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Detection of *Azadinium poporum* in New Zealand: the use of molecular tools to assist with species isolations

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Abstract A real-time PCR assay for the detection of species from the genera *Azadinium* and *Amphidoma* (family Amphidomataceae) was developed in order to screen field samples and to aid in the isolation of azaspiracid (AZA)-producing dinoflagellates. The assay was highly specific and sensitive and allowed the rapid detection of target species. Samples collected as part of the New Zealand Marine Phytoplankton Monitoring Programme were analysed using the Amphidomataceae real-time PCR assay. *Azadinium poporum* was detected in New Zealand for the first time, and a culture was successfully established. Extracts of this isolate proved to be of low toxicity to mice and did not contain AZA-1, -2 or -3. Field samples will continue to be screened with the aim of identifying AZA-producing species. The Amphidomataceae real-time PCR assay will be a useful tool for monitoring programmes and taxonomic surveys worldwide.

Keywords *Azadinium poporum* · Azaspiracids · New Zealand · Real-time PCR

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Introduction

The lipophilic, polyether azaspiracids (AZAs) are synthesised by species within the family Amphidomataceae. The first species described, and the first to be shown to produce AZAs, was *Azadinium spinosum* Elbrächter and Tillmann (Krock et al. 2009; Tillmann et al. 2009). Since then, more species have been described and several shown to produce AZAs, including *Azadinium poporum* Tillmann and Elbrächter (Krock et al. 2012; Gu et al. 2013), *Azadinium dexteroporum* Percopo and Zingone (Percopo et al. 2013) and *Amphidoma languida* Tillmann, Salas and Elbrächter (Krock et al. 2012). *Azadinium* species and/or AZAs have now been detected from various locations worldwide with new isolates demonstrating a high variability of AZA profiles, even within species (Krock et al. 2014; Tillmann et al. 2014). The AZAs cause a diarrhetic shellfish poisoning syndrome. The first recorded incident occurred in the Netherlands in 1995 when diners consumed contaminated blue mussels (*Mytilus edulis*) cultured in Ireland (Satake et al. 1998; Twiner et al. 2008). Further incidents occurred in Europe, including Ireland itself, over the next 5 years, all from the consumption of mussels produced in Ireland (James et al. 2004; Twiner et al. 2008).

The AZAs are regulated as a combined concentration of AZA-1, -2 and -3 at a limit of 0.16 mg kg^{-1} in shellfish in New Zealand, the same regulatory level as implemented in Europe. However, more than 30 AZA isomers and analogues have now been reported (Twiner et al. 2008) with new compounds regularly discovered as new species or strains are isolated (Krock et al. 2012). Toxicological information for the various AZA analogues is needed, in particular the determination of acute toxicities by oral administration (Munday 2014). AZAs produced by the dinoflagellates have been shown to undergo metabolism to other analogues in mussel tissue, as seen in the rapid conversion of AZA-1 and -2 to

AZA-17 and -19, respectively (Salas et al. 2011). In recent years, AZA-1 and -2 have been detected at low concentrations in New Zealand shellfish (<0.04 mg kg^{-1}), although the responsible organism has not yet been identified.

The morphological identification and/or isolation of *Azadinium* and *Amphidoma* species from field samples is difficult due to their small size (roughly 10–15 μm) and because most distinctive morphological characteristics require high magnification light microscopy or even electron microscopy (Tillmann et al. 2009). Due to these difficulties, species-specific molecular assays have been developed for the first three described species *A. spinosum*, *A. poporum* and *A. obesum* (Toebe et al. 2013). However, due to the high number of species presently known, variability in AZA production and lack of knowledge of the species present in New Zealand, it was necessary to design an assay that would enable the detection of a wide range of species from the genera *Azadinium* and *Amphidoma* (i.e. species from the family Amphidomataceae). In this study, we developed a real-time PCR assay that can detect all currently described species *Azadinium* species and *Amphidoma languida*. Cells from field samples that were positive using the real-time PCR assay were isolated with the aim of determining which Amphidomataceae species are present in New Zealand and to establish unialgal cultures.

