were compared with those of healthy zooxanthellae collected from coral tissue. Elution profiles of pigments of shrunk and healthy zooxanthellae are shown in Figure 6. As shown, HPLC analysis enabled the separation of more than 30 peaks and among them 27 pigment species were identified. The results of pigment identification are summarized in Table 3. In samples composed mainly by shrunk zooxanthellae, a noticeable peak of pigment at 31.03 min, which has a maximum absorption peak at 686 nm in the red band, was identified as cPPB-*a*E from its retention time and absorption spectrum. This absorption spectrum was also found in previous report (Goerick et al. 2000). Absorption spectra of cPPB-*a*E and other general chlorophylls are shown in Figure 7.

Pigment analysis of zooxanthellae in coral tissue showed that the concentration increased at both 27°C and 32°C incubation compared to initial. Concentration of chlorophyll *a*, peridinin and chlorophyll c_2 each were almost similar at 32°C compared to 27°C (Fig. 8). However, concentration of cPPB-*a*E was much higher at 32°C compared to 27°C (Fig. 8) despite the decrease in cell numbers at 32°C (Fig. 4), therefore the increase in the pigment concentrations resulted from the increase in zooxanthellae cell size.

Pigment contents of expelled zooxanthellae are shown in Figure 9. Chlorophyll *a* and

chlorophyll c_2 were low at both temperatures. However, cPPB-*a*E was the most abundant pigment in extract of expelled zooxanthellae at 27°C (Fig. 9) in which number of shrunk zooxanthellae were higher than healthy ones (Fig. 5).

Discussion

Although our incubation experiment showed that the number of expelled zooxanthellae from coral tissue during the experiment period was less than 1% of the total, the number of cells expelled at 27°C was seven times higher than at 32°C (Table 1 and 2).

It was reported that zooxanthellae were also expelled from *Pocillopora damicornis* (Stimson and Kinzie 1991) and other corals (*Acropora muricata, Pocillopora eydouxi, Porites lutea, Acropora* cf. *grandis, Favites abdita, Cyphastrea serailia* and *Acropora nobilis*; Yamashita et al. 2011) in similar rates. Since the amount of expelled cells was very low, it seems that the expulsion of zooxanthellae from coral is a natural physiological phenomenon of coral and may not be the main mechanism of coral bleaching.

Despite low expulsion of zooxanthellae from coral under thermal stress, the abundance of zooxanthellae greatly decreased inside coral tissue. Therefore the decrease of zooxanthellae at 32°C cannot be explained only by expulsion. It is likely that these

zooxanthellae might have been degraded inside the coral tissue. Previous study also reported that digestion of the algal symbiont by the coral host is a common process (Titlyanov et al. 1996). Similar symbiont digestion processes were observed in the sea anemone *Phyllactis flosculi* (Steele and Goreau 1977), giant clams (Fankboner 1971) and the marine hydroid *Myrionema ambionense* (Fitt and Cook 1990). Moreover, it was reported that high temperature produced a significant decline in maximum electron transport rate (ETR max) without any change in F_v/F_m (Bhagooli and Hidaka 2006) causing increasing levels of oxidative stress and oxidative damage in the larvae of *Acropora intermedia* (Yakovleva et al. 2009). The hydrogen peroxide is generated in the zooxanthellae cell under thermal stress and it may be a signal for triggering coral bleaching (Smith et al. 2005). Downs et al. (2002) reported that zooxanthellae were digested by coral under oxidative stress and removed by host coral in symbiophagy (xenophagic-like process) (Downs et al. 2009, 2013).

In our experiment, a large number of shrunk zooxanthellae were observed in coral tissue and the outlet water. Zooxanthellae with shrunk cytoplasm and reduced chloroplast were also observed under thermal stress (Fukabori 1998). Similar zooxanthellae were frequently observed under thermal stress condition in several corals species: *M. digitata* (Titlyanov et al. 1996, Papina et al. 2007), *Stylophora pistillata* (Titlyanov et al. 1996,

Kuroki and van Woesik 1999, Titlyanov et al. 2001), *Galaxea fascicularis* (Bhagooli and Hidaka 2002), *Zoanthus sansibaricus* (Reimer et al. 2007). They were also observed in planulae of *S. pistillata* (Titlyanov et al. 1998). Although these zooxanthellae were said to be degraded (Titlyanov et al. 1998, Downs et al. 2009, 2013), few studies have described the mechanism by which these shrunk zooxanthellae are formed.

Our experiment showed that the shrunk zooxanthellae cells are accumulated in coral tissue when the corals are under thermal stress. In contrast at normal temperature shrunk zooxanthellae are expelled especially during daytime. Titlyanov et al. (1996) also reported that most of expelled zooxanthellae cells under normal condition had degraded shape. Our results of pigment analysis showed the presence of cPPB-*a*E when there were abundant shrunk zooxanthellae. At the same time, small amount chlorophyll *a* and peridinin were observed. In addition, pheophorbide *a* and pheophytin *a*, which are the degradation products of chlorophyll *a*, and (13^2 *R*/*S*)-hydroxychlorophyllones *a*, which are the products of biotic processing (Aydin et al. 2003, Mawson et al. 2008) and/or abiotic oxidation products (Louda et al. 2000) of cPPB-*a*E, were also detected from expelled zooxanthellae. However, healthy zooxanthellae extracted from non-stress coral had no cPPB-*a*E and those degraded pigments. Recently, cPPB-*a*E was reported as a degradation product of chlorophyll *a* of phytoplankton and is commonly present in

aquatic environments (Kashiyama et al. 2012). The cPPB-*a*E is a chlorophyll-like pigment which is frequently found in marine and lacustrine sediment (Chillier et al. 1993, Harris et al. 1995, Ocampo et al. 1999, Louda et al. 2000) and has been identified in sponges (Karuso et al. 1986), bivalves (Sakata et al. 1990, Yamamoto et al. 1992, Watanabe et al. 1993, Louda et al. 2008) and protozoa (Goericke et al. 2000). Production pathway of cPPB-*a*E remained unknown for long time, but recently, it has been found that herbivorous protozoa produced cPPB-*a*E when they grazed and digested microalgae (Kashiyama et al. 2012). From these results, it is evident that some degradation processes occurred in shrunk zooxanthellae and that cPPB-*a*E was generated from chlorophyll *a* by the degradation pathway.

