



Effects of the bloom-forming alga *Trichodesmium erythraeum* on the pearl oyster *Pinctada maxima*

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Abstract

Farmed pearl oysters (*Pinctada maxima*) suffered high mortality in the Dampier Archipelago of Western Australia in 1996. The mortality event affected all oyster sizes and coincided with extensive blooms of the cyanobacterium *Trichodesmium erythraeum*. The potent neurotoxin saxitoxin was detected in small amounts in some of the affected adult oysters but was not detectable in *T. erythraeum*. *Vibrio* species were isolated from some of the affected oysters but not in patterns consistent with a primary disease and no virus-like particles were observed. Juvenile oysters were exposed to medium and high concentrations of *T. erythraeum* in experimental aquaria for 7 days. No mortality of juvenile oysters occurred but individuals exposed to *T. erythraeum* at 10^5 cells/ml were less healthy than those fed upon the diatom *Chaetoceros calcitrans*. Histopathology of adult oysters from the affected farm and juvenile oysters exposed to *T. erythraeum* in the aquarium experiments were similar and included dilation of digestive gland lumens, sloughing of epithelial cells and granulocytes under the epithelial layer. These symptoms suggest that the *T. erythraeum* blooms in the archipelago were not a suitable food source for the oysters and may have contributed to the observed mortalities.

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1. Introduction

The pearling industry, based on *Pinctada maxima* Jameson, has been active off the Western Australian coastline since 1861 (Edwards, 1994). Today's industry is valued at over \$100 million US and uses both wild-caught and hatchery-reared animals for the

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production of pearl oysters (O'Sullivan and Dobson, 2002). In 2000, Australia was the 6th largest global producer with 250 tonnes grown (FAOSTAT, 2000). While biomass production by pearl oysters is relatively small compared to other seafood species, single pearls can obtain extraordinary prices. Since pearl oysters can produce pearls several times over their productive lifespan, each oyster is highly valuable livestock and understanding mortality issues for this industry is therefore of paramount importance.

In the 1970s and 1980s, there were extensive mortality events, due to poor handling and husbandry techniques (Dybdahl and Pass, 1985; Pass et al., 1987). Today, with improved methodology and hygiene, mortality of farmed oysters is usually restricted to juvenile (15–50 mm) animals. From June to October 1996, a large mortality event occurred in the Dampier Archipelago, Western Australia. Mortality was highest (~ 100%) in small pearl oysters (< 30 mm), high (50–90%) in medium (30–100 mm) sized oysters and lowest (20–50%) in large (>100 mm) pearl oysters (Bunter personal observation). A definitive cause was not elucidated, but oysters sampled from the vicinity of the mortalities were found to have dilated digestive gland lumens, sloughing of epithelial cells and large numbers of residual bodies and with increased numbers of brown cells and granulocytes under the epithelial layer.

Extensive blooms of the marine cyanobacterium *Trichodesmium* sp. were observed in waters of the Dampier Archipelago during the latter months of 1996. Blooms were most conspicuous in calm conditions, with trichomes forming dense mats or rafts on the surface of the ocean. Satellite imagery and empirical observations have shown such blooms also occur in Thailand, New Caledonia, Vanuatu, Fiji, Tonga, Gulf of Mexico, the tropical Atlantic coast of South America, the coast of the Indian sub-continent, Arabian and China Seas (Devassy et al., 1979; Dupouy et al., 1988; Suvapepun, 1989; Dupouy, 1992; Capone et al., 1998; Carpenter et al., 1999; Chang et al., 2000). These regions encompass most of the world's pearl oyster producing countries (FAOSTAT, 2000).

Two pelagic species, *T. erythraeum* and *T. thiebauti*, are recognised in tropical waters (Hallegraeff and Jeffrey, 1984) and these may be harmful to oysters in a variety of ways. Decaying blooms of *Trichodesmium* sp. may lead to anoxic conditions and mortality, as has been reported for oysters in India (Chellham and Alagarwami, 1978) and fish and shrimp in Thailand (Suvapepun, 1989). *Trichodesmium* spp. has also been described as non-toxic, toxic or sometimes toxic to a range of organisms (Devassy et al., 1979; Hawser et al., 1991, 1992; O'Neil and Roman, 1994; Guo and Tester, 1994). A third possibility is that the cyanobacterium may not provide enough nutrition to maintain healthy oysters. This phenomenon was demonstrated for prawn larvae fed exclusively on *Trichodesmium* sp. cells (Preston et al., 1998). In this study, we examined the toxicity of *Trichodesmium* sp. and the histopathology of hatchery-raised juvenile *P. maxima* exposed to *Trichodesmium* in laboratory experiments.

