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Changes in zooxanthellae density, morphology, and mitotic index in hermatypic corals and anemones exposed to cyanide

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Abstract

Sodium cyanide (NaCN) is widely used for the capture of reef fish throughout Southeast Asia and causes extensive fish mortality, but the effect of NaCN on reef corals remains debated. To document the impact of cyanide exposure on corals, the species *Acropora millepora*, *Goniopora* sp., *Favites abdita*, *Trachyphyllia geoffrio*, *Plerogyra* sp., *Heliofungia actiniformis*, *Euphyllia divisa*, and *Scarophyton* sp., and the sea anemone *Aiptasia pallida* were exposed to varying concentrations of cyanide for varying time periods. Corals were exposed to 50, 100, 300, and 600 mg/l of cyanide ion (CN⁻) for 1–2 min (in seawater, the CN⁻ forms hydrocyanic acid). These concentrations are much lower than those reportedly used by fish collectors. Exposed corals and anemones immediately retracted their tentacles and mesenterial filaments, and discharged copious amounts of mucus containing zooxanthellae. Gel electrophoreses techniques found changes in protein expression in both zooxanthellae and host tissue. Corals and anemones exposed to cyanide showed an immediate increase in mitotic cell division of their zooxanthellae, and a decrease in zooxanthellae density. In contrast, zooxanthellae cell division and density remained constant in controls. Histopathological changes included gastrodermal disruption, mesogleal degradation, and increased mucus in coral tissues. Zooxanthellae showed pigment loss, swelling, and deformation. Mortality occurred at all exposure levels. Exposed specimens experienced an increase in the ratio of *gram-negative* to *gram-positive* bacteria on the coral surface. The results demonstrate that exposure cyanide causes mortality to corals and anemones, even when applied at lower levels than that used by fish collectors. Even brief exposure to cyanide caused slow-acting and long-term damage to corals and their zooxanthellae.

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1. Introduction

Since the early 1960s, coral reefs have been increasingly exploited by fishermen who use cyanide to capture live reef fish and sell them to the restaurant and aquarium trades (Rubec, 1986, 1997). Fishermen stun the live reef fish by squirting sodium cyanide (NaCN)

onto coral heads and into crevices in coral reefs (Fig. 1). While this method is designed to capture living fish, it results in high mortality rates. Approximately 50% of the NaCN-exposed aquarium fish die while still on the reef, and >80% of the remainder die before they are sold by retailers in North America and Europe (Rubec, 1986; Rubec and Soundararajan, 1991). The NaCN tablets used by collectors in the Philippines and Indonesia weigh about 20 g each (Johannes and Riepen, 1995). Fishermen who are collecting ornamental fish for aquarium tanks generally place one or two tablets of cyanide into a 1-l plastic squirt bottle filled with seawater, while food-fish collectors use three to five tablets (Rubec et al., 2001). The tablets sequentially dissolve in the squirt bottle as collectors proceed to spray the reefs,

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Fig. 1. Photo of a cyanide fisherman squirting cyanide from a squirt bottle to capture fish.

making it difficult to determine the cyanide ion (CN^-) concentrations being applied. It has been suggested that fish collectors use concentrations ranging from 1500 to 120,000 mg/l (Johannes and Riepen, 1995; Barber and Pratt, 1998; Pet and Djohani, 1998; Jones et al., 1998). Since not all of the cyanide applied from squirt bottles dissolves, it is sometimes visible underwater as a whitish plume (Rubec et al., 2001). Not all fishermen limit themselves to squirt bottles. Reports from the Philippines assert that 55-gallon drums of cyanide have been dumped onto reefs to kill and capture food fish (del Norte et al., 1989; Johannes and Riepen, 1995).

The various forms of cyanide fishing contribute to degraded, lifeless coral reefs (Rubec, 1986; Johannes and Riepen, 1995; Barber and Pratt, 1998) and force fishermen to relocate to more pristine locations. Unfortunately, fishermen usually bring cyanide techniques with them, thus repeating the cycle of destruction of the coral reefs and associated fish communities.

Although illegal in most Southeast Asian countries, cyanide fishing is widespread and is being introduced to other regions (Johannes and Riepen, 1995; Barber and Pratt, 1997).

Fishermen in the Philippines, Indonesia, Malaysia, Vietnam, and Papua New Guinea have reported cyanide use (Rubec, 1986; Johannes and Riepen, 1995; Barber and Pratt, 1998). Squirting cyanide onto a reef is an easy way to force fish out of coral crevices. Stressed and poisoned, they swim to open-water areas, where they are then captured. Although some fishermen acknowledge that cyanide fishing kills corals, others deny that such damage occurs (Galvez et al., 1989; Hingco and Rivera, 1991).

In a field visit to devastated reefs in the Philippines, former cyanide fishermen showed the author individual coral heads that had died following one application of cyanide (Cervino and Goreau, personal observation). Furthermore, an unpublished study by the Philippine Bureau of Fisheries and Aquatic Resources found that

corals situated on test quadrants off Mactan Island near Cebu died after two applications of CN^- spaced 4 months apart (Rubec, 1986, 1997).

There have been several studies regarding the deleterious effect of cyanide exposure to corals. Dr. Robert Richmond of the University of Guam found that *Pocillopora damicornis* colonies exposed to 4000 mg/l CN^- for 10 min began to bleach within 4 days (Johannes and Riepen, 1995). Nine out of 10 *Pocillopora* specimens died within 4 days. When exposed to CN^- concentrations of 100 mg/l for 30 min, *Pocillopora* specimens bleached within 3–4 days and tissue loss began after 9 days. Likewise, *Pocillopora damicornis* and *Porites lichen* were exposed to a 5200 mg/l CN^- concentration caused 100% mortality (Jones, 1997; Jones and Steven, 1997) with exposure times of 10, 20, or 30 min. Tissues coating the skeletons of *Pocillopora damicornis* sloughed off within 24 h. *Porites lichen* colonies became covered with a thick mucus-like tunic. After 7 days, the tunics of all the *Porites* colonies lifted off, leaving bare skeletons. Shorter exposure times (1 or 5 min) and lower CN^- concentrations (52 or 520 mg/l) induced bleaching (loss of pigment) in both coral species within 7 days (Jones and Steven, 1997).

Cyanide was also shown to inhibit photosynthesis and calcification rates of *Acropora formosa* and *Acropora cervicornis* in corals exposed to concentrations of 5 mg/l (Chalker and Taylor, 1975). Photosynthesis and calcification were not inhibited at the highest concentration tested (5 mg/l), suggesting the existence of cyanide-resistant respiration in either the zooxanthellae or the host (Barnes, 1985). Research by Jones et al. (1998) showed that photo-inhibition from exposure to cyanide resulted in an almost complete cessation of photosynthetic electron transport from algae in tissues of *Stylophora pistillata* and *Acropora aspera*. Cyanide was applied to small branch tips of these species at concentrations estimated to occur during cyanide fishing. Pulse-amplitude-modulation (PAM) chlorophyll fluorescence was used to examine photo-inhibition and photosynthetic electron transport in the symbiotic algae (zooxanthellae in the tissues of the corals). Jones and Hoegh-Guldberg (1999) have suggested that cyanide acts as an inhibitor of the dark reactions of the Calvin cycle; specifically, as an inhibitor of ribulose-1,5-bisphosphate carboxylase/oxygenase.

No known studies have been published investigating the direct effects on cyanide on symbiotic algae, the focus of this paper. Related work by Dr. R. Richmond indicated that minor concentrations of cyanide can have a deleterious effect on the relationship between symbiotic algae and host tissue in cnidarians, resulting in death. But Richmond's in vitro experiment was limited to filming the expulsion and behavior of zooxanthellae from corals following cyanide exposure (personal communication, 1999).

2. Materials and methods

Corals were imported to the United States from the Indo-Pacific region packed in fresh seawater and kept at a holding facility. The samples were held in two 750-l tanks for 4 weeks prior to each of the three experiments. During the first segment of this experiment, 150 fragments (5 ± 3 cm) of *Acropora millipora* were removed from larger parent colonies. The coral heads were fragmented 1 week prior to the experiment to allow the corals to recover from any damage/stress caused by the fragmentation process. Fragments that bleached and/or died from post-fragmentation damage/stress were not included in the experiment.

