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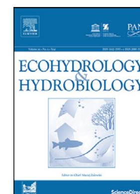

Ichthyotoxicity of bloom-forming dinoflagellates in an Egyptian saline lake: potential role of PUFA

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ABSTRACT

Lake Qarun, the third-largest saline lake in Egypt, has been plagued with algal blooms and experienced decline in fisheries. However, the toxicity of these blooms has not explored yet. Therefore, the present study is the first to evaluate the toxicity of the most frequently recorded harmful algal blooms (HABs) in Lake Qarun. The results showed an occurrence of dinoflagellate bloom in site1 located near El-Bats drain (1.4×10^8 cells L^{-1}) and site 2 located near El-Wadi drain (1.9×10^8 cells L^{-1}), but not found in site 3 located away from drains. This bloom associated with high nutrient concentrations and low salinity in these sites. Both intact cells and cell lysate of bloom samples and cultured species, but not cell-free supernatants, exhibited toxicity towards *Artemia salina* and cytotoxicity for the gill assay, with higher toxicity incurred by lysed cells. The cytotoxicity varied significantly among bloom-constituting species, with highest obtained by the most dominant species; *Protoperidinium quinquecorne* (LC_{50}= 445 cells $ml^{-1}$for lysed cells), *Prorocentrum micans* (LC_{50}= 757 cells ml^{-1}), *Gymnodinium lantzschii* (LC_{50}= 1151 cells ml^{-1}) and *Amphidinium carterae* (LC_{50}= 1289 cells ml^{-1}). These LC_{50}s showed greater correlation with the percentages of the polyunsaturated fatty acids (PUFA): octadecatetraenoic (OTA), eicosapentaenoic (EPA) and docosahexaenoic (DHA) in these microalgae, indicating the role of these fatty acids in ichthyotoxicity. No detectable levels of known phycotoxins were found in bloom or cultured species. This study suggests regular monitoring and systematic assessment of HABs and their toxins in Lake Qarun to mitigate their occurrence and maintain fisheries and seafood safety.

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1. Introduction

Lake Qarun is one of the largest lakes in the North Africa, and the oldest natural lake in Egypt (Al-Afify et al., 2019). The lake has turned from an ancient freshwater system into a marine system in the late 20th century (Abu-Zied et al., 2007; Flower et al., 2006). This increase in salinity in the lake has been attributed to the evaporative concentration of agricultural drainage water entering the lake through El-Wadi and El-Bats drains. (Abdel Wahed et al., 2014). This has led to the obvious invasion of marine algal species in Lake Qarun (Abd El-Karim, 2012). Moreover, the discharge of nutrients and chemical fertilizers has also caused eutrophication of Lake Qarun (Fathi and

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Flower, 2005; Zaher and Ibrahim, 2018). The trophic level index indicated that most sites of Lake Qarun lie at hypereutrophic level (Napió rkowska-Krzebietke et al., 2016). Such eutrophication conditions have induced algal proliferation and promoted formation of harmful blooms in the lake. Several studies reported the occurrence of different types of algal blooms in Lake Qarun including cyanobacterial blooms (Mohamed, 2016), dinaoflagellate blooms (Zaher and Ibrahim, 2018; Ibrahim et al., 2021), diatom blooms (Abd El-Karim, 2012) and green algae blooms (Ibrahim et al., 2021). Being living in the same environment, fish and seafood organisms could be influenced by the presence of HABs in Lake Qarun (Napió rkowska-Krzebietke et al., 2016). These blooms can clog fish gills and deplete oxygen from water, leading to fish kills (Mahmudi et al., 2020). Otherwise, fish mortality may occur as a result of poisoning by phycotoxins, particularly neurotoxins (e.g., saxitoxins and neosaxitoxins) produced by bloom-forming algae (Mohamed, 2018; Yurga, 2022). Additionally, fish gill damage may be due to the production of the so-called ichthyotoxins, which their production often differs from the well-studied paralytic, diarrheic, amnesic and neurotoxic phycotoxins (Hallegraeff et al., 2017). Furthermore, phycotoxins can accumulate in edible tissues of aquatic organisms such as fish and shellfish, leading to human poisoning upon consumption of contaminated seafood (Kantiani et al., 2010; Hinder et al., 2011). Based on the symptoms of human poisoning, these toxins include paralytic shellfish poisoning toxins (PSP) toxins (e.g., STXs), diarrhetic shellfish poisoning (DSP) toxins (e.g., okadaic acid), neurotoxic shellfish poisoning (NSP) toxins (e.g., brevetoxins), ciguatera fish poisoning (CFP) toxins (e.g., ciguatoxin and maitotoxin), azaspiracid shellfish poisoning (AZP) toxins (e.g., azaspiracids), and amnesic shellfish poisoning (ASP) toxins (e.g., domoic acid) (Camacho-Muñoz et al., 2021). Although dinoflagellate blooms have been frequently recorded in Lake Qarun (Ibrahim et al., 2021), to our knowledge, toxins produced by these blooms have never been identified yet. Additionally, there is no evidence of human intoxication through seafood consumption from the lake. This is because of lack of knowledge for public and water authority about these toxins. Based on a field Observation of fish mortality at bloom areas in Lake Qarun, we hypothesized that the algal blooms could be the factor causing these massive mortalities of fishes in the lake.

