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Toxin Profile of the Dinoflagellate Gymnodinium Catenatum Bloom in Libyan Mediterranean Coastal Waters

Zakaria A. Mohamed[1,*], Adel S. Ben Omran[2], Hana Abohbell[3], Hoida A. Badr[1]

[1] *Department of Botany and Microbiology, Faculty of Science, Sohag University, Sohag, 82524, Egypt.*
[2] *Tripoli University, Faculty of Agriculture, Aquaculture Department, Libya.*
[3] *Department of Botany, Faculty of Science, University of Sabratha, Libya.*
[*] *Email: mzakaria_99@yahoo.com*

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Abstract: This study describes the occurrence of the toxic dinoflagellate *Gymnodinium catenatum* bloom in Libyan Mediterranean coastal water. G. *catenatum* exhibited high abundance (3.5x105 cells L-1) in Tajoura coastal water during September 2023. The high abundance of G. *catenatum* associated with the increase in nutrient concentrations in sea water caused by anthropogenic discharge. Cysts of G. *catenation* were also identified (125 cysts g-1) in sediment samples collected from the study area during the bloom event. The analysis of high-performance liquid chromatography with fluorescence detection (HPLC-FLD) revealed that of G. *catenatum* bloom was able to produce paralytic shellfish toxins (PSTs) with a profile consisting of saxitoxin (STX), decarbamoyl-STX (dcSTX), neosaxitoxin (NeoSTX), gonyautoxin-1 (GTX1), GTX2, GTX3, GTX4, and C1/C2 toxins with varying concentrations (11.2, 5.3, 3.3, 2.4, 1.9, 18.2, 16.3, 41fg cell-1, respectively). Similarly, a strain culture of G. *catenatum* isolated from this bloom produced the same toxin profile as the bloom, but with different proportions of toxin variants. Cultured cells produced approximately 1.5 times more toxin than cells from bloom samples. The results of our study contribute to the knowledge of the toxicity of G. *catenatum* bloom in Libya coastal waters and provide valuable information on the persistence of G. *catenatum* cells and cysts which could lead to yjr bloom recurring in the water column. Therefore, the study suggests monitoring programmes for harmful dinoflagellates and their cysts in Libyan coastal waters to protect the marine ecosystem and seafood animals from exposure to such potent toxins.
Keywords: Algal blooms, environmental pollution, marine toxins, Mediterranean Sea.

1. Introduction

Harmful algal blooms (HABs) are widely distributed in coastal waters around the world and have been primarily related to a variety of factors, including upwelling systems, ocean fronts, and anthropogenic discharges [1,2,3]. Bloom events are particularly concerning because bloom-forming algae produce toxins, which have negative consequences for marine ecosystems, human health and the economy [2]. Dinoflagellate species have significant contribution to HAB events reported worldwide with 35% of the events associated with seafood toxins and human poisoning syndromes [4]. Among these toxins, paralytic shellfish toxins (PST) are produced by species belonging to three different dinoflagellate genera: *Gymnodinium, Alexandrium and Pyrodinium* [5,6]. PSTs comprise saxitoxin (STX) and its analogues, with carbamoyl (STX), neosaxitoxin (NEO), gonyautoxins (GTX) being the most potent, followed by decarbamoyl (dcSTX, dcNEO, dcGTX) and deoxydecarbamoyl analogues (doSTX, doGTX2, doGTX3), while N-sulfocarbamoyl (C toxins) are the least toxic [7]. The PST can block Na^+ conductance by binding to voltage-gated sodium channels, interfering with Na^+ transient permeability and causing extremities numbness, breathing difficulty and even full paralysis or death [8]. Many countries across the world have paid substantial attention towards PST-producing HABs to guarantee the protection of human health and ensure water quality, aquaculture and other coastal-related economic activities [9,10].

Among the gymnodinoid dinoflagellates, the chain-forming G. *catenatum* is known to be the only species capable of producing PST [11]. Reports of new occurrences on the distribution of G. *catenatum* have increased in coastal waters worldwide and such increases have been linked to many different factors including ballast water transport, coastal eutrophication and climate change [12,13].