2. Materials and methods

2.1. *P. maxima* collection and histopathology

Oysters submitted for histology were collected from an affected farm in the Dampier Archipelago (20°28S, 116°49E) and were flown directly to the Animal Health Laboratory

in Perth. The sample consisted of 10 adults (>110-mm diameter) with “fully retracted mantles” (Fig. 1B), 10 adults with “partially retracted mantles”, 3 wild healthy adult oysters (Fig. 1A) and 7 rock oysters (*Saccostrea echinata*). Pearl oysters were subdivided. “Retracted” (5/10), “partially retracted” (5/10) and “wild” (3/3) oysters were individually opened, a sample of haemolymph was extracted from the heart, pieces of tissue were then fixed immediately in seawater buffered 10% formalin, and a swab of the cut unfixed tissue was taken for bacteriology. The remaining pearl oysters and the rock oysters were not sampled for bacteriology but were immediately fixed on opening. After 12-h fixation, samples were embedded in paraffin, sectioned at 3 μm and stained with haematoxylin and eosin using standard techniques.

Haemolymph samples and smears were plated on MSA-B (Tryptone Soya agar with 2% NaCl and 3% horse blood) and incubated at 24 °C. Resulting bacterial colonies were identified to species level. Small pieces of digestive gland epithelium from five of the

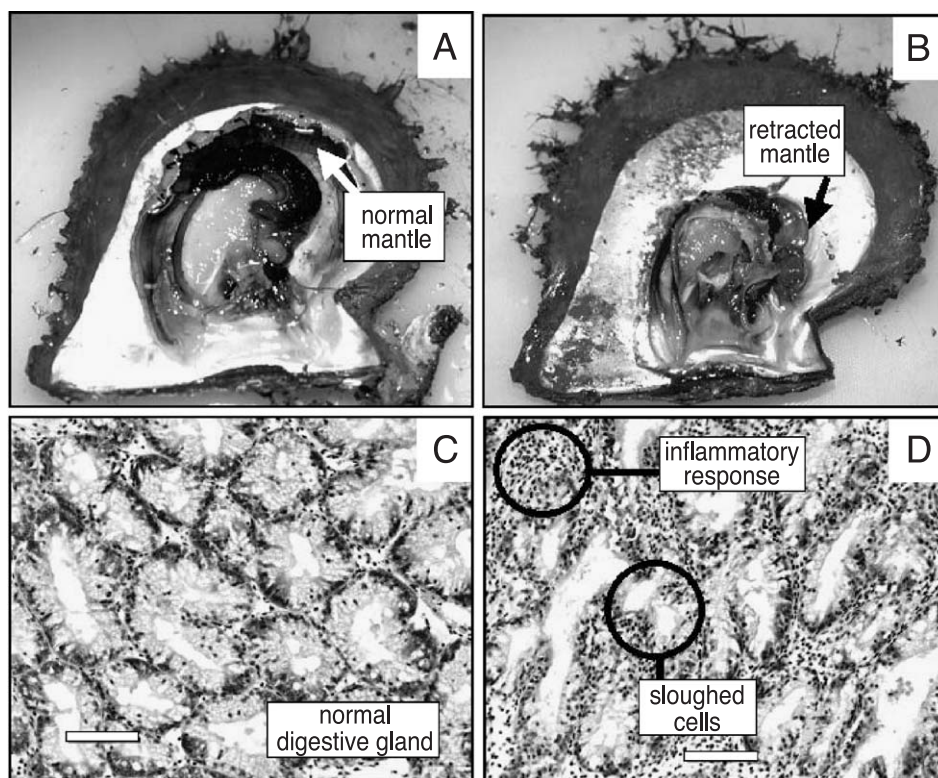


Fig. 1. Photographs illustrating (A) normal and (B) retracted mantles in 11-cm adult *P. maxima* sampled during the 1996 mortality event. Micrographs illustrating (C) normal digestive glands in *P. maxima* exposed to low concentrations of *C. calcitrans* for 7 days and (D) abnormal sloughing of digestive epithelial cells and inflammatory response in *P. maxima* exposed to low concentrations of *C. calcitrans* and *T. erythraeum* for 7 days (scale bar=0.065 mm).