Therefore, the present study was undertaken to determine the toxicity and toxins contained in dinoflagellate blooms prevalent in Lake Qarun. The data obtained from this study is expected to significantly contribute to lake management in order to reduce the risks of these toxins to fisheries and human health.

2. Materials and Methods

2.1. Study area and water samples

Lake Qarun is a closed saline basin situated about 95 km southwest of Cairo with an elevation of ≈ 43 m below sea level. It is located between longitudes of 30° 24′ and 30° 49′ E and latitudes 29° 24′ and 29° 33′ N. The lake surface area is about 215 km^2 (40 km length and 5.7 km width) with water volume about 1,100,000000 m^3 (Ahdy et al., 2011). It is a shallow lake with a depth varying between 5 m in the east to 12 m in the west. The lake is surrounded by desert at the northern region and cultivated land slopes steeply towards the lake which makes it a natural sink for agricultural drainage water (Abdel Wahed et al., 2015). Lake Qarun receives agricultural wastewater via two main drains, El-Bats at the most east part, and El-Wadi at the middle of the southern side. The total water discharging annually into the lake is about 395 million cubic meters. Lake Qarun represents one of the important sectors in the Egyptian fisheries, for a large number of economically important species (Napió rkowska-Krzebietke et al., 2016).

Water samples were collected from Lake Qarun at 3 sites: site 1 is located at the eastern part of the lake near El-Bats drain, site 2 is located at middle of the Lake near El-Wadi Drain, and site 3 is located at the western part of the lake (Fig. 1). Water samples were collected in July, 2021, which was approximately one week after the bloom was observed by fishermen. At each site, water and phytoplankton samples were collected in triplicate at 0.5 m below the water surface by using a Van Dorn water sampler. Bloom samples were passed through a 60 µm mesh to remove larger organisms and then divided into three parts. One part was preserved with 1% Lugol's iodine and used for the identification and counting of phytoplankton. Another part was used for isolation and culturing of dinoflagellate species. The remaining part was kept in polyethylene bottle and used for evaluating the bloom ichthyotoxicity.

2.2. Physico-chemical and phytoplankton analysis

Water temperature, salinity and pH were measured *in situ* using a multipurpose-probe meter (WTW Digit 88), and dissolved oxygen with an O_2-meter. Nutrients such as ammonium, nitrate phosphate and silicate were determined in GF/C filtered water samples by the standard analytical methods (APHA, 1995). All parameters were determined in triplicate. Dinoflagellate species constituting Lake Qarun bloom were enumerated in the Lugol-preserved samples and freshly collected samples (less than 6 hours after sampling) using Sedgwick Rafter cell counter under light microscope. Phytoplankton species in bloom samples were identified based on morphological characteristics according to Popovský and Pester, 1990, Anderson et al. (1995), Landsberg et al. (2002). The current status of the scientific names of the species were periodically checked and rearranged according to the website of AlgaeBase (2021).