In the Mediterranean Sea, G. *catenatum* was recorded for the first time in the NW Alborán Sea [14]. The species was probably transported by currents from the Atlantic Ocean according to the circulation through the Strait of Gibraltar [15]. Nowadays, this species has become an abundant and well-established in different basins of the Mediterranean Sea including Alboran Sea [16], the Algerian Basin [17], Alexandrian coast [18]), Morocco [19,20], and the Gulf of Gabes and Tunisia [21]. However, no study has investigated the occurrence of G. *catenatum* in Libyan Mediterranean coastal waters. Hence, this study is the first to report the presence of G. *catenatum* and its cysts in the Libyan basin of the Mediterranean Sea. The study also determined the toxin profile of PSTs produced by a cultured strain and natural bloom of this dinoflagellate.

2. Materials and methods

2.1 Sampling

The present study was carried out in Tajoura Mediterranean coast (Fig. 1), about 20km east of Tripoli, Libya (13°22′00″E, 32°54′00″N). The study site is a sheltered environment with a rocky shore and a macroalgal community dominated by the red macroalga Acanthophora spicifera. Samples were collected on September 2023 during G. *catenatum* bloom along the Tajoura coast. Water samples were collected from the bloom area with 500-mL plastic bottles at 0.5m depth.

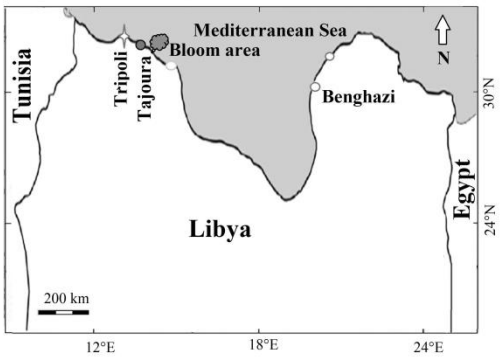

Figure 1. A map showing the bloom area (sampling location) in the Libyan Mediterranean coast.

All samples were kept in an ice box for transport to a laboratory in Libyan Marine Biological Research Center. Meanwhile, surface sediment samples were collected in triplicate at the bloom site using a Van Veen grab (Size:15×30 cm) to test the presence of G. *catenatum* cysts. Sediments were then stored in the dark, in a plastic pouch, at 4°C until processing.

2.2 Physico-chemical and phytoplankton Analysis

Environmental parameters including temperature, pH, salinity and dissolved oxygen were measured in situ with a multiparameter probe (HI 991300 pH/EC/TDS Temperature, HANNA, Italy). Concentrations of nutrients (NO_3^-, NH_4^+, PO_4^{3-} and SiO_2) were determined in water samples after filtering through Whatman GF/C filters, according to the standard analytical methods [22]. For phytoplankton analysis, one liter of water samples was preserved with acid Lugol solution (4%). Phytoplankton species in preserved samples were then identified under Seiz light microscope with digital camera, according to several taxonomic publications [23,24]. Counting of phytoplankton cells was carried out in preserved samples using a hemocytometer and Sedgewick Rafter counting chamber under a light microscope [25]. Abundance of G. *catenatum* was then calculated and expressed as cells per liter of seawater. A fraction of one liter of bloom sample was filtered through a glass fiber membrane (GF/C, Whatman) and frozen at -20°C until analysis of paralytic shellfish toxins (PSTs). The dinoflagellate cyst analysis (i.e., identification and counting) was made according to Matsuoka and Fukuyo [26] and the cyst abundance was expressed as cysts g^{-1} dry weight sediment.

2.3 Strain isolation and culturing

A strain of G. *catenatum* was isolated from live bloom samples collected in the Tajoura Mediterranean coast during bloom event in September 2023. The strain monoculture was established by pipetting single cells or single chains from bloom samples under light microscope. The single cells were then transferred into 24-well polystyrene cell culture plates containing f2/medium and incubated at 20°C and light intensity of 100µmol photons m^{-2} s^{-1} in a light:dark cycle of 12 h:12 h., following the growth conditions established by Costa et al.[27]. To obtain a large biomass, the cultures were scaled up by transferring the cells in plate wells into a conical one-liter flask containing fresh f2/medium and maintained under the same conditions outlined above for 15 days (i.e., the late exponential growth phase). Cells were then harvested by centrifugation (2000×g, 5min, 15°C). The cell density of the cultured strain was 4.2×105 cells L^{-1}.

