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Publisher's version / Version de l'éditeur:
https://doi.org/10.1016/j.hal.2019.101626
Harmful Algae, 85, pp. 1-10, 2019-07-09

Phylogenetic analysis and yessotoxin profiles of *Gonyaulax spinifera* cultures from the Benguela Current upwelling system

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Abstract

The Benguela Current in the Atlantic is one of the four major upwelling systems on the Eastern boundary of the world ocean. Thus the coastal regions off Namibia are prone to high primary productivity that can lead to Harmful Algae Blooms as this nutrient rich water reaches the euphotic zone. Yessotoxins (YTXs) produced by *G. spinifera* were detected in Namibian phytoplankton field samples in 2011. Isolation of *G. spinifera* cultures from this location in 2012 enabled molecular genetics work and further liquid chromatography-mass spectrometry assessment of toxin profiles. The molecular work grouped the Benguela *G. spinifera* with other toxic *G. spinifera* strains originating from Italy and New Zealand. The main YTX analogs present in the Benguela *G. spinifera* are homo-YTX, YTX and a hydroxylated analogue. This work adds important knowledge on the occurrence of Harmful Algae Blooms in this region and is of relevance for safety.

Keywords: Benguela Current, *Gonyaulax spinifera*, OH-YTX, Namibia

1. Introduction

Harmful Algal Blooms (HABs) from the proliferation of phytoplankton are the result of a combination of biological, chemical and physical factors. From a global perspective, the frequency of HAB events has increased significantly over recent years (Anderson et al., 2012; Trainer et al., 2010; Hallegraeff, 2010). This is explained through natural spread of species by currents and storms, dispersal through human activities (e.g. ballast water discharge), improved detection of HABs and associated toxins, improved communication among scientists, increased aquaculture operations in

coastal waters, and eutrophication or climate change (Hallegraeff, 2003, Anderson et al., 2012).

As one of the four major coastal upwelling currents on the eastern boundary of the world ocean the Benguela Current in the South Atlantic subtropical gyre (Peterson and Stramma 1991) contributes to the prominence of HABs in this region (Trainer et al., 2010, Kudela et al., 2005). High primary productivity by photosynthetic and chemosynthetic autotrophs is a result of the nutrient rich water reaching the euphotic zone (Pitcher et al., 2010). Thus, Namibia's developing mariculture sector is ideally situated to benefit from the Benguela Current as it straddles its coastline. These mariculture activities are concentrated in the coastal towns of Walvis Bay and Luderitz (Fig. 1) due to their natural coastal embayment's (Anderson et al., 2004; Silke, 2011). Coastal embayments such as this are frequently affected by HABs (Cembella et al., 2005). These blooms have had adverse effects in Walvis Bay by aiding the spread of hydrogen sulphide eruptions, resulting in significant kill-off of sea life (Emeis et al., 2004; Currie et al., 2007). Some HABs are made up of toxin producing phytoplankton (Smayda, 1997; Hallegraeff, 2003). As shellfish filter feed on such phytoplankton they accumulate toxins that pose a risk for human consumption. To ensure consumer safety, the Namibian Ministry of Fisheries and Marine resources (MFMR) implemented a shellfish sanitation program in 2004 (Currie et al., 2007). Routine samples are taken for microbiological, phycotoxin and phytoplankton analysis.

YTXs are polyether phycotoxins first isolated in Japan from the scallop *Patinopecten yessoensis* (Murata et al., 1987). YTXs are produced by a number of dinoflagellates including *Lingulodinium polyedrum*, *Protoceratium reticulatum* and *Gonyaulax spinifera* (Satake et al., 1999; Paz et al., 2004; 2008; Alvarez et al., 2011). More than 90 different YTX analogues have been identified in some species of algae

(Miles et al., 2005). Varying YTX producing algae and a range of YTX concentrations shellfish have been reported from around the world, including Norway, Italy, New Zealand, Russia and the USA (Table 1). Howard et al. (2008) reported concentrations up to 0.1 µg g^{-1} in mussel samples from waters off California that had been exposed to *L. polyedrum*. Levels as high as 14.8 µg g^{-1} and as low as 0.05 µg g^{-1} were measured in Norway and in Russia, respectively (Aasen et al., 2005; Vershinin et al., 2006). *G. spinifera* was first conclusively shown to produce YTX using an ELISA test on single cells from New Zealand (Rhodes et al., 2006). The toxin content detected was 176 and 200 pg YTX $cell^{-1}$. Knowledge of YTX production by *G. spinifera* is relatively recent with the only other previous occurrence reported in Italy (Riccardi et al., 2009). *G. spinifera* was shown to be the most toxic of the three genera of YTX producers to date with YTX levels being 20 and 600 times higher than *P. reticulatum* and *L. polyedrum,* respectively (Paz et al., 2004; 2008; Caron et al., 2010).

During phytoplankton sampling in Walvis Bay in March 2011, *Gonyaulax spinifera* was detected and shown to produce YTX and tentatively identified 45-OH-YTX and homoYTX analogs (Chikwililwa et al., 2013). YTXs were also detected in collected mussel samples. Unfortunately no *G. spinifera* cultures were established at that time.

Molecular phylogeny has become of great importance not only for determining the phylogenetic relationship between phytoplankton species, but also in discerning taxonomic classifications (Töbe et al., 2010; Grzebyk et al., 1998). This is emphasized for *G. spinifera* due to the species 'complex' (Ellegaard et al., 2002; Rochon et al., 2009). Previous genetic characterisation of *G. spinifera* has suggested a high level of diversity in the *G. spinifera* complex not seen from a morphological perspective (Pistocchi et al., 2012; Howard et al., 2009; Riccardi et al., 2009). Therefore,

phylogenetic positioning of the Benguela *G. spinifera* would help determine its relationship to other toxic and non-toxic *G. spinifera* strains from various geographical areas and would enhance the existing knowledge for this species.

This study details establishment of *G. spinifera* cultures from the Walvis Bay area in March of 2012. LC-MS analysis of the Benguela *G. spinifera* cultures were conducted to determine the YTX profile. The Benguela *G. spinifera* was also compared with toxic and nontoxic YTX producing strains from other geographical areas. This enables evaluation of the phylogenetic positioning of all *G. spinifera* strains in relation to their YTX toxin production.

2. Materials and methods

2.1 Micro-algal isolation, culture and identification

G. spinifera cells were isolated from a net sample collected from Walvis Bay (Fig. 1) in March 2012 using the capillary pipette method (Hoshaw and Rosowski, 1973) into natural seawater (salinity of 35) fortified with nutrients at f/2 concentrations (Guillard, 1975) to make monoclonal non-axenic cultures. Cultures were maintained in an incubator at 17 ºC and 70-100 µmol m^{-2} s^{-1} (14:10 h L: D cycle).

2.2 SEM observations

G. spinifera cultures were fixed with formaldehyde (5% final concentration). Culture aliquots were fixed onto polycarbonate filters and dried at 60 C. The filters were placed on a stub and sprayed with gold-palladium for observation under a Quanta 400 scanning electron microscope (SEM).