2.3. Establishment of dinoflagellate Cultures

Unialgal cultures of dinoflagellate species were established from cells of bloom samples collected in July 2021 at sites 1 and site 2 in Lake Qarun. Single cells of each species were isolated by capillary pipetting under a Carl Zeiss inverted microscope and transferred to F/2 medium without silica (Andersen et al., 1997) prepared in filtered and sterile Lake Qarun water with salinity adjusted to 30

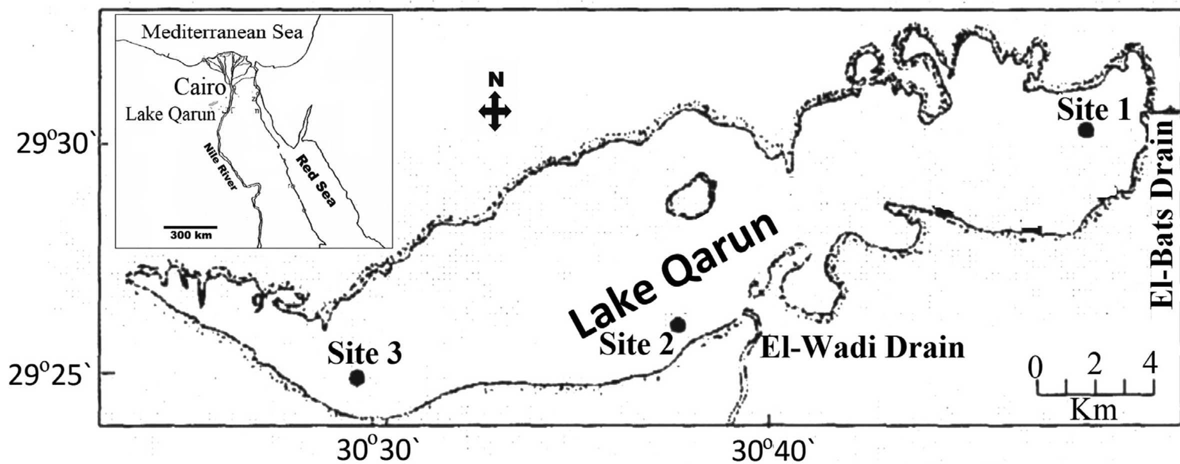

Fig. 1. Map showing the location of the study sites on Lake Qarun.

PSU. The cultures were then incubated at 26°C, with a photon flux of 50 µmoles photons of cool white fluorescent light and a 12:12 light: dark (LD) cycle. After 3 weeks, these monoclonal cultures were transferred into 100 ml culture flasks containing 50 ml F/2 medium and incubated under the above conditions for 21 days (i.e., the time of exponentially growth phase). The cell concentration was monitored every two days using a Sedgwick Rafter cell counter. Algal cultures were then divided into two parts; one part was used for testing ichthyotoxicity by *A. salina* and fish gill cell line, while the other part was used for fatty acid analysis.

2.4. Artemia salina toxicity assay

Three different algal suspensions; intact cells, cell lysate (obtained by sonication of the cultures), and cell-free supernatants were prepared from the cultures in the exponential growth phase at six different cell concentrations (100, 200, 500, 1000, 2000 and 5000 cell mL^{-1}), and used for ichthyotoxicity assays (i.e., *Artemia salina* and fish gill cell line assays). The *A. salina* assay was carried out using freshly hatched *A. salina* L nauplii according to Kiviranta et al. (1991). The mortality was estimated after 48h and expressed as the difference (%) between mortalities in the tested and control samples following this formula—%mortality = [(Test−Control)/(1−Control)] × 100. The lethal concentration causing death of 50% (LC_{50}) with 95% confidence limits was calculated by the probit analysis method (Finney, 1971)