Materials and methods

Real-time PCR assay targeting the Amphidomataceae species

The target positions for forward and reverse primers were designed using a multiple alignment of the ribosomal internal transcribed spacer (ITS) region (ClustalW) (Thompson et al. 1994) of sequences from *Azadinium* species and *Amphidoma languida*, and other peridinioid dinoflagellates obtained from GenBank (http://www.ncbi.nlm.nih.gov/genbank/). The specificity of the primer sequences was then confirmed using Basic Local Alignment Search Tool (BLAST) at National Centre for Biotechnology Information (NCBI). The assay was optimised on a Rotor-Gene 6000 (Corbett, Sydney, NSW, Australia), using genomic DNA extracted from an exponentially growing culture of *A. poporum* (strain AZDY06) isolated from the South China Sea (Krock et al. 2014). Genomic DNA was extracted using PowerSoil DNA isolation kits (Mo Bio, USA) following the manufacturer's instructions. Published primers and probes specific for *Azadinium poporum* and *Azadinium spinosum* (Toebe et al. 2013) were also optimised for the Rotor-Gene 6000 using DNA extracted from *A. poporum* (strain AZDY06) and *A. spinosum* (strain 3D9, North Sea). The hydrolysis minor groove binder (MGB) probes for these assays were synthesised (Integrated DNA Technologies (IDT), Singapore) with 6-FAM reporter dye at the 5′-end and Black Hole Quencher 1 at the 3′-end. The optimised assays consisted of a 20 μL reaction containing 10 μL of either Platinum SYBR Green qPCR SuperMix-UDG or Platinum Quantitative PCR SuperMix-UDG (Invitrogen, USA), 0.8 μg non-acetylated bovine serum albumin (BSA; Sigma-Aldrich, New Zealand) and 10 ng of DNA template. Primer and probe sequences, optimised concentrations and annealing temperatures for each assay are shown in Table 1. All PCR reactions in this study were set up manually and all included no template control reactions. Assays were run in clear 0.2-mL thin-wall PCR tubes (Axygen, USA). PCR cycling conditions were as follows: 50 °C for 2 min, 95 °C for 2 min and 30 cycles of 95 °C for 15 s and 59/62 °C for 60 s. The specificity of the Amphidomataceae assay was verified using DNA from various *Azadinium* species, *Amphidoma languida* and other related species (Table 2). DNA from each species (10 ng) was used in the real-time

Table 1 Sequences of primers and probes used in this study including optimised final concentrations and annealing temperatures for real-time PCR assays

Target	Type	Sequence	Product size	Annealing temperature	Final concentration	Reference
Amphidomataceae						This study
Amp240F	Forward primer	CAA CTT TCA GCG ACG GAT GTC TCG	179 bp	62°C	200 nM	
Amp418R	Reverse primer	AAG CYR CWGGCA TKA GAA GGT AGW GGC			200 nM	
Azadinium spinosum						Toebe et al. 2013
Asp48F	Forward primer	TCG TCT TTG TGT CAG GGA GAT G	72 bp	59°C	900 nM	
Asp120R	Reverse primer	GGA AAC TCC TGA AGG GCT TGT			900 nM	
Aspin77T	Probe	CGC CCA AAA GGA CTC CT			200 nM	
Azadinium poporum						Toebe et al. 2013
Apop62F	Forward primer	GAT GCT CAA GGT GCC TAG AAA GTC	68 bp	59°C	900 nM	
Apop148R	Reverse primer	CCT GCG TGT CTG GTT GCA			900 nM	
Apop112	Probe	TTC CAG ACG ACT CAA A			200 nM	

Table 2 Strains used for cross-reactivity testing of the Amphidomataceae real-time PCR assay. Results show either positive (+) or negative (−) for the duplicate PCR assays

