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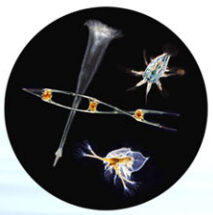

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Molecular detection and quantification of the azaspiracid-producing dinoflagellate *Amphidoma languida* (Amphidomataceae, Dinophyceae)

STEPHAN WIETKAMP[1]*, URBAN TILLMANN[1], DAVE CLARKE[2] AND KERSTIN TOEBE[1]

[1]ALFRED-WEGENER-INSTITUTE, HELMHOLTZ CENTRE FOR POLAR AND MARINE RESEARCH, AM HANDELSHAFEN 12, BREMERHAVEN D-27570, GERMANY AND [2]MARINE INSTITUTE, RINVILLE, ORANMORE, GALWAY H91 R673, IRELAND

*CORRESPONDING AUTHOR: stephan.wietkamp@awi.de

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Species of the planktonic dinoflagellates *Azadinium* and *Amphidoma* are small, inconspicuous and difficult, if not impossible to be identified and differentiated by light microscopy. Within this group, there are some species that produce the marine biotoxin azaspiracid (AZA) while others are non-toxigenic, therefore a requirement exists for precise species identification. A quantitative polymerase chain reaction (qPCR) assay for molecular detection and quantification of one of the toxigenic species, *Amphidoma languida*, was designed and extensively tested. The assay was highly specific and sensitive to detect and quantify down to 10 target gene copies (corresponding to ca. 0.05 cells) per reaction. DNA cell quota and copy number cell^{-1} were constant for four different *Am. languida* strains, and for one strain they were shown to be stable at various time points throughout the growth cycle. Recovery of known cell numbers of *Am. languida* spiked into natural samples was 95–103%, and the assay was successfully tested on field samples collected from Irish coastal waters. This new qPCR assay is a valuable tool for routine monitoring for the prevention of AZA-shellfish-poisoning caused by the consumption of contaminated shellfish and is a supportive tool for studies on the biogeography of this AZA-producing species.

KEYWORDS: *Amphidomataceae*; quantitative real-time PCR; *Azadinium*; azaspiracid shellfish poisoning (AZP); molecular probes

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INTRODUCTION

The azaspiracids (AZA) were identified in 1998 (Satake *et al.*, 1998) as new marine biotoxins, causing the serious seafood toxicity syndrome AZP (azaspiracid shellfish poisoning) in humans. AZA accumulate mainly in shellfish and crabs, and associated symptoms after consumption of contaminated seafood include mainly gastrointestinal health problems, like cramps, vomiting, nausea and severe diarrhea (Botana, 2014; Twiner *et al.*, 2014). AZA levels above the regulatory limit and extended shellfish harvest closures are a recurrent and serious problem mainly in Ireland (Salas *et al.*, 2011). In 2009, the small photosynthetic dinoflagellate *Azadinium spinosum* was described as a new species in a newly erected genus from the North Sea off the Scottish coast and identified as the first source organism producing AZA (Tillmann *et al.*, 2009). Since then, intense research has led to the description of different new AZA congeners and new species of *Azadinium*. To date, 13 *Azadinium* species have been described (Tillmann and Akselman, 2016), from which only three, *A. spinosum*, *A. poporum* and *A. dexteroporum* are known AZA producers (Krock *et al.*, 2012; Rossi *et al.*, 2017). However, AZA are not only produced by *Azadinium*. In 2012, the newly described *Amphidoma languida* was identified morphologically and phylogenetically as a close relative of the genus *Azadinium*, and both, *Azadinium* and *Amphidoma*, are now combined in the family Amphidomataceae (Tillmann *et al.*, 2012). Interestingly, *Amphidoma languida* produces AZA as well. To date, the azaspiracids AZA-2, -38, -39, -43, -52 and -53 with strain-specific AZA profiles have been found in *Am. languida* (Tillmann *et al.*, 2017).

Due to their small cell size (10–15 µm in cell length), most species of Amphidomataceae are difficult to detect and identify by light microscopy. A reliable morphological identification requires enhanced microscopic techniques like electron microscopy and the respective expertise. Thus, it is a time-consuming task, especially when other species of similar size and shape, such as *Heterocapsa* spp., are present in the samples (Tillmann *et al.*, 2009, 2010, 2012). This is probably the main reason why AZA-producing species have been discovered just recently. However, species identification is required for Amphidomataceae because toxigenic and non-toxigenic species are very similar in size and shape as well, and are known to co-occur in the same area (Tillmann *et al.*, 2014, 2015). Therefore, molecular tools are an ideal alternative method for rapid and routine identification of AZA-producing species in field samples. For the first three described *Azadinium* species (*A. spinosum*, *A. poporum* and *A. obesum*), Toebe *et al.* (2013) designed quantitative polymerase chain reaction (qPCR) assays, targeting the large subunit (LSU/28S) region of the ribosomal DNA (rDNA). Three years later, Smith *et al.* (2016) added a general Amphidomatacean real-time PCR assay, which allowed the detection of all described Amphidomatacean species that were known until that time, including *Amphidoma languida*.

Although the specific probes for two of the AZA-producing species are available (*A. spinosum* and *A. poporum*; Table I) and in use (Kim *et al.*, 2017; Tillmann *et al.*, 2018a), specific qPCR assays for the toxigenic *A. dexteroporum* and *Am. languida* are still lacking. While toxigenic *A. dexteroporum* have not been identified outside the Mediterranean (Tillmann *et al.*, 2015), *Am. languida* seem to be widely distributed in the North Atlantic (Tillmann, 2018) and have recently been identified as the causative agent of shellfish contamination above the EU regulatory limit in Spain (Tillmann *et al.*, 2017).

The aim of this study is to design and validate a real-time PCR assay for the identification and quantification of the AZA-producing dinoflagellate *Amphidoma languida* within environmental field samples for monitoring applications and to support biogeographical studies on this species.