2.1. Water conditions for observation and exposure tanks

Water quality was monitored and kept within the following ranges: temperature (28 ± 2 °C, ammonia = 0.1 mg/l, nitrite = 0.2 mg/l, nitrate = 3.5–5.0 mg/l, pH 8.1–8.3, salinity 35 ± 2 g/l, dissolved oxygen 8.0 ± 0.1 mg/l, and calcium 450 ± 50 mg/l. Lighting was provided by metal halide lamps (175 W, 6500 K) emitting approximately 17,000 lux. Filtration was conducted using biological/mechanical “box” type filters inoculated with nitrifying bacteria from established aquaria.

2.2. Dosing concentration and rate

This in vitro lab analysis was to determine if CN^- concentrations of 50, 100, 300, and 600 mg/l impaired normal cell physiology, leading to the impairment of coral symbiosis resulting in death. To prepare cyanide solutions, NaCN was dissolved in seawater. In seawater, the CN^- complexes form hydrocyanic acid (HCN) at pH values less than 8.5 (Leduc, 1984). The HCN molecule is very toxic to fish because it is rapidly absorbed across cell membranes (Duodoroff, 1980). Presumably,

HCN is the form of cyanide toxic to corals in seawater. (To simplify, we refer to CN^- concentrations rather than HCN concentrations.)

Exposures of the coral fragments to cyanide for 60 or 120 s were conducted in 4 l aquaria situated inside a chemical fume hood. A stock solution of NaCN (94.2% pure) was prepared prior to exposure. Control tanks (4 l) were set up with seawater accordingly. The exposure tanks were set up by adding 4 l of seawater containing various cyanide concentrations. Solutions of 600, 300, 100, and 50 mg/l CN^- were created by respectively adding 4.8, 2.4, 0.8, and 0.4 g of NaCN to 4 l of seawater. The corals were exposed to cyanide for 60 or 120 s by directly dipping them into cyanide solution, wearing surgical gloves. Following the cyanide dips, all specimens were lifted out of the exposure tank, rinsed in seawater for 30 s, and immediately placed back into the cyanide-free holding tank. Controls and cyanide-exposed colonies were used (1 cm length tips) to extract tissue containing symbiotic algae. Fragments of *Acropora millepora* were exposed to 50 mg/l CN^- for 60 or 120 s and observed for changes in zooxanthellae densities and mitotic indices in host tissue after 24 h. The remaining fragments were examined after 1 month.

During the final stages of this experiment, exposed hard and soft whole-coral colonies of *Acropora millepora*, *Goniopora* sp., *Favites abdita*, *Heliofungia actiniformis*, *Euphyllia divisa*, *Trachyphyllia geoffrio*, *Plerogyra* sp., *Scarophyton* sp., and the Caribbean sea anemone *Aiptasia pallida*. These species were exposed using the same methods (although at different doses) used for the *Acropora* fragments studied during the first round of experiments. For accuracy, this procedure was conducted three times per species. The tables represent the numbers of corals used for each trial. Controls and cyanide-exposed animals were dipped for 120 s in solutions of 100, 300, or 600 mg/l CN^- (Tables 1 and 2).

Table 1
Responses of corals exposed to 50,100 & 300 ppm of NaCN for 1 to 2 min

| Species | Dose (mg/l) | Time after dose (weeks) | Survival and morphological description |
|--------------------------------------|-------------|-------------------------|---|
| <i>Acropora millepora</i> 9 colonies | 50 | 4 | 4 died 4 lived after 4 weeks; high MI, lower alga density, mild bleaching, tissue detachment, and swollen tissue |
| <i>Aiptasia pallida</i> 10 animals | 50 | 12 | 3 died, 7 remained alive; all had high MI, lower alga density, (with abnormalities), mild bleaching, and swollen tentacles |
| <i>Acropora millepora</i> 6 colonies | 100 | 3 | 4 died, 2 lived after 3 weeks; high MI, lower alga density, all exhibited mild bleaching, slight tissue detachment, and swollen tissue |
| <i>Aiptasia pallida</i> 10 animals | 100 | 12 | 5 died, 5 survived; high MI, lower alga density, mild bleaching, and swollen tentacles |
| <i>Acropora millepora</i> 9 colonies | 300 | 3 | 7 died, 2 survived; high MI, lower alga density, severe bleaching, and detachment |
| <i>Aiptasia pallida</i> 10 animals | 300 | 6 | 10 died after 6 weeks; high MI, lower alga density; all died tissue exploded all tentacles retracted, and tissue necrosis |
| <i>Goniopora</i> sp. 9 colonies | 300 | 8 | 2 died, 2 are alive with polyps retracted, and 5 remained alive; high MI, lower alga density, bleached slowly, tentacles retracted and expanded during different time of the day, 1 survived; partial detachment was evident before death |

Table 2
Responses to corals exposed to 600 ppm of NaCN for 1 to 2 min

| Species | Dose (mg/l) | Time after dose (weeks) | Survival and morphological description |
|---|-------------|-------------------------|--|
| <i>Plerogyra</i> sp. 4 animals | 600 | 9 | 2 died, (with abnormalities), extreme swollen tissue, mild bleaching and detachment in two specimens exposed; 2 survived and appear healthy at 9 weeks |
| <i>Heliofungia</i> 9 animals | 600 | 2 | 9 died, no bleaching, tissue detachment and retracted tentacles, mucus production and swollen tissues; all were infected and showed signs of necrotic lesions |
| <i>Trachyphyllia geoffrio</i> 9 animals | 600 | 5 | 9 died tissue detachment, swollen tissue, constant expansion and contraction, swollen tissue; 2 survived for 4 weeks, followed by mild bleaching and detachment |
| <i>Euphyllia divisa</i> 9 animals | 600 | 9 | 5 died, with tissue detachment, all exhibit swollen tissue and excess mucus production; 4 remained swollen until the 9th week, death was due to infection of an overlying microbial mass |
| <i>Goniopora</i> sp. 9 animals | 600 | 9 | 5 died after 9 weeks; 3 died of tissue detachment, all exhibit swollen tissue, mild bleaching took place followed by death; 1 appeared to remain healthy after the 9th week |
| <i>Scarophyton</i> 4 animals | 600 | 9 | 3 died, tissue pigment darkened, 50% tentacle retracted tentacles, swollen tissue; 1 appears to remain healthy as of the 9th week |
| <i>Favites abdita</i> 4 animals | 600 | 9 | 2 died 2 remain alive, swollen tissue, mild bleaching, and pigment change |
| <i>Acropora millepora</i> 9 animals | 600 | 1 | 4 died within the first 48 h, 5 died within the first week; tissue detachment, bleaching, and slight microbial infection near lesion |
| <i>Aiptasia pallida</i> 20 animals | 600 | 6 | 4 died after 1 week, 16 died after 6 weeks, mild bleaching, extreme swollen tissue, constant expansion and contraction, excessive mucus production |

Methods to determine cellular impairment and necrosis included: (a) gel electrophoresis to determine changes in protein synthesis; (b) histology to observe visual tissue damage to the cellular structure and integrity of host and symbiotic zooxanthellae; and (c) enumeration of densities of zooxanthellae and mitotic indices (cell division of the zooxanthellae). The mitotic index has been measured previously with corals subjected to elevated or reduced temperatures (Suharsono and Brown, 1992; Wilkerson et al., 1983), and in toxicity tests using elevated dosages of copper on *Acropora formosa* (Jones, 1997).

2.3. Preparation of corals to determine mitotic indices and protein analysis

To estimate the rate of cell division of zooxanthellae for the coral and anemone species in the present study, we used a Pasteur pipette and light suction to capture zooxanthellae expelled from the gastrovascular cavity. Mitotic indices and zooxanthellae densities were analyzed for the corals *Acropora millepora*, *Goniopora* sp., *Favites abdita*, *Heliofungia actiniformis*, *Trachyphyllia geoffrio*, *Euphyllia divisa* and the Caribbean sea anemone *Aiptasia pallida* at 24 h, 30 and 60 days following cyanide exposure. *Trachyphyllia geoffrio*, *Euphyllia* sp., *Acropora* sp., *Aiptasia* sp. and *Heliofungia* sp. were also examined 1 and 2 months after exposure to CN⁻.