Also, chlorophyll's red fluorescence of shrunk zooxanthellae cells were almost quenched (Fig. 1). Chloroplasts of diatoms grazed by protozoa were also shrunk and small and had no fluorescence of chlorophylls (Kashiyama et al. 2012). Loose of fluorescence may indicate that ROS are not produced by free chlorophylls from damaged chloroplasts. Free chlorophyll *a* released from broken chloroplast becomes generator of singlet oxygen when exposed to light, promoting the formation of ROS (Perl-Treves and Perl 2002). The protozoans which feed on microalgae have transparent bodies and therefore always exposed to light. Therefore they had developed a strategy to detoxify free chlorophyll

a by degrading it into the non-fluorescence product cPPB-*a*E (Kashiyama et al. 2012). Corals also have transparent bodies and they live symbiotically with zooxanthellae. Therefore corals are always exposed to damage of oxidative stress from ROS (Lesser et al. 1990, Dykens et al. 1992, Downs et al. 2002). Oxidative damage becomes more severe with increasing UV radiation and water temperature (Lesser et al. 1990). At the same time, under thermal stress, broken chloroplasts are difficult to be rebuilt and producing more ROS (Bhagooli and Hidaka 2006). Therefore, it is likely that corals may use the same detoxification strategy as herbivores protists by degrading chlorophyll *a* into cPPB-*a*E. We suggest that decreased cell numbers of zooxanthellae in coral tissue is the result of degradation of zooxanthellae and one of the important mechanism of bleaching to reduce the production of ROS. Our results indicate that coral bleaching is a physiological process which is used as a survival strategy to avoid oxidative damage. Under normal conditions, corals maintain the number of their symbiotic zooxanthellae by releasing excess cells especially the unhealthy ones. Under thermal stress, corals save themselves from oxidative stress through bleaching accompanied by degradation of their zooxanthellae. From our results, *M. digitata* seems to be resistant against thermal stress. However, even zooxanthellae are degraded in coral tissues, the degradation process is still unclear. There are some reports that corals can degrade their symbiotic zooxanthellae

(Downs et al. 2009, 2013). On the other hand, it is reported that zooxanthellae have the ability to generate cPPB-*a*E (Yamada et al. 2013). In any case, coral probably uses strategic bleaching (degradation of zooxanthellae by coral or zooxanthellae themselves) to survive under thermal stress. Different environmental stressors (cold water, changes in salinity, bacterial infection among others) which are the trigger for bleaching may develop throughout different mechanisms. Furthermore some kinds of corals under thermal stress generate bleached or pale zooxanthellae rather than the shrunk type (Mise and Hidaka 2003). These corals might have other mechanism for saving themselves from these stresses. However, in the case of *M. digitata*, it seems that strategic bleaching by degrading chlorophyll *a* to non-fluorescent pigment is used as strategy to fight oxidative stress. Moreover, our results suggest that cPPB-*a*E appears only in shrunk zooxanthellae and can be used as an indicator of thermal stress in corals before reaching the stage of visible bleaching. Monitoring of cPPB-*a*E can be a new tool for predicting and understanding more about bleaching mechanism in corals.

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Figure Legends

Figure 1. Types of zooxanthellae observed in coral tissue. (a and b) Healthy cells with spherical shape and expanded chloroplast. (c and d) Shrunk cells with dark color, reduced cell size and partially broken chloroplast. (e) Bleached cells with pale and uncolored chloroplast. (f) Three different types of zooxanthellae. (g and h) Photograph (g) and fluorescence image (h) of healthy and shrunk cells. Shrunk cells are indicated with arrowheads.

Figure 2. Aspect of coral branches after 4 days of incubation at: (a) normal temperature, 27°C and (b) stress condition, 32°C.

Figure 3. Maximum quantum yield (F_v/F_m) of corals after 4 days of incubation. Error bars represent standard deviations. ($N = 27$)

Figure 4. Zooxanthellae density and composition in coral tissue at initial and after 4 days of incubation at 27°C and 32°C. Cell numbers were normalized to surface area of coral branch. Error bars represent standard deviations. ($N = 18$)

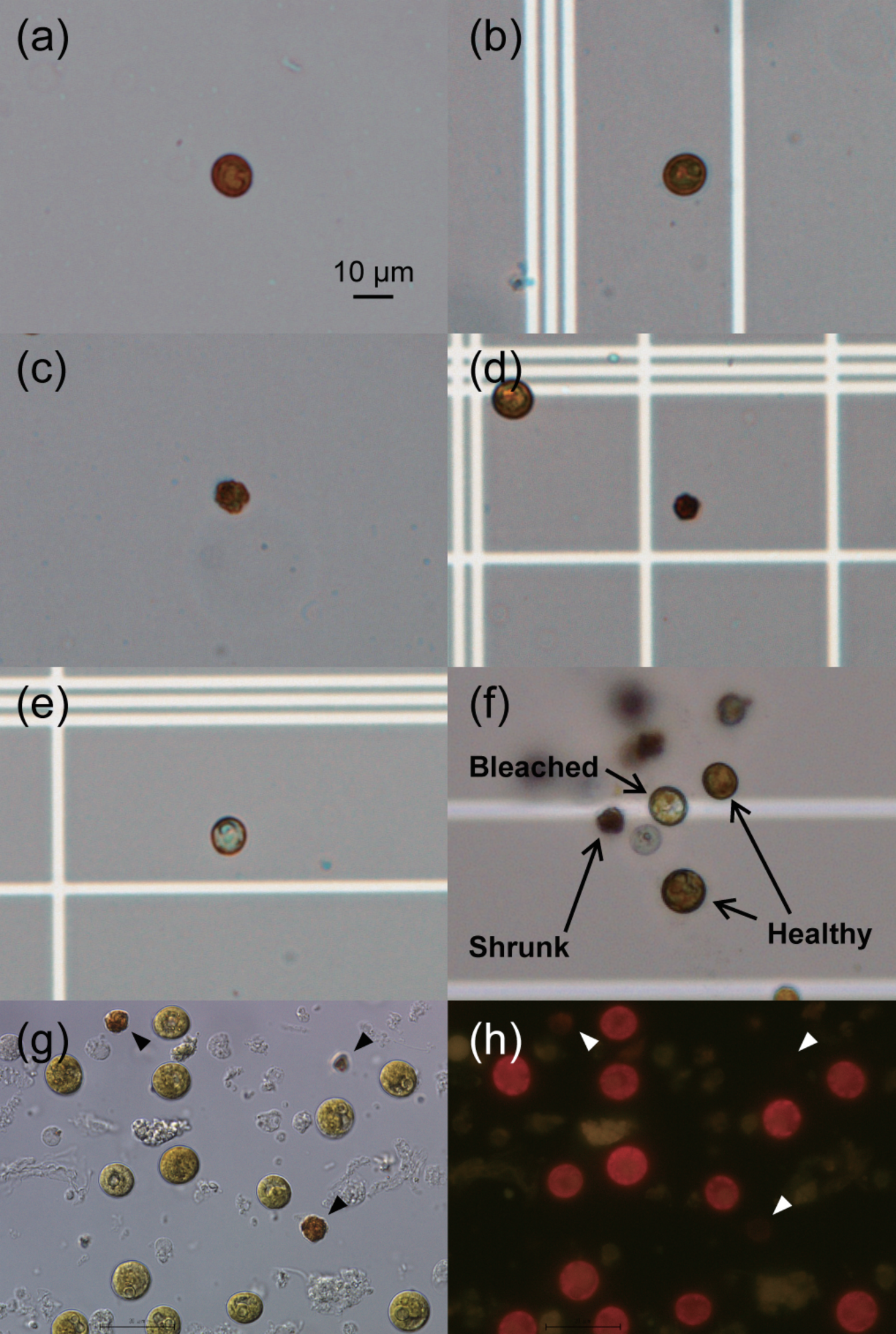
Figure 5. Zooxanthellae density and composition in outlet water during 12 h up to 4 days. (a) normal temperature, 27°C and (b) stress condition, 32°C. Cell numbers were normalized to surface area of coral branch. Error bars represent standard deviations. ($N = 3$)

