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A fish kill associated with a bloom of *Amphidinium carterae* in a coastal lagoon in Sydney, Australia

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Abstract

We report on a dense bloom ($\sim 1.80 \times 10^5$ cells mL⁻¹) of the marine dinoflagellate species *Amphidinium carterae* (Genotype 2) in a shallow, small intermittently open coastal lagoon in south eastern Australia. This bloom co-occurred with the deaths of >300 individuals of three different species of fish. The opening of the lagoon to the ocean, as well as localized high nutrient levels, preceded the observations of very high cell numbers. *A. carterae* is usually benthic and sediment-dwelling, but temporarily became abundant throughout the water column in this shallow (<2 m) sandy habitat. Histopathological results showed that the *Anguilla reinhardtii* individuals examined had damage to epithelial and gill epithelial cells. An analysis of the bloom water indicated the presence of a compound with a retention time and UV spectra similar to Luteophanol A, a compound known from a strain of *Amphidinium*. Assays with a fish gill cell line were conducted using a purified compound from cells concentrated from the bloom, and was found to cause a loss of 87% in cell viability in 6 h. The fish deaths were likely due to the low dissolved oxygen levels in the water and/or the presence of Luteophanol A-like compounds released during the bloom.

Keywords

Amphidinium carterae; Harmful algal bloom; Amphidinol; Fish kill; Lagoon; Luteophanol

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.hal.2015.08.003.

1. Introduction

Amphidinium species are amongst the most abundant benthic dinoflagellates in intertidal or estuarine marine sandy sediments in tropical, sub-tropical and temperate ecosystems (Murray and Patterson, 2002; Hoppenrath, 2000; Flø Jørgensen et al., 2004). Common species such as *Amphidinium carterae* Hulburt have a cosmopolitan distribution, occur worldwide, and grow readily and comparatively quickly. They are therefore commonly present in culture collections and have often been the subjects of physiological and genetic studies (i.e., Damjanovic et al., 2000; ten Lohuis and Miller, 1998).

Species of *Amphidinium* produce many different types of bioactive compounds (reviewed in Murray et al., 2012), with a wide range of toxicological impacts. Polyketides produced by *Amphidinium* species include macrolides, short linear polyketides, and long-chain polyketides. Amphidinols are one common type of polyketide compound produced by *Amphidinium carterae* and closely related species, with approximately 20 analogues known, displaying a variety of properties including haemolytic and antifungal activity (Satake et al., 1991; Paul et al., 1995, 1997; Morsy et al., 2005, 2006; Echigoya et al., 2005; Meng et al., 2010). A similar polyhydroxy compound, named luteophanol, was isolated from an uncharacterised species of *Amphidinium* inhabiting the acoel flatworm *Pseudaphanostoma luteocoloris* (Doi et al., 1997). The first polyketide synthase gene cluster sequenced from a dinoflagellate was from a strain of an unidentified species of *Amphidinium* (Kubota et al., 2006). Several unique and very divergent genotypes occur within the species *A. carterae* and the highly morphologically similar species *Amphidinium massartii*, including intraspecific variation of up to 38% in ITS rDNA sequences (Murray et al., 2004, 2012). Due to the presence of several cryptic species, molecular genetic sequencing is necessary for unequivocal identification of species and genotypes. Unfortunately, the vast majority of toxicological studies of species of *Amphidinium* have been conducted with unidentified strains (e.g., Tsuda et al., 2007).

Sudden mortalities of large numbers of fish and other marine life in coastal or estuarine habitats are common in south eastern Australia, with estimates of ~20 incidents annually reported to the Department of Primary Industries in New South Wales over the past 40 years, involving finfish, molluscs and crustaceans (NSW DPI, 2010). The causes of these fish kills are largely unexplored. In a small number of cases (~12%), chemical pollution or acid water inflow was detected and determined as the cause (Sammur et al., 1995; Roach, 1997). An extremely widespread fish kill in Jervis Bay, south eastern Australia in 2011 was due to a bloom of the toxic dinoflagellate *Karlodinium veneficum* (SM, unpub. data), a species that has previously caused fish kills in the region, in Lake Illawarra (Hallegraeff, 2002). In other Australian regions, fish kills have been attributed to blooms of the raphidophytes *Chattonella marina*, *Heterosigma akashiwo* or *Karenia* species (Hallegraeff, 2002). Generally, the causes of fish kills in Australian sites have not been fully investigated, although factors such as low dissolved oxygen levels, a change in salinity, temperature or pH were observed in some cases (~25% of cases) (NSW DPI, 2010).