2.3 Toxin analysis in cultures

2.3.1 Chemicals, reagents and standards

MeOH and CH_3CN (LC-MS grade) were purchased from Prochem LGC standards (GmbH, Wesel, Germany) and Caledon (Georgetown, ON, Canada). Ammonium hydroxide (25%) was from Merck (Darmstadt, Germany) and ammonium acetate was from Sigma Aldrich (Oakville, ON, Canada). YTX (CRM-YTX-b 5.6 ± 0.2 µg/mL) and homoYTX (CRM-hYTX 5.8 ± 0.3 µg/mL) calibration standards were obtained from the National Research Council (Halifax, Nova Scotia, Canada).

2.3.2 Toxin extraction

Batch cultures were established for each strain by inoculating 10 mL in a 500 mL culture flask. Samples were taken when cells reached the exponential growth phase. To determine the toxin profile 50 mL was passed through GF/F filters. Lipophilic toxins were extracted from filters using 0.8 mL of 80% MeOH, sonicating in an ice-bath for 10 min and centrifuging (10,950 × g, for 10 min). This was repeated once more combining supernatants and adjusting the final volume to 2 mL.

2.3.3 Liquid chromatography-mass spectrometry

Quantitative LC-MS/MS analysis was conducted using a Thermo Accela HPLC coupled to a Thermo TSQ Vantage quadrupole MS. Separation was on a Gemini C18 column (3.0 µm, 150 mm × 2.0 mm; Phenomenex, Aschaffenburg, Germany) at 40°C with a flow rate of 0.350 mL min^{-1} and 10 µL injection volume. A binary mobile phase of H_2O (A) and CH_3CN–H_2O (90:10) (B), each containing 6.7 mM ammonium hydroxide (pH 11) was used (Gerssen et al., 2009), with a gradient from 10 to 90% B over 9 min and held at 90% B for 3min, before returning to 10% B in 2 min and equilibrating for 4 min before the next injection. Tuning and calibration of the MS was done using the YTX and hYTX CRMs. YTX analogues were monitored in negative ion selected reaction monitoring (SRM) mode (YTX: *m/z* 570.4→396.4/467.4; homo-YTX

m/z 577.4→403.4/474.4; 45-OH-YTX *m/z* 578.4→396.4/467.4; 45-OH-homo-YTX *m/z* 585.4→403.4/474.4).

For further characterization of YTX profiles an Agilent 1200 LC system (Agilent Inc., Palo Alta, CA, USA) was connected to an API4000 QTRAP mass spectrometer (AB Sciex, Concord, ON, Canada) equipped with a turbospray ionization source. Separation was on a Kinetex C18 column (1.7 µm, 2 × 50 mm, Phenomenex, Torrance, CA, USA) at 20°C with a flow-rate of 0.15 mL/min with 1–5 µL injections. A binary mobile phase of H_2O (A) and CH_3CN–H_2O (95:5) (B), each containing 5 mM ammonium acetate (pH 6.8) was adapted from McCarron et al., 2011a running a gradient from 25 to 100% B over 19 min at 0.15 mL/min and held at 100% B for 3 min before re-equilibration for the next run. The mass spectrometer was operated in negative ion mode using a variety of scan functions. In negative ion SRM mode a series of transitions were monitored, with a dwell time of 30 ms for each (*m/z* 1141.5→1061.5; *m/z* 1157.5→1077.5; *m/z* 1155.5→1075.5; *m/z* 1171.5→1091.5; *m/z* 1173.5→1093.5; *m/z* 1187.5→1101.5; *m/z* 1175.5→1095.5).

Neutral-loss scanning was performed by fragmenting precursor ions selected in Q1 with nitrogen in Q2, while scanning Q1 and Q3 in parallel from *m/z* 800 to 1500 with a loss of 80 amu corresponding to a loss of SO_3. Enhanced product ion (EPI) spectra of YTXs were acquired in negative ion mode selecting precursor $[M-H]^-$ in Q1, fragmenting in Q2 (90-100 eV collision energy), and scanning from *m/z* 100-1200 in Q3. General MS parameters were collision energy -65 eV, declustering potential -60 V, electrospray voltage -4500 V, and source temperature 350 °C.

For high-resolution accurate mass measurements the Accela High Speed LC connected to an Exactive Orbitrap mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA), equipped with a heated electrospray ionization probe (HESI-II) was used. The instrument was calibrated and operated as previously described (Blay et al., 2011). The LC conditions described above for the identification of the YTXs were used. The MS was run in positive ion mode with a 3.3 kV spray voltage, sheath and auxiliary gas flows of 45 and 10 (arbitrary units), respectively, 250°C capillary temperature, and 300°C heater temperature. Separate scans with and without higher energy collisional dissociation (90 eV HCD) were obtained at the 50K resolution instrument setting (2 Hz scan rate).

2.4 Molecular analysis

2.4.1 DNA extraction, PCR amplification and sequencing

Cultures were grown until the mid-exponential stage and aliquots (100 mL) were gravity filtered gently on 0.45 µm polycarbonate filters, collected and centrifuged at 6000×g for 10 min. The resulting pellet was frozen in liquid nitrogen and stored at -80°C until analysis. Genomic DNA was extracted using a QIAGEN kit (QIAGEN, Hilden Germany) for plant tissue, according to the manufacturer's instructions. Approximately 1 ng of genomic DNA was amplified using 20 µL PCR master mix containing Taq buffer (2.0 µL) two gene specific primers (Table S1) (0.4 µL), dNTP (0.2 µL) and DNA polymerase (0.2 µL). All amplifications were carried out using an Eppendorf master cycler with the following conditions: initial template denaturation for 5 min at 94°C, followed by 33 cycles at 94°C for 30 s, 56°C for 30 s and extension at 72°C for 1 min and 15 seconds, followed by a final extension at 72°C for 7 min. The quality and specificity of PCR products were assessed by agarose gel electrophoresis. The PCR product was then purified using a Qiagens MinElute PCR purification kit

(QIAGEN, Hilden, Germany) in accordance with the manufacturer's instructions. The purified PCR product (1 µL) was sequenced using the following master mix (5x big dye buffer containing polymerase dNTP and ddNTP (1.5 µL), big dye (1 µL), primer (1 µL), and DNase free H_2O (6.5 µL)) with the primers specified (Table S1).. The sequence cycle was: 96°C for 1 min, 96°C for 10 s, 50°C for 5 s and 60°C for 4 min. This was repeated 25 times. The sequencing reaction product was purified up using AGENCOURT CleanSEQ kit (BeckmanCoulter GmBH, Krefeld, Germany) according to the manufacturer's instructions. The purified reaction product was then transferred into MicroAmp Optical 96 well reaction plates and loaded into the sequencer. A 3130xl genetic analyser (Applied Biosystems, Darmstadt, Germany) with a 16 capillary array was used for Sanger sequencing. DNA fragments were separated in POP 7 polymer within the 80 cm capillary allowing for sequence reads of up to 1000 base pairs.