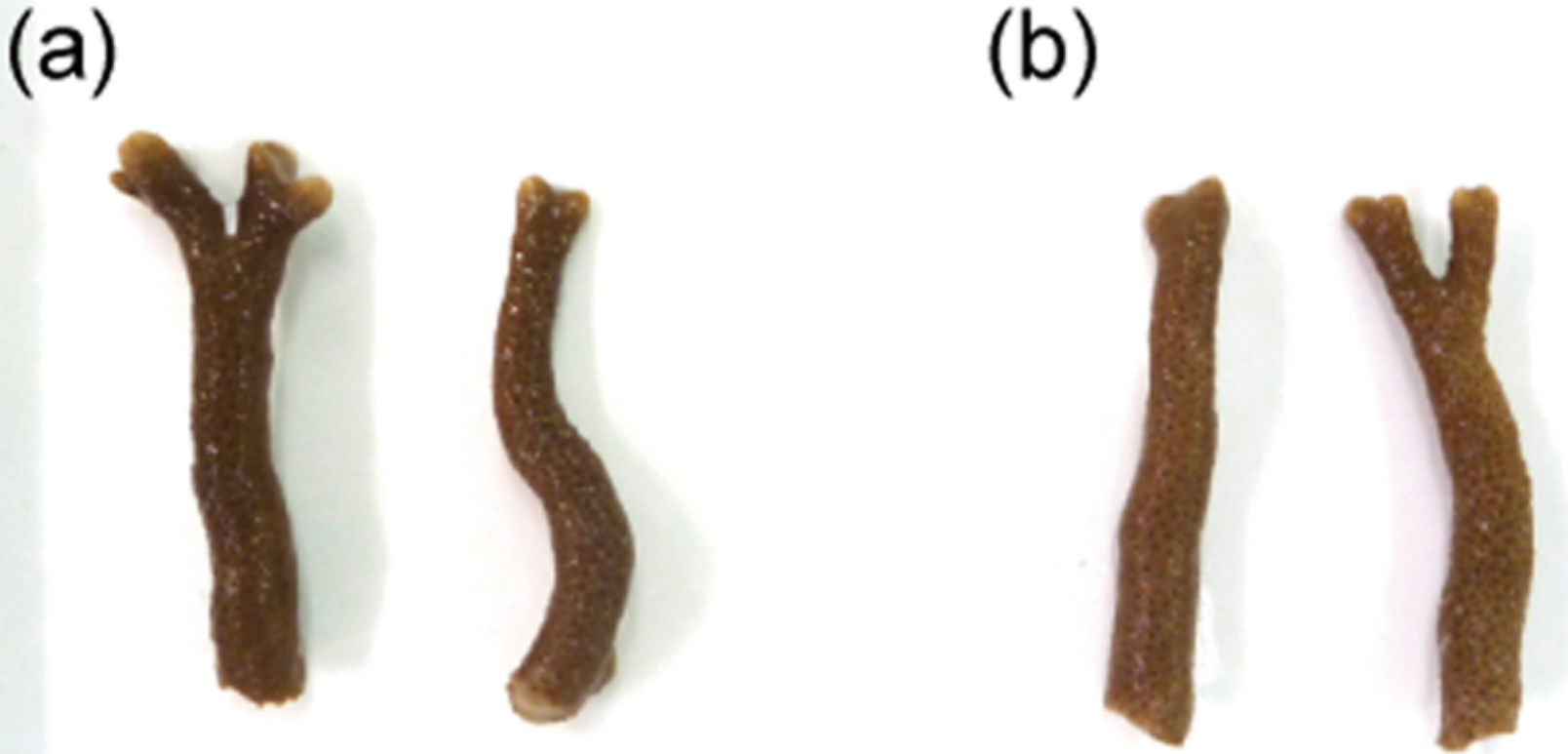
Figure. 6. Elution profiles of expelled zooxanthellae at 27°C (upper) and retained zooxanthellae at initial condition (lower). Peak numbers in elution profiles correspond to those in the identification table (Table 3).