Species of *Amphidinium* have very rarely been reported to be involved in pelagic harmful algal blooms (HABs) in shallow habitats (Lee et al., 2003; Sampayo, 1985; Gárate-

Lizárraga, 2012). One reported bloom of *Amphidinium carterae* (Genotype 2) occurred in sedimentation ponds fed by effluent water from fish farming in Israel (Lee et al., 2003). The authors studied the physiological ecology of the *A. carterae* strain and found it to be eurytrophic, and able to tolerate a very wide range of salinities, temperatures, and pH, with a capacity for luxury consumption of nitrate and phosphate for several generations (Lee et al., 2003). Recurrent seasonal blooms of *A. carterae* in shallow fish ponds in Sado, Portugal have been reported to correlate with fish kills (Sampayo, 1985). The chemical structure of amphidinols (Meng et al., 2010) and luteophanols (Doi et al., 1997) are very similar to that of karlotoxins (Van Wagoner et al., 2008, 2010), which are produced by the unarmoured dinoflagellate *Karlodinium veneficum*, and can lead to the deaths of fish (Deeds et al., 2002, 2006; Kempton et al., 2002). It is possible that the fish kills observed in Portugal were related to the presence of compounds produced during the bloom.

In September 2012, a dense bloom of the species *Amphidinium carterae* was found in a coastal lagoon in Curl Curl on the northern side of Sydney, in south eastern Australia. This bloom occurred concurrently with the deaths of at least three different species of fish, bream (*Acanthopagrus australis*), sea mullet (*Mugil cephalus*) and longfin eels (*Anguilla reinhardtii*). In this study, we investigated the water quality conditions at the time of the bloom, the identity and abundance of the phytoplankton present, the histopathology of the fish, and the presence of polyhydroxyl compounds in the water, in order to determine the cause of the fish deaths.

2. Materials and methods

2.1. Sampling site

Curl Curl Lagoon, including Greendale Creek, foreshores, and the bed of the lagoon is Crown land, and Warringah Council is the appointed reserve trust manager. The land is managed according to a Plan of Management adopted by Council and Department of Lands under the Crown Lands Act 1989. Warringah Council provided permission for sampling for this study, and notified a range of offices of the bloom as part of its duty of care, in particular, the NSW Environment Protection Authority and the NSW Department of Primary Industries, Fisheries. Fish sampling was conducted under a Section 37 permit from NSW DPI Fisheries, and overseen by a representative from NSW Fisheries. No protected or endangered species of fish were collected during the sampling conducted for this study.

Intermittently open coastal lagoon (ICOLL) systems are common along the subtropical and warm temperate east coast of Australia (Haines et al., 2006). Curl Curl Lagoon is a small, shallow (<2 m depth) ICOLL system, with a sandy bottom sediment, approximately ~0.06 km² with a volume of ~48 mL (Fig. 1). Much of its catchment is urbanized. The bottom sediment of the lagoon is comprised of marine sand, east of Griffin Rd Bridge. To the west of the bridge, the central basin is a mud flat during periods where the entrance is open.

Within the lagoon, the opening and closing of its entrance is the dominant physical process (BMT WBM, 2011). The entrance is closed approximately 80% of the time by a sandy berm. During closure periods, rainfall, catchment runoff and groundwater contribute to rising water levels in the lagoon. This eventually leads to overspill and the natural opening

of the entrance, approximately 12–16 times per year. During these periods, the lagoon becomes tidal and catchment runoff drains into the sea. Occasionally, the entrance has been artificially opened by local residents.

2.2. Phytoplankton sampling

In response to reports of water discolouration and fish deaths, triplicate 500 mL phytoplankton samples were taken on 17th September 2012 and fixed in Lugol's iodine solution. Samples were counted in a Sedgewick Rafter Cell. A live phytoplankton sample (200 mL) was also taken on the 20th September and observed within 6 h using an inverted compound light microscope (Nikon, Tokyo, Japan) and 100× magnification in order to verify species identification. Three additional live samples (4 L) were taken on 20th September for DNA extraction and chemical analysis.

2.3. Environmental variables

Samples to estimate the quantity of heavy metals, nutrients and other water quality variables were analysed. Nutrients were analysed at the Sydney Water Analytical Services Laboratory, according to American Public Health Association for the Examination of Water & Wastewater (APHA) reference methods, were: pH (APHA 4500-H⁺), ammonia (APHA 4500-NH₃ H), oxidised Nitrogen (APHA 4500-NO₃ I), total nitrogen and phosphorus (APHA 4500-NP H), NH₃ (APHA 4500-NH₃ H), and NH₄ (method not accredited). The following heavy metals were analysed: total arsenic, total cadmium, total chromium, total copper, total lead, total mercury, total nickel, and total zinc according to the reference method USEPA 6020.