“fully retracted” oysters were fixed in 2.5% gluteraldehyde in 0.22- μm filtered seawater for 1 h, postfixed in 1% osmium tetroxide in 0.1 M phosphate buffer for 1 h, washed, dehydrated and embedded in Epon. Ultra-thin sections were stained with uranyl acetate and lead citrate and examined with a Philips CM10 transmission electron microscope for signs of virus.

2.2. *Trichodesmium* collection

Integrated water samples (0–20-m depth) were collected adjacent to the affected pearl farm approximately seven days after the mortality event using a 20- μm nylon net. The samples comprised over 60 species of phytoplankton, including the potentially harmful *Trichodesmium erythraeum* Ehrenberg at 1800 cells/l.

Fresh *T. erythraeum* was collected daily from sub-surface water off the Dampier shoreline (Western Australia, 20°37'S, 116°45' E) using a 100- μm nylon net. This cyanobacterium was immediately transported to the nearby Sam's Creek Pearl Hatchery with gentle aeration. The colonies were gently washed with a large volume of 0.5- μm filtered, UV sterilised seawater and counted using a haemocytometer. The cells were diluted to $(1.00 \pm 0.19) \times 10^6$ cells/ml (\pm S.D., $n=7$ days) stocks for daily feeding. Colonies consisted almost exclusively of rafts of *T. erythraeum* filaments and were tentatively identified using cell and colony size and morphology (Fogg, 1982).

2.3. Feeding experiment

Although *T. erythraeum* is sometimes fed to larvae in hatcheries and has been assumed to be a suitable food for pearl oysters, the value of *T. erythraeum* as a food source has not been verified experimentally. Four-month-old (25–40-mm diameter) oysters were exposed to stock feeds of *T. erythraeum* and/or the control diatom *Chaetoceros calcitrans*, diluted in 0.5- μm filtered, UV sterilised seawater. Nine treatments were performed with low (10^4 cells/ml) and high (10^5 cells/ml) algal concentrations of each species as well as mixed species feeding and starvation treatments in separate 2 l aquaria, each gently aerated (Table 1). Fresh seawater and algae were introduced to each aquarium daily for 7 days. The temperature, dissolved O₂ and pH in the treatments were logged and ranged from 26 to 30 °C, 4.5 to 5.0 ppm and 8.1 to 8.2, respectively, throughout the exposure. Three oysters were also exposed to a water extract of the cyanobacterium (purple water containing water-soluble pigments phycoerythrin and chlorophyll) equivalent to 10^6 cells/ml to examine the possible toxic effect of water-soluble components on the oysters (Treatment 9). Clearance rates were estimated by comparing cell counts in the aquaria at the beginning and end of each 24-h period. Data were compared by ANOVA (Statistica V 6., Statsoft).

2.4. *Trichodesmium* toxin testing

T. erythraeum cells were extracted using two methods to examine the possibility that they may contain either water-soluble toxins such as the paralytic shellfish toxins (PST) or the lipophilic ciguatoxin-like compounds. Cells collected as above were concentrated

Table 1
Exposure of *P. maximus* to various concentrations of *T. erythraeum* and *C. calcitrans* over 7 days including a summary of histopathological examination

Treatment	Number of oysters	Cell numbers ($\times 10^4$)		Gut cells sloughing	Gill cells sloughing	Not feeding	Oedema	Haemocyte response	Overall score	Number of individuals affected
		<i>T. erythraeum</i>	<i>C. calcitrans</i>							
T1	8	1	–	0/8	1/8	3/7	2/8	0/8	6/39	3/8
T2	8	10	–	2/8	4/8	6/8	5/8	2/8	19/40	6/8
T3	8	–	1	2/8	4/8	8/8	0/8	2/8	16/40	7/8
T4	8	–	10	0/8	0/8	3/8	0/8	1/8	4/40	2/8
T5	7	1	1	2/7	4/7	3/6	0/7	1/7	10/34	4/7
T6	8	10	10	5/8	5/8	8/8	0/8	8/8	26/40	8/8
T7	8	–	–	4/8	6/8	3/7	1/8	5/8	19/39	7/8
T8 ^a	0	1	1							
T9 ^a	0	10	10							
T10 ^b	3	10	10	0/3	0/3	0/3	0/3	0/3	0/15	0/3

Treatment details can be found in Table 1. Each fraction represents the number of oysters testing positive out of the total available oysters. The lower the score, the better condition the animal.

