Pergamon

Marine Pollution Bulletin Vol. 38, No. 10, pp. 864–874, 1999

Printed in Great Britain

PII: S0025-326X(98)00160-X

0025-326X/99 $ - see front matter

PAM Chlorophyll Fluorometry: a New *in situ* Technique for Stress Assessment in Scleractinian Corals, used to Examine the Effects of Cyanide from Cyanide Fishing

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Sodium cyanide is being used on reefs in the Asia–Pacific region to capture live fish for the aquarium industry, and to supply a rapidly growing, restaurant-based demand. The effects of cyanide on reef biota have not been fully explored. To investigate its effect on hard corals, we exposed small branch tips of *Stylophora pistillata* and *Acropora aspera* to cyanide concentrations estimated to occur during cyanide fishing. Pulse amplitude modulation (PAM) chlorophyll fluorescence techniques were used to examine photoinhibition and photosynthetic electron transport in the symbiotic algae (zooxanthellae) in the tissues of the corals. These measurements were made *in situ* and in real time using a recently developed submersible PAM fluorometer. In *S. pistillata*, exposure to cyanide resulted in an almost complete cessation in photosynthetic electron transport rate. Both species displayed marked decreases in the ratio of variable fluorescence (F_v) to maximal fluorescence (F_m) (dark-adapted F_v/F_m), following exposure to cyanide, signifying a decrease in photochemical efficiency. Dark-adapted F_v/F_m recovered to normal levels in ~6 d, although intense tissue discolouration, a phenomenon well-recognised as coral 'bleaching' was observed during this period. Bleaching was caused by loss of zooxanthellae from the coral tissues, a well-recognised sub-lethal stress response of corals. Using the technique of chlorophyll fluorescence quenching analysis, corals exposed to cyanide did not show light activation of Calvin cycle enzymes and developed high levels of non-photochemical quenching (q_N), signifying the photoprotective dissipation of excess light as heat. These features are symptomatic of the known properties of cyanide as an inhibitor of enzymes of the Calvin cycle. The results of this *in situ* study show that an impairment of zooxanthellar photosynthesis is the site of cyanide-mediated toxicity, and is the cue that causes corals to release their symbiotic zooxanthellae following cyanide exposure. This study demonstrates the efficacy of PAM fluorometry as a new tool for in situ stress assessment in zooxanthellate scleractinian corals. © 1999 Elsevier Science Ltd. All rights reserved.

Keywords: coral; cyanide; chemical pollution; photochemistry; bleaching; pollution monitoring.

In a report commissioned by The Nature Conservancy (USA) and the South Pacific Forum Fisheries Agency, Johannes and Riepen (1995) revealed the widespread misuse of cyanide in the Asia–Pacific region, associated with the live reef fish trade. The use of cyanide on reefs originated in the Philippines during the early 1960s with the collection of tropical aquarium fish and invertebrates, principally for export to the United States, the United Kingdom, Germany and France (Rubec, 1986; Dufour, 1997). More recently, "cyanide fishing" has been used to supply a rapidly expanding restaurant-based market for live reef fish, primarily rock cod and grouper (*Epinephelus* spp.), coral trout (primarily *Plectropomus* spp.), barramundi cod (*Cromileptes altivelis*), Napoleon wrasse (*Cheilinus undulatus*) and also lobster (*Panulirus* spp) (Johannes and Riepen, 1995; Erdmann and Pet-Soede, 1996). Live reef fish captured in the Asia–Pacific region are transferred to central collection points, where they are distributed via live fish transport vessels or by air, to markets in Hong-Kong, Singapore, Taiwan, Malaysia, and mainland China (Johannes and Riepen, 1995; Riepen, 1997).

The short-term economic rewards offered by live reef fishing can be considerable (Cesar *et al.*, 1998). Live fish can fetch prices up to 25 times the price of equivalent dead ones (Erdmann and Pet-Soede, 1996). Premium

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species such as the Napoleon (Maori) wrasse can be sold live for US$9–11/kg by fishers in Indonesia, US$40–50/kg by local exporters, and US$70–90/kg by wholesalers to the restaurant trade (Johannes and Riepen, 1995; Erdmann and Pet-Soede, 1996). In Hong Kong restaurants, the same species can fetch US$180/kg. The live reef fish market in southeast Asia has an estimated annual retail value of US$1.2 billion, of which US$1 billion is associated with the food fish trade and US$200 million is associated with the export of aquarium fish (Barber and Pratt, 1997). The returns associated with the live reef fish trade have led to the development of several destructive fishing practices, including dynamite (blast) fishing (fish bombing), muro-ami (where fish are driven towards nets by long weighted lines which are rhythmically dropped on the reef), and the use of fish poisons. Sodium cyanide, quinaldine, chlorine, diesel fuel, and poisons derived from the leaves, berries, roots and seeds of trees, have been used to tranquilize fish hiding in holes in the reef matrix, thereby facilitating their capture (Rubec, 1986; Eldredge, 1987; Sadovy, 1992; McManus *et al*., 1997; Pet, 1997). More recently, the use of mixtures of sand and insecticides, including Endrin and Thiodan® (active ingredient endosulfan) has been reported during fish capture in Indonesia (Pet, The Nature Conservancy, Indonesia, personal communication, 1998). Cyanide is the predominant poison used in live reef fishing. Cyanide fishing has been confirmed in at least 15 countries or island territories including Indonesia, Malaysia, Maldive Islands, Papua New Guinea, the Philippines, Sri Lanka, Thailand, and Vietnam (Johannes and Riepen, 1995; Barber and Pratt, 1997; McManus, 1997).

Cyanide fishing techniques vary considerably. In the most common technique, sodium cyanide is dissolved in seawater in plastic bottles ('squirt bottles'). The milky solution is then squirted at fish, which are usually hidden in holes in the reef or within coral thickets or colonies. Fishers also squirt clouds of cyanide, which are wafted by hand movement to where the fish are located. Cyanide tablets may be secured to sticks and held close to a fish (McManus *et al*., 1997), or cyanide is mixed with baits and thrown overboard. In extreme cases, cyanide is pumped onto the reef from surface boats, using the turbulence from the propellers to mix it into the water column (McManus, International Center for Living Aquatic Resource Management (ICLARM), personal communication, 1995). Temporarily stunned fish are placed in hand-nets or attached to lines and hauled to surface support boats.