2.4.2 Sequence alignment and phylogenetic analysis

Sequences were aligned using clusteralX (version 8) and adjusted manually. The resulting sequences were aligned with the existing small subunit (SSU) and large subunit (LSU) rDNA sequences available from GenBank using Molecular Evolutionary Genetics Analysis (Mega, version 5.05). The phylogenetic relationships were inferred based on pairwise differences (p-distance) of nucleotides derived from the alignment, ignoring gaps with the species listed in Table 2. The p-distance represents the number of nucleotide differences divided by the total number of nucleotides compared. Maximum parsimony estimation was used for the phylogenetic analysis and subsequent phylogenetic tree. This was done for both the LSU and SSU of the rDNA. The *G. spinifera* SSU sequences in GenBank were derived from three different areas, namely Adriatic Sea (two strains EU805590 and DQ867107), Malaysia (AF052190) and the North Atlantic (AF022155). In the previous work only YTX production in the Adriatic

strains was analyzed (Table 3). The LSU sequences were derived from three areas: Adriatic Sea (EU805591 and EF416284); New Zealand (DQ151557 and DQ151558); and the North Atlantic (EU532478 and AY154960). The North Atlantic strains were nontoxic (Table 4).

3 Results

3.1 Micro-algal isolation and culture

G. spinifera was conclusively identified using Dodge's classification characteristics and included a prominent and descending cingulum, a short apical horn (Fig. 2a) and two short antapical spines (Fig. 2b). Cell surface is reticulated with the basic thecal arrangement of *G. spinifera* detailed by Dodge, 1985 (Fig. 2a-c). The 6" plate on the epitheca has a distinctive triangular shape (Fig. 2a). The cell size varies between 24-40 µm in length and 20-30 µm in width.

3.2 Yessotoxin production by *G. spinifera*

Three Bengeula *G. spinifera* cultures were analysed for the presence of YTXs by LC-MS/MS. SRM transitions were included for YTX analogues previously observed as biosynthetic products of algal and as shellfish metabolites. The major YTXs present were observed in transitions for homo-YTX, YTX, and 45-OH-YTX. The sum of these three analogues is referred to as total cellular YTX content in the subsequent text. To screen for the presence of additional YTX analogues the samples were analysed using a neutral-loss scan, which did not indicate the presence of any major additional YTX analogues. To confirm identities the retention times of the YTX analogues in the samples were matched against reference materials, and product ion spectra were acquired. The retention time of the analogues in the YTX and homo-YTX SRM transitions matched those of the respective CRMs, with added confirmation by high-

resolution mass spectrometry (Δppm <5 for observed compared to theoretical masses). However, the retention time of the YTX analogue observed in the *m/z* 1157>1077 SRM transition from analysis of the *G. spinifera* extract did not match that of 45-OH-YTX confirmed in a mussel tissue reference material containing YTXs (McCarron et al., 2011b; McCarron et al., 2017) (Fig. 3). The product ion spectrum showed a characteristic YTX fragmentation however, it was not entirely consistent with 45-OH-YTX (Fig. 4). The presence of the *m/z* 575 ion for the hydroxylated YTX in the *G. spinifera* strain, not otherwise present for 45-OH-YTX, suggests that the –OH group is not located at C45. Fragmentation inferred from the compounds product ion spectrum indicates the position of the –OH group on the G ring (Fig. 4). HRMS determined an accurate mass of 1157.46566 +/- 0.00099 (Δ 0.85 ppm for $C_{55}H_{81}O_{22}S_2$), indicating an elemental composition consistent with OH-YTX. In addition, the measured accurate mass for the *m/z* 575 fragment ion following HCD was in good agreement with the theoretical value for this proposed G-ring fragmentation (*m/z* 575.21649 = Δ 0.5 ppm for $C_{27}H_{39}O_{12}S$).

All three Benguela *G. spinifera* strains produced varying total cellular concentrations of YTXs (Table 5). Under the described culture conditions homo-YTX was the dominant toxin produced by all three strains, accounting for 65-79% of the cellular content with a concentration of 106.3 pg cell^{-1}for NamWB012. The OH-YTX analog was the second most abundant toxin present accounting for 17-28% of the total cellular YTX content. NamWB012 produced the highest concentration of YTX. YTX accounted for only 3-7% of the total measured YTX content.

3.3 DNA sequencing and phylogeny

All three Benguela strains had identical SSU and LSU sequences with a genetic divergence (p-distance) of 0.00, thus they were denoted collectively as NamWB *G.*

spinifera in the resulting phylogenetic trees (Fig. 5, 6, S1 and S2). The SSU was 1673 base pairs long. All three Benguela strains clustered with YTX producing strains EU805590 and DQ867107 isolated from the Adriatic Sea (Italy) with high bootstrap values of 100 and 99 % in both neighbourhood joining (NJ) (Fig. 6) and maximum likelihood analysis (Fig. 7). The p-distance between NamWB and EU805590 and DQ867107 was low (p-distance values of 0.007 and 0.092, respectively). All known YTX producing *G. spinifera* species formed a cluster and were distinct and highly divergent from the nontoxic Malaysian (p-distance of 0.253) and North Atlantic (p-distance of 0.206) *G. spinifera*. These nontoxic strains grouped with other species within the genus *Gonyaulax*.

The partial LSU was 793 bp long and as with the SSU analysis, all three Benguela *G. spinifera* strains clustered with YTX producing strains EU805591 (Adriatic Sea) and DQ151558/DQ151557 (New Zealand) with bootstrap values of 100% in both NJ (Fig. S1) and maximum likelihood (Fig. S2) trees. All five *G. spinifera* YTX producers formed a distinct clade. The genetic divergence between NamWB and EU805591 was low (p-distance 0.002). The p-distance between NamWB and both New Zealand strains was 0.005. NamWB was highly divergent from the second Adriatic strain (EF416284) with a p-distance of 0.211. The non-toxic *G. spinifera* strains from the North Atlantic, namely AY154960 and EU532478 were highly divergent from NamWB with p-distances of 0.500 and 0.563, respectively.

4 Discussion

The identification and confirmation of YTX producing *G. spinifera* in the Benguela region indicate that it is a recurring and prominent concern to the Namibian shellfish mariculture industry (Chikwililwa et al., 2013).

The YTX profiles of the isolated strains were dominated by homo-YTX, YTX and a hydroxylated YTX analogue. The retention time of the hydroxylated YTX analogue did not match that of 45-OH-YTX present in a mussel tissue reference material (Fig. 3). While previous studies have indications the production of 45-OH-YTX by *G. spinifera* at low levels has been indicated previously (Ciminiello et al., 2003), however this would be unexpected as this analogue is a known metabolic product of YTX in shellfish (Aasen et al., 2005). Accurate mass measurement confirmed a hydroxylated YTX analogue and comparison with the product ion spectra for 45-OH-YTX (Fig. 4) suggests hydroxylation on the G ring of the molecule. Future efforts will be directed towards bulk culturing of the Namibian *G. spinifera* strain for isolation and complete structure assignment by nuclear magnetic resonance. The occurrence of a novel YTX analogue in plankton has implications for subsequent metabolite profiles in shellfish feeding on this strain. This should be considered in analysis of shellfish samples from this region in the future.