METHOD

Laboratory cultures and DNA extraction

Genomic DNA was harvested from exponentially growing, unialgal strains grown in 1/10 strengh K medium (Keller *et al.*, 1987) at 15°C, a photon flux density of 70 µmol m^{-2} s^{-1} and a light:dark cycle of 16:8 h. Cells were collected by centrifugation in a 50 mL tube at 3220 × g for 15 min (5810R, Eppendorf, Hamburg, Germany). The supernatant was removed with a pipette. The pellet was resuspended in the remaining overlaying supernatant and subsequently transferred to a 1.5 mL tube and stored at −20°C. DNA was extracted using the NucleoSpin Soil DNA isolation kit (Macherey-Nagel, Düren, Germany) following the manufacturer's instructions. Instead of vortexing the bead tubes, the samples were shaken for 45 s and another 30 s at a speed of 4.0 m s^{-1} in a cell disrupter (FastPrep FP120, MP Biomedicals, Santa Ana, USA). DNA elution was performed twice using 100 µL of the provided elution buffer to increase the overall DNA yield. The DNA was stored at −20°C until further processing. Performance of the Soil kit was checked by analyzing DNA recovery/losses. Therefore, DNA extracts of known DNA concentrations were applied to the extraction procedure as described above. The NucleoSpin Soil kit revealed a ≥90% DNA recovery (Table S1) and was subsequently considered to be consistent. Reproducibility of DNA extractions was evaluated with six replicates each for four different strains and revealed relative standard deviations ranging from 4.9 to 8.2% (Table III).

Table I: Sequences of primers and probes for Amphidoma languida *(this study), the general Amphidomataceae assay and other* Azadinium *species from the literature*

Target species	Target gene	Oligonucleotide type	Sequence (5′-3′)	Product size (bp)	Reference
Amphidoma languida	LSU				
Alan509F		F-Primer	CGGTTCACAGGCGAGGAT	60	This study
Alan569R		R-Primer	GACATTCACACCTCCGTGGAA		
Alan528		TaqMan MGB probe	6FAM-CTTCTGAGGACATGGTAAC-MGB		
***Azadinium* and *Amphidoma* genera**	ITS				
Amp240F		F-Primer	CAACTTTCAGCGACGGATGTCTCG	179	Smith *et al.* (2016)
Amp418R		R-Primer	AAGCYRCWGGCATKAGAAGGTAGWGGC		
Azadinium spinosum	LSU				
Asp48F		F-Primer	TCGTCTTTGTGTCAGGGAGATG	72	Toebe *et al.* (2013)
Asp120R		R-Primer	GGAAACTCCTGAAGGGCTTGT		
Aspin77T		TaqMan MGB probe	6FAM-CGCCCAAAAGGACTCCT-MGB		
Azadinium poporum	LSU				
Apop62F		F-Primer	GATGCTCAAGGTGCCTAGAAAGTC	68	Toebe *et al.* (2013)
Apop148R		R-Primer	CCTGCGTGTCTGGTTGCA		
Apop112		TaqMan MGB probe	6FAM-TTCCAGACGACTCAAA-MGB		

A large batch of positive extraction-process-controls (EPC) with known cell numbers was prepared and stored at −20°C in 500 µL lysis buffer (buffer SL1, provided by the DNA isolation kit). Each EPC contained 10^3 cells of *Am. languida* (Z-LF-9-C9) and was extracted during each DNA extraction process to check DNA extraction efficiency and consistency. Reproducibility of EPC DNA extractions ($n = 14$) with a relative standard deviation of 7.9% was sufficiently high (Table S2).

Primer and probe design

The software Primer Express V.3.0 (Applied Biosystems by Thermo Fisher Scientific, Waltham, USA) was used to design species-specific primers and the probe, which target the large subunit (LSU/28S) region of the rDNA of *Amphidoma languida* in a real-time PCR assay (Table I). The probe was designed as a TaqMan minor groove binding (MGB)- probe with a 6-FAM reporter dye at the 5′-end and a Black Hole Quencher at the 3′-end (Applied Biosystems by Thermo Fisher Scientific, Waltham, USA). The target positions on the LSU were selected using multiple alignment comparisons within the software MEGA7 V7.0.26 (Kumar *et al.*, 2016). The sequences of the target species *Am. languida*, other Amphidomataceae and further related taxa were obtained from GenBank (https://www.ncbi.nlm.nih.gov/genbank/), as well as from unpublished sequences. To confirm the specificity of the designed primers and probe *in silico*, a sequence similarity search was performed using the Basic Local Alignment Search Tool (BLAST) of the National Center for Biotechnology Information (NCBI, https://blast.ncbi.nlm.nih.gov/Blast.cgi).

Conditions in real-time assays

A number of different primer sets, where no mismatch with the target-sequence and at least 5% mismatches with non-target sequences were observed, were preliminarily tested in a SYBR Green qPCR assay as a prescanning method. The 10 µL SYBR Green qPCR assay for the prescan primer tests contained 5 µL of Fast SYBR Green Master Mix (Applied Biosystems by Thermo Fisher Scientific, Waltham, USA), 0.25 µL of both primers (each 10 µm, for a final concentration of 200 nmol), 3.5 µL of high-grade PCR H_2O and 1 µL of template DNA (1 ng μL^{-1}). The SYBR Green assays were run in a StepOne Plus real-time PCR cycler (Applied Biosystems by Thermo Fisher Scientific, Waltham, USA) with the following conditions: Stage 1: hold 95°C for 20 s, followed by 40 cycles of Stage 2, Step 1: hold 95°C for 3 s, Step 2: hold 60°C for 30 s, followed by a Melt Curve Stage: Step 1: hold 95°C for 15 s, Step 2: hold 60°C for 60 s, Step 3: hold 95°C for 15 s.

The best performing primer set with the lowest C_T value was then tested for specificity in a TaqMan assay on DNA of various *Am. languida* strains (each at 1 ng μL^{-1} of DNA), as well as on extracted DNA from a range of non-target microalgae from different geographical regions (Table II). Assays were conducted either with stand alone DNAs or mixtures of DNAs spiked with *Am. languida* DNA.