Physicochemical data was measured with a YSI 6920 V2 multiparametre water quality sonde, and data was viewed and stored on a YSI 650 MDS data logger. Climate data was sourced from the Australian Bureau of Meteorology (BOM) online database (Australian Bureau of Meteorology, 2013). Air temperature data for the period preceding the bloom were sourced from BOM Station 066196 (Sydney Harbour, Wedding Cake West). BOM records daily measurements of global solar exposure (total solar radiation) (MJ m⁻²). Daily averages for September 2012 were downloaded from the BOM online archives, for the closest weather station to the lagoon Collaroy (Station No. 066126, ~3.7 km from lagoon). In order to gain an estimate of photosynthetically active radiation (PAR), 50% of the available daily solar radiation (Monteith, 1969) was used.

A water level buoy (Curl Curl Station Number: 213426) operated by Manly Hydraulics Laboratory was located near Griffin Road bridge (Fig. 1) and routinely recorded water levels at 15 min intervals.

2.4. Fish pathology

Dead bream (*Acanthopagrus australis*), sea mullet (*Mugil cephalus*), and longfin eels (*Anguilla reinhardtii*) were observed in the lagoon from 29th August to 26th September 2012. Five moribund eels were euthanized, immediately fixed in 10% neutral buffered formalin solution and kept cool on 20th September. Samples were sent via courier to the State Veterinary Diagnostic Laboratory at the Elizabeth Macarthur Agricultural Institute

where they were trimmed, embedded in paraffin and stained with haematoxylin and eosin for histology.

2.5. DNA extraction, PCR and sequencing

An aliquot of the water sample from 20th September 2012 was centrifuged at 3000 rpm for 10 min and the resulting pellet was extracted using a modified CTAB method (Doyle and Doyle, 1990). To amplify the internal transcribed spacer (ITS) region of the ribosomal RNA gene, the primers ITSfow (TTCCGTAGGTGAACCTGCGG), and ITSrev (ATATGCTTAAATTCAGCGGGT) were used (Murray et al., 2012). PCR reactions contained template, 0.5 μ M of each primer, 3 mM $MgCl_2$, 1 μ L BSA (NEB), and 10 μ L Immomix (Bioline, Tounton, MA), containing dNTPs, Immolase Taq polymerase, and reaction buffer. Hot start PCRs were performed with an initial denaturing step of 95 °C for 5–10 min, and 35 cycles of 30 s at 95 °C, 30 s at 60–65 °C, 30 s at 72 °C followed by a final extension step of 7 min at 72 °C.

Samples for DNA sequencing were prepared by adding 10 mM of primer to at least 400 ng of purified DNA. Samples were prepared in 20 μ L reactions of 1 μ L Big Dye Terminator Mix (Applied Biosystems, Carlsbad, CA) and the sequencing reaction was run using the following PCR program: 96 °C 3 min, 96 °C 10 s, 50 °C 5 s 60 °C 4 min for 30 cycles. The sequences were assembled using the software Geneious (Kearse et al., 2012). The sequences were submitted to GenBank (Accession Number KF356164).

The final alignment of 22 sequences of 434 nucleotides was initially aligned using MAFFT v6.814b (Kato et al., 2002) and manually inspected and checked. The program FindModel (Posada and Crandall, 1998) was used to test and determine optimal model-fitting of the sequence data. The PhyML 3.0 algorithm (Guindon and Gascuel, 2003) was used for Maximum Likelihood inference based on the optimal substitution model, the General Time-Reversible model with a gamma distribution. Five hundred bootstrap replicates were performed.