^a Treatments to examine the passive loss of *T. erythraeum* sp. filaments in the aquaria.

^b Oysters exposed to water extract of 10^5 cells/ml *T. erythraeum*.

using a 2-l separation funnel and the intact cells were freeze-dried. The aqueous (0.05 M acetic acid) extraction followed that described by Oshima (1995) and the lipophilic (acetone) extraction was performed as per Lewis (1995). In each case, 20 g of dried cells was extracted. The aqueous extract resulted in a single solution for testing and the lipophilic extract yielded three fractions (water, *n*-hexane and ether). Each of the fractions was freeze-dried and resuspended in sterile saline using a minimum amount of Tween 80 then injected (i.p.) into 20 g white mice according to the standard AOAC (1990) method. Two extract doses equivalent to 1 and 10 g *T. erythraeum*/mouse were administered for each extract fraction. High performance liquid chromatography (HPLC) was used to detect low levels of PST according to the method of Oshima (1995). The sodium channel and saxiphilin binding assays are highly sensitive for PSTs and tetrodotoxins and these techniques were also used to detect toxins in aqueous *T. erythraeum* extracts (Llewellyn et al., 1998).

2.5. Shellfish toxin testing

Adult pearl oysters (approx. 120 mm) from the 1996 mortality event were collected from a farm in the Dampier Archipelago and the viscera of each was extracted according to the AOAC (1990) method to determine the possibility that they may contain PST. The extracts were tested using HPLC and the mouse, saxiphilin and sodium channel assays as described above.

3. Results and discussion

3.1. Indicators of disease in adult *P. maxima*

Approximately 20–50% of the adult (>100 mm) *P. maxima* in the Dampier Archipelago were affected during the 1996 mortality event (Bunter personal observation). Oysters submitted for histology from the affected area exhibited dilated digestive gland lumens, sloughing of epithelial cells and large numbers of residual bodies into the lumen of the digestive gland and stomach, with increased numbers of brown cells and granulocytes under the epithelial layer. Both pearl oyster samples and rock oyster samples were similarly affected.

A mixed flora consisting of *Vibrio alginolyticus*, an unidentified *Vibrio* (*Vibrio* No. 3) and swarming *Vibrio* species and were isolated from all *P. maxima* (Table 2). In addition, two unidentified *Vibrios* (Nos. 1 and 2) were isolated from five of the “retracted” pearl oysters (Fig. 1B). Although there was a difference in the bacterial flora of the pearl oysters, the absence of *Vibrio* 3 and *Vibrio* 4 from most of the partially retracted oysters was not consistent with the histopathology or the “partial retraction” and was considered to be a secondary infection resulting from the severely compromised nature of these “retracted” oysters rather than a cause of the condition. Isolation of a mixed bacterial flora from the digestive gland, and from the haemolymph of immunocompromised oysters is not uncommon (B. Jones, personal observation). No virus-like particles were observed.

Table 2
Results of bacterial examination of adult *P. maxima* from the 1996 mortality event

Individual <i>P. maxima</i>	Sample	<i>V. alginolyticus</i>	<i>Vibrio</i> sp.1	<i>Vibrio</i> sp. 2	<i>Vibrio</i> sp. 3	<i>Vibrio</i> sp. 4	Swarming <i>Vibrio</i>
<i>Retracted</i>							
1	DG	+	++++	–	–	–	
	H	–	++++	–	–	–	
2	DG	–	–	++	–	–	
	H	+	++	+++	–	–	
3	DG	–	+++	++	–	–	
	H	–	++	+++	–	–	+
4	DG	–	++	–	+++	–	
	H	+	–	–	+++	–	
5	DG	–	+	–	++	–	+
	H	–	–	–	–	–	–
<i>Partially retracted</i>							
11	DG	–	–	–	–	+++	+
	H	–	–	–	–	–	–
12	DG	–	–	–	+	–	
	H	–	–	–	–	–	–
13	DG	–	–	–	++	–	+
	H	–	–	–	–	–	–
14	DG	+	–	–	–	–	++
	H	–	–	–	–	–	–
15	DG	–	–	–	++	–	
	H	–	–	–	++	–	
<i>Wild oysters</i>							
21	DG	+	–	–	++	–	+
	H	–	–	–	–	–	–
22	DG	+	–	–	+++	–	+
	H	–	–	–	+++	–	
23	DG	+	–	–	++++	–	+
	H	+	–	–	++	–	+

DG = digestive gland; H = haemolymph sample.