To find the optimal primer and probe concentrations for the TaqMan assay, six different primer concentrations (600, 700, 800, 900, 1000 and 1200 nM) and three different probe concentrations (100, 200 and 300 nM) were tested. The final 10 µL TaqMan qPCR assay contained 5 µL of 2 × TaqMan Universal PCR Master

Table II: Strains for cross-reactivity testing of the Amphidoma languida *qPCR assay*

Species	Strain	Origin	qPCR result
Amphidoma languida	**2 A11**	**North Atlantic, Iceland**	**+**
Amphidoma languida	**AND-A0920**	**North Atlantic, Spain**	**+**
Amphidoma languida	**N-01-01**	**North Atlantic Norway**	**+**
Amphidoma languida	**N-12-01**	**North Atlantic, Norway**	**+**
Amphidoma languida	**N-39-12**	**Noth Atlantic, Norway**	**+**
Amphidoma languida	**N-40-06**	**North Atlantic, Norway**	**+**
Amphidoma languida	**SM1**	**North Atlantic, Ireland**	**+**
Amphidoma languida	**Z-LF-14-E7**	**North Sea, Denmark**	**+**
Amphidoma languida	**Z-LF-14-F2**	**North Sea, Denmark**	**+**
Amphidoma languida	**Z-LF-14-G7**	**North Sea, Denmark**	**+**
Amphidoma languida	**Z-LF-9-C4**	**North Sea, Denmark**	**+**
Amphidoma languida	**Z-LF-9-C9**	**North Sea, Denmark**	**+**
Amphidoma parvula	H1-E9	South Atlantic, Argentina	–
Azadinium caudatum	AC 1	North Sea, Scotland	–
Azadinium concinnum	1 C6	North Atlantic, Irminger Sea	–
Azadinium cuneatum	3 D6	North Atlantic, Iceland	–
Azadinium cuneatum	35-A2	Northeast Pacific, Puget Sound	–
Azadinium dalianense	121-F6	Northeast Pacific, Puget Sound	–
Azadinium dalianense	48-1-F8	Northeast Pacific, Puget Sound	–
Azadinium dalianense	H-2-G7	South Atlantic, Argentina	–
Azadinium dalianense	N-38-03	North Atlantic, Norway	–
Azadinium dalianense	Z-LF-14-F7	North Sea, Denmark	–
Azadinium dexteroporum	1 D12	North Atlantic, Irminger Sea	–
Azadinium obesum	48-1-F2	Northeast Pacific, Puget Sound	–
Azadinium obesum	2E10	North Sea, Scotland	–
Azadinium obesum	AZA 2D	North Atlantic, Labrador Sea	–
Azadinium obesum	N-41-01	North Atlantic, Norway	–
Azadinium obesum	Z-LF-12-A12	North Sea, Denmark	–
Azadinium obesum	Z-LF-44-C3	Baltic Sea, Germany	–
Azadinium polongum	N-47-01	North Atlantic, Norway	–
Azadinium polongum	Shet B2	North Atlantic, Shetland Islands	–
Azadinium poporum	1 D5	South Pacific, Chile	–
Azadinium poporum	121-E10	Northeast Pacific, Puget Sound	–
Azadinium poporum	18 A1	South Atlantic, Argentina	–
Azadinium poporum	AZ-BH-03	South China Sea, China	–
Azadinium poporum	HJ-2011	East China Sea, Korea	–
Azadinium poporum	N-39-01	North Atlantic, Norway	–
Azadinium poporum	UTH C8	North Sea, Denmark	–
Azadinium poporum	Z-LF-14-E12	North Sea, Denmark	–
Azadinium spinosum	3D9	North Sea, Scotland	–
Azadinium spinosum	H-1-D11	South Atlantic, Argentina	–
Azadinium spinosum	H-4-A10	South Atlantic, Argentina	–
Azadinium spinosum	N-04-01	North Atlantic, Norway	–
Azadinium spinosum	N-05-01	North Atlantic, Norway	–
Azadinium spinosum	Shet F6	North Atlantic, Shetland Islands	–
Azadinium spinosum	SM2	North Atlantic, Ireland	–
Azadinium spinosum	UTH E2	North Sea, Denmark	–
Azadinium trinitatum	A2 D11	North Atlantic, Iceland	–
Azadinium trinitatum	N-39-04	North Atlantic, Norway	–
Alexandrium catenella	MX E11	North Atlantic, western Greenland	–
Alexandrium ostenfeldii	MX D1	North Atlantic, western Greenland	–
Gymnodinium sp.	H-1-A6	South Atlantic, Argentina	–
Heterocapsa minima	JK2	North Atlantic, Ireland	–
Heterocapsa steinii	UTK G7	Baltic Sea, Germany	–
Karlodinium veneficum	E11	Mediterranean, Spain	–
Prorocentrum balticum	CCMP1787	South Pacific, New Zealand	–
Prorocentrum micans	PM A4	Baltic Sea, Germany	–
Scripsiella precaria	SP14	North Sea, Scotland	–

Results show either a positive (+) or no (–) amplification.

Mix, with AmpliTaq Gold DNA polymerase and dNTPs and the passive reference dye ROX (Applied Biosystems by Thermo Fisher Scientific, Waltham, USA), 0.9 μL of both primers (as a final concentration of 900 nmol), 0.2 μL of the TaqMan MGB probe (as a final concentration of 200 nmol), 2 μL of high-grade PCR H_2O and 1 μL of template DNA (1 ng μL^{-1}). The TaqMan qPCR assay followed these steps: Stage 1: hold

Table III: LSU gene copy number and DNA content cell^{-1} in four different Am. languida *strains (n = 6)*

Strain	Origin	Mean LSU copy number (no. cell^{-1}) ± SD	Mean DNA content (pg cell^{-1}) ± SD
2A11	North Atlantic, Iceland	719 ± 48	2.73 ± 0.18
N-12-01	North Atlantic, Norway	830 ± 93	3.15 ± 0.35
AND-0920	North Atlantic, Spain	777 ± 38	2.95 ± 0.15
Z-LF-9-C9	North Sea, Denmark	829 ± 107	3.15 ± 0.41

95°C for 20 s, followed by 40 cycles of Stage 2, Step 1: hold 95°C for 1 s, Step 2: hold 60°C for 20 s. All reactions were carried out in triplicates within 0.1 mL MicroAmp Fast 96-Well Reaction Plates (Applied Biosystems by Thermo Fisher Scientific, Waltham, USA) on a StepOne Plus real-time PCR cycler (Applied Biosystems by Thermo Fisher Scientific, Waltham, USA) and a sample was considered as positive only if all three replicates of the sample showed a fluorescence signal above the threshold before cycle 37. No-template controls (NTC) containing high-grade PCR H_2O as well as extraction-process-controls (EPC) were present during all PCR runs. For C_T value (threshold cycle), baselines and thresholds were set manually before each qPCR analysis according to the guidelines from Applied Biosystems (Livak, 1997; Ruijter *et al.*, 2009).