2.6. Chemical analysis

A 2 L sample of the discoloured water in the lagoon was taken on 17th September 2012. The sample which contained 95,000 cells mL^{-1} was concentrated to a final volume of 4 mL by centrifugation (1000 rpm for 5 min) and then freeze dried. The cell pellet was extracted with 30% methanol in a sonication bath (20 mL) for 40 min, diluted to 200 mL with HPLC water and loaded on 3 mL packed volume C-18 solid phase extraction cartridge column (Waters Corporation, Massachusetts USA). After the extract was loaded onto the column it was washed with 12 mL of increasing concentrations of methanol/water from 0% methanol to 100% methanol in 20% increments. All fractions were collected, dried under a vacuum, resuspended in 1 mL of methanol, and filtered with a GF/B filter prior to HPLC–MS analysis. The eluates were injected onto a C8 (LiChrosphere 125 mm $\times$ 4 mm 5 μ m bead size RP-8, Agilent) column and subjected to a 1 $mL\ min^{-1}$ 10–95% methanol: water gradient over 25 min. using an Agilent 1100 HPLC. Toxin peaks were detected using an Agilent Diode Array Detector (Model#G1315B) with a micro high-pressure flow cell (G1315B#020; 6 mm pathlength, 1.7 μ L volume) over the wavelength range 190–950 nm.

The entire UV spectra were saved for each UV detectable peak. The eluate from the DAD detector was mixed with 10 mM ammonium formate in methanol prior to introduction to the electro-spray nozzle of the MS (Agilent G1956A SL) for ionization with the following spray chamber conditions using nitrogen as the drying gas: flow rate 10 L min⁻¹. Pressure 60 psi, temperature 350 °C, fragmentor voltage 350 V, and capillary voltage 3000 V. The 10 mM ammonium formate in methanol (0.1 mL min⁻¹) ensured appropriate pH conditions for negative mode ionization. The majority of the material was found in the 80% methanol fraction and was subsequently purified on a semipreparative C18 column (9.4 cm × 590 mm 5 µm Zorbax) using a 30–70% acetonitrile/water linear gradient.

2.7. Fish gill cell assay

The epithelial fish gill cell line RTgill-W1 was obtained from the American Type Culture Collection (CRL-2523) (Bols et al., 1994). This cell line was routinely cultured on 25 cm² culture treated flasks (690170, Greiner Bio-One) with Leibovitz's medium (L1518, Sigma) supplemented with 10% fetal bovine serum (v/v) (12003C, Sigma) and an antibiotic–antimycotic solution (A5955, Sigma). Cells were incubated at 19 °C in the dark. Confluent gill cells were detached with 0.25% trypsin–0.02% EDTA solution (59428C, Sigma) for subculturing and seeding purposes as described by Dorantes-Aranda et al. (2011).

Gill cells were detached, counted, and seeded at a concentration of 2×10^5 cells mL⁻¹ in quadruplicate in a 96-well microplate (655180, Greiner Bio-One) 48 h prior to the experiments (Dorantes-Aranda et al., 2011). An extract purified from the bloom of *Amphidinium carterae* was dissolved in 30% methanol and mixed with L-15/ex medium (Schirmer et al., 1997) for the exposure (final methanol concentration was 6.7%). Gill cells were exposed to the extract at 0.1, 1, 10, 50, and 100 µg mL⁻¹ for 2 and 6 h. After completion of this time period, experimental solutions were discarded and gill cells were rinsed with phosphate buffer saline (PBS). Control gill cells were exposed to L15/ex with methanol at 6.7%; preliminary experiments showed that 6.7% methanol does not have any effect on gill cell viability at 6 h.

Alamar blue (DAL1025, Invitrogen) in L-15-ex (5% v/v) was added to all wells and incubated for 2 h in the dark (Dayeh et al., 2005) to measure viability of gill cells. Fluorescence of metabolised alamar blue was measured with a microplate reader (FLUOstar OPTIMA, BMG Labtech, 413–3350), using excitation and emission filters of 540 and 590 nm, respectively.

3. Results

3.1. Water quality

The majority of the water samples from 17th September were found to have heavy metal concentrations below or just above detection limits. They were well below ANZECC (2000) guidelines for ecosystem health. Values of total nitrogen (TN—sum of all dissolved, particulate, organic, and inorganic nitrogen) were $2.7\text{--}7.75 \text{ mg L}^{-1} \pm 0.46$ ($193\text{--}250 \text{ µM N}$), while values of total phosphorus (TP) were $0.198\text{--}0.310 \text{ mg L}^{-1} \pm 0.058$ ($6\text{--}23 \text{ µM P}$) (Fig. 2). These values exceed ANZECC trigger levels for TN (0.12 mg L^{-1} (marine), 0.30 mg L^{-1}