There was a 10-fold difference in YTX production between the NamWB012 (134.3 pg YTX $cell^{-1}$) and NamWB011 (15.0 pg YTX $cell^{-1}$) *G. spinifera* isolates. This suggests that toxin production can vary even within populations under the same environmental conditions. However, it is important to balance this inference against the small sample size. From a global perspective, the total YTX levels of the Namibian *G. spinifera* strains are within the ranges measured elsewhere (Rhodes et al., 2006; Riccardi et al., 2009). The highest concentration of total cellular YTXs measured in the cultures was also relatively similar to that measured in field samples during the 2011 *G. spinifera* bloom (156.0 pg YTX $cell^{-1}$) (Chikwililwa et al., 2013). In all cultures homo-YTX was the dominant toxin (65-79% of the total cellular YTX content).

The three Benguela *G. spinifera* isolates have identical SSU and LSU sequences forming a distinct clade with the other known toxic *G. spinifera* strains. The Benguela *G. spinifera* was most closely related to the 2006 Adriatic Sea isolate (EU805591) that also produced homo-YTX as its dominant toxin (Riccardi et al., 2009). There was a low diversity distance between the Benguela, Adriatic Sea and the New Zealand isolates and these isolates formed a distinct group separated from the second Adriatic Sea isolate that only produced YTX (EF16284). The mechanism of YTX production is of interest due to the distinct separation between the homo-YTX and the YTX producers (p-distance of 0.211 between the Benguela *G. spinifera* and Adriatic Sea isolate, EF16284). This divergence is recent and can aid in determining the controls governing the production of other YTX derivatives as they contribute to the total toxicity of the cell. As there is a distinct separation between the toxic and non-toxic *G. spinifera*, the internal transcribed spacer (ITS) region should be assessed to determine if this area could provide any information regarding YTX production or lack thereof in the various strains. The Gonyaulacales group formed a clade in both the SSU and LSU phylogenetic trees, consistent with a previous study (Howard et al., 2009). Though YTX production is distributed within three different genera, all these species fell under the Gonyaulacales order and were widely distributed throughout the trees. Thus the occurrence of YTX producers in different distinct genera supports the hypothesis that YTX biosynthetic capacity arose early in the divergence of this order and consequently later in the evolutionary history of the Dinophyceae (Howard et al., 2009). There is a high level of interspecific variability in *G. spinifera*. This was also observed by Howard et al. (2009) and Riccardi et al. (2009), albeit using smaller data sets. This and the emergence of new biogenic YTX derivatives suggest that *G. spinifera* is undergoing diversification.

This diversification and biogenic origin of the novel YTX profile observed in the Namibian *G. spinifera* strains gives rise to questions on which controls govern the production of YTX and its derivatives. Polyketides similar to YTX and its derivatives can also be synthesized by a wide range of organisms such as bacteria, fungi, lichens and higher plants and vary in their molecular structure (Rein and Snyder, 2006). Though their production is encoded for in polyketide synthetase (PKS) genes, little is known about the polyketide biosynthesis in dinoflagellates (Kellmann et al., 2010; Eichholz et al., 2012). This is due to dinoflagellates large genome lacking normal chromosomal organization and experimental problems such as the difficulty in maintaining axenic cultures (Kellmann et al., 2010). Since these genes would control the production and type of YTXs biosynthesized, it would be of significant interest to categorize them for the *Gonyaulax* genus and to use this information to discern the evolution and mechanism of YTX production. The fact that the YTX producing *G. spinifera* aggregate into a clade suggests that there is a distinct ribotype for the toxic species. This information could be used to develop probes specific to this group allowing testing on field samples to conclusively identified YTX producing *G. spinifera*. This would be of great use as toxic and non-toxic *G. spinifera* strains cannot currently be distinguished from each other using microscopy. As toxic *G. spinifera* has only recently been identified in the Benguela and nearby regions (Pitcher et al. 2019), there is a need for knowledge on the probability of the co-occurrence of non-toxic species or the ratio of the various species included in the *G. spinifera* 'complex'. Whole cell hybridization could be used to investigate this. However, a probe that targets a specific genetic sequence/region shared by the toxin producers first needs to be developed. This probe could then be coupled to a fluorescent marker thus allowing identification of a specifically targeted organism using Fluorescence in situ Hybridisation (FISH) (Amann,

1995), as applied previously for harmful algae including *Pseudo-Nitzschia sp.* and *Alexandrium sp*. (Scholin et al., 1997; John et al., 2003; Anderson et al., 2005), including the *Alexadrium tamarense* 'complex' (John et al., 2005). This would be a valuable tool in an area such as the coastal waters of Namibia where rapid identification and enumeration of HABs species is needed in order to effectively evaluate risks posed to local mariculture activities, since the rRNA probes enable distinguishing between toxin and non-toxic species in field samples.

Acknowledgements

This study was funded by the Geochemistry and Ecology of the Namibian Upwelling System (GENUS) project. Special thanks are given to the staff in the Mariculture section of the Aquaculture directorate of Ministry of Fisheries & Marine Resources, Swakopmund: Richard Gaeb, Aljarreau Kotze, Jan Gei-Kaub and Gonzalo Murta. Support of Dr. Uwe John from the Alfred Wagner Institute Bremerhaven (AWI) for the molecular work is greatly appreciated. Nancy Lewis and Joseph Hui at the National Research Council of Canada are thanked for assisting with algae culture and LC-MS experiments.

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Tables

Table 1: Published concentrations of yessotoxin in shellfish and per cell in *Prorocentrum reticulatum*, *Lingulodinium polyedrum*, and *Gonyaulax spinifera.* n.d.- no quantities published.