Quantification experiments

For DNA-based quantification of cells, standard curves with known DNA concentrations are required in each qPCR run. Two types of standard curves were established: First, a standard curve of 10-fold dilution series of *Am. languida* DNA (10 ng μL^{-1} to 0.1 pg μL^{-1}) was generated. The DNA from 10^5 cells was collected from four exponentially growing strains of *Am. languida*. Cell density was estimated by light microscopy (Axiovert 200 M, Zeiss, Germany) counting of settled subsamples of 0.5 mL at a magnification of 400×. The DNA was extracted as described above. The amount of dsDNA of these extracts was measured using the Quantus Fluorometer (Promega, Fitchburg, USA) and DNA cell quota was calculated.

The second standard curve was a 10-fold dilution series of copies of the target amplicon (10^8 copies μL^{-1} to 10^1 copies μL^{-1}), which were prepared according to Perini *et al.* (2011). The 681 bp D1-D2 region of the LSU rRNA from purified genomic DNA of *Am. languida* was amplified in a qualitative PCR. Each 20 μL PCR reaction contained 2 μL of 10× HotMaster Taq buffer, 0.1 μL of HotMaster Taq DNA polymerase, 0.2 μL dNTP (10 μM), 0.2 μL of both primers (each 10 μM; Forward primer: D1R; Reverse primer: D2C; (Scholin *et al.*, 1994)), 16.3 μL of high-grade PCR H_2O and 1 μL of template DNA (10 ng μL^{-1}). PCR was carried out in an Eppendorf Mastercycler (Eppendorf, Hamburg, Germany). Initial denaturation (94°C, 2 min) was followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, elongation at 68°C for 2 min and a final extension at 68°C for 10 min. The amplicon was analyzed and quantified on a Bioanalyzer Instrument (2100 Bioanalyzer, Agilent, Santa Clara, USA) and the number of amplicon copy number per μL was calculated using the following equation:

$$\text{No. molecules } \mu L^{-1} = (A \times 6.022 \times 10^{23})(B \times 660)^{-1}$$

where A is the amplicon concentration (g μL^{-1}), 6.022×10^{23} is Avogadro's number, B is the number of base pairs of the amplicon and 660 is the average molecular weight of one base pair. Both, the DNA-based and the copy-molecule-based standard curve, were performed in triplicates in all qPCR runs. To account for intraspecific variability, both DNA content and copy numbers per cell were determined for four strains (Z-LF-9-C9, N-12-01, 2A11 and AND-0290).

To additionally test for potential intra-clonal variability in DNA content or DNA copy number cell^{-1}, one of the *Am. languida* strains (Z-LF-9-C9) was sampled in 10 mL duplicates at 10 am and 3 pm for a period of 17 days. For each sampling, the cell density of the culture was determined by microscopy enumeration. The DNA was extracted and measured as described above and the DNA content and copy number cell^{-1} was calculated.

Spiked seawater samples

To account for potential PCR inhibition effects of a natural seawater matrix, known cell numbers of *Am. languida* were spiked into a natural seawater sample. The sample was prepared from 1 L of water taken at Bremerhaven harbor additionally enriched with 50 mL of *A. poporum* (strain AZ-BH-03, 56.000 cells mL^{-1}) and 50 mL of *Lingulodinium polyedra* (strain 28-4C, 500 cells mL^{-1}). In triplicates, 10^5, 10^4, 10^3 or 10^2 cells of *Am.*

languida (strain Z-LF-9-C9) were spiked into 50 mL of the generated seawater matrix. Negative controls without *Am. languida* cells were prepared as well. The tubes were centrifuged and DNA extracted from the pellet as described above.

Subsequent qPCRs with DNA and target molecule based standard curves were performed and the *Am. languida* cell number was calculated as described above. A second spike experiment was performed 2 weeks later, using the same cultures and cell numbers for spiking as described above.

Application of the assay on field samples

In August 2017, a coastal survey (CV17022) of Irish waters was conducted by the Marine Institute (Ireland) on board the RV Celtic Voyager. In total, 66 stations were sampled along a number of transects from the Southeast coast, right round the South coast and up along the West coast (Fig. 4). At each station, 5 L water samples were collected from the observed chlorophyll-maximum-layer with Niskin bottles attached to the deployed CTD instrument. Samples were prefiltered through a 20 µm mesh, subsequently filtered onto 3 µm TSTP filters (47 mm, Merck, Darmstadt, Germany) and stored at −20°C until further analysis. To wash the collected cells off, the filters were cut into halves, with one half placed in individual 2 mL microtubes containing 1.5 mL of lysis buffer and vortexed for 1 min. The filter papers were discarded, the microtubes were centrifuged at 3220 × g for 5 min and the supernatant was discarded as well. DNA extractions were in accordance with the laboratories ISO-17 025 accredited internal procedures (available at the Marine Institute, SOP No. PHY-055 Vr 1.1). Quantitative PCR was performed on a Roche LightCycler 480 Vr I and II PCR instrument (Roche AG, Basel, Switzerland). Cell number per sample was calculated based on a standard curve of 10-fold dilutions of *Am. languida* DNA as described above using the associated software with the LightCycler instrument.

Statistical analysis

Statistical analyses were performed with parametric (analysis of variances; ANOVA) or non-parametric (Mann–Whitney, Kruskal Wallis, or Spearman correlation) tests, using RStudio ver. 1.1.419, with a $P < 0.05$ level of significance.