Corals for histology and mitotic indices were preserved in a 10% glutaraldehyde filtered seawater (FSW) solution. The corals were placed in sterile 100 ml poly-

ethylene bottles. The samples were kept in ice coolers until the beginning of the experiment. Specimens of *Acropora millepora* were used for protein analysis 24 h after exposure. Samples placed in sterile 100 ml polyethylene bottles containing FSW were stored in a freezer (-4 °C) until tissue processing was conducted. The coral tissue was removed from all specimens using a Water Pik (Johannes and Wiebe (1970)). The liquid portion containing tissue and zooxanthellae was collected and inserted into 2 ml Eppendorf tubes and centrifuged (5000 rpm Vari Hi-Speed Centricone; Precision Scientific) to separate host tissue from zooxanthellae. After centrifuging, the supernatant was discarded and a 20–30 µg pellet was extracted and homogenized on ice. A 500 µl dilution of FSW was added to the remaining pellet for mitotic index and protein analysis. Mitotic indices were observed under a phase contrast microscope 40× and 100× (Meiji Scientific, Japan), and counted using a Neubauer ruling hemocytometer (Levy Scientific).

For protein analysis of corals exposed to 50 and 100 mg/l CN⁻, observations were made 24 h post-exposure. The corals were collected and immediately prepared for protein analysis. A 5 µl of beta mercaptoethanol and a 14 µl of loading buffer was added to the sample containing the 20–30 µg pellet. Standard low molecular weight markers (Sigma) were used to compare molecular weights of proteins in controls and cyanide-exposed corals. A 2×-sample treatment buffer (0.5 Tris-HCl, pH 6.8, 10% w/v SDS, glycerol, 5 µl beta mercaptoethanol, 0.1% bromophenol blue, pH 6.8) was added to the 20–30 µg sample. Precast 4–20% tris-glycine gels (Novex) were

used for gel electrophoresis to recognize the relative abundances of expressed proteins in corals before and after exposure to cyanide. Homogenized pellet samples were placed in boiling water for 5 min and cooled before loading. Each of the 10 gel lanes was loaded with 14 μ l of sample (control or CN), and the remaining sample was reused. The following samples were loaded: (CN) cyanide-dosed (100 mg/l CN⁻); (C) control (0 mg/l CN⁻); and (M) Marker (known low-molecular weights). Upon completion, a running buffer (0.025 M Tris, 0.192 M glycine, 0.1% SDS, pH 8.3) was poured over the gels. The gels were run on a Hoffer Mighty Small II, SE 250/260 electrophoresis apparatus at 155 V for 1 h. The gel was removed and placed overnight in Coomassie Brilliant Blue R (Sigma) stain solution. The initial destaining process took place 12 h later using 40% methanol and 7% acetic acid. Destain II containing 7% acetic acid and 5% methanol was then poured onto the gels. The same procedure was conducted with host tissue samples lacking symbiotic zooxanthellae, prepared by centrifugation, and then homogenized.

2.4. Preparation of corals for light microscopic histology

All tissues examined using light microscopic histology were fixed in a 3% solution of glutaraldehyde in FSW. Fixation was extended for several days, and the tissues were then transferred to 70% ethanol. The tissues were left in alcohol for several more days and then decalcified in 70% alcohol mixed with a solution containing ethylene-diamine tetra-acetic acid (EDTA), tannic acid and hydrochloric acid. Decalcification was continued until release of gaseous carbon dioxide from the tissues had terminated. The tissues were then dehydrated through a graded series of alcohols to absolute ethanol. After gradual transfer to propylene oxide, tissues were embedded in low viscosity Spurr plastic resin. The plastic blocks were cured at 60 °C for 48 h before the specimens were trimmed and prepared for sectioning. One-to-two micron thick sections were cut on an LKB microtome using a diamond knife. The sections were adhered to clean glass microscope slides, stained with aqueous Toluidine blue. Then, a cover slip was mounted over the section before photomicroscopy. Pictures were taken on a B&L Balplan microscope with 35 mm photographic attachment. Fuji print film (ASA 100) was used to generate the images shown.

3. Results

3.1. Visual observations

We found cyanide to be lethal to hermatypic corals, both in situ and in vitro. Immediately upon exposure to 50, 100, 300, or 600 mg/l CN⁻ solutions, all corals se-

creted a substantial amount of mucus while retracting their tissue and tentacles. Mucous production at much lower levels was also evident from cyanide-free (controls) corals, and ended 6 h after exposure to FSW. This mucus was a stress response to the handling of corals.

All of the cyanide-exposed corals, with the exception of *Favites abdita* and *Plerogyra* sp., released mucus on a daily basis throughout the experiment. Mucus production in *Plerogyra* sp. and *Favites abdita* ceased after 1 month, and some colonies survived 7 months after the cyanide exposure (Table 2). Although most *Plerogyra* sp. and *Favites abdita* specimens survived exposure, three of the 12 colonies tested revealed necrotic tissue that sloughed off small portions of the coral head. *Acropora millepora* and *Scarophyton* sp. appeared lighter in color after 24 h, continued producing mucus, and never fully extended their polyps. *Goniopora* sp., *Favites abdita*, *Trachyphyllia geoffrio*, *Plerogyra* sp., *Heliofungia actiniformis*, *Euphyllia divisa*, and the Caribbean sea anemone *Aiptasia pallida* all appeared darker in color after exposure and were engulfed with mucus. Their tentacles were fully extended for the majority of the day and evening hours. In some cases, mucus with zooxanthellae dislodged from the oral cavity and hung on the tips of the tentacles. Over time, the tissues of *Aiptasia pallida*, *Trachyphyllia geoffrio* and *Heliofungia* sp. became somewhat lighter in color, but appeared to have recovered after 30 days, except for a slight residual paling. Some corals exposed to CN⁻ concentrations ranging from 50 to 600 mg/l died and exhibited tissue detachment from the skeleton (Tables 1 and 2). Gram staining revealed a higher overall percentage of gram-negative bacteria within cyanide-exposed coral samples compared to unexposed samples. *Euphyllia divisa*, *Heliofungia actiniformis* and *Goniopora* sp. showed signs of infection, in some cases exhibiting a brown microbial mat before death occurred. Comparison of controls and cyanide-treated corals indicated severe morphological changes (Fig. 2a–f).

3.2. Zooxanthellae abundance and mitotic index

Our results reveal higher than normal mitotic indices (MI) and decreased zooxanthellae densities upon exposure to CN⁻ concentrations of 50, 100, 300, or 600 mg/l.

Acropora sp. Control samples had higher counts of symbiotic algae cells ($n = 4972$; $P < 0.05$) within host tissue compared to test samples. In samples exposed to 50 mg/l CN⁻, the density was ($n = 4424$) per 2.5 sq cm. This represents an 11% decrease compared to control after 24 h (Figs. 3a,b and 4). The MI increased from 2.3% in controls to 3.8% in exposed samples ($P < 0.05$). Slight bleaching was evident. *Acropora* tips exposed to CN⁻ for 1–2 min and analyzed after 30 days, showed a zooxanthellae density of ($n = 977$) compared to ($n = 1395$) in controls, a 30% decrease. The MI was