2.4 Extraction of PSTs

Cell pellets of strain culture and frozen GF/C filters with attached cells of G. *catenatum* bloom were extracted in 5mL of 0.05M acetic acid using a probe sonicator at 25W, 50% pulse duty cycle (Branson Sonifier 450, Danbury, NH, USA) for 4 min on ice, following the protocol of Costa et al. [27]. Cell lysis was verified using light microscopy. The extract was then centrifuged (4000×g for 10min) and the supernatant was cleaned by solid phase extraction (SPE) using an Supelclean LC-18 cartridge (Supelco, Sigma Aldrich, Cairo, Egypt), which previously conditioned with 3mL methanol followed by 3mL water. The eluate containing hydrophilic PSTs was collected and dried with liquid nitrogen. The dried material was reconstituted in 2ml water and filtered through a syringe membrane filter (0.22µm) before HPLC-FLD analysis.

2.5 HPLC-FLD analysis

All samples and PST standards were oxidized with periodate and peroxid prior to analysis in order to allow toxin detection.

The periodate oxidation enables the detection of both hydroxylated and non-hydroxylated toxins; while the peroxide oxidation enables only the detection of non-hydroxylated toxins (dcGTX2,3; C1,2; dcSTX; GTX2,3; GTX5; STX). PSTs were analyzed according to the conditions described in Leal et al. [28], using the high-performance liquid chromatography with fluorescence detection (HPLC-FLD, model Prominence-i LC-2030C Plus, Shimadzu). The system consists of a refrigerator autosampler, a spectrofluorometric detector RF-20A XS, a column oven and a quaternary pump. Both reversed-phase C18 column (25cm x 0.46 (5µm particle size) ultraguardTM column (10×3.2mm) were used for toxins separation. The temperature of the detector, column oven and autosampler was adjusted at 30°C, 25°C, and 10°C, respectively. Two mobile phases were used to elute PSTS oxidation products including ammonium formate 0.1M adjusted to pH 6 with 0.1M CH_3COOH as mobile phase (A) and CH_3CN as a mobile phase (B). the toxins were eluted in a

gradient mode as follow: 1–5% CH_3CN during the first 6 minutes, 5–28% CH_3CN from 6 to13min, 28–1% CH_3CN from 13 to 16 min. and maintain 1% CH3CN for 3 min before the next injection. The flow rate was 1.5ml min^{-1}. The excitation and emission occurred at wavelengths of 340nm and 395nm, respectively. The injection volume was 30µL for solutions oxidized with peroxide and 100µL for solutions oxidized with periodate. Toxins in bloom and culture extracts were identified based on the retention times (Rt) of toxin standards and quantified using the corresponding toxin calibration curve.
PST standards including STX, dcGTX2^{+3}, C1^{+2}, C3^{+4}, GTX1^{+4}, GTX2^{+3}, GTX5, GTX6, NEO, dcNEO and dcSTX were purchased from the National Research Council Canada (Halifax, Canada) through Egyptian chemical corporation
LOQ (limit of quantification) of these toxins ranged between 0.02 and 0.42µM, and their LOD (limit of detection) ranged between 0.01-0.13µM.

2.6 Statistical analysis

All data were processed statistically using SPSS17 software for Window. Variations in the abundance of G. *catenatum* and toxin concentrations in cultures and bloom samples were compared using ANOVA ($p<0.05$). The Spearman test was used to investigate the correlations between the abundance of G. *catenatum* and environmental factors.

Table 1. Water characteristics of Libya Mediterranean coastal waters before and during *G. catenatum* bloom event.