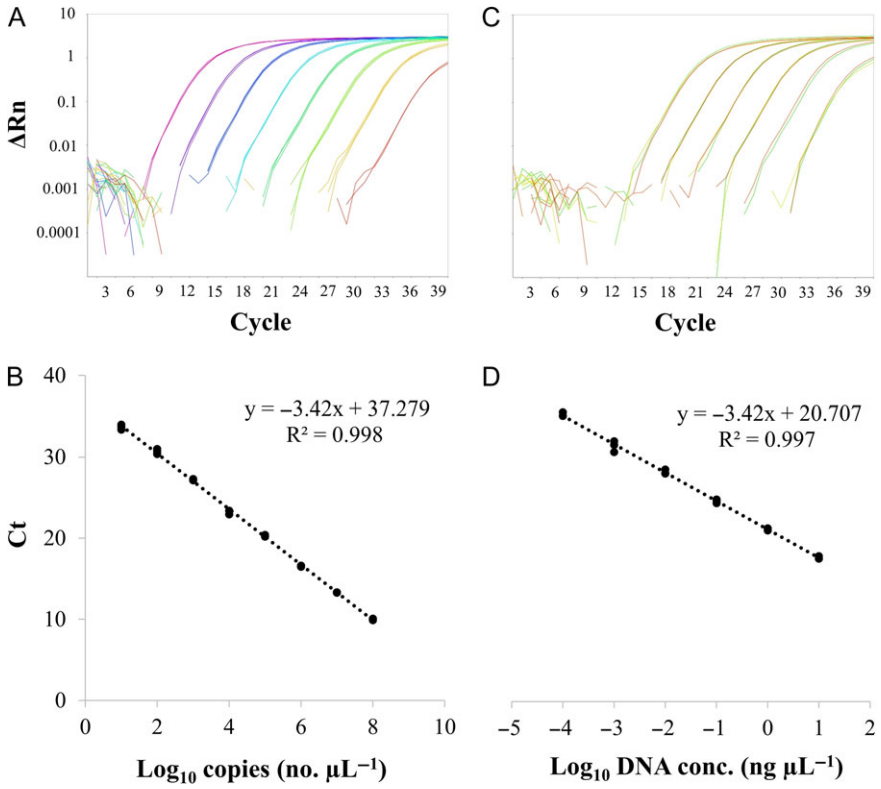

Fig. 1. Amplification of two types of standard curves in the qPCR for *Am. languida* (strain 2A11). The amplification plot and the corresponding standard curve of serial dilutions of rDNA copies produced via PCR amplification of the target region (**A**, **B**) and serial dilutions of extracted DNA from known cell numbers (**C**, **D**). Measurements have been performed in triplicates, dots may overlap.

RESULTS

Assay and assay specificity

The primer and probe sequences and the amplicon sizes of the newly developed qPCR assay for the detection of *Amphidoma languida* along with the respective information for other AZA-producing species are presented in Table I.

Specificity of the new qPCR assay was tested with target and non-target DNA of a large collection of microalgal species and strains (Table II). All 12 strains of *Am. languida* from different areas of the North Atlantic were detected as single strains or in mixed samples, where no cross-hybridization with any non-target microalgae (multiple species and strains of other Amphidomatacea, and a representative set of species of other dinophycean genera), neither with single-testing nor within mixed samples. No inhibition of the assay was observed in any of the reactions.

Quantification of *Am. languida* cells

Two types of standard curves were established for quantification of *Am. languida* cells. The first was based on a 10-fold dilution series of target gene copies and the second based on a 10-fold dilution series of DNA extracts of *Am. languida*. The standard curve of target gene copies yielded C_T values between 11.5 ± 0.05 (10^8

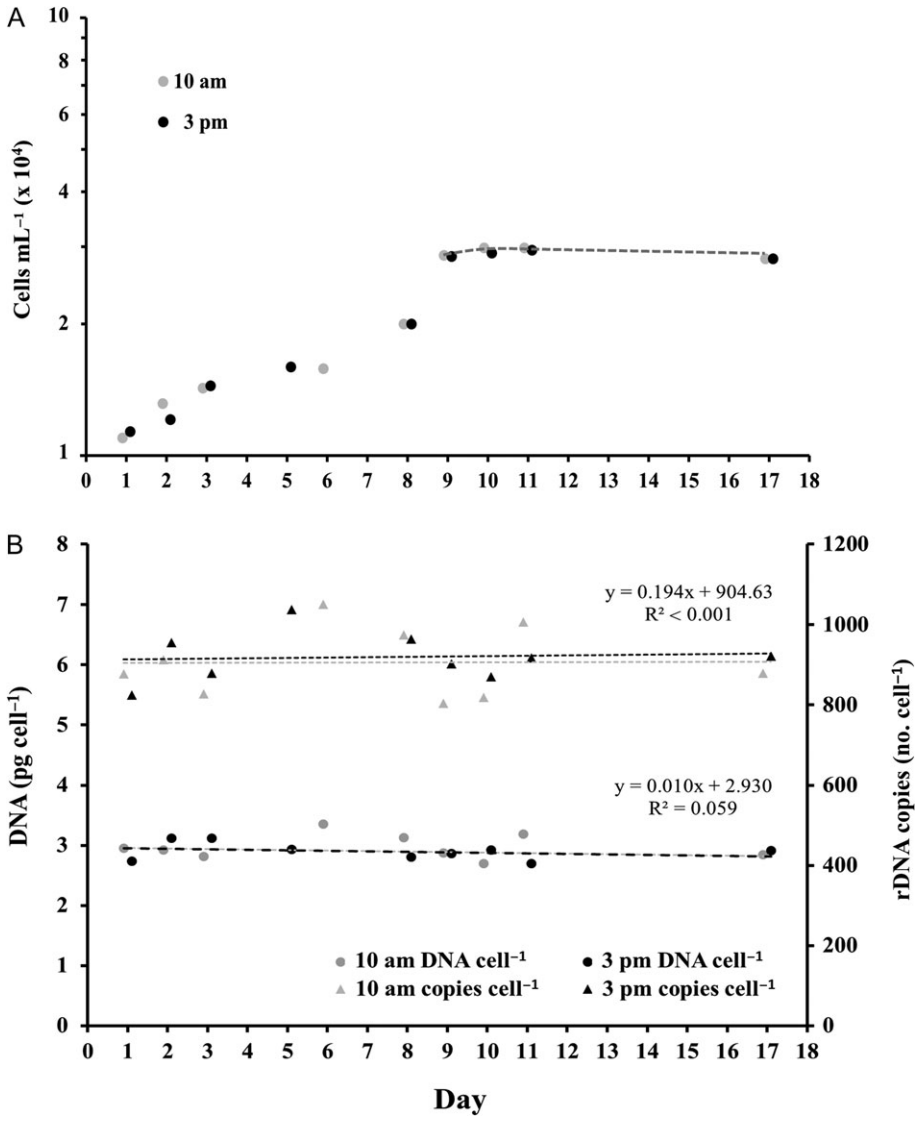

Fig. 2. Culture observation of *Am. languida* over 17 days. Cell density (**A**), as well as variations in rDNA copy number (triangles) and DNA content (circles) cell^{-1} (**B**) over 17 days.