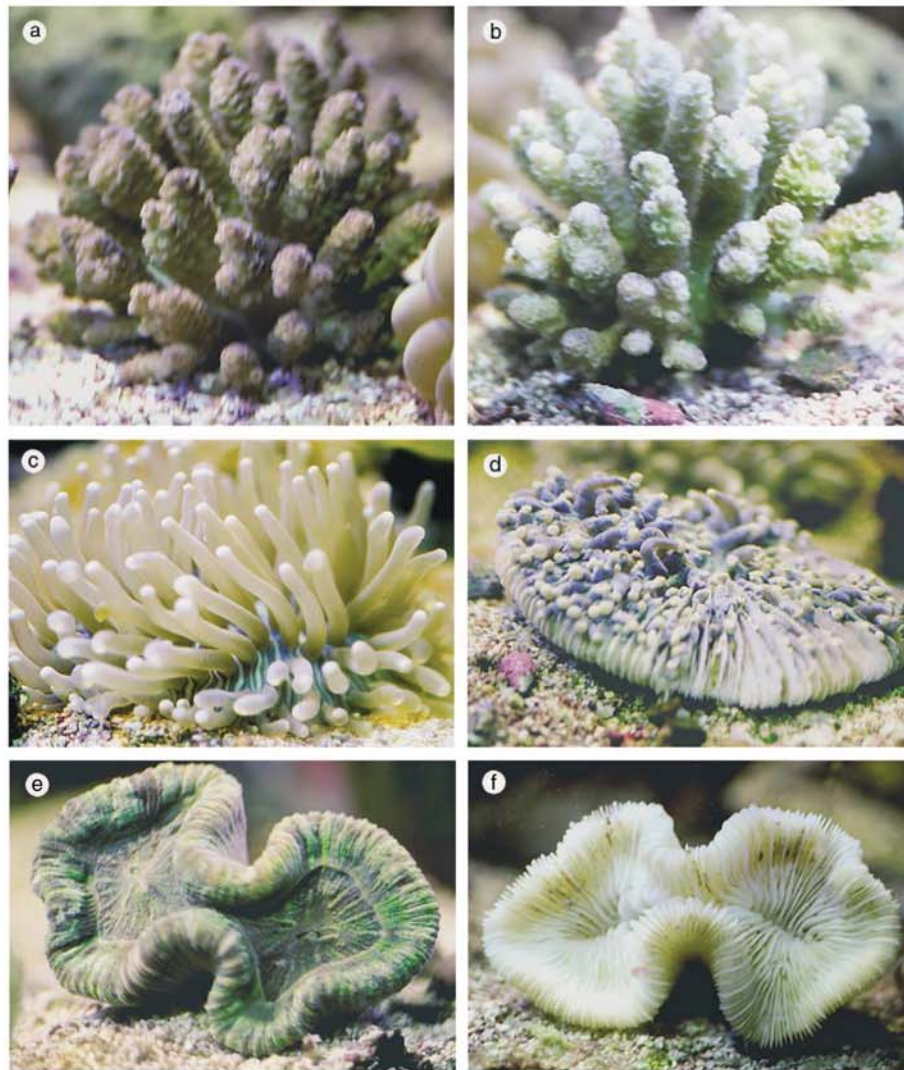


Fig. 2. All are exposed to 600 mg/l of CN^- : (a) *Acropora millepora* control; (b) *Acropora millepora* 24 h after exposure; (c) *Heliofungia* control; (d) *Heliofungia* 72 h after exposure; (e) *Trachyphyllia geoffrio* control; (f) *Trachyphyllia geoffrio* fully bleached 3 weeks after exposure.

2.7% in cyanide-exposed samples, compared to 2.0% in controls.

Favites abdita. Control samples had ($n = 864$; $P < 0.05$) symbiotic algae within host tissue. The density of zooxanthellae in samples exposed to 600 mg/l CN^- was ($n = 1713$) after 24 h, a 50% increase. The MI increased from 1.16% in controls to 1.44% ($P < 0.05$). Mild bleaching was evident (Fig. 5a and b). The animals that survived remained healthy 7 months post-exposure.

Heliofungia actiniformis. Controls had ($n = 4532$; $P < 0.05$) symbiotic algae within host tissue. The density of zooxanthellae in samples exposed to 600 mg/l CN^- was ($n = 2590$) after 24 h, a 43% decrease. The MI increased from 0.7% in control samples to 1.00% ($P < 0.05$) with cyanide-exposed samples (Fig. 6a and b). The same sample, 30 days post-exposure, showed the zooxanthellae density was ($n = 818$; $P < 0.05$), an 82%

decrease. The MI for the 30-day-exposure sample increased from 0.7% in controls to 3.67% in CN^- exposed specimens.

Euphyllia divisa. Control samples had counts of ($n = 2934$) symbiotic algae within host tissue. Zooxanthellae density in test samples exposed to 600 mg/l CN^- was ($n = 1314$) after 24 h (55% decrease). The MI increased from 1.53% in controls to 5.03% in test corals. The same sample, 8 weeks post-exposure, showed the zooxanthellae density had decreased to ($n = 797.6$), a 73% decrease. The MI increased from 1.53% in controls to 8.06% in test corals (Fig. 7a and b).

Trachyphyllia geoffrio. Control samples had ($n = 2821$) symbiotic algae within host tissue. The zooxanthellae density in test samples exposed to 600 mg/l CN^- was ($n = 1460$), a 48% decrease. The MI increased from 2.0% in control samples to 2.3% in test corals. Eight

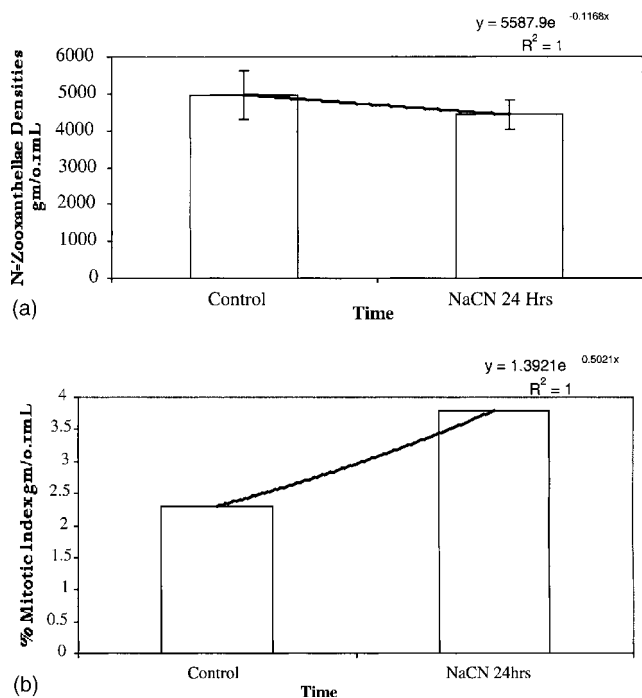


Fig. 3. (a) Zooxanthellae densities in *Acropora millepora* exposed to 50 mg/l of CN ($P < 0.05$). (b) Mitotic index (MI) of *Acropora millepora* exposed to 50 mg/l of CN. Twenty four hours after exposure at 120 s ($P < 0.05$).

weeks post-exposure, the zooxanthellae density with the same test sample was ($n = 884$), a 69% decrease. The MI increased to 6.7% compared to controls. (Fig. 8a and b).

Aiptasia pallida. Twenty four hours control samples had ($n = 42,740$; $P < 0.05$) symbiotic algae within host tissue. The zooxanthellae density in samples exposed to 600 mg/l CN^- was ($n = 2267$), a 47% decrease. The MI increased to 2.7% compared to 1.2% in control samples. The same test sample, 8 weeks post-exposure, showed zooxanthellae density was ($n = 1397$; $P < 0.05$), a 67% decrease. The MI increased to 4.4% compared to controls (Fig. 9a and b).

3.3. Protein electrophoresis

Gel electrophoresis was used to examine whether *Acropora millepora* exposed to 52 mg/l CN^- underwent changes in protein expression compared to control samples. Results show that expression of low molecular weight proteins is inhibited in cyanide-exposed samples, and expression of other proteins is increased (Fig. 10).