–, Designates no colonies isolated; +, designates relative number of colonies isolated.

3.2. Juvenile feeding experiment

When oysters were not present (Fig. 2; T8, T9), approximately 70–90% of the *T. erythraeum* and *C. calcitrans* cells added to each of the treatments remained in the water column after 24 h. This was not statistically significant from the concentrations at the start of each day ($p > 0.05$). In aquaria containing pearl oysters (Fig. 2; T1–T6), 90–95% of either the cyanobacterium or diatom cells were cleared from the water column after each 24-h period ($P < 0.01$) indicating active feeding on both species by *P. maxima*.

No mortality was recorded among juvenile oysters fed exclusively *T. erythraeum* or *T. erythraeum* in combination with the diatom *C. calcitrans*. The oysters exposed to the purple water extract of *T. erythraeum* (Table 1; T10) also survived for 7 days. Histopathological examination of each of the juvenile oysters revealed less indicators of

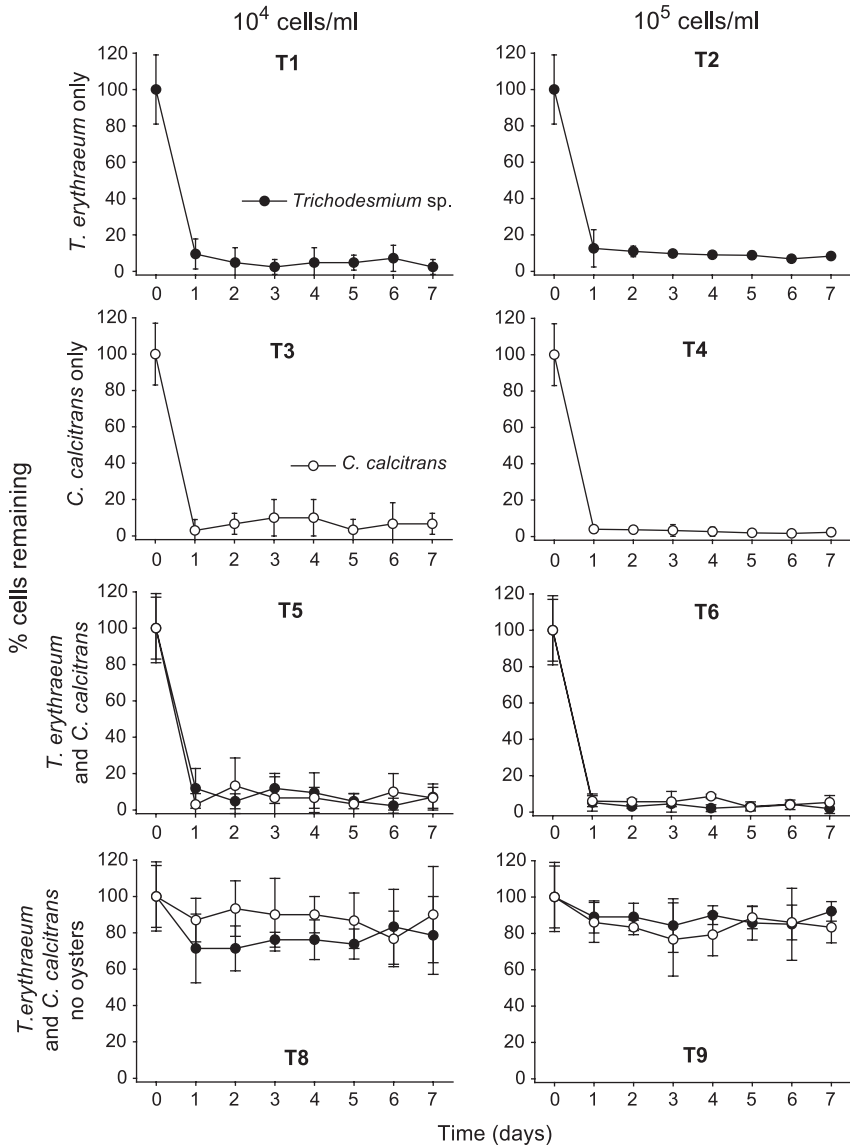


Fig. 2. Proportion of *T. erythraeum* and *C. calcitrans* cells remaining after 24-h feeding periods. Oysters were fed 10^4 or 10^5 cells/ml each day and the proportion expressed as a percentage of the starting concentration. Error bars are ± 1 S.D. from the mean at the end of each 24-h exposure ($n = 3$).