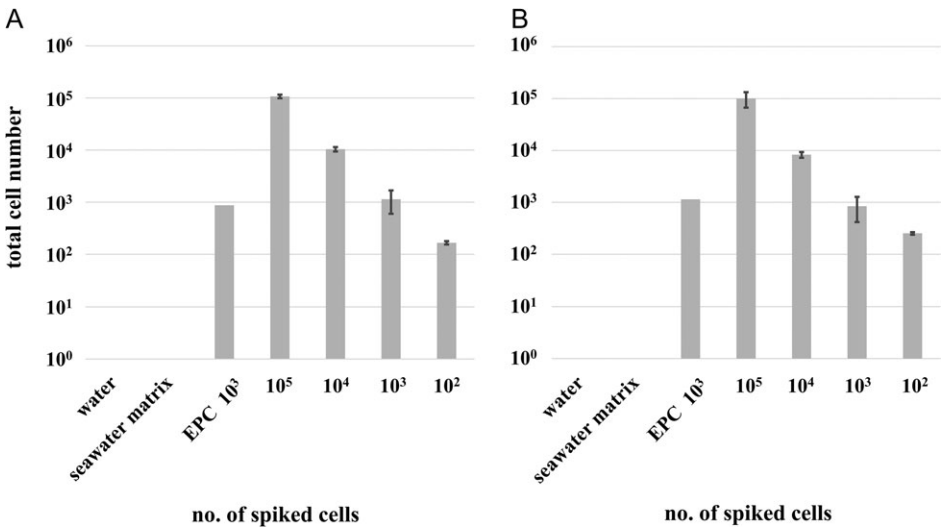

Fig. 3. Cell recovery from C_T values of known cell numbers in qPCR for two independent experiments (**A** and **B**). Spiked samples were prepared in 10-fold dilutions. Milli-Q water and the seawater matrix without *Am. languida* were used as negative controls, EPCs with 10^3 cells of *Am. languida* were included to check for variations between different DNA extractions. Bars represent mean ± 1 SD ($n = 6$).

copies μL^{-1}) and 36.1 ± 0.56 (10^1 copies μL^{-1}), with an amplification efficiency $E = 96.1\%$ (Fig. 1A and B), calculated according to Bustin *et al.* (2009). The DNA standard curve yielded C_T values between 18.7 ± 0.09 (10 ng μL^{-1}) and 35.9 ± 0.11 (10^{-4} ng μL^{-1}), with an amplification efficiency $E = 96.1\%$ (Fig. 1C and D).

DNA copy number and DNA content cell^{-1}

The mean copy number of four different *Am. languida* strains ranged from 719 to 830 cell^{-1} (Table III) and was not significantly different between strains ($F = 0.057$, $P = 0.981$). Likewise, the mean DNA cell quota ranged from 2.73 to 3.15 pg cell^{-1} (Table III), without significant differences between strains ($F = 1.705$, $P = 0.218$).

Temporal variability in copy number and DNA content

Potential temporal variability of rDNA copy number and DNA cell quota was extensively analyzed for one strain of *Am. languida* (Z-LF-9-C9) over a time period of 17 days. In batch culture mode, cell density increased from 10×10^3 cells mL^{-1} to 30×10^3 cells mL^{-1} after 17 days of observation, with stationary growth reached at approximately day 9 (Fig. 2A). The rDNA copy number ranged from 805 to 1050 copies cell^{-1} and did not change significantly with time or cell density (*t*-test, $P = 0.521$). The DNA cell quota ranged from 2.70 to 3.35 pg cell^{-1} and did not change significantly with time or cell density either (*t*-test, $P = 0.473$). Likewise, there was no significant difference between samples taken at 10 am versus samples taken at 3 pm for both, the rDNA copy number (*t*-test, $P = 0.476$) and DNA cell quota (*t*-test, $P = 0.549$) (Fig. 2B).

Limit of quantification and limit of detection

The limit of quantification (LOQ) and the limit of detection (LOD) were estimated from analysis of replicate standard curves ($n = 8$) according to Forootan *et al.* (2017). In the present study, the criterion for LOQ that 95% of the measured values have to be within the interval of mean ± 2 SD was valid for a target concentration of 10 copies μL^{-1} or 0.1 pg target DNA μL^{-1}, respectively. The respective criterion for LOD, i.e. the lowest target concentration for which at least 95% of replicates are positive, was 10 copies μL^{-1} or 0.1 pg target DNA μL^{-1} as well. With the next dilution below these concentrations (1.0 copies or 0.01 pg target DNA μL^{-1}), no consistent amplification between replicates (≥95%) was observed.