3.4. Histology

Histological examination indicated that during exposure to 50 and 100 mg/l CN^- , cellular damage occurred in *Acropora millepora* (Fig. 11a–c). The control tissue revealed darkly pigmented zooxanthellae with distinct organelles. Light microscopy revealed retraction

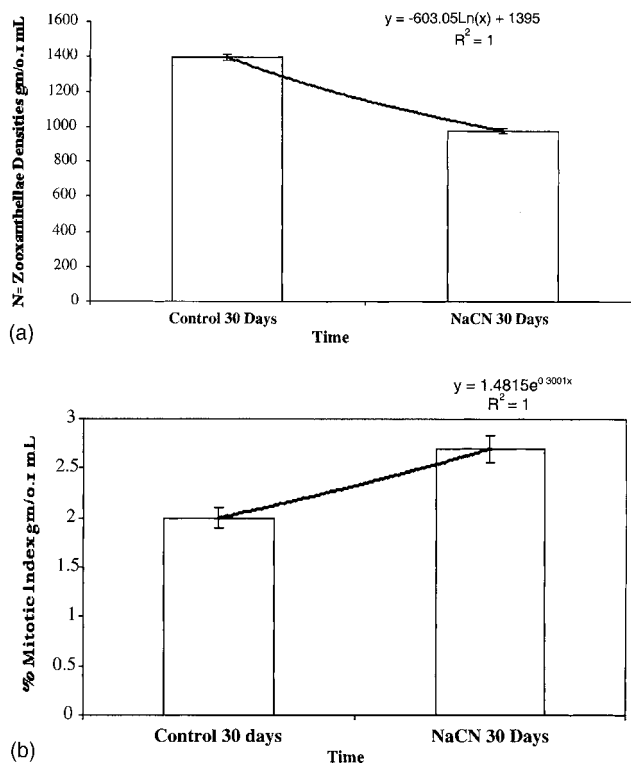


Fig. 4. (a) Zooxanthellae densities in *Acropora millepora* exposed to 50 mg/l of CN after 30 days. (b) MI of *Acropora millepora* exposed to 50 mg/l of CN after 30 days.

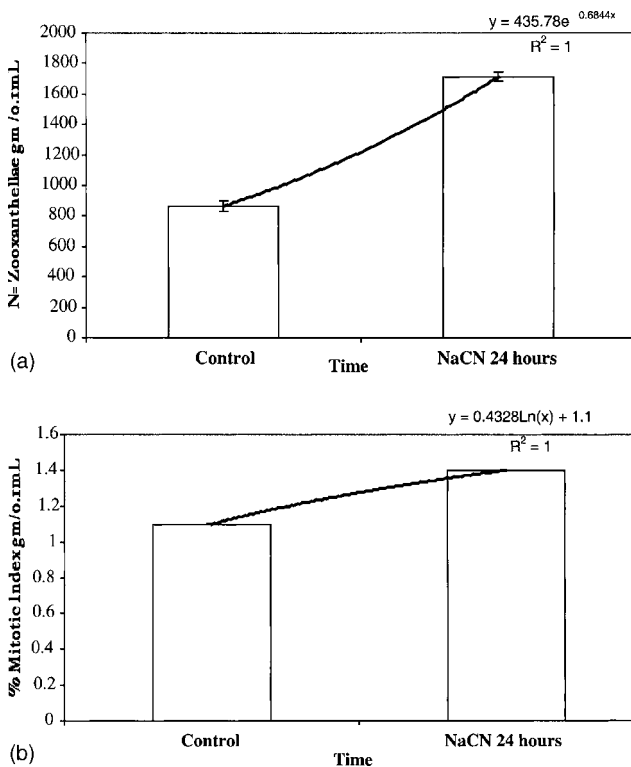


Fig. 5. (a) Zooxanthellae densities in *Favites abdita* exposed to 600 mg/l of CN ($P < 0.05$). (b) MI of *Favites abdita* exposed to 600 mg/l CN ($P < 0.05$).

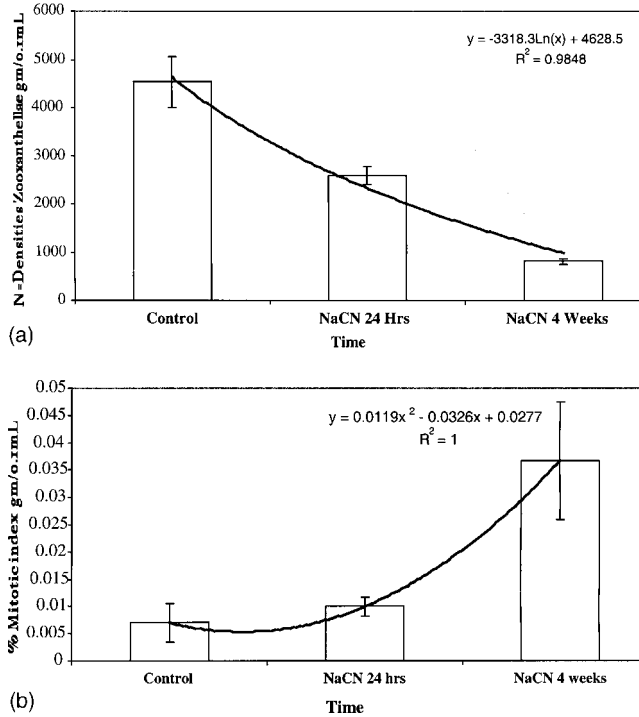


Fig. 6. (a) Zooxanthellae densities in *Heliofungia actiniformis* exposed to 600 mg/l CN ($P < 0.05$). (b) MI of *Heliofungia actiniformis* after exposure to 600 mg/l CN ($P < 0.05$).

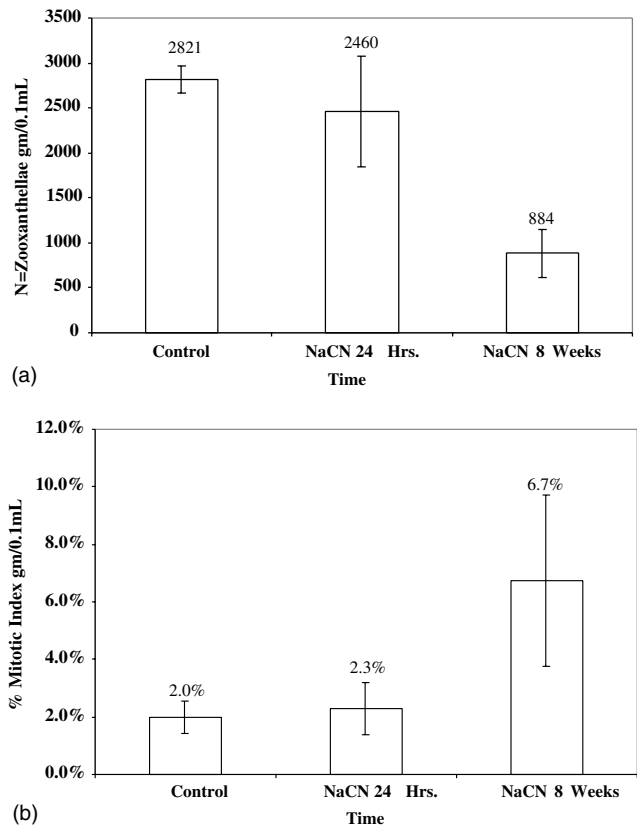


Fig. 8. (a) Zooxanthellae densities in *Trachyphyllia geoffrio* exposed to 600 mg/l of CN. (b) MI of *Trachyphyllia geoffrio* exposed to 600 mg/l CN.

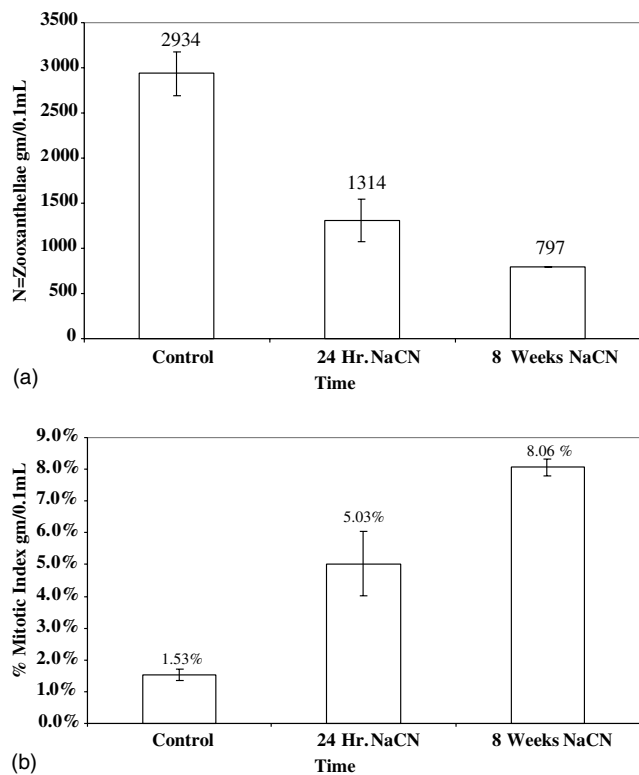


Fig. 7. (a) Zooxanthellae densities in *Euphyllia divisa* exposed to 600 mg/l CN. (b) MI of *Euphyllia divisa* exposed to 600 mg/l CN.