(estuaries)), TP (0.025 mg L^{-1} (marine), and 0.03 mg L^{-1} (estuaries)). While there is no criteria within the guidelines for TN:TP ratio and eutrophication, these values are over an order of magnitude higher than what would be considered to be a “slightly disturbed ecosystem” (ANZECC, 2000). The molar ratio of TN to TP was approximately 26:1 over the three sampling stations. This was greater than the nominal Redfield ratio of 16:1 and suggested that phosphorus was limited within the lagoon. Levels of oxidised nitrogen (NO_2^- and NO_3^-) and ammonia (NH_4^+ and NH_3) were below detectable limits ($<0.01 \text{ mg L}^{-1}$). Dissolved oxygen levels on the 18th September were low, $10.5\% \pm 0.2$ ($0.91 \text{ mg L}^{-1} \pm 0.02$). Temperature was $18.5^\circ\text{C} \pm 0.47$ and salinity was $12.6\% \pm 2.44$ on 18th September; pH was $9.0\text{--}9.4 \pm 0.06$ on 18th September.

3.2. Environmental variables

On August 28th 2012, the mouth of the lagoon was illegally opened, causing it to drain to the sea. In the lead up to, and during the bloom event, water level data recorded by MHL (Griffin Rd data buoy) in the lagoon showed a gradual increase from 1.2 m on 10th September to 1.7 m on 30th September, but it had not reached the natural spill level of ca. 2 m. Depending on light conditions within the water column (Parslow et al., 1999), PAR could range between 78 and $475 \mu\text{E m}^{-2} \text{ s}^{-1}$ within a 2 m water column, based on 50% of the available solar radiation (18 MJ m^{-2}). Rainfall data from the Collaroy gauge was 0 mm in the weeks preceding the bloom, indicating that sudden changes in salinity are unlikely to have occurred related to rainfall. Daily air temperature was $16.0\text{--}26.7^\circ\text{C}$ in the two weeks preceding 15th September (Sydney Harbour, Wedding Cake West). These temperatures are within the normal range experienced in September in this area. Based on the local weather conditions, water column turbulence was assumed to be low within the lagoon.

3.3. Phytoplankton abundance

The phytoplankton samples taken from the bloom on 17th September 2012 were found to be dominated by the single species *Amphidinium carterae*, which showed an abundance of $36\text{--}186 \times 10^3 \text{ cells mL}^{-1}$ (Fig. 2). During the bloom the water was a yellow-brown colour. No distinct odours were reported. In the period following the bloom, it appeared that the cells of *Amphidinium* had sunk and settled on the bottom of the lagoon.

3.4. Identification of the species

As several species of *Amphidinium* are morphologically highly similar, and as four distinct genotypes of *Amphidinium carterae* exist, we conducted a detailed morphological examination of the samples and molecular genetic sequencing studies. Cells of *A. carterae* were approximately oval, $8\text{--}12 \mu\text{m}$ long ($n = 20$), with a very small, left deflected epicone, and a yellow-brown plastid, which filled the cell.

In order to determine which genotype of *Amphidinium carterae* was present, we amplified and sequenced the ITS rRNA region directly from DNA from a water sample from the lagoon taken on 20th September, using PCR primers that amplify those of any eukaryote. A sole clean sequence was recovered, and was found to be highly similar (99.5%) to Genotype 2 of *A. carterae*. Phylogenetic analysis was performed, and sequences belonging to *A.*

carterae formed a clear monophyletic group, with three well-supported clades representing three of the four different genotypes of *A. carterae* (Fig. 3), which is in accordance with previous studies (Murray et al., 2012). The query sequence clustered with other reference sequences belonging to Genotype 2 of *A. carterae*, further confirming the identity of the species (Fig. 3).

3.5. Fish pathology

Approximately 300 dead fish were found and removed from the lagoon from 17th to 26th September. These consisted of ~50% sea mullet (*Mugil cephalus*), 30% bream (*Acanthopagrus australis*) and 20% longfin eels (*Anguilla reinhardtii*). Five individuals of *A. reinhardtii* were submitted for histopathology examination. All eels displayed similar changes in varying severities. Consistent findings on histology included diffuse, sometimes marked epithelial hypertrophy, associated with epithelial hyperplasia, lamellar fusion, and mild infiltrates of predominantly lymphocytes. Other findings included a mild increase in mitoses of gill epithelial cells, individual cell degeneration, necrosis, surface bacterial proliferation, and accumulates of cellular debris among lamellae.