Shellfish	YTX range (µg YTX g^{-1})	Country	Reference
Blue mussels (*Mytilus edulis*)	0.5-14.8	Norway	Lee et al., 1988; Ramstad et al., 2001; Aasen et al., 2005
Blue mussels (*Mytilus galloprovincialis*)	0.1-9.62	Italy	Ciminiello et al., 1997, Draisci et al., 1999; Riccardi et al., 2009; Visciano et al., 2013
Greenshell mussels (*Perna canaliculus*)	1.6-3.2	New Zealand	Yasumoto and Takizawa, 1997, MacKenzie et al., 2001, MacKenzie et al., 2002
Blue mussels (*Mytilus chilensis*)	n.d.	Chile	Yasumoto and Takizawa, 1997
Blue mussels (*Mytilus edulis*)	n.d.	Japan	Yasumoto and Takizawa, 1997
Blue mussels (*Mytilus edulis*)	0.053	Russia	Vershinin et al., 2006
Scallops (*Patinopecten yessoensis*)	n.d.	Japan	Murata et al., 1987, Yasumoto and Takizawa, 1997
Scallops (*Patinopecten yessoensis*)	0.44-0.79	Japan	Koike et al., 2006
Shellfish (unspecified)	n.d.	Ireland	Howard et al., 2008
California sea mussels (*Mytilus californicus*)	0-0.1	USA	Howard et al., 2008
Blue mussels (*Mytilus galloprovincialis*)	n.d.	Russia	Morton et al., 2007
Mussel, oyster and clams	2.0	Spain	Arevalo et al., 2006
Dinoflagellate	**YTX (pg $cell^{-1}$)**	**Country**	**Reference**
P. reticulatum	5.0	Canada	Stobo et al., 2003
P. reticulatum	5.83	Italy	Boni et al., 2002
	18-79	Norway	Samdal et al., 2004
	0.9-14.0	Japan	Satake et al., 1999, Eiki et al., 2005

	3.0-13.0	New Zealand	Satake et al., 1996, 1999 MacKenzie et al., 2002, Mitrovic et al., 2005, Rhodes et al., 2006
	0-2.6	Spain	Paz et al., 2004, 2007
	0.3	UK	Stobo et al., 2003
	0-2.1	USA	Paz et al., 2004, 2007,
	0.2-0.4	Chile	Alvarez et al., 2011
	0.075	South Africa	Krock et al., 2008 Trainer et al., 2010
L. polyedrum	0.3	Ireland	Howard et al., 2008
	1.5	Italy	Tubaro et al., 1998 Draisci et al., 1999
	0	Norway	Ramstad et al., 2001
	0, 0.3	Spain	Riobo et al., 2002, Paz et al., 2004
	0-0.02	UK	Stobo et al., 2003
	0-0.005	USA	Armstrong and Kudela, 2006, Howard et al., 2008
G. spinifera	0-200	New Zealand	Rhodes et al., 2006
	0	UK	Stobo et al., 2003
	0	USA	Howard et al., 2008
	3.6-33.4	Italy	Ciminiello et al., 2006, Riccardi et al., 2009

Table 2: List of sequences used in the LSU region rDNA phylogenetic analysis from GenBank.

Species	GenBank accession #.
Alexandrium catenella	AF200667
Alexandrium margalefii	AY154957
Alexandrium minutum	AF033532
Alexandrium ostenfeldii	AF033533
Alexandrium pseudogoniaulax	AY154958
Alexandrium tamarense	AY438021
Cochlodinium polykrikoides	AF067861
Gonyaulax baltica	AY154962
Gonyaulax digitale	AY154963
Gonyaulax elongata	AY154964
Gonyaulax membranacea	AY154965
Gonyaulax polygramma	DQ162802
Gonyaulax cf. spinifera	AY154960
Gonyaulax spinifera	DQ151557
Gonyaulax spinifera	DQ151558
Gonyaulax spinifera	EF416284
Gonyaulax spinifera	EU532478
Gonyaulax spinifera	EU805591
Heterocapsa triquetra	AF260401
Lingulodinium polyedrum	EF613357
Lingulodinium polyedrum strain	EU532472
Lingulodinium polyedrum strain 104A	EU532471
Prorocentrum lima	AJ567459
Prorocentrum micans	AF260377
Prorocentrum minimum	DQ662402
Protoceratium reticulatum	EF613362
Protoceratium reticulatum	EU532476
Protoceratium reticulatum	EU532477
Pyrodinium bahamense var. compressum	AY154959

Table 3: List of *Gonyaulax* species and YTX toxicity used in the phylogenetic analysis. . Toxic and non-toxic cultures are denoted by (+) and (-), respectively.

Species	Genbank accession #	Origin and Reference	YTX toxicity
Gonyaulax cochlea	AF274258	Rhodes Island, USA, Saldarriaga et al., 2001	-
Gonyaulax fragilis	56384899	Adriatic Sea, Italy, Riccardi et al., 2007	-
Gonyaulax polyedra	AF377944	South Korea, Lee et al., 2001	-
Gonyaulax polyedra	AJ415511	Norway, Shalchian, 2001	-
Gonyaulax polygramma	AJ833631	South Korea, Jeong et al., 2005	-
Gonyaulax spinifera	AF022155	Maine, USA, Saunders et al., 1997	-
Gonyaulax spinifera	AF052190	Malaysia, Usup et al., 1998	-
Gonyaulax spinifera	DQ867107	Adriatic Sea, Italy, Riccardi et al., 2009	+
Gonyaulax spinifera	EU805590	Adriatic Sea, Italy, Riccardi et al., 2009	+
Gonyaulax verior	AY443013	Unknown, Saldarriaga et al., 2004	-

Table 4: List of *Gonyaulax* species and YTX toxicity used in the phylogenetic analysis of the LSU. Toxic and non-toxic cultures are denoted by (+) and (-), respectively.

Species	Genbank accession #	Origin and Reference	YTX toxicity
Gonyaulax baltica	AY154962	Sweden, Ellegard et al., 2003	-
Gonyaulax digitale	AY154963	Canada, Ellegard et al., 2003	-
Gonyaulax elongata	AY154964	United Kingdom, Ellegard et al., 2003	-
Gonyaulax membranacea	AY154965	Ireland, Ellegard et al., 2003	-
Gonyaulax polygramma	DQ162802	South Korea, Kim et al., 2006	-
Gonyaulax cf. spinifera	AY154960	Unknown, Ellegard et al., 2003	-
Gonyaulax spinifera	DQ151557	New Zealand, Rhodes et al., 2006	+
Gonyaulax spinifera	DQ151558	New Zealand, Rhodes et al., 2006	+
Gonyaulax spinifera	EF416284	Adriatic Sea, Italy, Riccardi et al., 2009	+
Gonyaulax spinifera	EU532478	Massachusetts, USA, Howard et al., 2008	-
Gonyaulax spinifera	EU805591	Adriatic Sea, Italy, Riccardi et al., 2009	+

Table 5: Percentage and cellular concentrations of YTX analogues in *G. spinifera* strains NamWB002, NamWB011 and NamWB012.

***G. spinifera* Strain**	**YTX (% of total)**	**homo-YTX (% of total)**	**OH- YTX (% of total)**	**Total YTX (pg cell^{-1})**
NamWB002	7	65	28	37.2
NamWB011	7	71	22	15.0
NamWB012	4	79	17	134.3

Figure captions

Fig. 1. Location of working area Aquapark 1 within Walvis Bay of the coast of Namibia. The site of Aquapark 1 used for shellfish cultivation.

Fig. 2. Scanning Electron Micrographs of *G. spinifera* from field samples taken on the 16th March 2011 from Walvis Bay: A) Apical dorsal view, B) Apical dorsal view with cingulum and antapical spines and C) Dorsal antapical view. All plates are labeled according to the Kofoidean numerical notation.