of mesenterial filaments, gastrodermal disruption, excess mucus production, and loss of pigment as well as swelling and disfigurement of the zooxanthellae cells. Minimal doses of CN^- caused an immediate stress response by *Acropora millepora*. The slight loss of pigment and excess mucus release are the first signs of a stress-response (Hayes and Goreau, 1998). Swelling of the zooxanthellae cells was visible macroscopically. Cyanide-exposed corals and anemones showed changes in cellular morphology compared to control samples. The average diameter of cells in control tissues was approximately 1.14 μm at a print magnification of 1250 \times . This means that the unit diameter of the zooxanthellae was about 0.01 mm. At 48 h after cyanide dosing for 60 s, the zooxanthellae had lost some of their staining intensity and the cytoplasm appeared disorganized. At 48 h after a 120-s exposure, the same trend was apparent. The cytoplasm was poorly stained and the chloroplasts appeared less dense. At 48 h after the 120-s exposure, the cytoplasm appeared vacuolated and the chloroplasts appeared less dense. No changes in the morphology of the pyrenoid body or the nucleus were apparent. The average diameter of the zooxanthellae cells seemed to be reduced by 5–9% in cross-sectional area. This result may

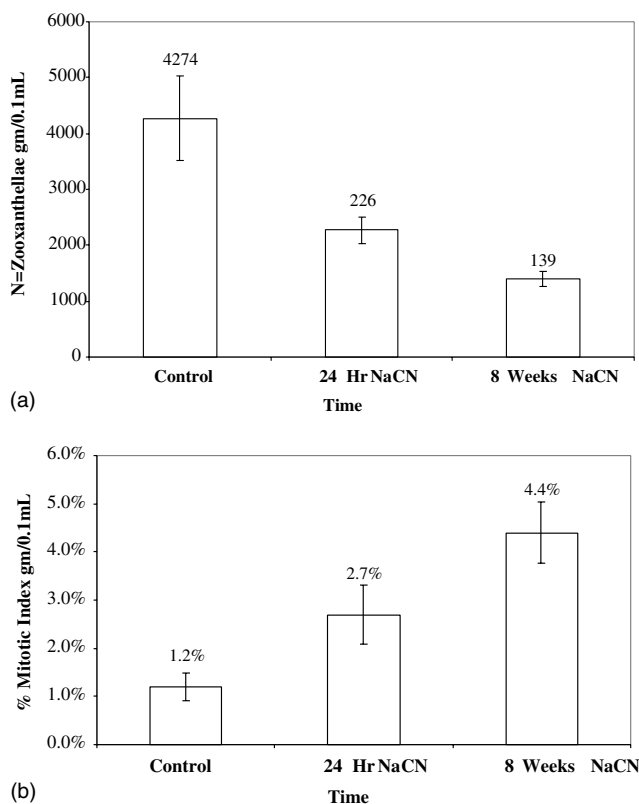


Fig. 9. (a) Zootaxanthellae densities in *Aiptasia pallida* exposed to 600 mg/l of CN ($P > 0.46$). (b) MI of *Acropora pallida* exposed to 600 mg/l CN ($P < 0.05$).

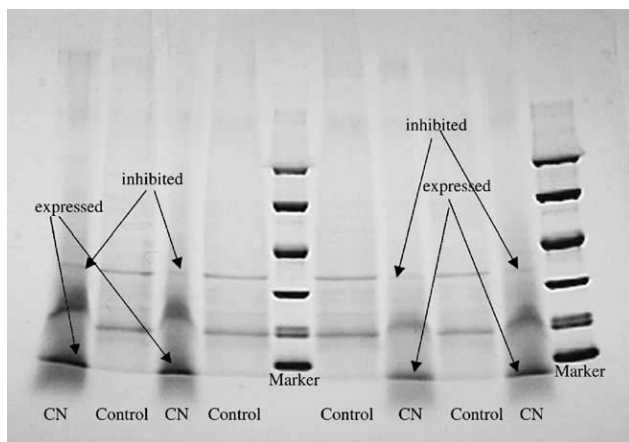


Fig. 10. Results of gel electrophoresis on the symbiotic algae in *Acropora millepora* exposed to 100 ppm of CN. Lanes are labeled accordingly; control for non-exposed and CN for cyanide-exposed samples. M is for the low molecular weight marker. Expressed arrows indicate a production of proteins that are not evident in the control lanes. Inhibited arrows indicate a loss of protein content or a failure of enzyme activity that is evident in the control samples.

be attributed to reduced cell volume or to changes in roundness of the cells during cyanide exposure.

Corals exposed to CN^- for either 60 or 120 s were examined 15 days post-exposure. The zooxanthellae

from the corals exposed for 60 s had cross-sectional areas close to control values, but those exposed for 120 s were about 7% reduced in size in comparison to controls. After 7 days, the integrity of the gastrodermis began to disappear in the 60- and 120-s exposures (Fig. 11b and c). The gastrodermal tissue exposed for 60 s showed irregularity of the luminal border as well as significant release of mucus, leaving a vacuolated cytoplasm. Blebbing of the luminal membrane of the gastrodermal cells may account for the release of zooxanthellae into the gastrocoele. Disruption was more obvious in the 120-s exposure. Zooxanthellae were liberated from the gastrodermis and the integrity of the epithelial sheet was further advanced (Fig. 11c). The same structural change was apparent in the mesenterial filament, which discharged their zooxanthellae and evidenced filament disruption. After 15 days, the 60-s-exposed tissues showed further disintegration of the gastroderm and persistent loss of mucus. The 120-s-exposed tissues showed disruption of the gastrodermis, both on the cellular and tissue levels. A 300 \times magnification image of the cyanide-exposed *Heliofungia actiniformis* showed three cross-sections of the epidermis, mesoglea and gastrodermis (Fig. 12a). Unusual configurations of zooxanthellae within gastrodermal tissue were evident. Images taken after two days showed zooxanthellae being released from the gastrodermis (Fig. 12b). *Heliofungia actiniformis* showed ghosts of zooxanthellae, and the gastrodermis was observed to release mucus (Fig. 12b). The effect increased with duration of exposure to cyanide, as well as dosage level. We observed a gradual loss of gastrodermal integrity over time.

Apical blebbing or vesiculation of the epidermis was also evident. In comparison to control tissues, the 72-h exposure to CN^- appeared to result in disruption of the normal integrity of the apical epidermal cells. Not only were mucous contents released from these cells, but the epidermal border and the cell surfaces were irregular and lacking in normal membrane projections (Fig. 13).

Following cyanide exposure, the zooxanthellae in the tissue are pale. Some appear homogeneous and empty of organelles, while other zooxanthellae are distorted or irregular in shape (configuration). Based upon the histopathology of *Heliofungia actiniformis*, CN^- exposure has severely damaged tissue morphology and function. Also, the zooxanthellae within the gastrodermis appear abnormal. The other coral genera appear to be similarly degenerated following cyanide exposure, with the worst effects in corals exposed for the longest duration. Even brief exposure to low cyanide levels resulted in long-term and irreversible damage to corals. In *Plerogyra* sp. and *Goniopora* sp., slight bleaching was evident following exposure, and polyps remained retracted on certain areas on the colony while fully extended in other areas. *Plerogyra* sp. and *Favites abdita* survived the longest