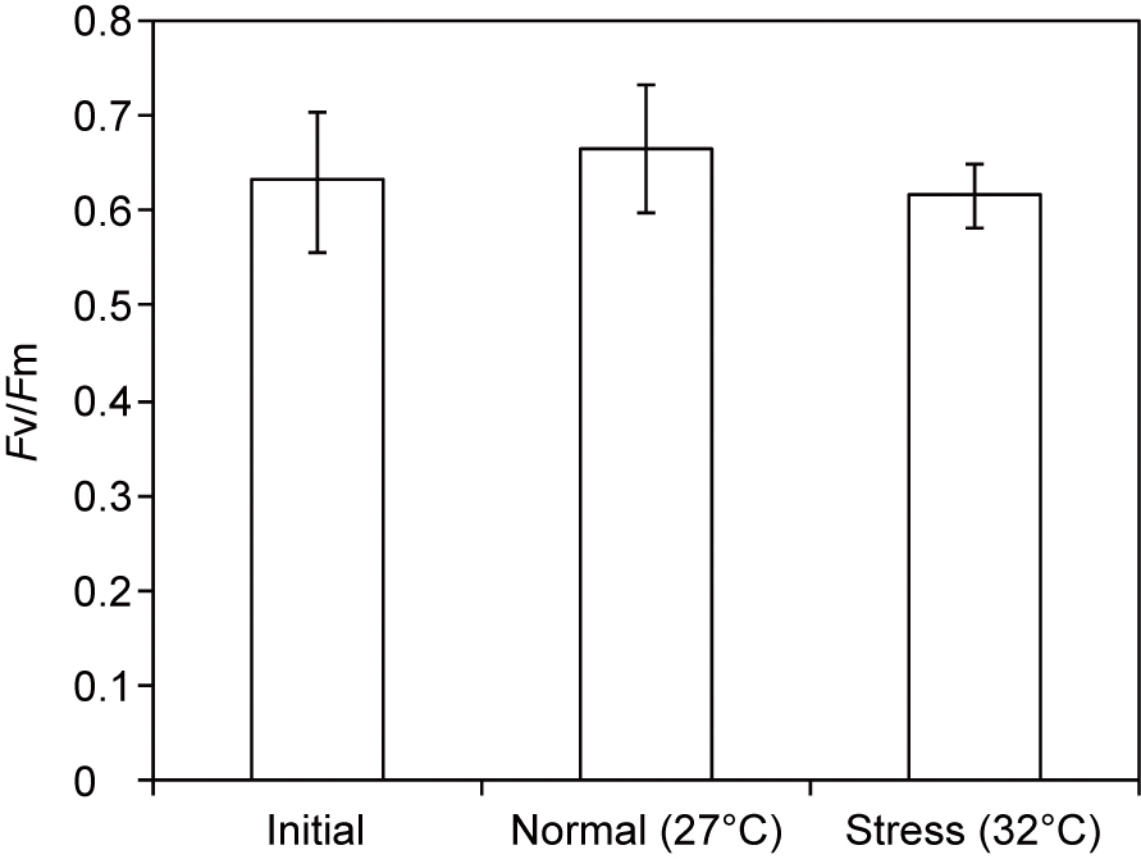
Figure. 7. Absorption spectra of cPPB-*a*E and other pigments (chlorophyll *a*, *b*, c_2, *d*, *f* and pheophytin *a*).

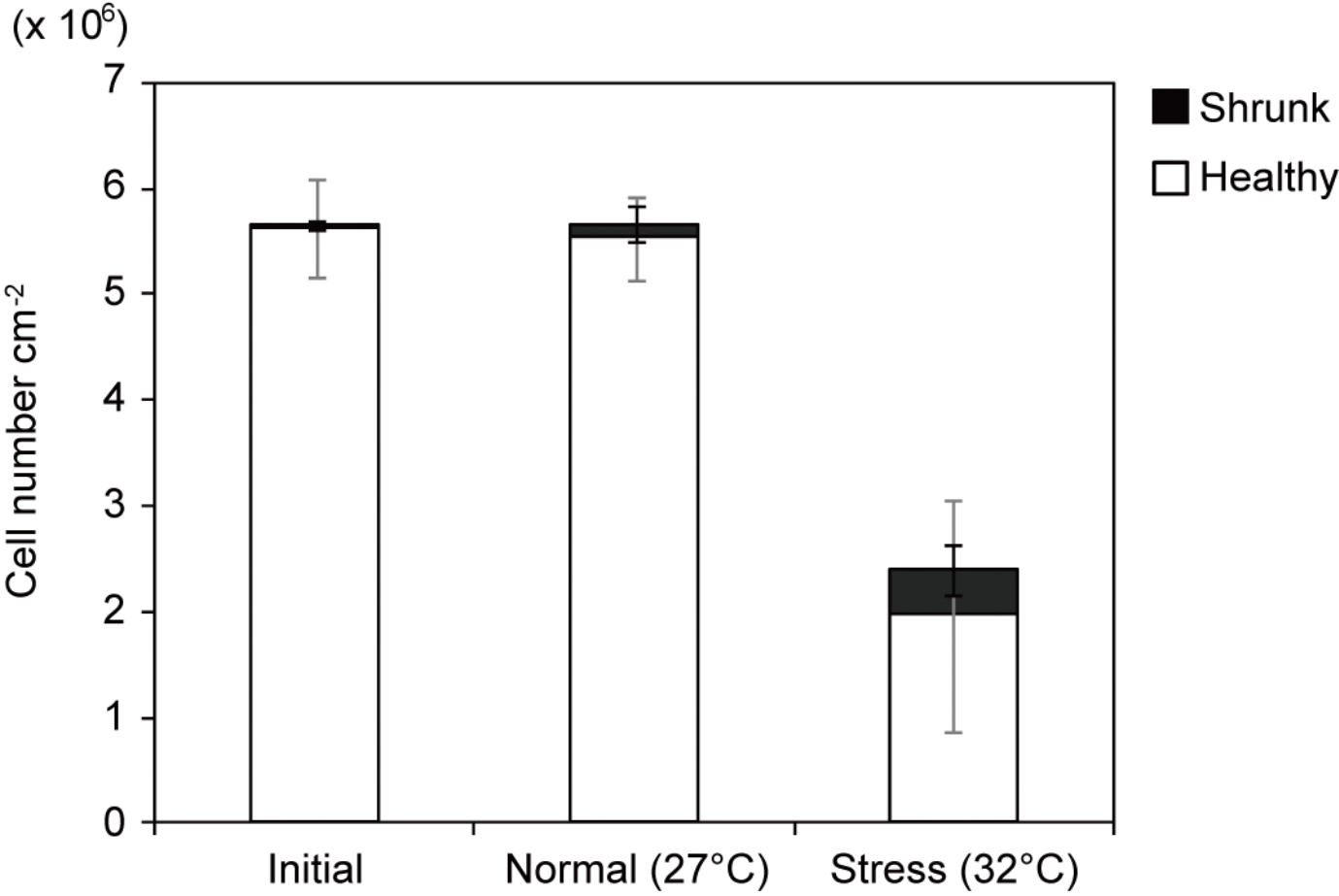
Figure 8. Pigments composition of zooxanthellae retained in coral tissue at initial and after 4 days of incubation at 27°C and 32°C. Pigment contents were normalized to surface area of coral branch. Error bars represent standard deviations. ($N = 9$)