The cyanide used by fishers comes from the silver and gold mining and electroplating industries. It is purchased in the form of a tablet or powder. Between 1991 and 1995, 290 000 tonnes of cyanide was legally imported into the Philippines, 70 000 tonnes in 1995 alone (Barber and Pratt, 1997). Estimating the concentration of cyanide used by fishers in squirt bottles is difficult. For example, some fishers report the potency of the cyanide varies, suggesting it has been diluted by distributors before sale (Johannes and Riepen, 1995). Some fishers have been observed biting cyanide tablets with their teeth to break them into smaller pieces and allow placement into squirt bottles. The concentration of cyanide in the squirt bottles naturally decreases (is diluted) as repetitive applications are made. To accommodate this, cyanide fishers use several cyanide tablets to create a saturated solution (McManus (ICLARM) personal communication, Pet and Djohani, 1998). Excess cyanide dissolves as successive applications are made, thereby reducing the need to return to the support boat to replace the spent cyanide. Given these considerations, the concentration of cyanide in the squirt bottles is not known. Analyses of cyanide concentrations in squirt bottles seized on cyanide fishing vessels in Indonesia indicated concentrations of 2 g/l (Pet and Djohani, 1998). Whilst this result confirms the high cyanide concentrations used during cyanide fishing, the bottles may have been used previously, and the concentrations an underestimate. Suggested cyanide concentrations in squirt bottles vary by almost an order of magnitude: 13 g/l (Pet, 1997), 100 g/l (Barber and Pratt, 1997), 30–120 g/l (Johannes and Riepen, 1995).

Cyanide fishing has been banned in many countries although widespread illegal use continues. Concern has been raised over the collateral damage associated with cyanide fishing on reefs and, in particular, its effect on the hard corals, which provide the reef framework. In early studies of the environmental effects of cyanide on coral, it was shown that one of the first effects was to cause loss of the symbiotic algae (zooxanthellae) from the coral tissues (Jones and Steven, 1997). The zooxanthellae supply the host (animal) with photosynthetic products (sugars and amino acids) in return for key plant nutrients (ammonia and phosphate) from host waste metabolism (Trench, 1979; Muscatine, 1990). This mutualistic association is thought to be the key to the success of reef-building corals in tropical water. Loss of zooxanthellae causes corals to turn white, as the coral skeleton becomes visible through the relatively transparent animal tissue. The term bleaching has been used to describe the discolouration phenomenon. Corals can recover from loss of considerable quantities of zooxanthellae (i.e. it is a sublethal response), but in a bleached state they display reduced growth rates, and are unable to complete gametogenesis.

In laboratory-based studies, cyanide has been reported to affect respiration of the intact association, and photosynthesis of the zooxanthellae in the tissues. Despite brief exposure to comparatively high cyanide concentrations (1×10^{-1}, 1×10^{-2} M NaCN) respiration rates of the coral *Pocillopora damicornis* returned to pre-exposure levels in 2–3 h (Jones and Steven, 1997). Using Pulse amplitude modulation (PAM) chlorophyll fluorescence techniques, we observed a decrease in the photosynthetic efficiency of the zooxanthellae of cyanide-exposed *Plesiastrea versipora* that lasted for several

days (Jones and Hoegh-Guldberg, in press). Loss of zooxanthellae (bleaching) from *P. versipora* was closely correlated to the decrease in the photosynthetic efficiency of the zooxanthellae. We also observed a light dependent effect of cyanide, whereby a decrease in photosynthetic efficiency and loss of zooxanthellae only occurred in corals exposed to cyanide in the light (Jones and Hoegh-Guldberg, in press). We proposed that cyanide caused bleaching by affecting zooxanthellar photosynthesis, and that changes in photosynthesis could be used to assess the environmental impact of cyanide on corals during controlled releases in situ.

The chlorophyll fluorescence techniques used to determine the effect of cyanide on coral photosynthesis in the laboratory study are comparatively new in the study of aquatic plants and algae. The underlying principle, and recent progress in the applied use of the techniques, has been reviewed in Krause and Weis (1991) and Schreiber and Bilger (1993). In photosynthesis, antenna pigments absorb light and excitation energy is transferred to reaction centres of the two photosystems where it drives the photochemical reactions that initiate photosynthetic energy conversion. A small proportion of excitation energy is dissipated by the emission of fluorescence, stemming almost exclusively from chlorophyll *a* of photosystem II. Fluorescence emission competes with two other de-excitation processes that deactivate the excited chlorophyll states. These processes reduce (or quench) the amount of fluorescence, and are referred to as photochemical quenching (q_P) and non-photochemical quenching (q_N). Photochemical quenching reflects useful photochemistry (i.e. assimilatory or non-assimilatory electron flow), and depends upon the presence of oxidized Q_a (a quinone-type primary electron acceptor in photosystem II). When Q_a is oxidized, it can accept electrons from the photosystem II reaction centre, and pass these along the photosynthetic electron transport chain. This leads to oxidation of water, oxygen evolution, the reduction of $NADP^+$ to NADPH, membrane proton transport, ATP synthesis and eventually to the reduction of CO_2 to carbohydrate in the dark reactions of photosynthesis (Calvin cycle). The other means by which excited chlorophyll states can be deactivated is non-photochemical quenching, which reflects photoprotective dissipation of excess absorbed energy as heat in the light-harvesting antennae (Demmig-Adams, 1990; Horton and Ruban, 1994).

Differentiating between the two main quenching components (quenching analysis) can provide useful insights into regulatory processes that occur within the photosynthetic apparatus, especially under stress conditions (Schreiber *et al*., 1994). This can be achieved by applying pulses of strong light (saturation pulses). These cause the temporary reduction of Q_a and reduce photochemical quenching to zero; all remaining chlorophyll fluorescence quenching is then attributed to non-photochemical quenching (Bradbury and Baker, 1981). The pulse amplitude modulation principle is a new technique that allows separation of the fluorescence signal from the much stronger excitation light (Schreiber *et al*., 1986). Light is provided by a modulated measuring beam given by a high frequency light-emitting diode (LED). Fluorescence from a sample is measured by a selective window amplifier that is highly selective for pulse fluorescence signals against non-modulated and scattered light. This allows measurements of the efficiency of photosystem II electron transport to be made in full sunlight (see Schreiber *et al*., 1986).