3.6. Chemical analysis

The results of the chemical analysis on the extracted material showed the presence of a single abundant compound (Fig. 4, lower panel), with retention time and UV spectra (Fig. 4, upper panel) consistent with a Luteophanol-A like compound, and not an amphidinol. The UV spectra indicated the presence of conjugated diene chromophore (Lambda max 232 nm) not a conjugated triene chromophore (Lambda max 280 nm) expected for amphidinol. The negative electrospray ionization mass spectra for the peak showed a quasi-molecular ion peak at m/z 1471.4 ($M-H$)⁻ and 1487.3 ($M-H$)⁻ (Fig. 5). The presence of a sulphate ester was suggested by intense fragment ions observed [m/z 120 ($NaHSO_4^-$) and 103 ($NaSO_3^-$)].

3.7. Fish gill cell assay

Gill cells were sensitive to material purified from Curl Curl Lagoon bloom (Fig. 6). Gill cells showed a decrease of viability of 27% after exposure to this compound at 0.1–50 $\mu\text{g mL}^{-1}$ for 2 h. The same effect was observed after 6 h of exposure but only at concentrations of 0.1, 1, and 10 $\mu\text{g mL}^{-1}$ (no significant differences were observed). A major decrease in cell viability was observed at 50 $\mu\text{g mL}^{-1}$ between 2 and 6 h (72.6 versus 36.4%). Gill cells were 48% ($\pm 2.7\%$) viable upon exposure to 100 $\mu\text{g mL}^{-1}$ for 2 h, which went down to 13% ($\pm 4.5\%$) at 6 h. Median lethal concentration (LC50) was 95 and 23.5 $\mu\text{g mL}^{-1}$ at 2 and 6 h, respectively (Fig. 6).

4. Discussion

Phytoplankton samples collected during the Austral spring/summer period at Curl Curl Lagoon from 2011 to 2013 have been found to comprise a mixed assemblage of diatoms (e.g., *Thalassiosira*, *Navicula*, and *Pseudo-nitzschia* spp.) with some presence of dinoflagellates (e.g., *Ceratium* spp., *Scrippsiella* spp., and *Protoperidinium* spp.) (Murray, 2012, 2013). Species of the genus *Amphidinium* had not previously been identified during

routine phytoplankton monitoring of the lagoon, which is not unexpected, as characteristically *Amphidinium* spp. are sand-dwelling rather than planktonic (Murray and Patterson, 2002). The observation of $\sim 180 \times 10^3$ cells mL⁻¹ (corresponding to a biovolume of 256 mm³ L⁻¹) is substantially higher than the abundance of other species observed during the routine monitoring program (Murray, 2012, 2013), as other species were found to reach maximum cell concentrations of 10 cells mL⁻¹ for individual species (e.g., *Prorocentrum minimum*).

At its most abundant, the bloom caused an extensive yellow-brown water discolouration in this shallow sandy lagoon system. This density of *Amphidinium carterae* is in the range of similar blooms of this species reported from Pakistan, Portugal, and Mexico (Sampayo, 1985; Gárate-Lizárraga, 2012; Baig et al., 2006). In the Sado estuary of Portugal, *A. carterae* has been found to occur in high abundances seasonally in fish ponds, corresponding with fish die-offs (Sampayo, 1985). Intertidal pools in the North Arabian Sea along the coast of Pakistan were reported to have blooms of *A. carterae* with concentrations of 12×10^3 cells mL⁻¹ (Baig et al., 2006). In Mexico, densities of *A. carterae* were also high ($28\text{--}64 \times 10^3$ cells mL⁻¹), however, no fish die-offs were observed (Gárate-Lizárraga, 2012). A maximum growth rate ($\mu_{\max}$) of 2.89 d⁻¹ for *A. carterae* has been reported (Smayda, 1997). Similar findings of $\mu_{\max}$ 2.7 d⁻¹ have also been reported (Ismael et al., 1999). Applying the formula $N_0 \times e^{\mu t}$ where N_0 was an initial cell concentration (10 cells mL⁻¹), cell concentrations of 180×10^3 cells mL⁻¹ could be achieved within a very short time period (t) of 3–4 days.

Species of *Amphidinium* are typically found in marine shallow sediments, however, some species are known to undergo vertical migration, and are occasionally found in the water column (Eaton and Simpson, 1979). *Amphidinium carterae* does not generally exhibit diel vertical migration, but has been reported to maintain its position within a 1 m range in the water column (Kamykowski and Zentara, 1977; Eggersdorfer and Hader, 1991). Similarly, recurrent blooms in the Sado Estuary in Portugal were in water depths of ~ 0.8 m (Sampayo, 1985). This life strategy may be of benefit during optimal nutrient conditions, during which *A. carterae* may have the capacity for “luxury consumption” of nutrients and the ability to store reserves of both phosphorus and nitrogen (Lee et al., 2003). High pH levels during dinoflagellate blooms are not uncommon, and blooms of *Amphidinium* have been found to tolerate high pH levels (>8.5) (Lee et al., 2003; Hinga, 1992). In Curl Curl lagoon, the elevated pH levels may have been a result of fertilizer input or the uptake of carbon dioxide by the bloom during photosynthesis.