Species	Strain	Origin	Result	Melt temperature
Azadinium poporum	CAWD230	Marlborough Sounds, New Zealand	+/+	84.2
Azadinium poporum	AZDY06	South China Sea	+/+	83.8
Azadinium poporum	UTH C5	North Sea, off Denmark	+/+	83.2
Azadinium poporum	UTH C8	North Sea, off Denmark	+/+	84.0
Azadinium poporum	UTH D4	North Sea, off Denmark	+/+	83.0
Azadinium poporum	AZBH 03	South China Sea	+/+	83.2
Azadinium polongum	Shet B2	North Sea, Shetland Islands	+/+	83.0
Azadinium spinosum	3D9	North Sea, off Scotland	+/+	84.2
Azadinium spinosum	UTH E2	North Sea, off Denmark	+/+	83.3
Azadinium spinosum	Shet F6	North Sea, Shetland Islands	+/+	83.3
Azadinium spinosum	SM2	Irish Coast, Bantry Bay	+/+	83.3
Azadinium obesum	2 E10	North Sea, off Scotland	+/+	83.3
Azadinium caudatum var. *margalefii*	AC 1	North Sea, off Scotland	+/+	83.5
Azadinium trinitatum	4 A8	Irminger Sea	+/+	83.3
Azadinium trinitatum	A2 D11	Irminger Sea	+/+	83.3
Azadinium trinitatum	4 B11	Irminger Sea	+/+	83.3
Azadinium cuneatum	3 D6	Irminger Sea	+/+	83.3
Azadinium concinnum	1 C6	Irminger Sea	+/+	82.7
Azadinium dexteroporum	1 D12	Irminger Sea	+/+	83.3
Amphidoma languida	SM1	Irish Coast, Bantry Bay	+/+	83.7
Amphidoma languida	2 A11	Irminger Sea	+/+	83.9
Heterocapsa minima	JK2	Irish Coast, Bantry Bay	−/−	NA
Heterocapsa minima	Heincke 2014	North Sea, Norwegian coast	−/−	NA
Heterocapsa sp.	Laz 83	Tasman Bay, New Zealand	−/−	NA
Heterocapsa niei	C29	Coromandel, New Zealand	−/−	NA
Heterocapsa sp.	CAWD88	Kerikeri, New Zealand	−/−	NA
Heterocapsa sp.	Laz 82	Marlborough Sounds, New Zealand	−/−	NA
Biecheleriopsis adriatica	L41	Marlborough Sounds, New Zealand	−/−	NA
Biecheleriopsis adriatica	L42	Marlborough Sounds, New Zealand	−/−	NA
Prorocentrum sigmoides	CAWD120	Marlborough Sounds, New Zealand	−/−	NA
Prorocentrum micans	CAWD142	Coromandel, New Zealand	−/−	NA
Gymnodinium beii	Laz 31	Marlborough Sounds, New Zealand	−/−	NA

PCR assays as described above. The amplification efficiency of the assay was determined by using 10-fold serially diluted DNA extracts from an *A. poporum* (strain AZDY06) culture with known cell concentration. The amplification efficiency was calculated from the slopes of the regression curve derived from the standard curve. Three separate DNA extracts were analysed in triplicate and ranged from approximately 900 to 0.09 cells per reaction.

Environmental sampling, real-time PCR analyses and cell isolations

Sea water samples (100 mL) were collected from around New Zealand as part of the New Zealand Marine Phytoplankton Monitoring Programme by personnel from the New Zealand Food Safety Authority (Ministry for Primary Industries, Wellington, New Zealand) and the Marlborough Shellfish Quality programme (MSQP) during routine weekly phytoplankton monitoring. Cell counts of species that morphologically resembled *Azadinium* species by light microscopy were determined using the Utermöhl technique and reported as cf. *Azadinium* sp. Samples containing live cf. *Azadinium* cells were split, and 50 mL was filtered (Durapore membrane filters, 0.45 μm, Millipore, USA). Genomic DNA was extracted from the filter papers as described above. The genomic DNA samples were then analysed using the Amphidomataceae real-time PCR assay (Table 3, Fig. 1). Reactions were deemed positive if an increase in fluorescence was detected before 30 cycles, and

the melt temperature of the product was between 82.5 and 84.5 °C. Positive samples were then analysed using the *Azadinium poporum* and *Azadinium spinosum* real-time PCR assays. The remaining 50-mL subsample was examined using a CK-2 microscope (Olympus, Japan) and *Azadinium*-like cells isolated using micropipettes and transferred to 12-well tissue culture plates (Corning, USA). Medium (F/2; Guillard and Ryther 1962) was added to the wells, and the cells were incubated at 18 °C, 90 μmol photons m^{-2} s^{-1} under a 12:12 L/D cycle. Positive environmental samples were also PCR amplified using the real-time PCR primers to confirm results. PCR amplifications were carried out in 50-μL reaction volumes containing i-Taq 2× PCR master mix (25 μL; Intron, Korea), both forward and reverse primers (0.4 mM) and template (ca. 50–150 ng of DNA). Thermocycling conditions were the same as for real-time PCR. Amplification products were purified (AxyPrep PCR cleanup kits, Axygen, USA) and sequenced (Sanger sequencing) in both directions using the primers from real-time PCR assay by an external contractor (University of Waikato DNA Sequencing Facility, Hamilton, New Zealand). The resulting sequences were compared to existing sequences in GenBank using the BLAST online software.