Fig. 3. LC-MS/MS (SRM) analysis of YTXs in A) a mussel tissue reference material extract and, B) in an extract of *G. spinifera* culture harvested in Namibia.

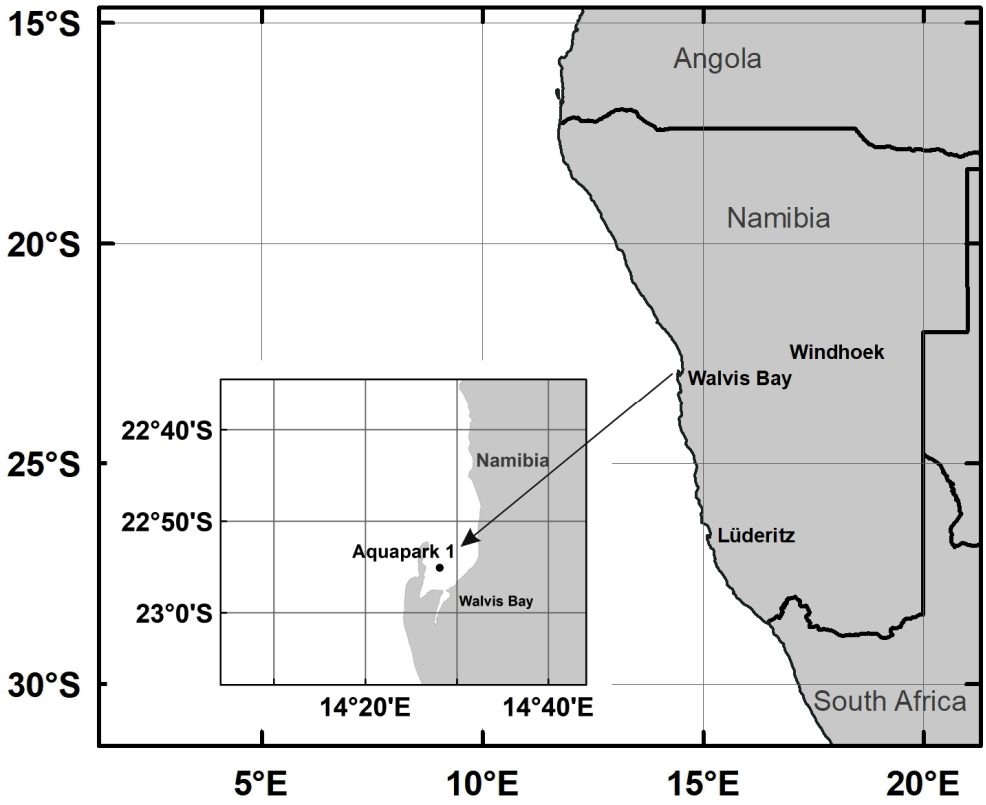
Fig. 4: Product ion spectra of 45-OH-YTX acquired from a mussel tissue reference material (A) and OH-YTX from extract of Namibian strain of *G. spinifera* (B). Collision induced dissociation of the novel OH-YTX from Namibian strain of *Gonyaulax spinifera* (C) tentatively indicating position of the hydroxyl on the G-ring.

Fig. 5. Neighbourhood joining (NJ) analysis of the SSU. Bootstrap values (1000 replicates) are listed as percentages of 100 and only values greater than 50 are shown. YTX producers are denoted by (+) and nontoxic *G. spinifera* strains are denoted by (-).

Fig. 6. Maximum likelihood analysis of the SSU. Bootstrap values (1000 replicates) are listed as percentages of 100 and only values greater than 50 are shown.

Figures

Fig. 1: Location of working area Aquapark 1 (inset) within Walvis Bay of the coast of Namibia. The site of Aquapark 1 used for shellfish cultivation.



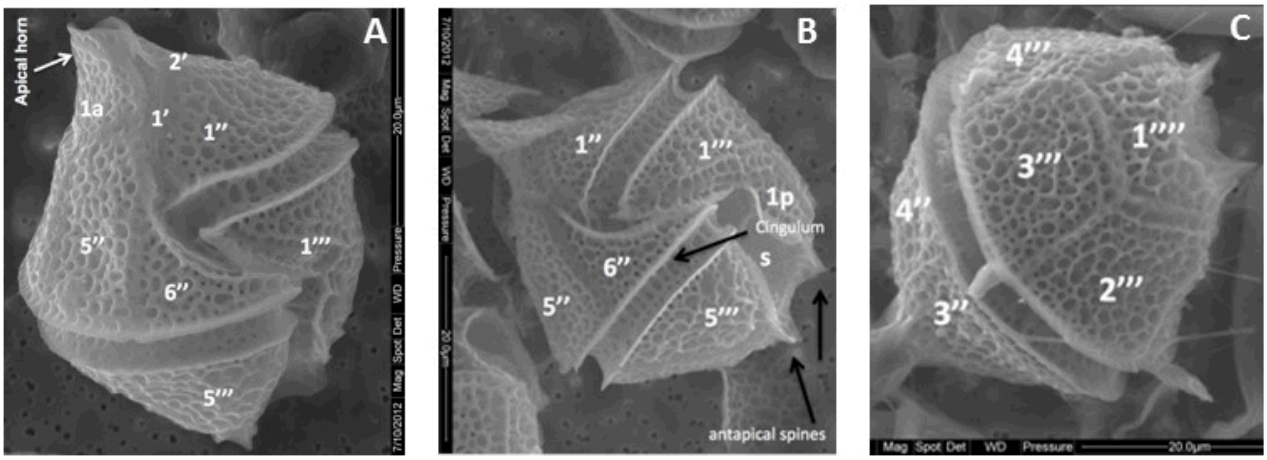


Fig. 2: Scanning Electron Micrographs of *G. spinifera* from field samples taken on the 16th March 2011 from Walvis Bay: A) Apical dorsal view, B) Apical dorsal view with cingulum and antapical spines and C) Dorsal antapical view. All plates are labeled according to the Kofoidean numerical notation.