Here, we further investigate the physiological and environmental effects of cyanide fishing on reef corals, by measuring the effects of cyanide on coral photosynthesis. We describe a series of experiments conducted *in situ* using modulated chlorophyll fluorescence techniques with a newly developed submersible PAM fluorometer. Experiments were designed to simulate the exposure of coral to a pulse of cyanide from a cyanide fisher's squirt bottle. We examine whether: (1) photosynthetic electron transport rate in the zooxanthellae is affected by the plume of cyanide; (2) a reduction in quantum yield of the zooxanthellae occurs *in situ* as a result of cyanide exposure; and (3) these physiological insults precede loss of zooxanthellae in the bleaching response. In so doing, we evaluate the efficacy of PAM chlorophyll fluorometry as a means of assessing stress in corals, such as that arising from the use of cyanide to capture fish.

Materials and Methods

Study site

Experimental work was carried out at One Tree Island (23°30′S, 152°06′E) on the Great Barrier Reef (Australia, Fig. 1) using the reef-building corals *Stylophora pistillata* and *Acropora aspera*. To reduce the possible environmental impact of cyanide on the reef, and to satisfy the permit requirements of the Great Barrier Reef Marine Park Authority (GBRMPA), experimental dosing of corals was conducted in a small sand gully (Fig. 1). Dosing experiments were conducted with single branches of coral (30–40 mm in length) and larger fragments (100 × 100 mm) collected from separate parent colonies located at 1–2 m depth at the top of the inner reef slope in the One Tree Island lagoon. Larger fragments possessed a common stem but divided to 10–20 terminal branches. Samples were mounted into small cylindrical polypropylene holders using non-toxic modeling clay, and maintained at 2 m depth at the dosing site for 2 d before experimentation.

Photo-respirometry study

To determine the photokinetic characteristics of *Stylophora pistillata* from 1 to 2 m depth, changes in respiratory oxygen consumption and photosynthetic oxygen production were measured in four colonies using an automated underwater photo-respirometer, similar in design to that described by Cheshire *et al*. (1995).

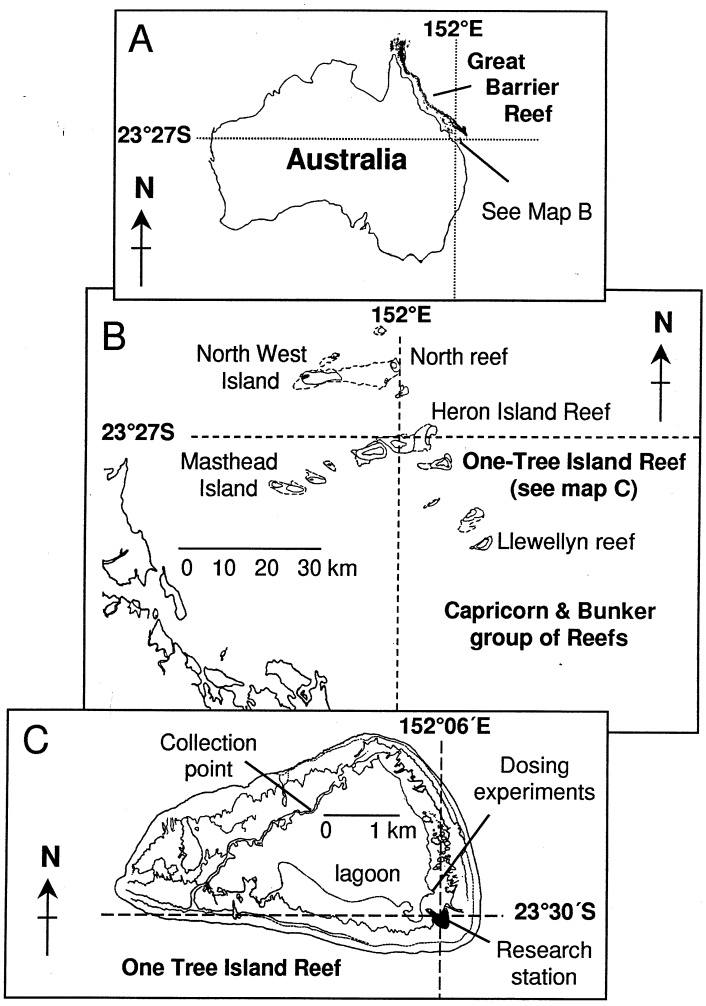

Fig. 1 Location map showing One-Tree Island Reef (23°30′S, 152°06′E, Map C), in the Capricorn and Bunker groups of reefs (Map B), in the southernmost section of the Great Barrier Reef, Australia (Map A).

Changes in oxygen levels, light intensity and temperature were recorded in 2 l acrylic chambers housing individual corals. Experiments were conducted in situ at a mean depth of 2 m. Data from each deployment were used to generate *PI* (*P*hotosynthesis versus *I*rradiance) curves, and a hyperbolic tangent curve (Chalker *et al.*, 1983) fitted to each set of raw data to determine the photokinetic parameters: $P_{m(gross)}$ (maximum gross photosynthetic rate), I_k (sub-saturating light intensity) and R (dark respiration rate; Eqn. (1)). Residual variances between predicted and observed values were minimized with multiple iterations of altered parameter sets using the Solver Utility of Microsoft Excel 1997. The compensation light intensity (I_c; the light intensity where net photosynthesis = 0) was determined from the results obtained from the computation of the model.

The model has the form:

$$P = P_{m(gross)} \cdot \tanh(I/I_k) + R, \quad (1)$$

where P is the production at any photon irradiance.