DNA sequencing of isolated unialgal cultures and phylogenetic analyses

Unialgal cultures were centrifuged (20 mL, 542×*g*, 15 min, room temperature) and DNA extracted as above from the resulting pellet. The D1-D3 region of the large subunit ribosomal RNA gene (LSU rDNA) was amplified using primers D1R-F (Scholin et al. 1994) and D3B-R (Nunn et al. 1996) as described previously (Rhodes et al. 2014). The PCR amplifications were carried out using i-Taq 2× PCR master mix (25 μL; Intron, Korea), forward and reverse primers (0.4 μM) and template (1.0 μL) containing ca. 50–150 ng of DNA. Thermocycling conditions and purification of amplification products were carried out as described previously (Rhodes et al. 2014), and sequencing was performed at Genetic Analysis Services, University of Otago (Dunedin, New Zealand). Sequences were aligned using the ClustalW algorithm (Thompson et al. 1994) in Geneious v6.0.4 (Drummond et al. 2011) and conflicts resolved by manual inspection. Sequences were aligned using the ClustalW algorithm (Thompson et al. 1994) in Geneious with publically available sequences from GenBank (www.ncbi.nlm.nih.gov). The D1–D3 sequence data matrices were truncated to 582 positions.

Table 3 Real-time PCR assay results for field samples. These assays were used to screen samples to target for cell isolations

Site	Collection date	Amphidomataceae	*Azadiniumpoporum*	*Azadiniumspinosum*
Hallam Cove	15/09/2014	+	+	−
Laverique	15/09/2014	+	+	−
West Beatrix	17/09/2014	+	+	−
Laverique	22/09/2014	+	+	−
Crail Bay	20/10/2014	−		
Laverique	20/10/2014	−		
West Beatrix	20/10/2014	+	+	−
Laverique	30/10/2014	−		
Cannon Bay	17/11/2014	−		
Nydia Bay	17/11/2014	−		
Waitaria	17/11/2014	−		
Anakoha	24/11/2014	−		
Forsyth	12/1/2015	−		
East Bay	20/01/2015	+	+	−
Port Taranaki	23/01/2015	−		
Port Hardy	26/01/2015	+	+	−
Port Taranaki	26/01/2015	+	+	−
Port Taranaki	3/02/2015	−		
Port Hardy	17/02/2015	+	+	−
Laverique	23/02/2015	−		
Laverique	2/03/2015	−		
West Beatrix	2/03/2015	−		
West Beatrix	4/03/2015	−		
Crail Bay	9/03/2015	−		
Richmond Bay	9/03/2015	−		

Bayesian analyses were carried out in Geneious using MrBayes 3.1.2 (Huelsenbeck and Ronquist 2001) using the evolutionary model (general time reversible with gamma-shaped among-site variation, GTR+G). Analyses of alignments were carried out in two simultaneous runs with four chains each 2×10^6 generations, sampling every 1000 trees. A 50 % majority-rule consensus tree was drawn from the last 1000 trees.

Toxin analyses

Dinoflagellate cultures (ca. 200 mL; 10–20,000 cells mL^{-1}) were centrifuged (3000×*g*) to pellet cells. These were extracted with analytical grade methanol (1 mL) and centrifuged (3000×*g*) to pellet any insoluble debris. Analysis was completed using a Waters Xevo TQ-S triple quadrupole mass spectrometer with an electrospray ionization source coupled to a Waters Acquity UPLC i-Class with flowthrough needle sample manager. A Waters Acquity BEH Shield RP18 1.7 μm 50 mm× 2.1 mm (column temperature 40 °C, injection volume 1 μL, flow rate 0.5 mL min^{-1}) was eluted using a gradient with MilliQ water/acetonitrile (95:5 *v*/*v*) and MilliQ water/acetonitrile (5:95 *v*/*v*), with both mobile phases containing 50 mM formic acid and 2 mM NH3. Azaspiracid detection parameters were as follows: ESI source temperature (100 °C),