stress in oysters fed either high concentrations of *C. calcitrans* or low concentrations of *T. erythraeum* (Table 1). Those individuals exhibited very low instances of gut and gill cell sloughing (Fig. 1C). The least healthy groups were those that were starved or were exposed to high concentrations of *T. erythraeum* (Table 1). A high proportion of starved

oysters exhibited sloughing of the gut and gill cells as well as an inflammatory response. A similar pattern was observed for oysters exposed to *T. erythraeum* (Fig. 1D). Of the 16 oysters exposed to high (10^5 cells/ml) *T. erythraeum*, only 2 were observed to have fed immediately before fixation. No recognisable *T. erythraeum* cells were observed in the gut or digestive gland, indicating that the oysters may not have been able to digest the cells or had ceased to feed prior to fixation (Fig. 1D). It was therefore difficult to confirm the ability of the oysters to digest the cyanobacterium, however, the absence of intact cells in the faeces combined with consistent cell clearance after each 24-h period indicates that most of the *T. erythraeum* cells may have been consumed. In a similar exposure experiment, prawn larvae (*Penaeus merguienis*) were shown to feed on *T. erythraeum* but the nutritional value of the cyanobacterium was not sufficient for the larvae to develop beyond the protozoa stage (Preston et al., 1998). Symptoms of *P. merguienis* that had been starved or exposed to *T. erythraeum* included degradation of the gut cells, reflecting our observations for *P. maxima*. Mass mortality of Japanese pearl oysters, *Pinctada fucata martensi*, occurred when densities of the inedible diatom of *Nitzschia* spp. were high (Tomaru et al., 2001). It is believed that reduced health of the Japanese pearl oysters, caused by starvation, led to the animals being more susceptible to infection. In the absence of bacterial or viral infection, mortality of the same species of Japanese pearl oyster was found to increase after 70 days of starvation (Numaguchi, 1995).

3.3. Toxin content of adult *P. maxima*

Both healthy ($n=3$) and visibly diseased ($n=4$) adult *P. maxima* from the affected pearl farm were tested for water-soluble toxins such as PSTs and tetrodotoxins. Extracts equivalent to 1 g shellfish viscera were not toxic to mice (Table 3). Several of the extracts did however exhibit inhibition in the sodium channel and saxiphilin assays, indicating the presence of PST-like toxins (Table 3). HPLC detected 7.3 μg of saxitoxin, one of the most potent PSTs, per 100 g of viscera in one of the diseased pearl oysters. This amount of toxin is below the detection limit of the mouse bioassay for these toxins (Fernandez and Cembella, 1995). This toxin was identified by comparison of retention times with a standard along with spiking experiments (Onodera et al., 1997). This is the first time that saxitoxin has been detected in *P. maxima* but the concentration was low in comparison with other molluscs, which are able to concentrate several orders of magnitude more toxin without deteriorating health. The observation that only one of the four diseased oysters contained saxitoxin suggests that this toxin was not responsible for mortality in adult oysters.

3.4. Toxin content of *Trichodesmium* cells

Aqueous (0.05 M HOAc) extracts ($n=3$) of *T. erythraeum* cells were not toxic to mice at 1 g cells per 20 g mouse (Table 3), which equates to a very high dose of 50 g cells/kg. The more sensitive saxiphilin and tetrodotoxin assays and HPLC also failed to detect PSTs or tetrodotoxins in the cells, again indicating the absence of these common neurotoxins in the *Trichodesmium* samples tested. The toxicity of aqueous extracts of *T. erythraeum* in the present study was much lower than that reported for neurotoxic *Trichodesmium* spp. from the Virgin Islands (Hawser et al., 1992) and Carolina (Guo and Tester, 1994).