Parameter	Before the bloom event (17 Sep 2023)	During the bloom event (21 Sep 2023)
Temp (°C)	28	25
pH	8.3	8.6
Salinity (psu)	40±3.2	34±8
NO_3 (mg L^{-1})	5.7±1.1	3.3±0.7
NH_4 (mg L^{-1})	3.4±0.9	5.3±1.3
PO_4 (mg L^{-1})	1.2±0.3	2.3±0.5
SiO_2 (mg L^{-1})	0.13±0.04	0.87±0.1
Cysts (cysts g^{-1})	431±46	125±16
Vegetative cells (cells L^{-1})	136±19	350000±51000

3. Results and Discussion

3.1 Environmental conditions sssociated with *G. catenatum* Bloom

The results of the field study through regular monitoring of microalgae in Libyan coastal waters, revealed the first appearance of G. *catenatum* in Tajoura coastal water on 17 September 2023 with low cell abundance (135 cells L^{-1}). For days later, on 21 September, a sudden bloom of G. *catenatum* had been observed in this area with high cell density (5.3x105 cells L^{-1}), exceeding the HAB threshold (>105cells L^{-1}), which were previously shown by Guo et al. [29] to correspond to the onset of visibly discolored water. G. *catenatum* presented morphological characteristics: vegetative cells are 24-35µm width forming distinctive chains of 2-4 cells and presence of sexual stages in samples of living cells (Fig. 2), fully consistent with the morphology reported for the species by Hallegraeff et al. [30] and Zardoya et al. [31].

During this bloom, coastal waters in the impacted area (Tajoura coast) were characterized by a rise in nutrient concentrations (NO_3, NH_4, PO_4 and SiO_2) compared to their levels during the pre-bloom period (Table. 1). Our results are thus in line with previous studies showing that G. *catenatum* has a proliferation preference during early autumn following the enrichment of the environment with nutrients released from the sediments by upwelling [19,32,33]. In this respect, it has been reported that upwelling is an important physical process that brings nutrient-rich deep waters to the upper surface water throughout the summer, providing the necessary nutrients for the development of algal blooms in early autumn [12,34]. Additionally, coastal waters in the impacted area showed a decrease in water temperature (25°C) and salinity (34psu) compared to their values during the pre-bloom period of G. *catenatum* (28°C, 40psu, respectively).

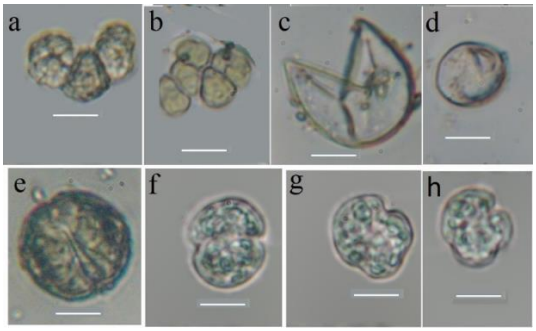

Figure2. Light micrographs of *Gymnodinium catenatum* collected form Libyan Mediterranean coastal waters. **a,b)** vegetative cells forming two and 3-celled chains; **c)** empty cyst; **d)** small cyst; **e)** large cyst with large chasmic archeopyle; **f,g,h)** Planktonic cells germinated from cysts. Scale bars:15µm.

Most reported blooms of G. *catenatum* occurred in coastal waters at temperatures between 18-26°C and salinity of 34-35psu [35,36]. In this regard, three ecotypes have been suggested for bloom populations of G. *catenatum* based on water temperature: warm ecotype growing at water temperatures >26°C, cold ecotype growing at water temperatures between 12-17°C and moderate ecotype growing at temperatures between 18-20°C [36,37]. This indicates that our strain of G. *catenatum* belongs to the moderate ecotype. Therefore, our results support the hypothesis of previous studies that environmental factors such as temperature and nutrient supply are probably the major constraints on the success of blooms [12,36].