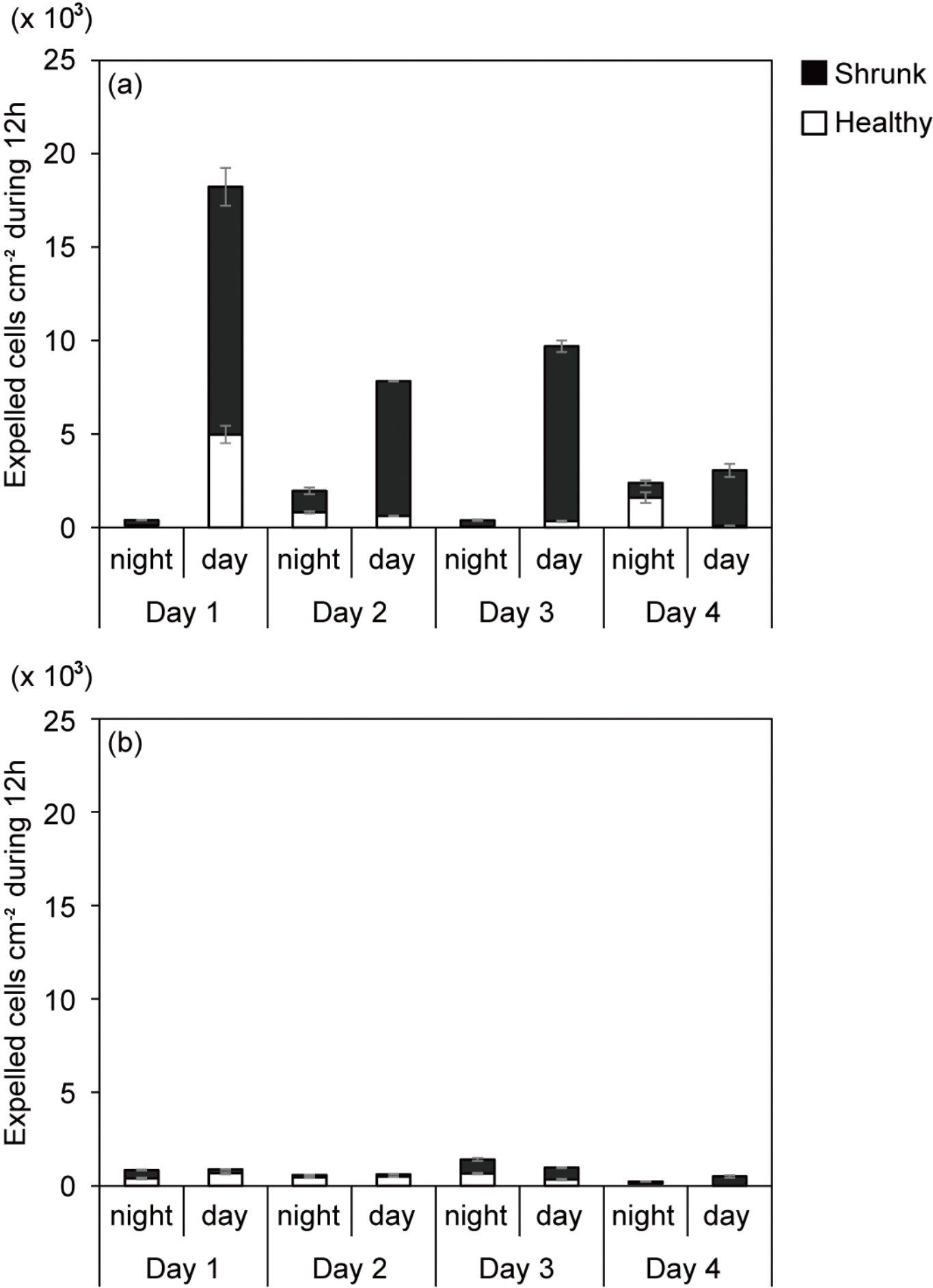
Figure 9. Pigments composition of expelled zooxanthellae during 12 h up to 4 days. (a) normal temperature, 27°C and (b) stress condition, 32°C. Pigment contents were normalized to surface area of coral branch. Error bars represent standard deviations. ($N = 3$)