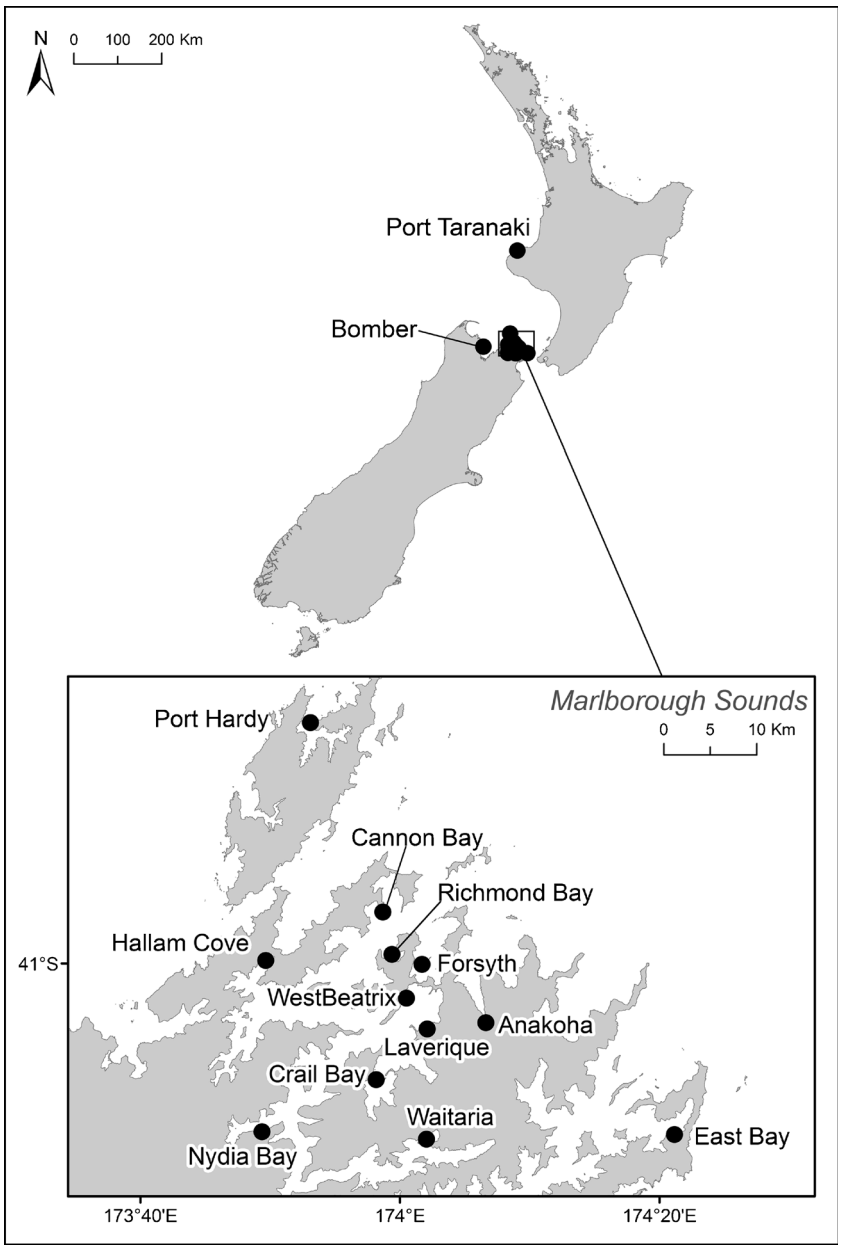

Fig. 1 Map of sample sites in New Zealand that were analysed using the real-time PCR assays

with N_2 nebulisation (7 bar), desolvation temperature (600 °C), cone gas (150 L h^{-1}), collision gas (0.15 mL min^{-1}), capillary voltage (2.0 kV), cone voltage (50 V). Data was acquired with the following +ESI MRM transitions: AZA-1, m/z 842.5→654.5 (CE 55V); AZA-2, m/z 856.5→645.5 (CE 45V); AZA-3, m/z 828.5→640.5 (CE 55V). Using this method, the limit of detection for AZA-1,-2 and -3 was 0.1 ng mL^{-1}.

Toxicity

Freeze-dried cell pellets from *Azadinium* batch cultures (CAWD230) were extracted five times with ethanol (4 mL), using a Potter-Elvehjem homogeniser. After centrifugation, the combined extracts were evaporated to dryness in vacuo at 40 °C. The residue was taken up in MeOH and aliquots evaporated under N_2 and then freeze-dried overnight. The dried extract was suspended in 1 % Tween 60 in saline and administered to Swiss albino mice (initial body weight 18–20 g, bred at Ruakura) by intraperitoneal (i.p.) injection at doses up to 200 mg kg^{-1}. Mice were allowed unrestricted access to tap water and to food (Rat and Mouse Cubes, Specialty Feeds Pty Ltd, Glen Forrest, Western Australia) throughout the experimental period. All experiments were approved by the Institutional Animal Ethics Committee.