2.5. Gill cell line assay

The gill cell line RTgill-W1, an epithelial cell line derived from rainbow trout (*Oncorhynchus mykiss*) was obtained from the American Type Culture Collection (CRL-2523, ATCC). Cells were cultured and maintained in Leibovitz's L-15 medium supplemented with fetal bovine serum (10%) and antibiotic-antimycotic solution (penicillin,10,000 units mL^{-1}; amphotericin B, 25 mg mL^{-1} and streptomycin, 10 mg mL-1) (Dorantes-Aranda et al., 2011). The cytotoxicity assay was carried out according to Dorantes-Aranda et al. (2011). Briefly, gill cell cultures were trypsinized (0.25% trypsin-0.02% EDTA) for detachment, and the cells were counted using a hemocytometer and adjusted to a final concentration of 2 × 105 cells mL^{-1}) with L-15 medium. Gill cells were seeded in 96-well microplates (100 µL per well), and incubated at 17 ℃ in the dark for 48 hours. Thereafter, L-15 medium was discarded and the cells were washed with phosphate buffer saline (PBS). The gill cells were exposed to intact cells, cell lysate or cell-free supernatants of algal cultures and bloom materials for 2 hours, and the wells were washed twice with PBS. The viability of gill cells was determined by incubation in L-15 medium containing resazurin (5%) as an indicator dye (100 µL per well) for 2 hours in the dark. After that, the metabolic reduction of resazurin to resorufin by the remaining viable gill cells was quantified in a fluorescent plate reader (FLUOstar Optima, BMG Labtech) at excitation and emission wavelengths of 540 and 590 nm, respectively. Wells without gill cells were used as blank. Wells containing gill cells and F/2 medium but without any algal material were used as a control. The gill cell viability was expressed as a response percentage of the treatments relative to the control (% of control).

2.6. Analysis of saxitoxins and okadaic acid

Saxitoxins were analyzed by enzyme-linked immunosorbent assay (ELISA) using commercial microplate kits for these toxins (Abraxis, Westminster, PA, USA). The detection limit of this method was 0.02µg L^{-1} for saxitoxins. Diarrheic shellfish poisoning (DSP) toxins including okadaic acid and its analogues of dinophysistoxins (DTXs) were also analyzed by ELISA using relevant kits (Abraxis, Westminster, PA, USA) with detection limit of 0.45 µg L^{-1}.

2.7. Analysis of Fatty acids

An aliquot of bloom samples or algal cultures was filtered onto a pre-combusted Whatman GF/F-filter

Table 1
Physico-chemical Lake Qarun water at different study sites during the bloom event.

Parameters	Site 1 (Bloom area)	Site 2 (Bloom area)	Site 3 (Non-bloom area)
Water Temperature (°C)	26	29	36
pH	8.2± 0.06	8.1± 0.04	8.3± 0.05
Salinity (psu)	29.2±1.2	31.5±1.5	39.3±2.3
DO (mg/L)	5.3±0.7	6.2±0.6	7.7± 0.8
NO_3 (µg/L)	670.4± 23	632.9±19	111.52±13
Ammonium (µg/L)	286.4± 31	257.6± 27	78.6±8
PO_4 (µg/L)	323.71± 21	367.7±29	83.9±12
SiO_2 (µg/L)	19.6±3	24.1±4	11.6 ±2

(450°C for 2 h). Lipids were extracted from algal cells and analyzed following the method described by Hyun et al. (2016). Briefly, filters with attached algal cells were frozen at -20 ℃, and then extracted in dichloromethane: methanol (2:1, v/v) with sonication at 20 kHz for 10 min on ice. The resulting homogenate was centrifuged at 6000 × *g* at 4°C for 5 min. The supernatant was collected and dried under nitrogen. The dried extract was re-dissolved in a known volume of dichloromethane and methanol (2:1, v/v) and spiked with a known concentration of internal standard (n-nonadecanoic acid; C19:0; Sigma-Aldrich). The extracts were dried again under nitrogen, and the extracted lipids underwent transesterification with 0.5N methanolic potassium hydroxide (KOH) at 70°C for 30 min. The neutral lipids (alcohols and sterols) were removed using a mixture of hexane and diethyl ether (9:1 v/v), while the polar fraction (fatty acids) was acidified to pH < 2 and extracted with a mixture of hexane and diethyl ether (9:1 v/v). The extracted fatty acids were dried under nitrogen and converted to methyl esters (FAMEs) using boron trifluoride–methanol at 70°C for 30 min. FAMEs were analyzed by gas chromatography (GC; Agilent 6890, Agilent GmbH, Waldbronn, Germany) equipped with capillary column (60 m × 0.25 mm I.D.; film thickness: 0.25 µm; liquid phase: DB-FFAP (Agilent)) using temperature programming (Injection: splitless at 250°, detection: FID at 280° C). FAMEs were identified by comparing against the retention time of standards.