Chlorophyll fluorescence studies

At ambient temperatures, chlorophyll fluorescence in algae and plants emanates almost exclusively from antennae pigments of photosystem II. If a sample previously maintained in darkness (dark-adapted) is illuminated by a constant pulsed weak red-light source, chlorophyll fluorescence yield shows a characteristic change. The initial or constant fluorescence, F_0, of the samples signifies fluorescence when the reaction centres of photosystem II are fully oxidized. When a saturating pulse of white light is applied, to cause a closing (reduction) of the photosystem II reaction centres, fluorescence increases to a maximal value (F_m). The change in fluorescence from F_0 to F_m (ΔF) denotes the variable fluorescence, F_v (i.e. the fluorescence observed upon illumination). The ratio of variable to maximal fluorescence in a darkened sample (dark-adapted F_v/F_m, Eqn. (2)) is correlated to the quantum yield of photosynthesis and a convenient measure of the maximum potential quantum yield (Björkman and Demmig, 1987).

$$F_m - F_0/F_m = F_v/F_m = \Delta F/F_m. \quad (2)$$

In an illuminated sample, the F_0 and F_m values change, giving new values F and $F_{m'}$. The change in fluorescence $\Delta F/F_{m'}$ (Eqn. (3)) is lowered with respect to $\Delta F/F_m$ by partial closure of the reaction centres and a relative increase in non-radiative energy dissipation. The new value $\Delta F/F_{m'}$ is a measure of the effective quantum yield of photosystem II in an illuminated sample.

$$F_{m'} - F/F_{m'} = \Delta F/F_{m'}. \quad (3)$$

Since electrons leading to CO_2 reduction in the dark reactions of photosynthesis are derived from the splitting of water in photosystem II, photosynthetic electron transport rate (ETR) may be estimated from the effective quantum yield. Thus,

$$\text{Electron transport rate} = \text{ETR} = \Delta F/F_{m'} \times \text{PFD} \times 0.5. \quad (4)$$

where PFD = photosynthetic photon flux density of photosynthetically active radiation (400–700 nm), and an assumption is made that photosystem II absorbs half (0.5) the quanta of available light.

Cholorophyll fluorescence competes with two other processes that deactivate the excited chlorophyll states, photochemical quenching (q_P) and non-photochemical quenching (q_N). Separation of the q_P and q_N components of chlorophyll fluorescence is achieved by quenching analysis. During quenching analysis, samples are dark-adapted and F_0 and F_m determined. The sample is then illuminated and a series of saturation flashes applied at regular intervals to determine the new F_0 value (F) and F_m value ($F_{m'}$). The difference between $F_{m'} - F$ represents photochemical quenching (Eqn. (5)), and the difference between $F_m - F_{m'}$ represents non-photochemically quenched fluorescence (Eqn. (6)).

$$\text{Photochemical quenching} = q_P = (F_{m'} - F)/(F_{m'} - F_0), \quad (5)$$

$$\text{Non-photochemical quenching} = q_N = (F_m - F_{m'})/(F_m - F_0). \quad (6)$$

In the present study, chlorophyll fluorescence was measured using DIVING–PAM (Walz, Germany) and TEACHING–PAM (Walz, Germany) chlorophyll fluorometers. Specifications of these two new chlorophyll fluorometers are outlined by Jones *et al.* (1998) and Schreiber *et al.* (1997). All corals were dark-adapted for >20 min before measurements of chlorophyll fluorescence parameters. F_0 was determined after applying a modulated measuring beam of <1 μmol quanta m^{-2} s^{-1} and saturation pulses of white light (800 ms duration, >3500 μmol quanta m^{-2} s^{-1}) were used to determine F_m.

The effect of cyanide on electron transport rate in *Stylophora pistillata* was measured *in situ* using the DIVING–PAM fluorometer. Experiments were conducted between 12:00 and 16:00 h on clear, cloudless days. Corals were laid horizontally on a platform 0.1 m above the sand and a layer of 90% absorption shade cloth positioned over the experimental set-up to reduce the light levels to 100–200 μmol quanta m^{-2} s^{-1}. The fibre-optic cable of the fluorometer was then orientated at 60° to the plane of the coral, and moved to within 3–5 mm of the coral surface. PFD immediately adjacent to the coral was measured using the micro-quantum sensor on the fluorometer calibrated against a 2π cosine-corrected quantum sensor (LiCor 190 SA). Electron transport rates were calculated from the photochemical quantum yield of photosystem II in the light according to Eqn. (4). ETR was measured every 20 s for 5 min before exposing the corals to 50 ml of cyanide (2×10^{-1} M NaCN, or 2×10^{-2} M NaCN) or 50 ml of seawater (controls). Electron transport rate was then measured for a further 25 min. Cyanide solutions were prepared immediately before each experiment using analytical grade NaCN (Sigma Chemicals) dissolved in freshly collected seawater. A syringe was used to administer the cyanide or seawater to the corals. Only one measurement of electron transport rate could be made during each experiment; therefore, we alternated between experiments on control and cyanide-treated corals (either 2×10^{-1} M NaCN or 2×10^{-2} M NaCN), giving eight results for control corals and four results each for corals exposed to each cyanide concentration.

To examine the effects of cyanide dosing on dark-adapted F_v/F_m, nine prepared coral branches of *Stylophora pistillata* or *Acropora aspera* were oriented in a circle (radius 5 cm) and 50 ml of cyanide (2×10^{-1} M NaCN) applied to the centre of the circle using a medical syringe. Cyanide was expelled from the syringe continuously over a 10 s period. In each experiment, nine corals were dosed with cyanide and nine corals (controls) exposed to 50 ml of seawater. Three replicate treatments involving nine cyanide-treated or control corals were conducted with each species. Dark-adapted F_v/F_m of control and cyanide-treated corals was measured at 20:00 h, ~2 h after sunset (6 h after dosing). Dark-adapted F_v/F_m in the control and cyanide-treated corals was measured again 6 d after dosing, after which three randomly selected corals were chosen from each treatment replicate of nine control or cyanide-treated corals and frozen for biomass determination.

In addition to dosing experiments with single branches, we performed experiments with larger fragments of *Stylophora pistillata*. Fragments ($n = 3$) were each exposed to 50 ml of cyanide (2×10^{-1} M NaCN) applied from a medical syringe directly onto the tissue surfaces. Additional fragments were exposed to 50 ml of seawater only (controls). Dosing of the corals occurred between 11:00 and 12:00 h. After 1,3,4 and 6 d, dark-adapted F_v/F_m was measured at 20 randomly selected locations within each fragment, including upper and lower surfaces of branches in the interior and exterior parts.