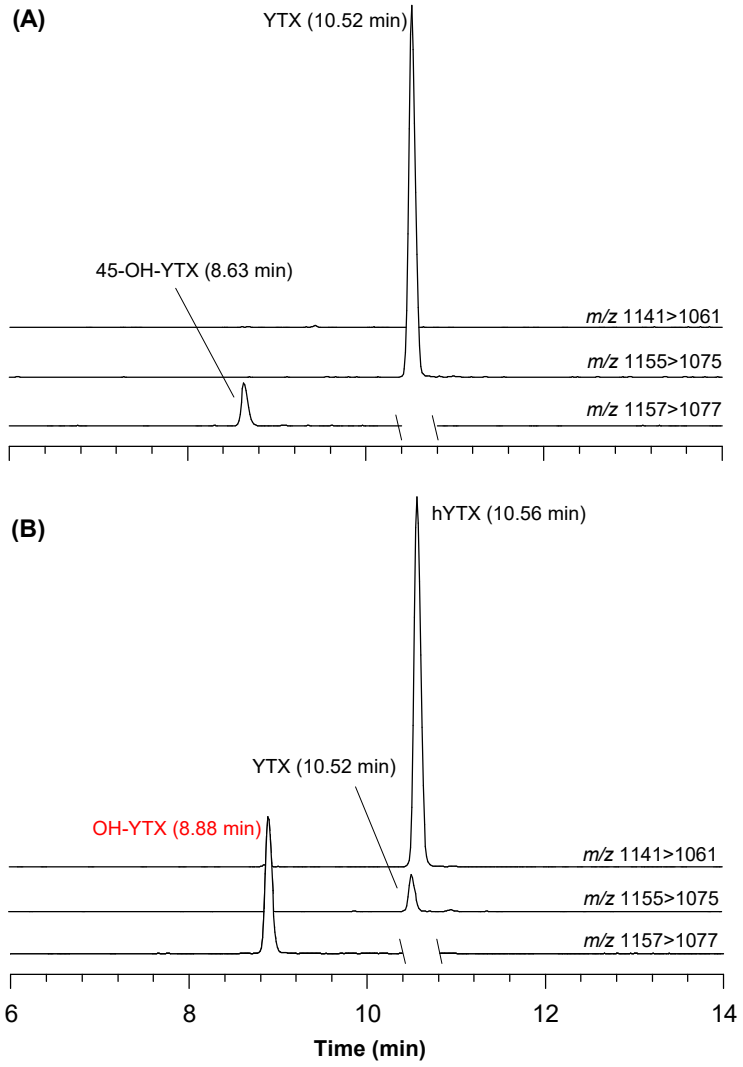


Fig. 3: LC-MS/MS (SRM) analysis of YTXs in A) a mussel tissue reference material extract and, B) in an extract of *G. spinifera* culture harvested in Namibia.

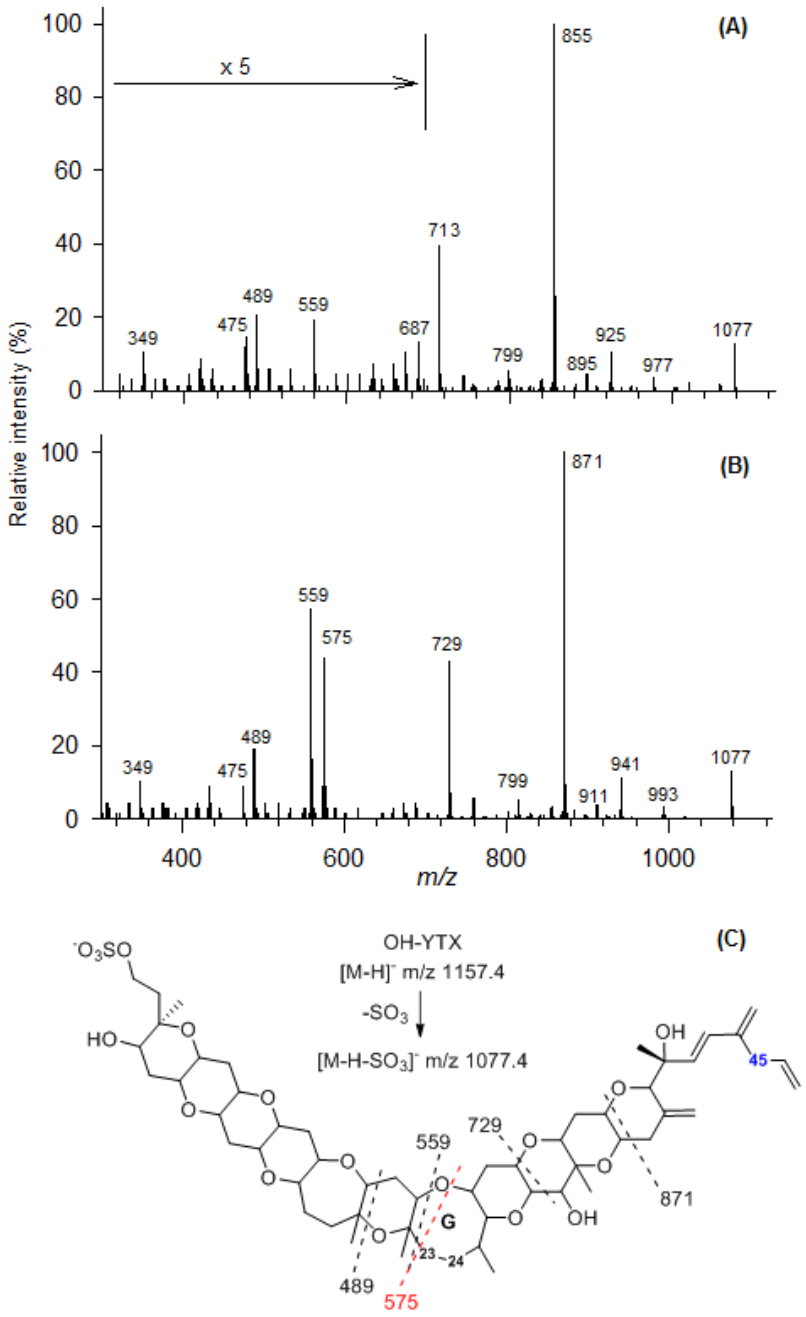


Fig. 4: Product ion spectra of 45-OH-YTX acquired from a mussel tissue reference material (A) and OH-YTX from extract of Namibian strain of *G. spinifera* (B). Collision induced dissociation of the novel OH-YTX from Namibian strain of *Gonyaulax spinifera* (C) tentatively indicating position of the hydroxyl on the G-ring.