Table 3

Toxicity of *T. erythraeum* and adult *P. maxima* to mice following i.p. injections of cell or tissue extracts (g/mouse)

	Extract (fraction)	Dose	Symptoms in mouse assay	Na channel assay	Saxiphilin assay	HPLC
<i>Lipophilic</i>						
<i>T. erythraeum</i>	water	1 g cells/mouse	Survived	BDL	BDL	BDL
		10 g cells/mouse	3 min death, neurotoxic	BDL	BDL	BDL
<i>T. erythraeum</i>	n-hexane	1 g cells/mouse	Survived	BDL	BDL	BDL
		10 g cells/mouse	16 min death, neurotoxic	BDL	BDL	BDL
<i>T. erythraeum</i>	ether	1 g cells/mouse	Survived	BDL	BDL	BDL
		10 g cells/mouse	Survived	BDL	BDL	BDL
<i>Aqueous</i>						
<i>T. erythraeum</i> (n=3)	0.05 M acetic acid	1 g cells/mouse	Survived	BDL	BDL	BDL
Adult <i>P. maxima</i> viscera						
Healthy (n=3)	0.05 M acetic acid	1 g/mouse	Survived	BDL	BDL	BDL
Diseased (n=4)	0.05 M acetic acid	1 g/mouse	Survived	+	+	7.3 µg/100 g saxitoxin ^a

BDL designates below detectable level (< 40 µg STXeq/100 g flesh for mouse assay and < 4 µg STXeq/100 g for sodium channel, saxiphilin and HPLC).

+, Designates toxicity detected in sodium channel and saxiphilin assays (>30% inhibition in each assay).

^a Saxitoxin was confirmed in a single animal of 4 tested.

Australian *Trichodesmium* has been linked with production of lipophilic toxins such as ciguatoxin (Hahn and Capra, 1992; Endean et al., 1993). None of the fractions of the lipophilic *T. erythraeum* extracts in the present study were toxic to mice at 1 g cells per 20 g mouse (Table 3). As more sensitive assays were not available, each fraction was tested at 10 times the original concentration. In this case, mortalities were recorded, but the dose equated to 500 g/kg mouse and therefore could not be considered toxic at realistic exposure levels.

The differences in neurotoxicity of *Trichodesmium* spp. between previous studies and the species tested here may be a result of differences in toxin synthesis between species and strains of this genus. The synthesis of toxins may be inherently different for each strain or may be influenced by external factors such as nutrition or the presence of bacteria (Paerl et al., 1989). It is also possible that zooplankton assays undertaken by Hawser et al. (1992) and Guo and Tester (1994) may be more sensitive to the toxins than the mouse and PST assays applied in the present study. It is worth noting that the ciguatoxin-like compounds reported in Australian *T. erythraeum* from the Great Barrier Reef (Hahn and Capra, 1992; Endean et al., 1993) have not been conclusively identified as ciguatoxins. Despite its high abundance and distribution, there has never been an established case of poisoning that could be reliably attributed to *Trichodesmium* in Australian waters.

A further potential effect of *Trichodesmium* is anoxic conditions that may arise from decaying blooms. This was the case for an Indian pearl oyster farm where the mortality of hundreds of pearl oysters awaiting seeding was recorded (Chellham and Alagarwami,

1978). Vast blooms of *T. erythraeum* in Thailand, similar to those seen along Australia's tropical coasts, have also resulted in fish and shrimp kills in farms due to low oxygen and high ammonia levels from the decaying microalgae cells (Suvapepun, 1989). However, it is highly unlikely that anoxia was involved in 1996 mortality event as the affected farms were situated in high current open water locations in the Dampier Archipelago.

In the natural environment, pearl oysters such as *P. maxima* are benthic filter feeders and would probably not be exposed to extreme densities of *Trichodesmium* nearer the surface in still conditions. The farming practice of suspending the oysters in the water column is very effective but may result in increased exposure to potentially harmful algae such as *Heterocapsa circularisquama* (Nagai et al., 2000) and *Trichodesmium*. The only precautions that could be taken would be to locate farms in high current areas to avoid anoxia and culture the oysters low in the water column to avoid buoyant algal blooms.

4. Conclusion

Although blooms of *T. erythraeum* were observed at the time of the pearl oyster mortality, this species was not found to be toxic. The histopathological symptoms at Dampier, in the absence of any correlation between observed signs and the presence of bacteria or other pathogens, and considering the results of the feeding trails reported here, suggests that the *T. erythraeum* blooms in the archipelago were not a suitable food source for the pearl oysters and may have contributed to the observed mortalities.

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