The effect of cyanide dosing on non-photochemical (q_N) and photochemical (q_P) quenching was determined in the laboratory with the TEACHING-PAM fluorometer. A small branch of *Stylophora pistillata* was laid horizontally on the bottom of a 300 ml beaker and illuminated with ~100 µmol quanta m^{-2} s^{-1} (PAR) by a 50 W quartz-halogen spotlight. Seawater in the container was stirred using a magnetically coupled stir-bar and fresh seawater was allowed to flow into the container at a rate of 100 ml/min. After 20 min, 1 ml of a 2×10^{-2} M NaCN solution was gently expelled onto the coral surface using a syringe and the coral left for 5 min. The coral was then dark-adapted for 20 min, removed from the incubation chamber and placed on a glass side with a few drops of seawater on the 2 mm exit point of fluorometer measuring head (see Schreiber *et al.*, 1997). F_0, F_m and F_v/F_m (Eqn. (2)) were determined, after which the actinic light was turned on and a series of saturation flashes applied at 20 and 40 s intervals to calculate $\Delta F/F_{m'}$ (Eqn. 3) and q_N (Eqn. (6)) on-line, using a software controlled pre-programmed protocol.

Biomass determination

Coral tissues were stripped from the skeletons with a jet of re-circulated filtered seawater (~100 ml) using a WaterPik™ (Johannes and Wiebe, 1970). The slurry produced from the tissue-stripping process was homogenised in a blender for 30 s and the volume of the homogenate recorded. The number of zooxanthellae in 10 ml aliquots of the homogenate was determined using a haemocytometer (8 replicate counts). Total zooxanthellae per coral were determined after correcting for the volume of the homogenate. The density of zooxanthellae was expressed as number per unit surface area. Coral surface area was determined using the paraffin wax technique (Stimson and Kinzie, 1991). The density of zooxanthellae in a subset of freshly collected corals (referred to as a 'Field Control') was also determined to allow an examination of whether handling and preparation caused any significant loss of zooxanthellae from the test corals.

All data are presented as means ($\bar{x}$) ± standard deviation (SD). To test the null hypothesis that the cyanide exposure had no effect on dark-adapted F_v/F_m, or density of zooxanthellae in the tissues, data were analyzed ($\alpha = 0.05$) using type 1 analysis of variance (ANOVA). Assumptions of normality (Shapiro-Wilks test) and homogeneity of variance (Welchs test) were tested before analyses.

Results

Photo-respirometry study

The photokinetic characteristics of *Stylophora pistillata* colonies from 2 m depth at One-Tree Island were determined using a submersible photo-respirometer. We measured a maximum gross photosynthetic rate ($P_{m(gross)}$) and respiration (R) of 9.3 ± 2 and −3.1 ± 0.4 µmol O_2 g buoyant wt^{-1} h^{-1}, respectively ($\bar{x}$ ± SD $n = 4$ colonies), corresponding to a $P_{m(gross)}/R$ ratio of 3. The

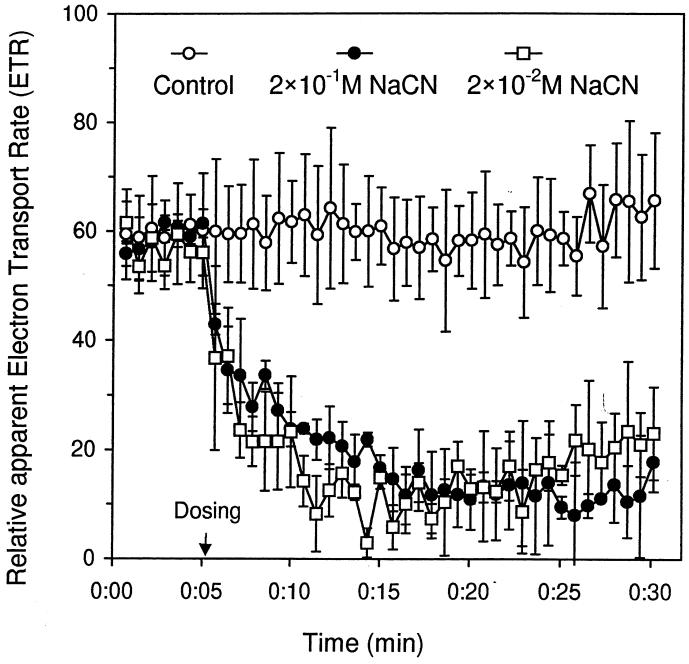

Fig. 2 *Stylophora pistillata*. Apparent relative electron transport rate (ETR; $\Delta F/F_{m'} \times \text{PFD} \times 0.5$) in *S. pistillata* before and after applying seawater (control), or 2×10^{-1} M NaCN or 2×10^{-2} M NaCN to the coral surface (dose administration indicated by an arrow). Experiments were conducted in situ under an irradiance intensity of 80–180 µmol quanta m^{-2} s^{-1}. Data are $\bar{x}$ ± SD, $n = 4$ corals (cyanide-treated), $n = 8$ (controls).

mean compensation light intensity (I_c) for the corals was 138 ± 40 μmol quanta m^{-2} s^{-1}.

The effect of cyanide on apparent relative electron transport rate in *Stylophora pistillata* was conducted under an irradiance intensity of 80–180 μmol quanta m^{-2} s^{-1}. The mean irradiance experienced by the corals (120 μmol quanta m^{-2} s^{-1}) was close to their I_c value determined using the photo-respirometer (see above). Applying 2×10^{-1} M or 2×10^{-2} M NaCN to the coral surface caused an almost immediate decrease in apparent relative electron transport rate (Fig. 2). We observed some contraction of coral polyps during exposure to cyanide; however, in most instances, the coral polyps were partially expanded in the corallites and retracted still further when gently touched. At the end of the 25 min monitoring period, electron transport rate was still drastically reduced at both cyanide concentrations.

Six hours after dosing corals with 2×10^{-1} M NaCN, dark-adapted F_v/F_m was 70% (*Stylophora pistillata*) and 60% (*Acropora aspera*) of values control corresponding to a significant difference between control and experimental groups (ANOVA $p<0.05$, Fig. 3). After 1 d, corals of both species exposed to cyanide appeared to be lighter in colour. After 3 d cyanide-treated corals had turned a brown light/colour and remained noticeably paler than the controls. There was no significant differences in dark-adapted F_v/F_m of cyanide-treated or control corals after 6 d (data not shown, but see Fig. 5).