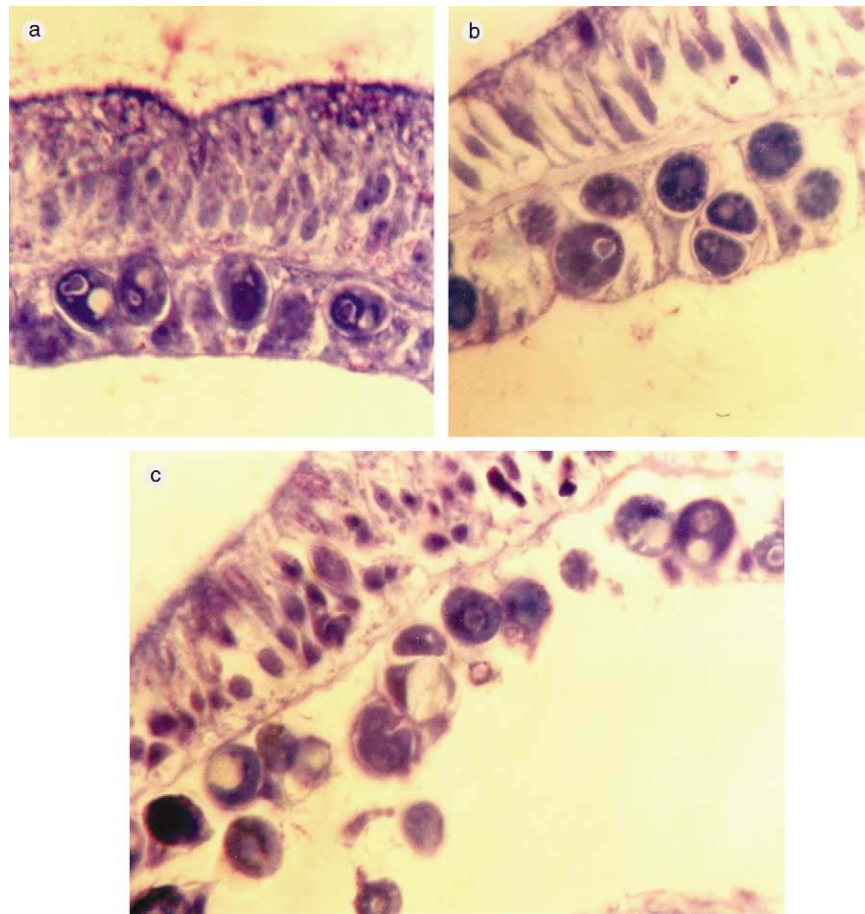


Fig. 11. *Acropora millepora* exposed to 50 and 100 mg/l. Photo on the upper left is the control, middle is 50 mg/l, and far right is 100 mg/l exposures. 50 and 100 mg/l exposures images reveal a complete loss of integrity within the epidermis, increase production of mucocytes, swelling of the mesoglea, and abnormal zooxanthellae released into the gastroderm. The average diameter of a cells in control tissues is approximately 1.14 μm at a print magnification of 1250 \times . This means that the unit diameter of the zooxanthellae is about 0.01 mm. The average diameter of the cells, however, seems to be reduced by 5–9% in cross-sectional area.

after exposure, whereas *Acropora millepora* bleached within the first week. *Heliofungia actiniformis* detached within 1 week after exposure, and *Euphyllia divisa* detached between 2 and 4 weeks post-exposure. *Scarophyton* sp. lost the ability to extend its tentacles fully compared to controls. The polyps remained fully retracted or partially extended until necrosis. The edges of *Scarophyton* after exposure were severely necrotic, developing a granular appearance as well as a loss of polyp extensions. Some species with thicker tissues such as *Goniopora* sp., *Plerogyra* sp. and *Euphyllia divisa* seemed hardier and better able to survive.

4. Discussion

This is the first medium-term study to monitor sub-lethal affects on corals from cyanide exposure. Corals and anemones exposed with 50–600 mg/l CN^- showed cellular impairment that dissociated the symbiotic relationship between host coral and resident alga (zooxan-

thellae). The CN^- dosage used by fishermen can be far greater, and therefore far more devastating to corals reefs. Estimates of the CN^- concentration used by fishermen range from 1500 to 120,000 mg/l (Johannes and Riepen, 1995; Pet and Djohani, 1998; Jones and Hoegh-Guldberg, 1999). This in vitro experiment indicates that minimal concentrations of cyanide on corals induce cellular damage and death.

Species of *Acropora* appear to be particularly targeted by cyanide fishermen because fish hide in *Acropora* branches. Our experiments indicated that *Acropora* was the genus most susceptible to cyanide, showing more rapid signs of stress and loss of zooxanthellae (*Acropora* sp. died in 24 h of exposure) compared to the other coral genera tested. The results showed that coral species with thicker tissue, such as *Plerogyra* sp., *Goniopora* sp., and *Favites abdita*, respond more slowly to cyanide exposure. *Favites* and *Plerogyra* sp. survived the longest (up to 7 months) after CN^- exposure. Swollen tissue in the subject corals was evident throughout the experiment and bleaching remained evident after 4 months in two of

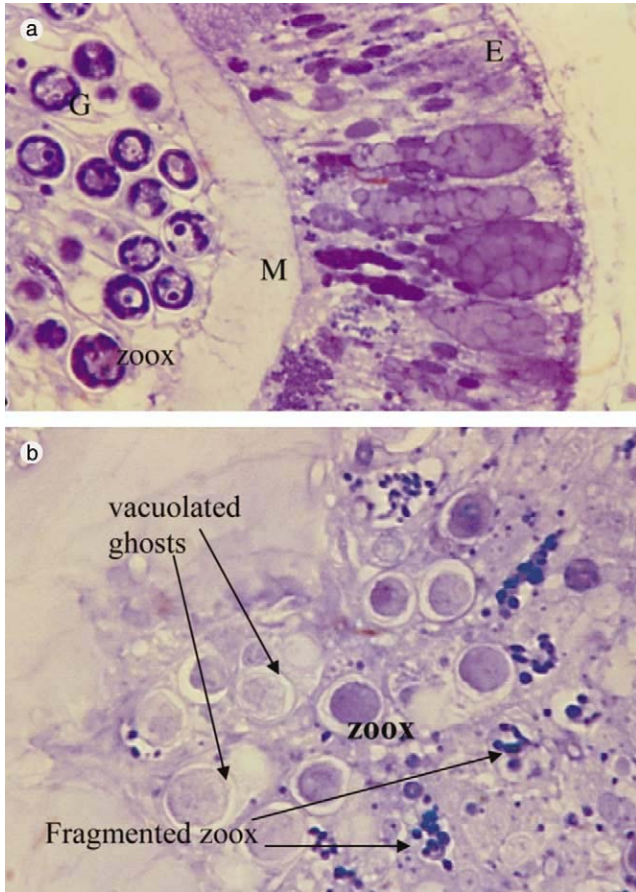


Fig. 12. (a) Histology of *Heliofungia* shows three profiles hours after exposure to 600 mg/l CN: the histological section *Heliofungia actiniformis* (300× magnification) depicts zooxanthellae in the basal gastrodermis showing signs of abnormal configuration. The mesoglea is swollen and irregular at 2 days after 120 s exposures. The changes are most evident in the coral gastrodermis and in the zooxanthellae. However, changes are also evident in the epidermis and in the mesoglea as well. (b) Image indicates swelling of the gastrodermal cells, leading to their progressive rupturing and the ultimate releasing of zooxanthellae. The swelling of the vacuoles encompassing the algal cell results in the loss of membrane integrity. There also appears to be the vacuolization of the algae themselves, as is seen after the 100 mg exposure.

the specimens tested. Our results indicate that changes in protein expression occur following cyanide stress. Some proteins showed increased density, while others decreased. Further, results show that prolonged exposure to low levels of cyanide may dissociate or alter the conformation of integral membrane proteins.