In addition to vegetative cells. cysts of G. *catenatum* have also been reported in sediments from Tajoura coastal waters during the present study. These cysts are brown, spherical with a fine microreticulate surface ornamentation and split along the edge of the cingulum (roughly in half) (Fig. 2), similar to G. *catenatum* cysts recorded in Tasmania coastal sediments [39] and identified following the description of Anderson et al. [44]. In the present study, cyst concentrations of G. *catenatum* observed in Tajoura sediments before the bloom was significantly higher than those detected during the bloom (431cysts g^{-1}), indicating the germination of these cysts into motile vegetive cells in the water column. This supports

supported the hypothesis that cysts are the 'seeds' that inoculate algal blooms coastal water [40,41], and cyst abundance in sediments represents crucial information to understand and possibly predict HABs [42].

With respect to Libyan coastal waters, unfortunately, no data regarding G. *catenatum* cyst distributions prior to the HAB event in September 2023 are available. However, recent studies have detected G. *catenatum* cysts in coastal sediments of the neighboring country, Tunisa [21]. Therefore, G. *catenatum* bloom and its cysts observed in Libyan coastal water could arise from cysts transported from Tunisian coastal waters through ballast water. In this regard, G. *catenatum* cysts are well known to be viable for at least two years, can move over great distances and durations, and continuously produce motile vegetative cells [12,36,43,44]. G. *catenatum* cysts were found to be transported in ballast water [43], resulting in an increased risk of cyst invasion in coastal waters worldwide. Another reason for the first emergence of G. *catenatum* in Libyan water is researchers' increased interest in monitoring harmful algae in Libyan coastal waters. Therefore, regular monitoring of toxic G. *catenatum* populations and cysts along the Libyan Mediterranean coast should be maintained and expanded.

3.2 Toxin profile of G. *catenatum*

Data obtained by HPLC-FLD revealed a similar profile of PSTs in bloom and cultured strain of G. *catenatum* from Libyan coastal water during the present study was constituted by the carbamoyl (STX, NeoSTX), decarbamoyl (dcSTX), N-sulfocarbamoyl gonyautoxins (GTX1–GTX4) and N-sulfocarbamoyl toxins (C1/2) (Fig. 3, chromatogram). However, cultured cells produced approximately 1.5 times more toxin (total PSTs=99.3 pg cell-1) than cells from bloom samples (total PSTs=130.9 pg cell-1). The presence of low levels of toxins in G. *catenatum* bloom samples may be related to the fact that the natural bloom may comprise both toxic and non-toxic strains (Cusick and Widder 2021) [45], whereas strain culture contains only monoclonal toxic cells. Several examples exist of both toxic and nontoxic strains occurring within monospecific blooms for different HAB species (Alpermann et al., 2010 [46]; Touzet et al., 2012 [47]; Cusick and Widder 2021 [45]). Specifically, natural populations of G. *catenatum* bloom have been demonstrated to consist of a combination of toxic and toxic strains (Hallegraeff et al. 2012) [40].

In our study, N-sulfocarbamoyl toxins (C1/2) were dominant among PST variants in both bloom and culture extracts (41 and 57 pg cell-1, respectively), followed by gonyautoxins (GTX1/4, up to 18.2 and 21.1 pg cell-1, respectively) and saxitoxin (STX, 11.2 and 16.7 pg cell-1, respectively) (Table 2). Other PST analogues such as NeoSTX and dcSTX were found with low concentrations in bloom (3.3 and 5.3 pg cell-1, respectively) and culture (4.9 and 7.5 pg cell-1, respectively) extracts during the present study.

The dominance of C1/2 toxins was also shown for G. *catenatum* strains from Moroccan Mediterranean water representing 57% (0.08 fg cell-1) [20] and Portuguese strains with 95% (0.09 fg cell-1) [48]. The production of other PST analogues such as STX (0.023 fg cell-1), dc-STX (0.034 fg cell-1), NeoSTX (0.004 fg cell-1) and GTX1-4 (0.014 -0.026 fg cell-1) was also assigned for Moroccan G. *catenatum* strain [20]. This indicates that our G. *catenatum* strain produced the same PST profile as Moroccan Mediterranean strain, but with different proportions of each toxin variant. Generally, our Libyan G. *catenatum* strain produced much more toxins than Moroccan strain. This finding corroborates the hypothesis that G. *catenatum* seems to have a relatively conservative genetic profile [49], but the relative abundance of each PST variant produced by this dinoflagellate differ between strains of different geographic origins [28].