Results

The primers designed in this study reliably amplified only the target species, as determined via cross-reactivity testing with strains listed in Table 2. The assay had a linear range of detection of five orders of magnitude with a limit of detection of less than one cell (Fig. 2). The amplification efficiency of the assay when targeting *A. poporum* was 93 % (Fig. 2). When the primer sequences were aligned with the ITS sequence from *Amphidoma languida* strain SM1 (GenBank accession number JQ247699), the forward primer showed 100 % identity, but the reverse primer had five mismatches (81 % identity). Despite the number of mismatches, the assay could still detect the presence of *Amphidoma languida*, albeit at lower efficiency (23 %; data not shown).

Twenty-five samples from 14 sites around New Zealand with cf. *Azadinium* cells were analysed using the real-time PCR assays (Fig. 1, Table 3). Nine samples were positive using the Amphidomataceae assay. All nine samples were also positive using the *A. poporum* assay and negative using the *A. spinosum* assay (Table 3). From these samples, an isolate of *A. poporum* (CAWD230) was successfully cultured from Laverique Bay, Marlborough Sounds, New Zealand (Fig. 1). Identification was confirmed by phylogenetic analyses using the LSU rDNA gene (Fig. 3).

A pellet of ca. 2,000,000 cells was analysed for AZA-1, -2 and -3 using LC-MS/MS analyses with a limit of detection of 0.1 ng mL^{-1}. The isolate contained <0.05 fg of AZA1, -2 and -3 per cell (i.e. below of the limit of detection). A culture extract was tested for toxicity in mice (by intraperitoneal injection at doses up to 200 mg kg^{-1}) to determine whether other toxic compounds were present. Mice became lethargic, with abdominal breathing, soon after dosing, but their appearance normalised within an hour after administration of the extract and remained normal throughout the subsequent 14-day observation period.

Discussion

Currently, AZAs are reported additively in New Zealand (addition of AZA-1, -2 and -3, pers. comm. Brian Roughan, Ministry of Primary Industries, New Zealand), and AZA-1 and -2 have been detected at low concentrations in New Zealand shellfish. A phytoplankton trigger level, to alert farmers to stop harvesting and test shellfish, has been set at 30,000 cells L^{-1}. *Azadinium* spp. are reported regularly in New Zealand's coastal waters by the Cawthron Institute micro-algae monitoring laboratory, but, due to the difficulty of differentiating *Azadinium* from other morphologically similar species by light microscopy, it is reported as cf. *Azadinium* sp. More than 50 isolates were cultured during 2013 and 2014, but most proved to be small *Heterocapsa* species (in particular *Heterocapsa niei*) (data not shown), a genus which is superficially similar in size, shape and swimming mode to *Azadinium*. Due to these difficulties, a real-time PCR assay

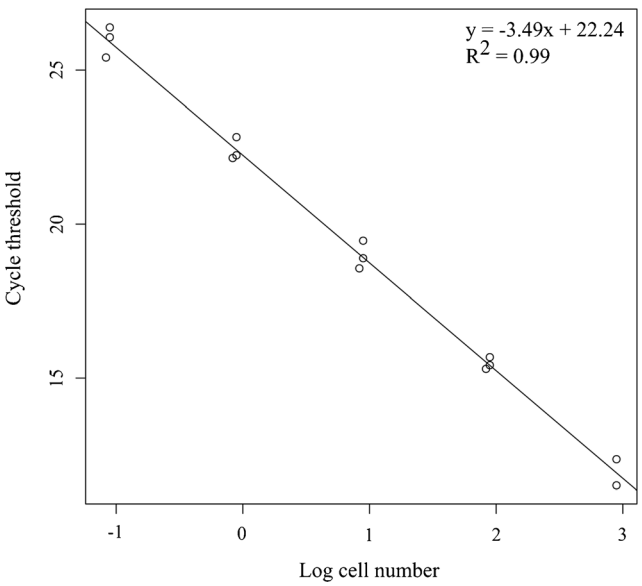

Fig. 2 Standard curve for the Amphidomataceae real-time PCR assay constructed with 10-fold serial dilutions of genomic DNA extracts from an *Azadinium poporum* culture (strain AZDY06)