The salinity was observed to be low on the 17th September during the initial identification of the *Amphidinium carterae* bloom (12.6‰) and some freshwater microalgae were also observed (*Chlamydomonas* spp., *Oocystis* spp., and *Chroomonas* spp.; Fig. 2), though marine species (e.g., *Ceratium* spp. and *Scrippsiella* spp.) were also found. Very little research has been conducted on the impact of salinity on growth or bloom formation of *A. carterae*. In the only studies conducted, strains of *A. carterae* grew at a wide range of salinities, from 14‰ to 50‰, with an optima from 20‰ to 32‰ (Brand, 1984; Lee et al., 2003; Zimmermann, 2006). Light, salinity, and temperature have been shown to affect haemolytic activity by a strain of *A. carterae* in culture, with higher salinities and temperatures being associated with the highest haemolytic activity (Zimmermann, 2006).

The analysis of the water samples showed that heavy metals were not present at high concentrations, and that pH was not in the range to indicate the presence of an acid inflow. A single abundant compound was found which had the same retention time and UV spectra as Luteophanol A (Doi et al., 1997) (Fig. 4). Luteophanol A is a polyhydroxy linear carbon chain compound that was first isolated from an uncharacterised species of *Amphidinium*, which had been isolated as symbiotic within a flatworm (Doi et al., 1997). Further investigation of this *Amphidinium* strain led to isolation of Luteophanols B, C, and D (Kubota et al., 1998, 2005). Polyhydroxyl compounds produced by dinoflagellates, such as amphidinols, have been reported to have haemolytic and antifungal activities (Echigoya et al., 2005, Meng et al., 2010). Luteophanol D is the only luteophanol compound investigated to date for its biological activity, and it has been reported to have antibacterial effects (Kubota et al., 2005).

Exposure of fish gill cells to the bloom extract from *Amphidinium carterae* caused 27–35% decrease of cell viability at low concentrations ($0.1\text{--}10\text{ }\mu\text{g mL}^{-1}$), which increased with time and concentration (maximum of 87% at $100\text{ }\mu\text{g mL}^{-1}$). *Amphidinium carterae* has high levels of intraspecific genetic variation, and consists of four known genotypes (Murray et al., 2004, 2012; Rhodes et al., 2010). Strains of only two of these genotypes have been examined for their toxicological effects, Genotypes 2 and 4. A strain of Genotype 2 (strain number CAWD 57) has shown haemolytic effects due to its production of amphidinols (Echigoya et al., 2005). A raw extract of a Genotype 4 strain was found to be highly toxic by mouse bioassay (Rhodes et al., 2010). An unidentified Genotype of *A. carterae* was found to have impacts on sea urchin larval development, strong impacts on *Artemia salina* nauplii, and some haemolytic activity (Pagliara and Caroppo, 2012). Studies conducted over the past 50 years have shown toxic effects of strains of *A. carterae*, using mouse, fish, cell-based, or *Artemia* assays (Ismael et al., 1999; Nakajima et al., 1981; Nayak et al., 1997; Zimmermann, 2006). In these studies, the specific toxins involved were not characterized, and the species was identified using only microscopy.

A chemically closely related compound, karlotoxin, which belongs to a group of fish-killing toxins produced by *Karlodinium veneficum*, has been described (Van Wagoner et al., 2008; Peng et al., 2010). Karlotoxin (KmTx) 2 has proven to be toxic to fish gill cell cells as well; however, KmTx 2 showed a higher cytotoxicity than this compound, with LC50 values ranging between $203\text{ and }380\text{ ng mL}^{-1}$, compared to $\text{LC50} = 23.5\text{--}95\text{ }\mu\text{g mL}^{-1}$ (Place et al., 2012). Karlotoxins may function by non-specifically increasing the ionic permeability of biological membranes, resulting in osmotic cell lysis and damage to gill epithelial tissues (Deeds et al., 2006).