2.8. Statistical analysis

Differences in cell density, environmental parameters, LC_{50} values, percentages of fatty acids were compared by ANOVA using the Excel data analysis tool at the 0.05 significance level. Correlations between algal density and environmental parameters, as well as between toxicity and fatty acids were measured using Spearman rank correlation coefficients using the Excel data analysis tool.

3. Results

3.1. Environmental factors associated with dinoflagellate blooms

During the field study, red patches of algal bloom was observed on 5 July 2021 at site 1 and site 2, but not found at site 3 in Lake Qarun. Also, fish mortalities of tilapia, shrimp, eels and sea bream were observed at bloom sites (data not shown).

During this bloom event, physico-chemical parameters of lake Qarun water were measured at bloom sites (site1 & site2) and the non-bloom site (site3) in order to define the prevailing environmental parameter(s) favoring bloom formation in Lake Qarun. Water temperature varied significantly among the three sites (P=0.0001), was higher in the non-bloom site (site3) (36°C) compared to 26 and 29°C in bloom sites (Table 1). There was insignificant difference in pH values (P= 0.08) among the study sites. A slight decrease in DO was observed in bloom sites (5.3 & 6.2 mg L^{-1}) compared to 7.7 mg L^{-1} in the non-bloom site. Salinity showed a wide variation among the study sites (P =0.001) and was lower in bloom sites (29-31psu) than the non-bloom site (39 psu). Regarding nutrients, NH_4, NO_3, PO_4 and silicate varied significantly among the sites (P< 0.0001), with higher concentrations recorded at bloom sites (Table 1). The phytoplankton analysis of algal bloom observed in Lake Qarun demonstrated that this bloom was mediated by the rapid growth of dinoflagellate species. Phytoplankton samples taken from the bloom were found to be dominated by three dinoflagellate species (*Gymnodinium lantzschii, Prorocentrum aporum* and *Prorocentrum micans*), which showed cell densities in excess of 3.3 × 10^7 cells L^{-1} in bloom sites (Sit 1 and site 2), but they were totally absent in the non-bloom site (Site 3) (Table 2). Other dinoflagellate species such as *Amphidinium carterae, Gymnodinium simplex, Prorocentrum gracile Protoperidinium quinquecorne* were also found, but with lower cell densities (0.2 × 10^4 – 4.1 × 10^5 cells L^{-1}) in bloom sites (Table 2). Interestingly, these subdominant species were also recorded in the non-bloom site with cell densities of 0.4 × 10^4 – 0.8 × 10^4 cells L^{-1} (Table 2). Species of other phytoplankton groups including cyanobacteria, green algae, diatoms were not observed or represented in negligible cell numbers in both bloom and non-bloom sites (Therefore data are not shown). The statistical analysis revealed significant correlations between the abundance of bloom-forming species and physicochemical parameters of lake Qarun water determined during bloom event. The cell density of main bloom-forming species (*G. lantzschii, P. aporum* and *P. micans*) showed positive correlations with concentrations of NH_4 (r=0.8-0.9), NO_3 (r=0.8), PO_4 (r=0.8) and silicate (r=0.9) in lake water. On the other hand, the cell density of these species negatively correlated with temperature (r=-0.8) and Salinity (r=-0.9).

Table 2
List of dinoflagellate species and their cell densities (cells L^{-1}) recorded in bloom samples collected from different sites at Lake Qarun during the bloom event.