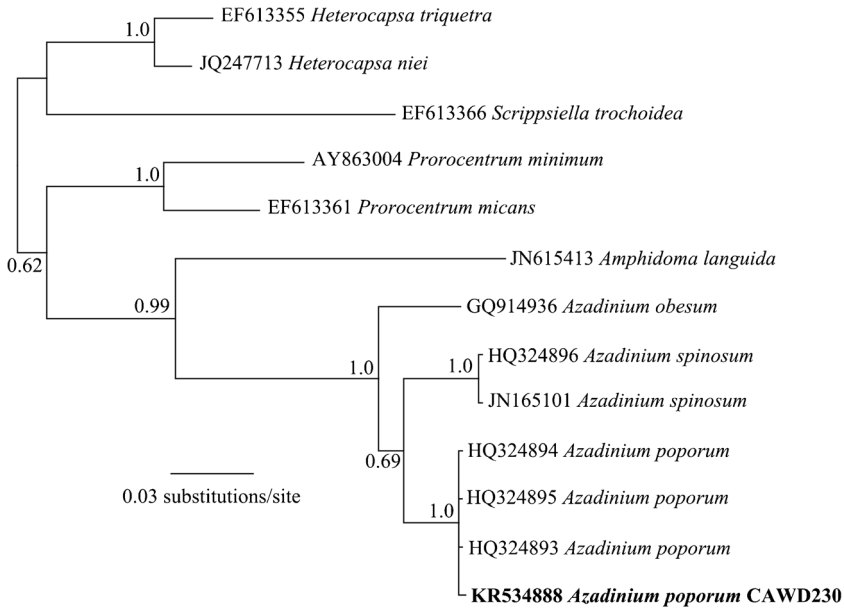

Fig. 3 Phylogenetic analysis of partial LSU rDNA sequences (D1–D3 region) from the *Azadinium poporum* strain (CAWD230) isolated in this study using Bayesian analyses. Values at nodes represent Bayesian posterior probability support. *Scale bar* is substitutions per site

was designed for the detection of *Azadinium* spp. and *Amphidoma languida* to ensure field samples reported as having cf. *Azadinium* sp. are in fact positive for that genus and to aid in the isolation of *Azadinium* cultures for the Cawthron Institute Culture Collection of Micro-algae (CICCM, http://cultures.cawthron.org.nz/ciccm/). The assay was tested against all currently described species of *Azadinium*, *Amphidoma languida* and other related species. The assay reliably amplified only species from the family Amphidomataceae.

Using this assay, we analysed 25 environmental samples from several sites around New Zealand where cf. *Azadinium* cells had been identified using light microscopy. Positive samples were then analysed using published species-specific assays for *A. poporum* and *A. spinosum* (Toebe et al. 2013). *A. poporum* was detected in nine samples, and an isolate was successfully cultured from the Marlborough Sounds. This isolate (CAWD230, GenBank accession number KR534888) was genetically similar (99.9 % identity, LSU D1-D2 region) to *A. poporum* isolates from the Danish coast of the North Sea (Tillmann et al. 2011). Extracts from the culture were analysed by LC-MS/MS, and regulated AZAs (AZA-1, -2 and -3) were not detected. This isolate of *A. poporum* was deemed to be of low toxicity by mouse bioassay, and therefore, extracts were not screened for additional AZA analogues.

The Amphidomataceae real-time PCR assay developed in this study demonstrated high specificity and sensitivity. The assay allowed rapid determination of positive environmental samples, enabling those with Amphidomataceae species present to be targeted for cell isolations. *Azadinium poporum* was detected in New Zealand for the first time, and a culture was successfully established. This culture was of low toxicity and lacked AZA-1, - 2 and -3, but we cannot exclude the presence of other (low toxixity) AZA analogues. Since low levels of AZA-2 have been detected in New Zealand shellfish, it is likely that some strains of *A. poporum* and/or other species present in New Zealand do produce AZAs. Field samples from the New Zealand Marine Phytoplankton Monitoring Programme will continue to be screened with the aim of isolating AZA-producing species. The assay will also be a useful tool for monitoring programmes and taxonomic surveys worldwide.

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