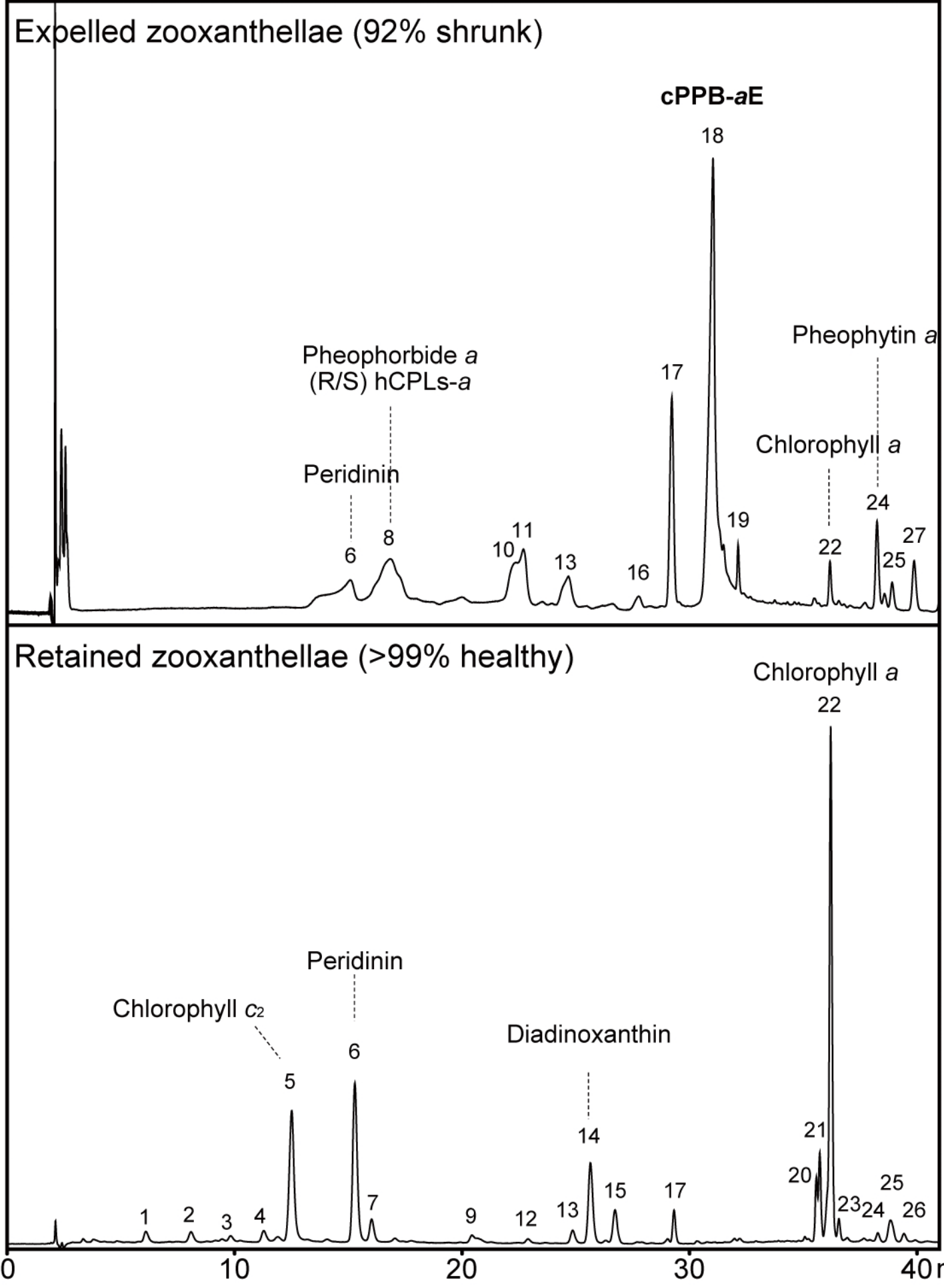

(a)
10 µm
(b)
(c)
(d)
(e)
(f)
Bleached
Shrunk
Healthy
(g)
(h)

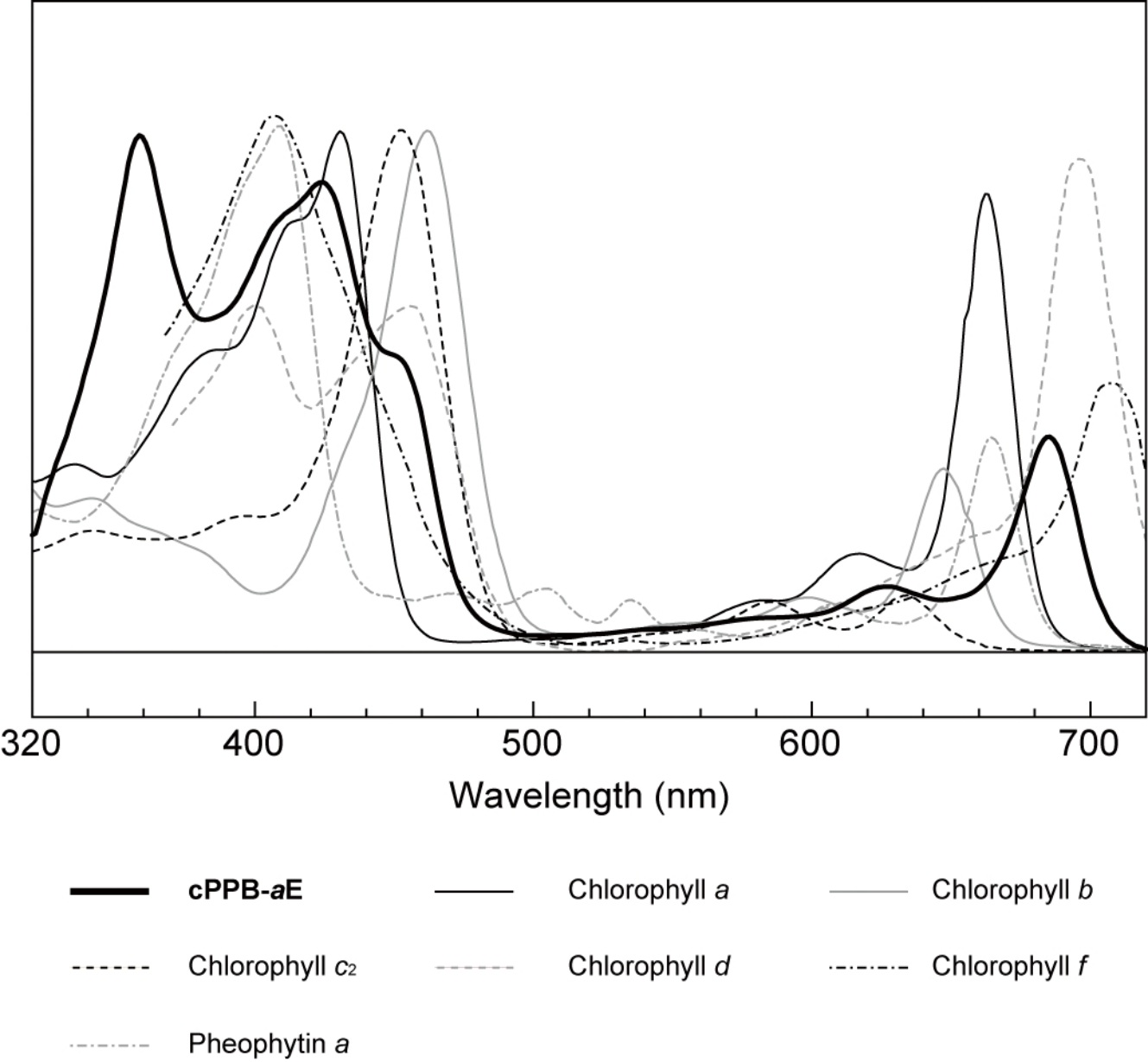

(a)
(b)