Cyanide is one of the most rapidly acting, toxic, and devastating poisons to biological systems. It acts by abruptly terminating cellular respiration through inhibition of key enzymes (Buchel and Garab, 1995). Electron transport in mitochondria is inhibited by cyanide due to disruption of the cytochrome oxidase enzyme system. Cyanide binds to the trivalent iron atom in the heme molecule of the cytochrome *a*, *a3* component during electron transport, preventing oxygen from re-

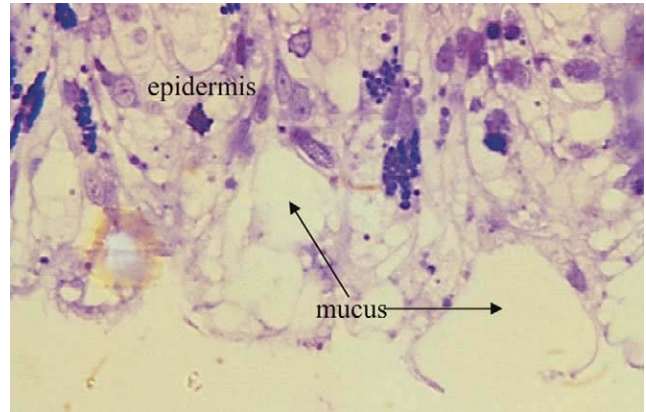


Fig. 13. In comparison to control tissues, exposure to CN appears to result in disruption of the normal integrity indicates apical blebbing of epidermal cells after 48 h. Not only is there release of mucous contents from these cells, but the microvillous border and the cell surfaces are irregular and lacking normal membrane projections. 48 h after CN exposure shows abnormal zooxanthellae within the gastrodermis (notice the fragmented degenerative alga). At 8 weeks, the epidermis is abnormal, however the gastrodermis is still affected.

acting with this terminal receptor (Buchel and Garab, 1995; Devlin, 1997). The resulting loss of mitochondrial respiration and energy production are responsible for cell death following cyanide exposure (Devlin, 1997). It is interesting to note that the zooxanthellae, which are symbiotic in corals, are so susceptible to cyanide.

Cyanide is also known to inhibit the normal function of carbonic anhydrase, the enzyme responsible for generating the carbonate ions required for the production of coral skeletal aragonite (Hayes and Goreau, 1977). Both carbonic anhydrase and cytochrome oxidase are membrane-bound enzymes. Cytochrome oxidase is an integral component of the inner mitochondrial membrane in animal cells and of the thylakoid membrane of the chloroplast in plant cells. Carbonic anhydrase in the coral epidermal cell associates with the plasma-lemmal membrane and associated cytoplasmic vesicles.

Cyanide appears to be disrupting the specialized deposition of calcium salts in epidermal surfaces contacting substrate skeleton. Anchoring desmocytes are cells that attach the calcicoblastic epithelium to the skeleton. This dynamic adhesion is joined by fibers that pass through the plasma membrane attaching to the skeleton (Muscatine et al., 1997). Cyanide may cause corals to lose the capacity to attach to the calcareous skeleton, leading to detachment of the tegument over time. Our results found that cyanide-exposed *Heliofungia actiniformis*, *Euphyllia divisa*, *Goniopora* sp., and *Trachyphyllia geoffrio* frequently exhibited detachment of tissue from skeleton (Fig. 14).

Increases in cell division (reproduction) of the zooxanthellae and lower zooxanthellae densities were evident even when the mildest dose of CN⁻ was used (50 mg/l). Those responses were most severe in corals

exposed to the highest dose (600 mg/l). Overall, zooxanthellae in controls divided approximately two times less frequently than those in corals exposed to 50–600 mg/l CN^- . Rapid increases in mitosis leading to expulsion of zooxanthellae were also evident during our experiment. This can leave the coral with reduced photosynthetic capabilities. Continuous cell division of algal populations may result from lower densities of zooxanthellae, a condition that may induce density-dependant division in order to restore original algal concentrations (Muscatine and Neckelmann, 1981; Hoegh-Guldberg et al., 1987; Jones and Yellowlees, 1997; Falkowski et al., 1993; Cervino's Masters Thesis, 1995).

Our results showed increased division of zooxanthellae despite decreased zooxanthellae densities. A similar outcome occurred in other experiments, when corals were exposed to various concentrations of copper (Jones and Yellowlees, 1997). In our experiment, only *Favites abdita* showed an increase in algal density in combination with a higher rate of cell division upon exposure to CN^- . The failure of *Favites* to expel the symbiotic algae, and the presence of thicker tissue deep into the skeletal matrix, may explain this occurrence. Why this coral species behaves differently than the others is unknown and requires further investigation. However, it has been hypothesized that density-dependant release factors during the recovery period following bleaching may account for the balance of host cells and symbiotic alga (Jones and Yellowlees, 1997). Following exposure to cyanide, *Favites* samples were observed to have symbiotic algae along the surface of the oral cavity packaged in mucus. The alga was being slowly released while rarely showing signs of tissue-bleaching. Post-exposure, we noticed the slowly expelled alga in good

condition. Jones and Yellowlees (1997) hypothesized that the presence of normal zooxanthellae in the coelenteron may act as a readily obtainable supply for algal-free host cells to replenish themselves in the actively growing areas. In the case of our experiment, during cyanide exposure, *Favites* may have been accumulating symbionts. During cyanide exposure, algal division frequency was inversely correlated with algal density. Jones and Yellowlees (1997) reported a similar response during the bleaching recovery period. The highest algal division frequency was in the growing white tips of *Acropora formosa* colonies. In those regions, the algal densities were at their lowest. In the segments adjacent to the tips, the zooxanthellae density sequentially increases and mitotic index decreases. Tissue samples containing symbiotic algae below the tips showed consistently higher MI and lower densities post-cyanide exposure. This may be due to an impairment of key enzymes that play a role in cell division. The control and regulation of algal division frequency and density may be due to mitogenic factors (Muscatine and Pool, 1979).

The disruption of the gastrodermis may also account for expulsion of zooxanthellae from the gastrodermis, and thus for the bleaching response of cyanide-treated tissues. Experiments conducted by Jones and Hoegh-Guldberg (1999) are consistent with our studies, which have shown that cyanide exposure will induce bleaching. This response is similar to the expulsion of zooxanthellae resulting from temperature-related coral bleaching events (Gates et al., 1992, 1995; Goreau and Hayes, 1994).

Gram-staining of corals and anemones subjected to CN^- had higher levels of gram negative bacteria on the coral surface microlayer (CSM) than controls. The CSM is heavily colonized by bacteria (DiSalvo, 1971; Ducklow and Mitchell, 1979a,b) and by other microorganisms (Lyons et al., 1998). The bacterial component on the CSM is important in nutrient recycling (Lyons et al., 1998), and responds to stresses applied to its coral host (Ducklow and Mitchell, 1979a,b).

5. Conclusion

Our results showed that a single exposure to cyanide at a concentration lower than that used by cyanide fisherman (Rubec et al., 2001) kills or severely impairs corals. Mortality was seen in all species tested at all CN^- concentrations used. We have shown that zooxanthellae densities decrease upon exposure, leading to bleaching or immediate death of the zooxanthellae, but that the rate of cell division increases. Changes occur in the structure of the zooxanthellae, coral host tissues, and in bacterial flora on the surfaces of the corals. Following cyanide exposure in this study, the coral species exposed to CN^- exhibited various cellular re-



Fig. 14. *Trachyphyllia geoffrio* tissue detachment from the skeleton 7 days after exposure.

sponses that involved changes in protein synthesis, a process dependant upon activation of transcription or repression of certain genes (Hayes and King, 1995; Black et al., 1995). Further molecular tests are necessary to identify the specific proteins being inhibited or expressed.

This experiment was unique from past in vitro experiments regarding CN^- exposure, due to the time frame of analysis and monitoring following exposure. Previous in vitro experiments were conducted for significantly shorter time periods than used in this study. We attempted to lengthen the experiment to monitor medium-term effects following exposure (R. Jones, personal communication, 2000).

Cyanide fishermen frequently revisit the same reefs. We conclude that even a single minor dose of cyanide kills corals or induces long term cellular impairments in surviving colonies. Corals are susceptible to microbial infection (Tables 1 and 2) and unable to control algal population densities, thereby leading to bleaching. We observed progressive tissue detachment with *Goniopora* sp., *Trachyphyllia geoffrio* and *Euphyllia divisa* until the tegument completely sloughed off from the skeleton, leading to death. Our results refute widespread claims by collectors of aquarium fish and live food fish that their cyanide use has little or no effect on corals. In fact, cyanide blocks respiratory electron transport, and impairs the dynamic between host corals and symbiotic zooxanthellae, as this study proves. Hence, cyanide fishing is detrimental to coral reefs. There is an urgent need for governments throughout the world to develop strategies to stop destructive fishing practices including the use of cyanide.

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