A comparison of the number of zooxanthellae per cm^2 between bleached (cyanide-treated) and normal (control) coloured corals six days after experimentation revealed a significant difference (ANOVA, $p < 0.05$, Fig. 4). The density of zooxanthellae in the cyanide treated coral was ~40% of the density in control corals, or freshly collected colonies (Field Control).

Larger fragments of *Stylophora pistillata* containing 10–20 terminal branches also bleached when exposed to

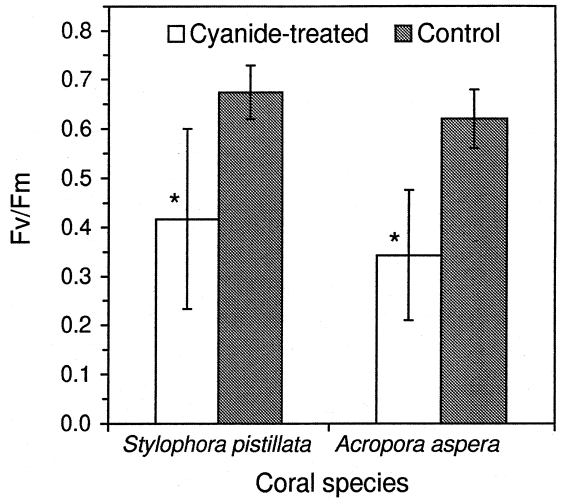

Fig. 3 *Stylophora pistillata*, *Acropora aspera*. Dark-adapted F_v/F_m in *S. pistillata* and *A. aspera* measured by a DIVING–PAM chlorophyll fluorometer 6 h after exposure to 50 ml of 2×10^{-1} M NaCN in situ. Data shown are $\bar{x}$ ± SD ($n=27$ corals, nine corals at each of three treatment replicates).

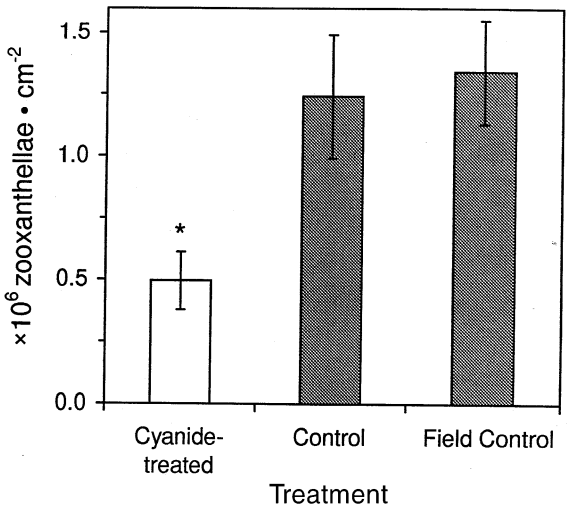

Fig. 4 *Stylophora pistillata*. Mean zooxanthellae • cm^{-2} in *S. pistillata* 6 d after exposure to 50 ml of 2×10^{-1} M NaCN in situ. Data are $\bar{x}\times10^6$ ± SD ($n=9$). Field Control refers to freshly collected samples.

cyanide. Bleaching of the tissues was first observed 24 h after cyanide exposure. At the end of the 6 d monitoring period the corals had discoloured to a light brown/pale yellow. Twenty-four hours after treatment, dark-adapted F_v/F_m in cyanide-treated corals was lower than in the control groups (ANOVA $p<0.05$, Fig. 5). After 6 d, dark-adapted F_v/F_m was directly comparable between groups.

Representative traces of the original fluorescence data of control and experimental corals are shown in Fig. 6. Maximum effective quantum yield ($\Delta F/F_{m'}$) and non-photochemical quenching (q_N) were determined by the saturation-pulse technique. In the control sample, when

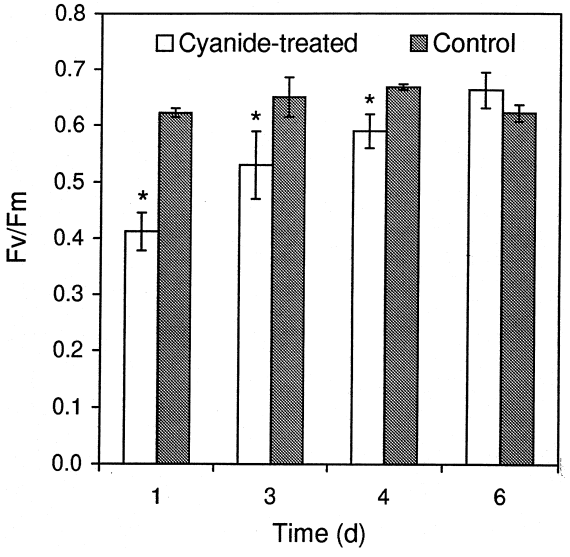

Fig. 5 *Stylophora pistillata*. Dark-adapted F_v/F_m in *S. pistillata* measured by a DIVING–PAM chlorophyll fluorometer 1, 3, 4 and 6 d after exposure to 2×10^{-1} M NaCN in situ. Data are $\bar{x}$ ± SD $n=4$ colonies. A mean of 20 measurements of dark-adapted F_v/F_m was taken on upper and lower branches in the interior and exterior for each colony.