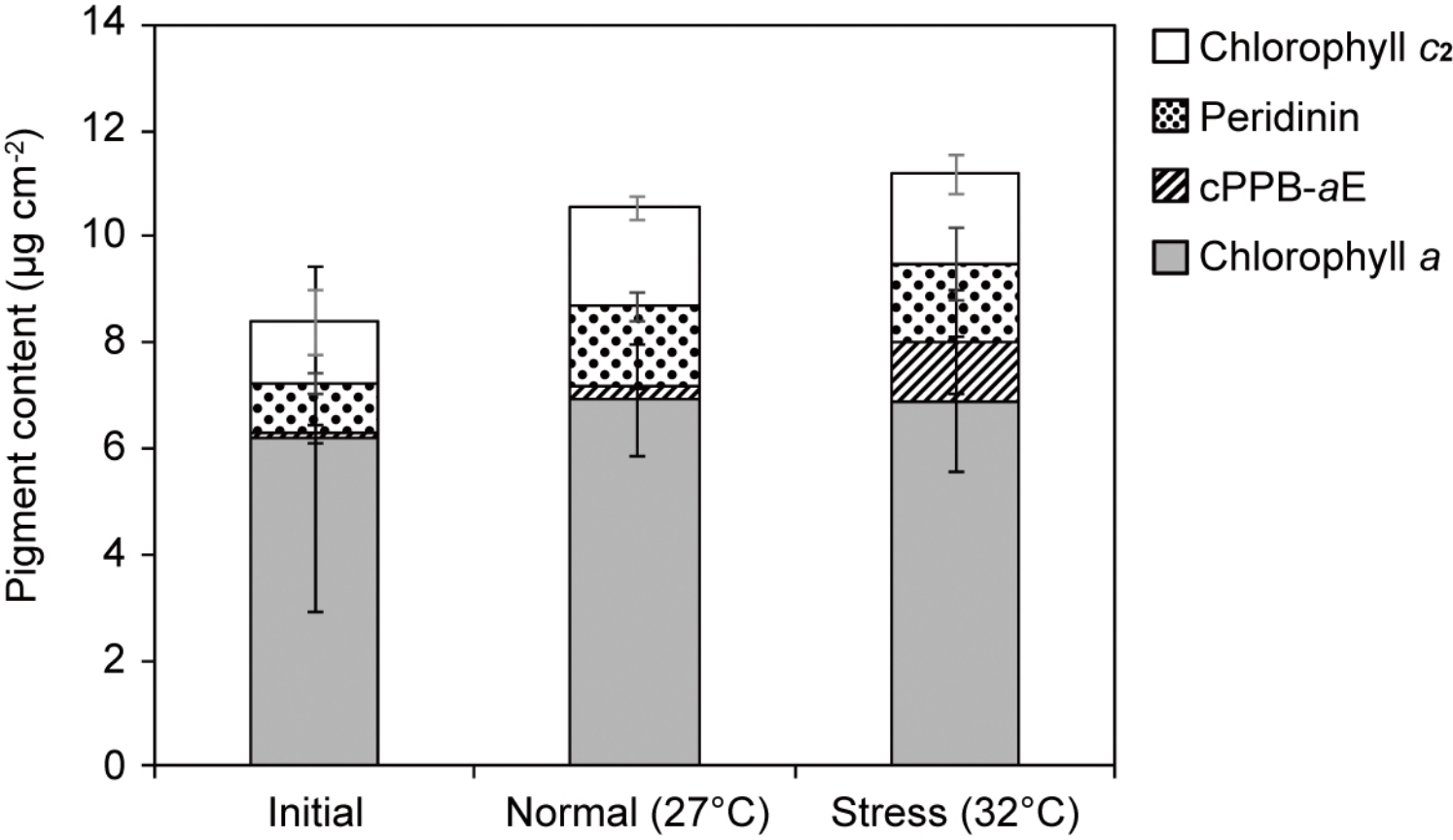

0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Fv/Fm
Initial
Normal (27°C)
Stress (32°C)


(x 10^6)
7
6
5
4
3
2
1
0
Cell number cm^-2
Shrunk
Healthy
Initial
Normal (27°C)
Stress (32°C)


(x 10³)
25
20
15
10
5
0
(a)
Expelled cells cm⁻² during 12h
Shrunk
Healthy
night
day
Day 1
Day 2
Day 3
Day 4
(x 10³)
(b)


Expelled zooxanthellae (92% shrunk)
cPPB-aE
18
Pheophytin a
Pheophorbide a
(R/S) hCPLs-a
17
Chlorophyll a
Peridinin
24
19
11
8
27
10
22
6
25
13
16
Retained zooxanthellae (>99% healthy)
Chlorophyll a
22
Peridinin
Chlorophyll c2
Diadinoxanthin
6
5
14
21
20
25
15
17
1
2
3
4
7
9
12
13
23
24
26
0
10
20
30
40 min


320
400
500
600
700
Wavelength (nm)
cPPB-aE
Chlorophyll a
Chlorophyll b
Chlorophyll c2
Chlorophyll d
Chlorophyll f
Pheophytin a


Pigment content (µg cm-2)
14
12
10
8
6
4
2
0
Initial
Normal (27°C)
Stress (32°C)
Chlorophyll c2
Peridinin
cPPB-aE
Chlorophyll a