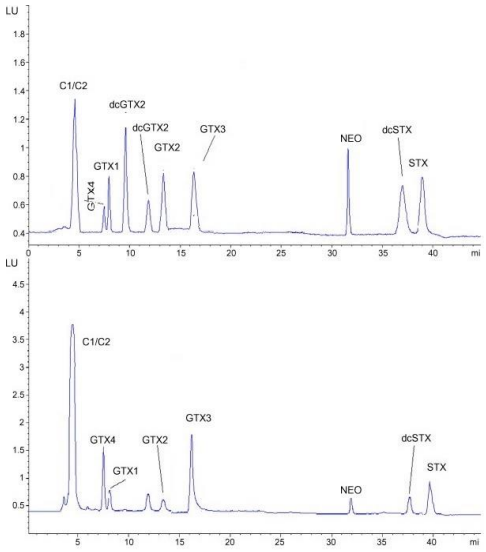

Figure 3. HPLC-FLD chromatograms of PST standards (top) and extract of bloom of *G. catenatum* from Libyan Mediterranean waters (bottom).

Table 2. Concentrations of PSTs produced by cultured strain and natural bloom of *G. catenatum* collected from Libyan Mediterranean coastal water during the present study.

Toxin	Toxin cell quota (fg cell^{-1})	
	Bloom	Culture
STX	11.2±2.2	16.7±4.3
dcSTX,	5.3±1.1	7.2±2.2
NeoSTX,	3.3±0.4	4.9±0.8
GTX1	2.1±0.5	2.9±0.6
GTX2	1.9±0.3	2.7±0.9
GTX3	18.2±4.1	21.1±5.7
GTX4	16.3±3.1	18.4±4.7
C1/C2 toxins	41±6.7	57±9.3

In addition to Mediterranean G. *catenatum* strains, several strains of this dinoflagellate from other geographical regions including Japan, Australia, Uruguay [50], Taiwan Strait [36], East China [51] and Iberian Peninsula (NE Atlantic) [28] also exhibited toxin profiles dominated by N-sulfocarbamoyl toxins, with significant amounts of other PST analogues.

4. Conclusions

To our knowledge, this is the first report of a G. *catenatum*

bloom in Libyan Mediterranean coastal waters. Temperature and nutrient enrichment could be the dominant drivers of G. *catenatum* occurrence in this region. Toxin analysis showed this bloom produced PSTS with a profile dominated by the least toxic N-sulfocarbamoyl toxins analogues (C1/2). The highest toxic carbamoyl analogues (STX, NeoSTX) were also detected with considerable concentrations. The study also revealed the presence of G. *catenatum* cysts in the impacted area of Libyan coasts. The cysts accumulate in the sediments and would be the seedbed responsible for the initiation and recurrence of the harmful bloom in the region. Furthermore, after deposition, the cysts can be transported by circulation and tidal processes from the original deposition area and relocate into other zones [**41**]. Given that anthropogenic nutrients concurrently with climate change could increase incidence of HABs in marine ecosystems [**52**], discharge of nutrients from aquaculture, agriculture and manufactory practices into coastal waters should be reduced. Furthermore, besides monitoring of HAB species, cyst survey would also help to predict potential future blooms and thereby reduce their negative impacts of future HAB events on aquatic ecosystem, seafood quality and human health.

CRediT authorship contribution statement:

"Conceptualization, Z.M and H.A.; methodology; software, H.A. and H.A.B; validation, Z.M., H.A.; formal analysis, H.A.; investigation, Z.M. and H.A..; resources, Z.A..; data curation, H.A.B.; writing—original draft preparation, H.A.; writing—review and editing, H.A. and A.S.B.; visualization, H.A. and H.A.B.; supervision, Z.M. and A.S.B.; project administration, A.S.B. All authors have read and agreed to the published version of the manuscript."

Data availability statement

The data used to support the findings of this study are available from the corresponding author upon request.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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