Species	Sites		
	Site 1 (Bloom area)	Site 2 (Bloom area)	Site 3 (Non-bloom area)
Amphidinium carterae Hulburt	$0.1 \times 10^5 \pm 2.1$**	$0.4 \times 10^5 \pm 2.8$**	$0.8 \times 10^4 \pm 1.8$*
Gymnodinium lantzschii Utermöhl	$3.3 \times 10^7 \pm 6.3$**	$5.7 \times 10^7 \pm 5.8$**	-
Gymnodinium simplex (Loh.) Kof. & Swezy	$0.3 \times 10^4 \pm 3.1$*	$1.2 \times 10^4 \pm 2.2$*	$0.6 \times 10^4 \pm 1.2$*
Prorocentrum aporum (Schiller) Dodge	6. $x10^7 \pm 3.4$**	$8.3 \times 10^7 \pm 7.1$**	-
Prorocentrum gracile Schutt.	$0.2 \times 10^4 \pm 4.2$*	$0.4 \times 10^4 \pm 5.1$*	$0.4 \times 10^4 \pm 1.1$*
Prorocentrum micans Ehrenberg	$4.2 \times 10^7 \pm 2.3$**	$4.8 \times 10^7 \pm 3.7$**	-
Protoperidinium quinquecorne (Abé) Balech	$3.2 \times 10^5 \pm 1.1$**	$4.1 \times 10^5 \pm 1.2$**	$0.7 \times 10^3 \pm 1$*

* SD is the value $\times 10^2$.
** SD is the value $\times 10^3$.

3.2. Ichthyooxicity of dinoflagellate blooms and cultured species

The results of lethality assay revealed that both whole living cells and lysate of dinoflagellate bloom collected from Lake Qarun during the present study exhibited toxicity towards *A. salina* with significant variation in LC_{50} values (P<0.0001). The bloom lysate was more toxic (LC_{50}=210-430 cells mL^{-1}) than intact cells (LC_{50} = 1300-2000 cells mL^{-1}) (Table 3). The toxicity of bloom materials (i.e., intact cells and cell lysate) also differed between the two bloom sites (P =0.01-0.001). the intact cells and cell lysate of stie2 bloom was more toxic to *A. salina* (LC_{50} = 1300 and 210 cells mL^{-1}, respectively) than those of site 1 bloom (LC_{50}=2000 and 430 cells mL^{-1}, respectively) (Table 3). Phytoplankton samples collected from the non-bloom site (site 3) showed toxicity to *A. salina*, but it was much lower (LC_{50}=4300 and 600 cells mL^{-1} for whole cells and lysate, respectively) than that of bloom samples of site1 and site2 (Table 3). For cultured dinoflagellate species constituting the algal bloom in Lake Qarun, the toxicity of the intact cells and cell lysate to *A. salina* varied significantly (P< 0.001) among these species. The highest toxicity was exhibited by *P. quinquecorne* (LC_{50} =300 and 140 cells mL^{-1} for intact cells and cell lysate, respectively), followed by *P. micans* (LC_{50} =800 and 200 cells mL^{-1}), *G. lantzschii* (LC_{50}=1200 and 270 cells mL^{-1}) and *A. carterae* (LC_{50}=1400 and 290 cells mL^{-1}) (Table 3). While the remaining species had lower toxicity to *A. salina* with LC_{50} values over 3000 and 200 cells ml^{-1} for intact cells and cell lysate, respectively (Table 3). On the other hand, neither the supernatants of the centrifuged bloom samples nor the cell-free medium of algal cultures caused mortality in *A. salina* (Table 3).

The results of gill cell line assay showed that the dinoflagellate bloom collected from Lake Qarun during the present study exhibited significant toxicity and reduced the viability of RTgill-W1 cells after a two- hour exposure to either intact or lysed cells. The loss of gill cells viability differed significantly (P=0.001-0.005) between phytoplankton samples collected from bloom sites (site1 and site2) and the non-bloom site (site 3) in Lake Qarun, but it did not differ significantly between samples of bloom sites (P=0.07-0.2). The highest losses of viability observed on gill cells were achieved by intact cells and cell lysate of site 2 bloom at a concentration of 5000 cells mL^{-1} (89 and 94%, respectively) (Fig. 2A,B). While the least loss of viability occurred by intact cells and cell lysate (72 and 82%, respectively) of phytoplankton samples collected from site3 at a concentration of 5000 cells mL^{-1} (Fig. 2C).

For cultured species isolated from Lake Qarun during the present study, The loss of gill cells viability varied markedly among species either for intact cells (P =0.0001) or cell lysate (P< 0.0001). Moreover, intact cells and cell lysate of the same species showed a great difference in the loss of viability towards the RTgill-W1 cells (P = 0.03-0.01).