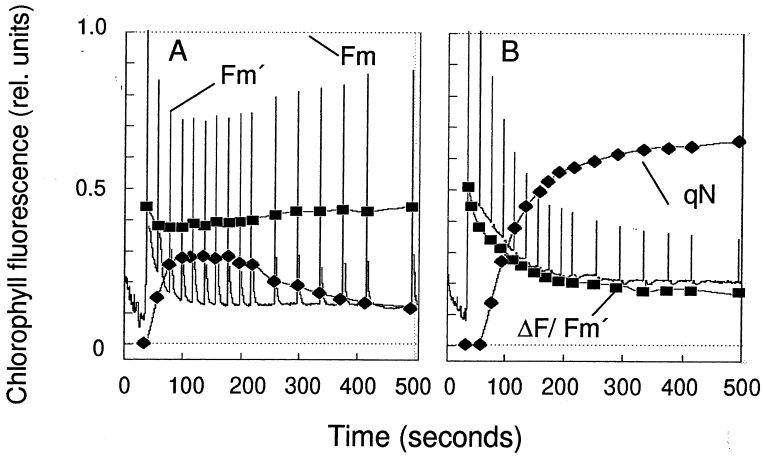

Fig. 6 *Stylophora pistillata.* Representative dark-light induction curves of control (A) and cyanide-exposed (B) *S. pistillata* with saturation pulse 'quenching' analysis (TEACHING–PAM, chlorophyll fluorometer) to determine non-photochemical quenching (q_N) and maximum effective quantum yield ($\Delta F/F_{m'}$). Corals were dark adapted for 20 min prior to determining F_0 and F_m). Corals exposed to cyanide were illuminated under 100 μmol quanta $m^{-2}s^{-1}$ PAR for 20 min, before injection of 2 ml of a 2×10^{-2} M NaCN solution onto the coral surface. Corals were illuminated for a further 5 min before dark-adaptation and quenching analysis.

actinic illumination was turned on, fluorescence rose to a peak and then declined to a steady-state level. Saturating pulses were applied at regular intervals to temporarily reduce Q_a and reduce q_P to zero. The new F_m values ($F_{m'}$) were lower than the original F_m because of non-photochemical quenching. During the first 3 min of illumination, q_N increased. This increase is associated with the light-driven formation of a proton gradient between the stroma and the thylakoid interior (i.e. the build up of a transthylakoid ΔpH) The activation of Calvin cycle enzymes causes utilization of the ΔpH and a corresponding decrease in q_N: this characteristic rise and fall is well known from previous studies on leaves of plants (e.g. Schreiber and Bilger, 1987). In cyanide-treated coral, light-induced non-photochemical quenching of fluorescence yield was considerably enhanced. Importantly, q_N did not diminish during illumination, but after an initial rise, further increased in a second slower phase. These features of cyanide exposed corals are symptomatic of a lack of induction of Calvin cycle activity following dark adaptation (Schreiber and Bilger, 1987).

Discussion

In this study, dosing experiments were conducted *in situ* with cyanide concentrations chosen to approximate those used in cyanide fishing. Applying a cloud of cyanide to the tissue of the coral *Stylophora pistillata* caused an almost complete cessation in the transport of electron down the photosynthetic electron transport chain of the symbiotic algae (zooxanthellae) in the coral's tissue. These measurements were conducted in real time, *in situ* on the reef, using a submersible chlorophyll fluorometer. Electron transport rates have been used to estimate photosynthetic carbon fixation because electrons in the water splitting process are used to fix CO_2 (Genty *et al.*, 1989). In our studies, the electron transport rate is only an 'apparent' rate, since the actual amount of quanta absorbed by photosystem II of the zooxanthellae is not known (Bilger *et al.*, 1995).

Applying cyanide to the corals *Stylophora pistillata* and *Acropora aspera* also caused a dramatic decrease in the ratio of variable to maximal fluorescence (dark-adapted F_v/F_m. Photoinhibition by excessive light is a well-known cause for a reduction of dark-adapted F_v/F_m (Krause and Weis, 1991, Long *et al.*, 1994), but other factors such as temperature stress, can also lead to a lowering of F_v/F_m signifying a reduction in photosystem II quantum efficiency. We have previously observed a short term, readily reversible dynamic reduction in F_v/F_m of corals (*Stylophora pistillata* and *Porites cylindrica*) at One-Tree Island associated with daily fluctuations in light levels (Hoegh-Guldberg and Jones, unpublished data). This was characterized by a decrease in dark-adapted F_v/F_m during daylight hours, but a recovery to normal levels before the start of the nextday. In cyanide-treated corals dark-adapted F_v/F_m remained markedly lower than that of control corals and was reduced for several days symptomatic of chronic photoinhibition (Osmond and Grace, 1995).

To further examine where cyanide affects photosynthesis of the zooxanthellae, we measured the levels of non-photochemical quenching in cyanide-exposed and control corals. Cyanide-exposed corals showed high levels of non-photochemical quenching, but did not

display the characteristic fall during illumination which signifies activation of Calvin cycle enzymes (see Fig. 6 and results section; Schreiber and Bilger, 1995). The lack of induction of Calvin cycle activity and the development of high levels of non-photochemical quenching, are consistent with the known properties of cyanide as an inhibitor of Calvin cycle enzymes, especially ribulose-1, 5-bisphosphate carboxylase/oxygenase (Wishnick and Lane, 1971). Non-photochemical quenching reflects photoprotective dissipation of excess absorbed energy as heat in the light-harvesting antennae once electron flow through to the Calvin cycle is slowed (Demmig-Adams, 1990; Schreiber and Neubauer, 1990). Non-photochemical quenching requires the formation of an acidic lumen via vectorial proton transport that accompanies light-driven electron flow through the photosystems (Mitchell, 1961). Previous studies on chloroplasts of higher plants have shown that electron flow to O_2 during the Mehler reaction is responsible for the formation of q_N during inhibition of CO_2 fixation by cyanide (Neubauer and Yamamoto, 1992). During cyanide fishing, corals may experience extremely high and also rapidly fluctuating cyanide concentrations depending upon the application procedures and conditions. Although our studies suggest an inhibition of Calvin cycle activity, at high concentrations cyanide is likely to have multiple effects on components of the electron transport chain including inhibition of the oxidation of plastoquinone-oxidoreductase (Buchel and Garab, 1995).

In corals where the zooxanthellae had been chronically photoinhibited by cyanide exposure, we observed tissue discolouration (bleaching). The bleaching was caused by a decrease in the density of zooxanthellae in the tissues. Similar results have also been reported for the coral *Plesiastrea versipora* (Jones and Hoegh-Guldberg, in press). Loss of zooxanthellae from coral (including *Stylophora pistillata*) has also been reported following a reduction in dark-adapted F_v/F_m after heat stress (Fitt and Warner, 1995; Jones *et al*., 1998). In the present study, we have used the high concentrations of cyanide, typical of cyanide fishing (Pet and Djohani, 1998). In previous studies, we report a significant decrease in photochemical efficiency and subsequent loss of zooxanthellae, in corals exposed to cyanide concentrations four orders of magnitude lower than those used in the present study (Jones Hoegh-Guldberg, 1998).