Seawater matrix effects

Primer and probe quantification performance under environmental conditions was tested by spiking different amounts of *Am. languida* (strain Z-LF-9-C9) into a natural seawater sample, which was additionally enriched with two non-target microalgae. The concentration of non-target DNA in the seawater matrix without spiked *Am. languida* was 234.7 ± 6.3 µg sample^{-1} ($n = 3$) for the first experiment (Fig. 3, A) and 219.7 ± 8.2 µg sample^{-1} ($n = 3$) for

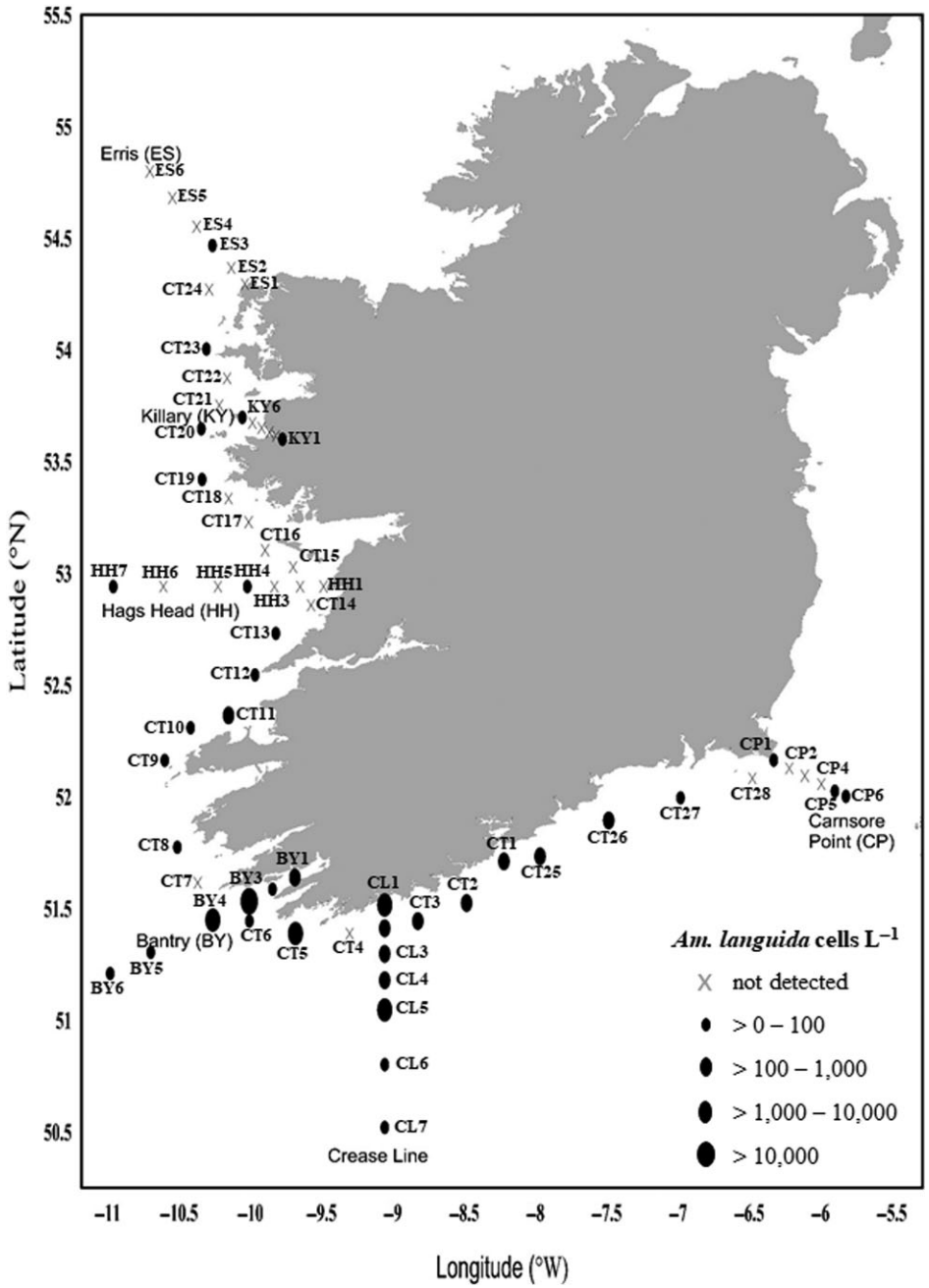

Fig. 4. Sampling locations of the coastal survey (CV17022) in Irish waters in 2017. Stations, where *Am. languida* has been found with the newly developed qPCR assay, are presented as filled circles. Circle size categorizes the amount of cells L^{-1}. Exact qPCR-based cell numbers estimated for each station are given in Table S3.

the second experiment (Fig. 3B). Calculated cell numbers were in good agreement with the actual number of spiked cells. Mean cell recovery rate was 103.0 ± 9.8% in the first experiment and 95.3 ± 12.7% in the second experiment with values above 100% mainly occurring at the lowest addition (100 cells).

qPCR application on field samples

On the survey off the Irish coast, *Am. languida* was determined to be present in a number of stations along the south and south-west coast in 2017 (Fig. 4). In particular, *Am. languida* was mainly detected along the southern and south-western sampling locations, with cell concentrations

generally in the range of 5-1000 cells L^{-1} (Table S3). Higher numbers were exclusively recorded from some stations in the south-east with highest cell concentrations of 8 720 cells L^{-1} (station BY4) and 22 720 cells L^{-1} (station BY3). In contrast, in the North-West the target species was detected only in some isolated spots, with cell concentrations not exceeding 20 cells L^{-1}.

DISCUSSION

In this study, a specific and sensitive qPCR assay for the detection and quantification of the AZA-producing species *Amphidoma languida* in field samples is presented.

In general, the quantitative PCR is a highly sensitive tool (Tahir *et al.*, 2017). There are several real-time PCR assay types used in different laboratories and for different research questions. SYBR Green assays contain target-specific primers and an intercalating fluorescent dye, which releases a detectable signal in the qPCR if the primers amplify any DNA amplicon. It is a more economical method compared to the TaqMan chemistry, but also less specific since SYBR Green assays tend to amplify also non-target DNA (Purcell *et al.*, 2011; Mohr *et al.*, 2015). Here, the TaqMan chemistry was chosen, because specific fluorescent probes (additionally to the target-specific primers) enable a highly specific and sensitive amplification of the target molecule. The MGB moiety increases the stability of the probe and raises the melting temperature, which allows the design of shorter, highly specific probes with the same annealing temperature compared to traditional TaqMan probes without the MGB motif (Kutyavin *et al.*, 2000). The new primers and probe for *Am. languida* were designed to amplify a target region on the large subunit (LSU, 28S) of the rDNA. This region worked well previously for the assays on *A. spinosum*, *A. poporum* and *A. obesum* (Toebe *et al.*, 2013). Targeting other regions, e.g. the internal transcribed spacer (ITS) region would be far less suitable, as ITS sequencing for that species revealed surprisingly large intra-genomic variability in this DNA region (Tillmann *et al.*, 2012).