The findings of the histopathological study of *Anguilla reinhardtii* collected at the site of the fish kill indicated epithelial hypertrophy, associated with epithelial hyperplasia, lamellar fusion, and a mild increase in mitoses of gill epithelial cells. Water quality analysis showed that the dissolved oxygen levels were very low, $10.5\% \pm 0.2$ (0.91 mg L^{-1}) on the 18th September, two days prior to the collection of the fish for histopathological analysis on 20th September. Historical information is available on the dissolved oxygen levels at Curl Curl Lagoon over a 6 year time scale, taken at 9 sites within the lagoon (Supplementary Fig. S1). This shows that levels are highly variable, and have been historically measured as

occasionally as low as 0–6 mg L⁻¹ at several sites over several years. There has been much research on the phenomenon of high biomass phytoplankton blooms leading to hypoxia in water bodies, due to the large respiratory oxygen demands of phytoplankton and accompanying bacterial communities (i.e., reviews are Diaz, 2001; Anderson et al., 2002; Diaz and Rosenberg, 2008). A study of dense blooms of *Prorocentrum minimum* in coastal embayments showed that the growth of *P. minimum* could reduce ambient dissolved oxygen levels of 10–12 mg L⁻¹ to 0.1 mg L⁻¹ within 4 days due to respiration demand in the dark (Brownlee et al., 2005). It has been shown that the growth of *Amphidinium* spp. in culture was highest at a low ambient oxygen concentration of 5%, suggesting that its growth is not inhibited by low dissolved oxygen levels (Kitaya et al., 2008). Dense phytoplankton blooms leading to low dissolved levels have been frequently shown to result in fish kills (Diaz, 2001; Diaz and Rosenberg, 2008; Jones and Rhodes, 1994, and references therein).

It has been shown that *Anguilla* spp. had 100% survival for 48 h at very low dissolved oxygen levels of 1 mg L⁻¹, the level reported in this study, and other fish species showed survival rates from 20% to 85% (Dean and Richardson, 1999). For this reason, we are cautious about attributing the cause of death of fish in the lagoon. Based on the observed gill damage, we suggest that it was likely due to the low dissolved oxygen levels, and the presence of the luteophanol-like compound may have contributed.

5. Conclusions

We report on a dense bloom ($\sim 180 \times 10^3$ cells mL⁻¹) of *Amphidinium carterae* (Genotype 2) in a shallow intermittently open coastal lagoon in south eastern Australia. The deaths of >300 individuals of three different species of fin fish occurred concurrently. *Anguilla reinhardtii* individuals had damage to epithelial and gill epithelial cells. An analysis of the bloom water indicated the presence of a compound with the retention time of a Luteophanol A-, which caused up to 87% decrease in cell viability in an assay of fish gill cells. The fish deaths may have been attributed to the low dissolved oxygen, and the presence of a Luteophanol Alike compound may have contributed.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.
(A) Map of the coast of New South Wales, Australia showing the location of Curl Curl Lagoon. (B) Map of Curl Curl Lagoon, showing the location of three sampling sites (1, 2 and 3).

Fig. 2.
(A) Abundance of different species of phytoplankton observed at three sampling sites in Curl Curl Lagoon on 17th September 2012 during the *Amphidinium* bloom. (B) Mean and standard deviations of nutrient for samples and abundance of *Amphidinium carterae* taken on 17th September 2012 from three sampling sites in Curl Curl Lagoon.

Fig. 3.

Phylogenetic analysis using maximum likelihood of ITS gene sequences obtained from *Amphidinium* bloom sample. Support values are bootstrap values based on 500 replicates. Reference sequence names are followed by the strain number, if available and then accession number. Sequence marked by an asterisk (KF356164) was obtained during this study.

Fig. 4. HPLC chromatography (Lower Panel) on a C8 reverse phase column of the methanol extracted material from the lyophilized bloom sample and the purified material (middle panel). The UV spectra for the purified material (Upper panel) is identical to that described for Luteophanol A (Doi et al., 1997).

Fig. 5.
The mass spectra for the purified material is consistent with two congeners (base plus a hydroxylated form) that is sulphated similar to Luteophanol A (Doi et al., 1997).

Fig. 6.

Effect of a pure extract from the *Amphidinium carterae* bloom on viability of fish gill cells RTgill-W1. Gill cells were exposed to the extract for 2 h (white circles) and 6 h (black circles). LC50 obtained for the extract purified from *A. carterae* from Curl Curl Lagoon was 95 and 23.5 $\mu\text{g mL}^{-1}$, at 2 and 6 h, respectively, which is represented by the line across the 50% viability level and the arrows signalling in the x -axis. Values represent the mean and error bars the standard deviation of cell viability from quadruplicate wells.