Dark-adapted F_v/F_m recovered in cyanide-treated corals over a period in which significant tissue discolouration occurred. We have previously suggested that the increase in dark-adapted F_v/F_m during the bleaching process is associated with the selective loss of damaged zooxanthellae (i.e. those with lower F_v/F_m), rather than repair processes within the zooxanthellae themselves (Jones and Hoegh-Guldberg, in press). It is important to recognize that despite having a 'normal' dark-adapted F_v/F_m ~6 d after exposure to cyanide, the corals had lost considerable quantities of zooxanthellae. Corals can recover from such extensive loss, but recovery of the algal population to a steady-state level may take between 16 and 24 weeks (Hayes and Bush, 1990; Jones and Yellowlees, 1997). In a bleached state, corals have reduced growth rates (Goreau and McFarlane, 1990), protein, lipid and carbohydrate concentrations (Glynn and D'Croz, 1990; Fitt *et al*., 1993), and are unable to complete gametogenesis (Szmant and Gassman, 1990). Thus, although loss of zooxanthellae from corals is a sublethal response, it has significant physiological and ecological consequences.

Cyanide-induced bleaching has now been documented in 5 species of corals: *Pocillopora damicornis*, *Porites lichen* (Jones and Steven, 1997), *Plesiastrea versipora* (Jones Hoegh-Guldberg, 1998), *Acropora aspera* and *Stylophora pistillata* (this study). These species encompass 4 families (Acroporidae, Faviidae, Pocilloporidae, and Poritidae), of the 17 extant families of zooxanthellate scleractinian corals. The species chosen include encrusting, massive and branching growth forms. This experimental evidence of bleaching is supported by observations of Erdmann and Pet-Soede (1996) who report bleached and dead corals surrounding holes or recesses on reefs where cyanide fishing had occurred. Presumably, the holes contained target fish that were collected using cyanide (Erdmann, personal communication, 1998). Interestingly, bleached corals were not observed in the interior of the recesses where the highest cyanide concentrations are likely to have occurred. It is interesting to speculate whether the pattern of bleached corals on the outside of the holes, as opposed to the inside where light levels are lower, was caused by the light-dependent effect of cyanide on loss of zooxanthellae reported previously (see Jones and Hoegh-Guldberg, in press).

Stress assessment in corals

Pulse amplitude modulation fluorometry is a new tool for stress assessment studies with marine plants and algae. In corals, it allows rapid and non-invasive assessment of the photochemical efficiency of photosystem II of zooxanthellae in the host tissues. Using recently introduced submersible instrumentation, these measurements can now be conducted *in situ* on a coral reef. It is becoming clear that in corals, external stressors such as elevated water temperature and cyanide, which cause an impairment of zooxanthellar photosynthesis, also cause corals to lose their zooxanthellae (Fitt and Warner, 1995; Jones *et al*., 1998). Other chemicals which are known to affect photosynthesis at low concentrations, such as diuron (3-(3′,4′-dichlorophenyl)-1,1-dimethyl urea (DCMU)) and copper, also cause loss of zooxanthellae from cnidaria (Suharsono *et al*., 1993; Jones, 1997). Similarly, increased irradiance intensity (Brown *et al*., 1994), and cold shock (Muscatine *et al*., 1991), which cause loss of zooxanthellae in corals, are known to cause an impairment of photosynthesis in algae and

plants. Recently, decreases in coral photosynthesis have been reported following reduced salinity (Moberg *et al*., 1997): bleaching following freshwater runoff has been frequently reported (for example Goreau, 1964). There is thus the engaging possibility that an impairment of zooxanthellae photosynthesis is the common (universal) cue that initiates the stress-related dissociation of the coral-algal symbiosis. If this is the case, PAM fluorescence techniques can be used to rapidly (i.e. within seconds) quantify the photochemical efficiency of zooxanthellae, and provide information of change that will ultimately lead to the dissociation of the symbiosis. Measurements of a reduction in photochemical efficiency, in combination with measurements of zooxanthellae loss (Jones, 1997), can therefore provide a powerful tool for stress assessment studies in corals in the laboratory and *in situ* on the reef. In addition, as demonstrated in the present communication, chlorophyll fluorescence techniques can provide a diagnostic analysis of the effect of a pollutant. This attribute may prove particularly useful in identifying which components of a complex mixture (i.e. drilling mud from the offshore oil and gas industry) are causing toxicity to corals.

The results of this study suggest that if a coral is exposed directly to a plume of cyanide from a cyanide fishers squirt bottle, there is an immediate disruption of photosynthetic electron flow in the symbiotic algae, possibly through the effect of cyanide on Calvin cycle enzymes. This leads to chronic photoinhibition of the algae and in turn results in their expulsion from the tissues causing the coral to bleach. Such loss of zooxanthellae is a well-known sublethal stress response of corals and has significant physiological and ecological consequences. Overall, the impact of the cyanide will depend upon irradiance intensities under which cyanide exposure occurs, and the product of cyanide concentration and exposure time. This in turn will be influenced by the proximity of corals to cyanide plumes and the local hydrological conditions (current speed etc.). The techniques described in the present communication may prove particularly informative in further evaluating the areal extent of environmental damage associated with cyanide fishing practices, especially when used in combination with modeling studies of the dispersal of cyanide plumes.

Considerable progress has been made in combating cyanide fishing in the Philippines by training fishers in alternative harvesting methods, developing alternative livelihoods for fishers and their families, detecting cyanide in fish, and efficient law enforcement (Vaughan Pratt, International Marinelife Alliance-Philippines (IMA) personal communication). However, under the influence of a rapidly increasing demand, destructive fishing practices have spread rapidly to many new locations where educational and environmental awareness programs are either in their infancy or are non-existent (Barber and Pratt, 1997).

RJJ would like to thank M.V. Erdmann, C.V. Barber, J.S. Pet, R.E. Johannes, Y. Sadovy, V.R. Pratt, J.W. McManus and H.S.J. Cesar for information and helpful discussion regarding the cyanide fishing issue in the Asia–Pacific region.

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