The primers and probe were thoroughly tested for specificity and yielded positive signals only for DNA of the different strains of *Am. languida*. Non-target strains in specificity testing included multiple strains covering almost all species of *Azadinium* and also the only other species of *Amphidoma* where DNA is available, i.e. *Amphidoma parvula* (Tillmann *et al.*, 2018b). Furthermore, for *in silico* probe design, all environmental sequences in GenBank that were identified as *Amphidoma* sp. in the phylogenetic tree presented in Tillmann *et al.* (2018b) were included. In the genus *Amphidoma*, there are 11 additional species, where DNA sequences are not yet available, and the probes presented here need to be continuously tested for cross-reactivity once new sequence data of other *Amphidoma* species become available. In addition, for species of *Azadinium*, such as *A. poporum*, *A. dalianense* and *A. spinosum*, recently established new strains revealed considerable intraspecific sequence variability and the presence of different ribotypes (Luo *et al.*, 2017; Tillmann *et al.*, 2018a). The same might be expected for *Am. languida*, so the availability of new strains will again require updates of specificity testing.

With a reliable and reproducible detection and quantification of down to 10 target gene copies (corresponding to ca. 0.05 cells) per reaction, the qPCR assay is highly sensitive. The corresponding final cell detection limit for field samples, of course, depends on a number of adjustable factors. With the precondition of our assay specifications (100 µL DNA extraction volume, 1 µL of extract within a 10 µL assay volume and filtering e.g. 1 L seawater), the limit of quantification would be 2 cells L^{-1}.

Early warning threshold values for *Amphidoma languida* or any other AZA-producing species are not yet defined due to a lack of knowledge and data. Anyhow, given the small cell size and the limited amount of AZA $cell^{-1}$, cell concentrations critical for AZP are expected to be orders of magnitude higher, i.e. in the range of at least hundreds of cells L^{-1}. Thus, the assay sensitivity is suitable for monitoring purposes and well suited in ecological studies aiming the detection of even low background concentrations. The challenges and problems of reliable quantification of microbial species using the qPCR are addressed in a number of studies (Galluzzi *et al.*, 2004; Godhe *et al.*, 2008; Erdner *et al.*, 2010). Three different issues for quantification are highlighted: (i) DNA extraction performance, (ii) variability in rDNA copy numbers and (iii) assay inhibition effects.

DNA extraction performance

There are several commercial kits on the market for DNA extraction from phytoplankton. DNA extraction performance is especially essential for quantification studies due to the very high impact of uncertainties, and there are ongoing discussions about this topic in the qPCR community (Yuan *et al.*, 2015; Brauge *et al.*, 2018). In the present study, the NucleoSpin Soil DNA isolation kit was used for DNA extraction and purification. Inclusion of EPC of defined *Am. languida* cell numbers for all extraction proceedings revealed a consistent extraction performance of this kit.

rDNA copy number

The qPCR method quantifies the number of target gene copies and therefore, any intraspecific variability and variation in copy number cell^{-1} is an outstanding factor to consider for qPCR-based quantification and makes enumeration more challenging (Créach *et al.*, 2006; Garneau *et al.*, 2011; Penna and Galluzzi, 2013; Wang *et al.*, 2017). Any mismatch in cell copy number between a field population and the strain used to prepare the qPCR standard curve will bias quantification. For some microalgal species and especially for dinoflagellates, which are known to increase their genome by incorporating copies of several DNA regions (Bachvaroff and Place, 2008), large variability in copy number has been reported for different strains, for different growth stages and culturing conditions (Godhe *et al.*, 2008; Galluzzi *et al.*, 2009).

However, for *Am. languida* the data presented here reveal the same copy number for four different *Am. languida* strains from different geographic origins. Moreover, one representative strain (Z-LF-9-C9) showed no major variability in target gene copy number over time within a batch culture growth cycle.

Inhibition

qPCR amplification of target molecules can be inhibited by several substances common in field samples, such as humic acids, polysaccharides, haem, proteins, polyphenols and others (Gallup, 2011), and it always has to be kept in mind that inhibitors within a field sample set may vary between sites. Inhibitory substances are reduced by washing buffers and spin columns of commercial DNA extraction kits to some extent (Fock-Chow-Tho *et al.*, 2017), but potential matrix effects in field samples are considered by quantifying seawater samples spiked with known amounts of target cells. With two independent experiments using natural seawater (even further enriched with non-target cells), it is shown here, that the qPCR recovers spiked *Am. languida* cells at almost 100% efficiency over a concentration range of four orders of magnitude.

Finally, to investigate the applicability of the assay for field samples, the assay was applied to a field data set of Irish coastal waters and yielded the first abundance data for this species. The assay confirmed the presence of *Am. languida* for the area and revealed the species to be widely distributed along the southern and western Irish coast. qPCR quantification further indicates higher abundance for the south-western part with peak densities off Bantry Bay, the location where the type strain of *Am. languida* was isolated (Tillmann *et al.*, 2012). Peak densities >22 000 cells L^{-1} indicate that *Am. languida* might substantially contribute to AZA contamination in Irish mussels and underline the need to include AZA produced by this species in routine seafood monitoring of AZA toxins.

CONCLUSION

Due to its high specificity and sensitivity, the quantitative real-time PCR is a very efficient and rapid tool for the detection and quantification of microorganisms. In this study, a newly developed TaqMan qPCR assay for the detection and enumeration of the AZA-producing marine dinoflagellate *Amphidoma languida* is presented. The high specificity and sensitivity were tested and confirmed, therefore the assay is well suited in monitoring programs. Moreover, it can be a supportive tool for detailed studies on biogeography and spatial and temporal distribution of this AZA-producing species. In the future, the new primers and probe may be integrated with other *Azadinium*-specific probes in a multiplex assay, allowing a simultaneous and thus time- and cost-effective detection and quantification of all known North-Atlantic AZA-producers, *Azadinium spinosum*, *A. poporum* and *Am. languida*.

SUPPLEMENTARY DATA

Supplementary data can be found online at *Journal of Plankton Research* online.

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