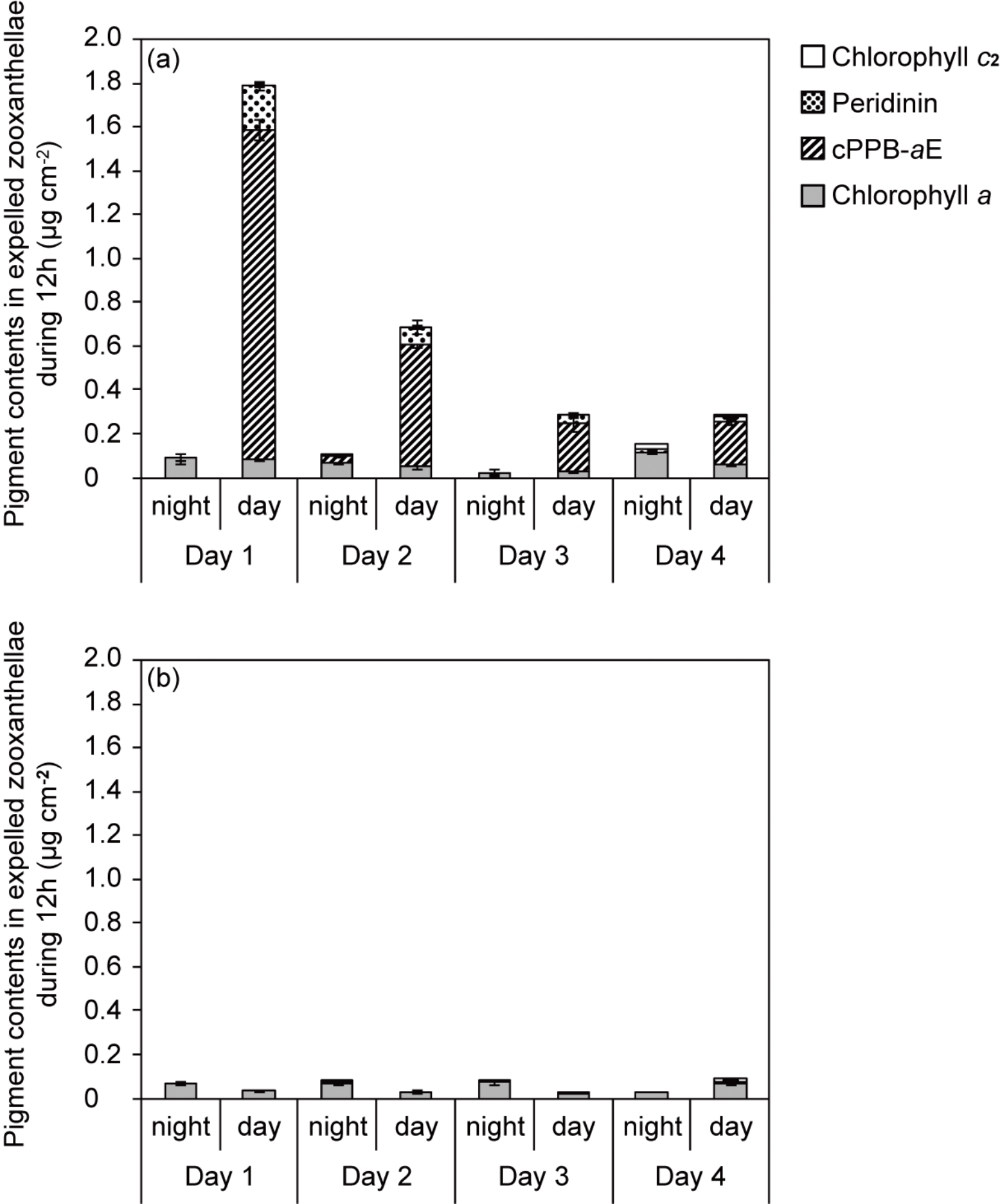

(a)
(b)
Pigment contents in expelled zooxanthellae during 12h (µg cm-2)
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
night
day
Day 1
Day 2
Day 3
Day 4
Chlorophyll c2
Peridinin
cPPB-aE
Chlorophyll a

Table 1. Expulsion rate of zooxanthellae from coral tissue during daytime and nighttime.

Expelled zooxanthellae (expelled cells per h)			
	Total	Healthy	Shrunk
Normal (27°C)			
Daytime	808.2	124.7	683.5
Nighttime	106.5	53.4	53.1
Stress (32°C)			
Daytime	61.5	30.6	30.9
Nighttime	63.5	33.6	29.8

(cells h^{-1} cm^{-2} coral surface)

Table 2. Number of zooxanthellae retained in coral tissue and expelled from coral tissue after 4 days of incubation

Retained zooxanthellae (initial and day 4)

	Initial	Day 4	
		Normal (27°C)	Stress (32°C)
Total	5.64×10^6	5.65×10^6	2.37×10^6
Healthy	5.60×10^6	5.52×10^6	1.95×10^6
Shrunk	3.78×10^4	1.25×10^5	4.25×10^5

(cells cm^{-2} coral surface)

Expelled zooxanthellae (total number during 4 days)

	Day 4	
	Normal (27°C)	Stress (32°C)
Total	4.39×10^4	6.00×10^3
Healthy	8.55×10^3	3.08×10^3
Shrunk	3.54×10^4	2.92×10^3

(cells cm^{-2} coral surface)

Table 3. Identification table of detected pigments

Peak no.	Retention (min)	Maxima in eluant (nm)			Identification
1	6.09	475			Peridinin like pigment
2	8.08	464			Peridinin like pigment
3	9.82	451		633	Chlorophyll c_2 species
4	11.27	430		663	Chlorophyllide *a*
5	12.50	452	584	633	Chlorophyll c_2
6	15.28	470			Peridinin
7	16.02	470			Peridinin species
8	**17.05**	**410**		**665**	**Pheophorbide *a* ***
9	20.43	455			Prasinoxanthin
10	**22.33**	**475**			**Peridinin species ***
11	**22.69**	**475**			**Peridinin species ***
12	22.89	446	469		19'-hexanoyloxyfucoxanthin
13	24.86	408	429	456	Diadinochrome
14	25.64	421	447	475	Diadinoxanthin
15	26.72	418	441	470	Dinoxanthin
16	**27.76**	**408**	**429**	**456**	**Diadinochrome species ***
17	29.22	453	480		Alloxanthin
18	**31.03**	**423**	**628**	**686**	**P686 ***

19	**32.13**	**452**	**480**		**Alloxanthin like pigment ***
20	35.58	420		660	Chlorophyll *a* like pigment
21	35.72	429	613	662	Chlorophyll *a* allomer
22	36.21	430	618	662	Chlorophyll *a*
23	36.57	429	616	662	Chlorophyll *a* epimer
24	38.28	406	504	665	Pheophytin *a*
25	38.89	452	477		β-carotene
26	39.43	432		667	Chlorophyll *a* like pigment
27	**39.93**	**409**		**668**	**Pyropheophytin *a* ***

***** Detected only in shrunk zooxanthellae