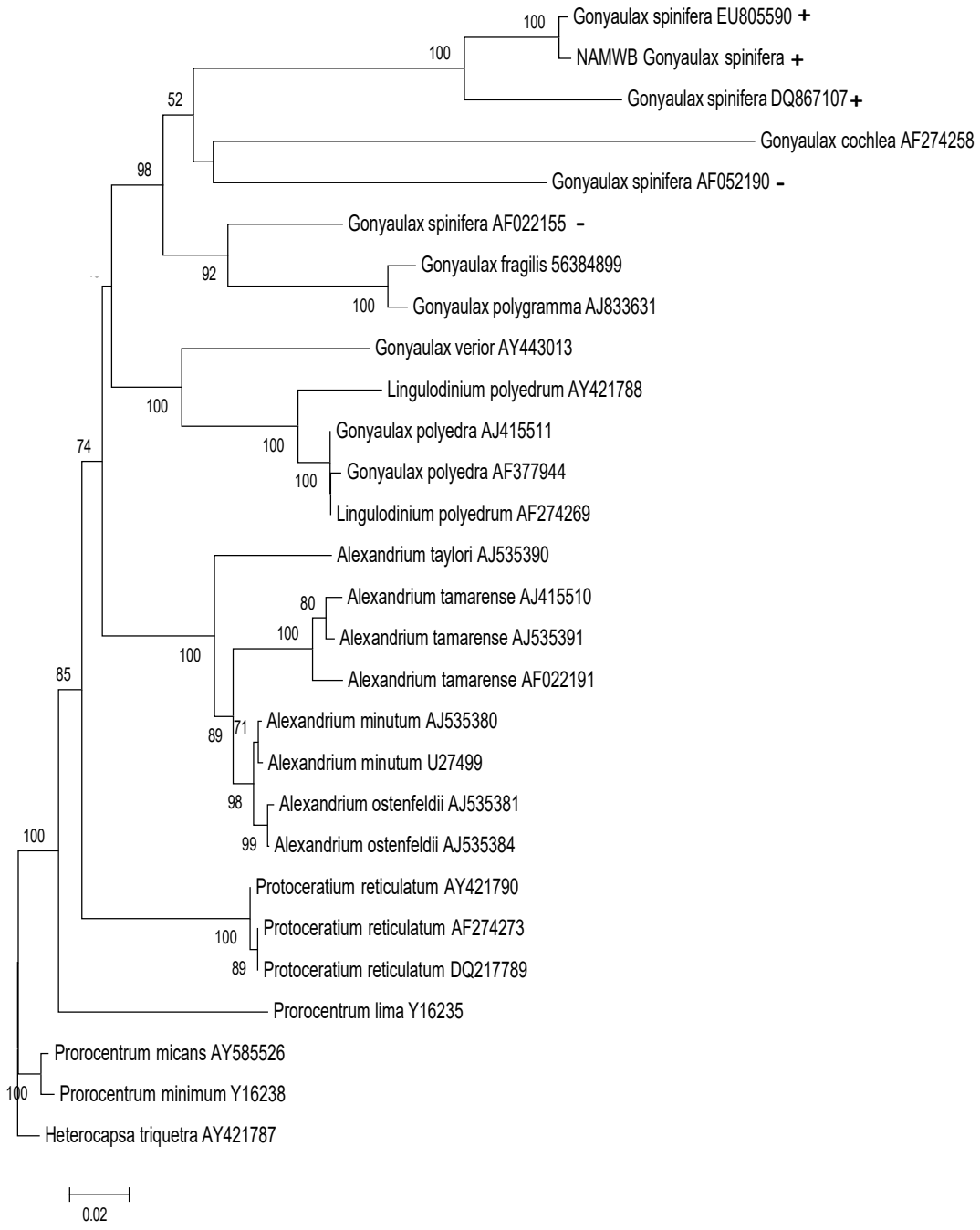


Fig. 5: Neighbourhood joining (NJ) analysis of the SSU. Bootstrap values (1000 replicates) are listed as percentages of 100 and only values greater than 50 are shown. YTX producers are denoted by (+) and nontoxic *G. spinifera* strains are denoted by (-).

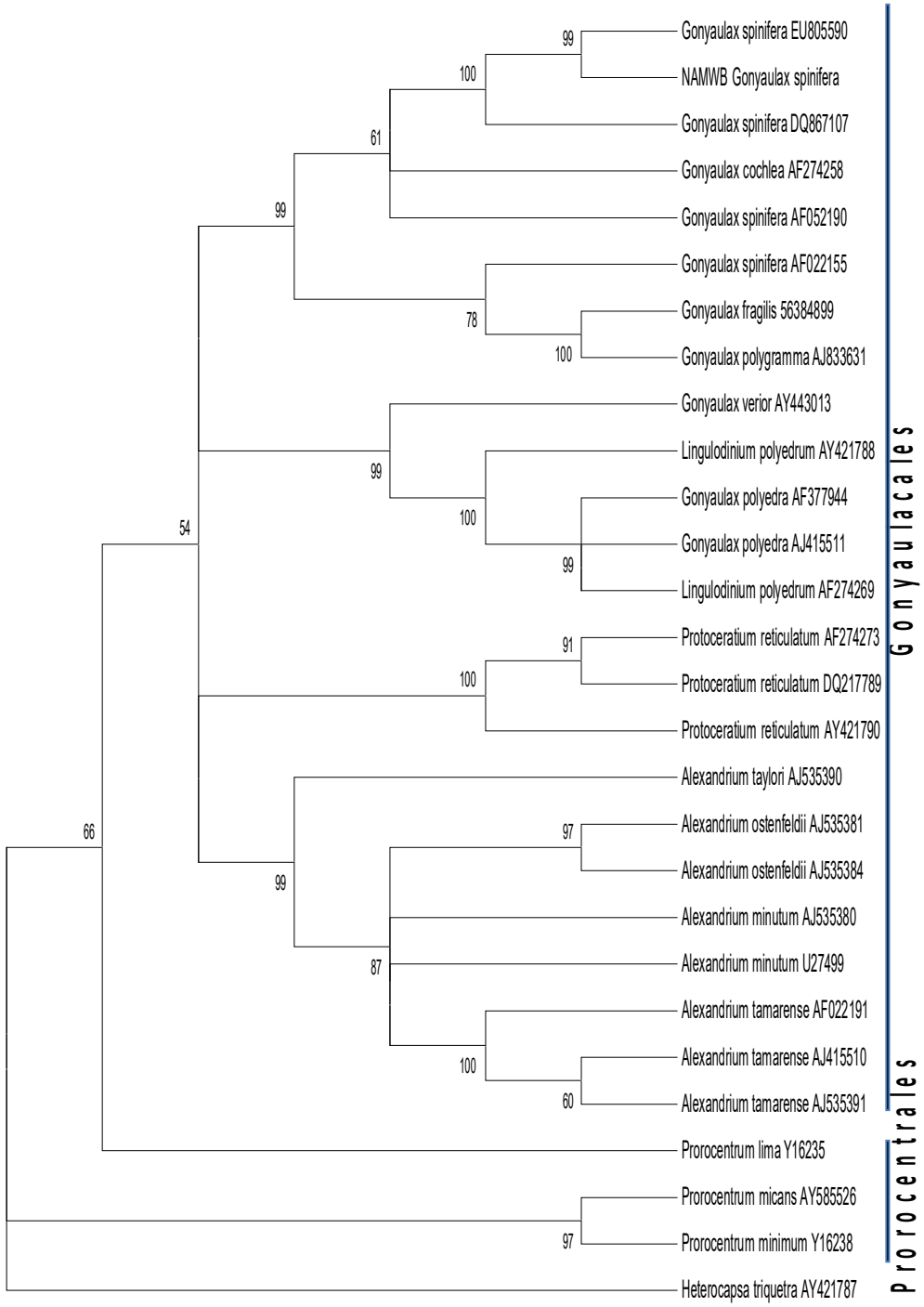


Fig. 6. Maximum likelihood analysis of the SSU. Bootstrap values (1000 replicates) are listed as percentages of 100 and only values greater than 50 are shown.