Table 3
Results of *Artemia salina* assay (LC_{50}, cells mL^{-1}) for intact cells, cell lysate and supernatant of bloom and algal cultures from Lake Qarun

Specimen	LC_{50} (cell equivalent)		
	Intact cells (cells mL^{-1})	cell lysate (cells mL^{-1})	Culture Supernatant (cells mL^{-1})
Sit 1 bloom	$2 \times 10^3 \pm 2.1$**	$4.3 \times 10^2 \pm 6.2$*	ND*
Site 2 bloom	$1.3 \times 10^3 \pm 2$**	$2.1 \times 10^2 \pm 3.6$*	ND
Site 3 phytoplankton samples	$4.3 \times 10^3 \pm 3$**	$6 \times 10^2 \pm 5.8$*	ND
Gymnodinium lantzschii culture	$1.2 \times 10^3 \pm 1.1$**	$2.7 \times 10^2 \pm 4.6$*	ND
Prorocentrum aporum culture	$3.3 \times 10^3 \pm 2.2$**	$2.2 \times 10^3 \pm 5.7$**	ND
Prorocentrum micans culture	$0.8 \times 10^3 \pm 1.1$**	$2 \times 10^2 \pm 3.8$*	ND
Protoperidinium quinquecorne culture	$0.3 \times 10^3 \pm 0.8$**	$1.4 \times 10^2 \pm 3.3$*	ND
Prorocentrum gracile culture	$4.9 \times 10^3 \pm 4.3$**	$2.8 \times 10^3 \pm 4.6$*	ND
Gymnodinium simplex culture	$4.7 \times 10^3 \pm 5.3$**	$2.1 \times 10^3 \pm 5.5$**	ND
Amphidinium carterae culture	$1.4 \times 10^3 \pm 4$**	$2.9 \times 10^2 \pm 3.9$*	ND

ND means that LC_{50} was not defined because no mortality was observed at the highest cell concentration used.
* SD is the value $\times$ 10.
** SD is the value $\times 10^2$.

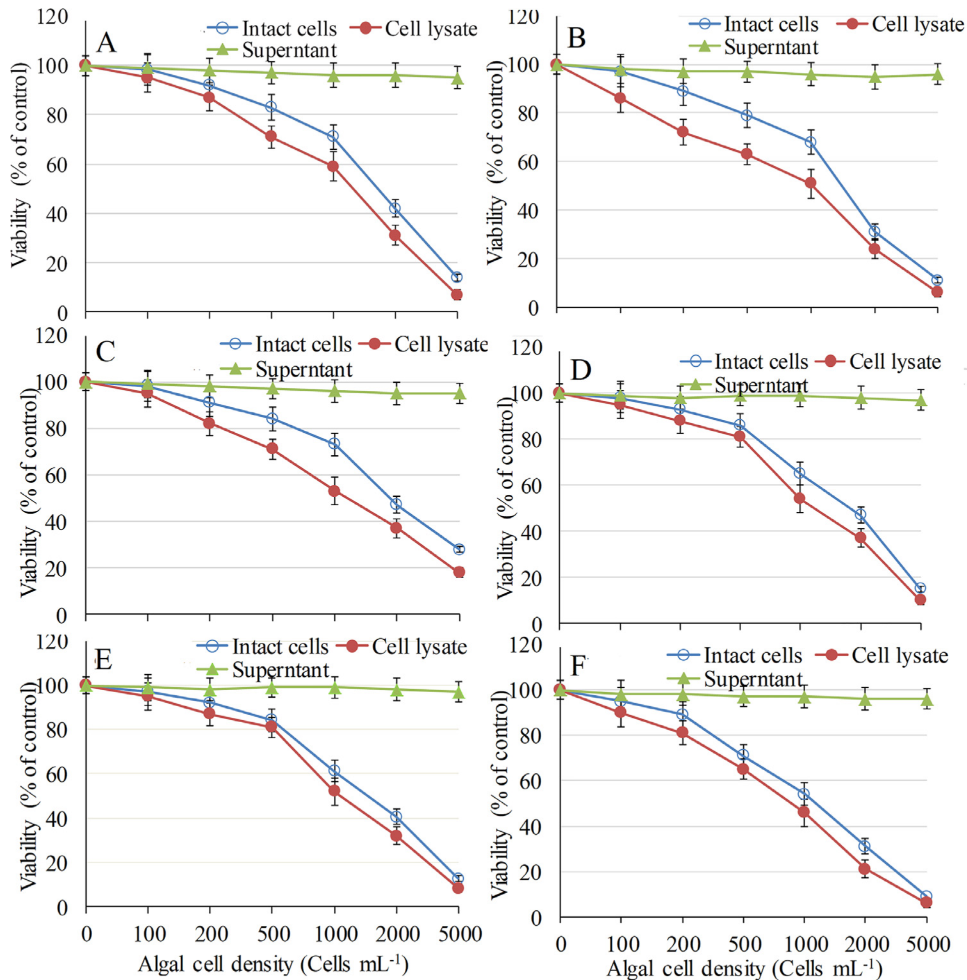

Fig. 2. Ichthyotoxicity to fish gill cells (% of viability) by bloom samples collected from site 1 (A), site 2(B) and site 3 (C), and by cultured dinoflagellates species isolated from Lake Qarun: (D) *Amphidinium carterae*, (E) *Gymnodinium lantzschii*, and (F) *Prorocentrum micans*.

The highest losses of viability (expressed as a reduction percentage) were exerted by intact and lysed cells at a concentration of 5000 cells mL^{-1} of four species: *A. carterae* (85% for intact cells and 90% for lysed cells), *G. lantzschii* (88 and 92%), *P. micans* (91 and 94%) and *P. quinquecorne* (94 and 98%) (Fig. 2C,D, E and F). The remaining species showed lower effect on RTgill-W1 cells, with loss of viability less than 50% for both intact cells and cell lysate at a concentration of 5000 cells mL^{-1} (Fig. 3 B,C and D). The gill cells did not exhibit any significant loss of viability when exposed to the supernatants of the centrifuged bloom samples or the cell-free medium of algal cultures (Figs. 2 and 3).

Based on LC_{50} values calculated by Probit analysis, intact cells and cell lysate of site 2 bloom had lower LC_{50} values (1345 and 842 cells mL^{-1}, respectively) towards RTgill-W1 cells (i.e., higher toxicity) compared to high LC_{50}s for cells of site 1 bloom (1593 and 1133 cells mL^{-1}) and the non-bloom site (LC_{50} =2086 and 1146 cells mL^{-1}) (Fig. 4). Similarly, cultured species also exhibited varying extents of toxicity towards RTgill-W1 cells, with highly variable LC_{50} values ($P < 0.0001$).

P. quinquecorne was the most cytotoxic species having the lowest LC_{50} values (787 cells mL^{-1} for intact cells and 445 cells $mL^{-1}$for lysed cells) followed by *P. micans* (LC_{50}=1070cells mL^{-1} and 757 cells mL^{-1}), *G. lantzschii* (LC_{50}=1509 cells mL^{-1} and 1151 cells mL^{-1}) and *A. carterae* (LC_{50}=1826 cells mL^{-1} and 1289 cells mL^{-1}) (Fig. 4). *P. gracile* was the least toxic species to gill cells, with highest LC_{50} values of 5143 cells mL^{-1} for intact cells and 3172 cells $mL^{-1}$for lysed cells). On the other hand, bloom samples exhibited lower toxicity to fish gill cells than cultured species (Fig. 4). Also, there was no significant difference in LC_{50} values of species strains originating from the study sites in Lake Qarun (therefore data are not shown).

The fatty acid composition (expressed as % of total fatty acids) based on GC-MS analysis of microalgae forming dinoflagellate blooms in Lake Qarun are listed in Table 4. In all species, the FA profile was dominated by polyunsaturated fatty acids (PUFAs) with low levels of monounsaturated fatty acids (MUFAs) and saturated fatty acids (SFAs) However, the % of PUFA varied significantly among species ($P < 005$) and correlated with their ichthyotoxicity towards RTgill-W1 cells. The highest the percentage of

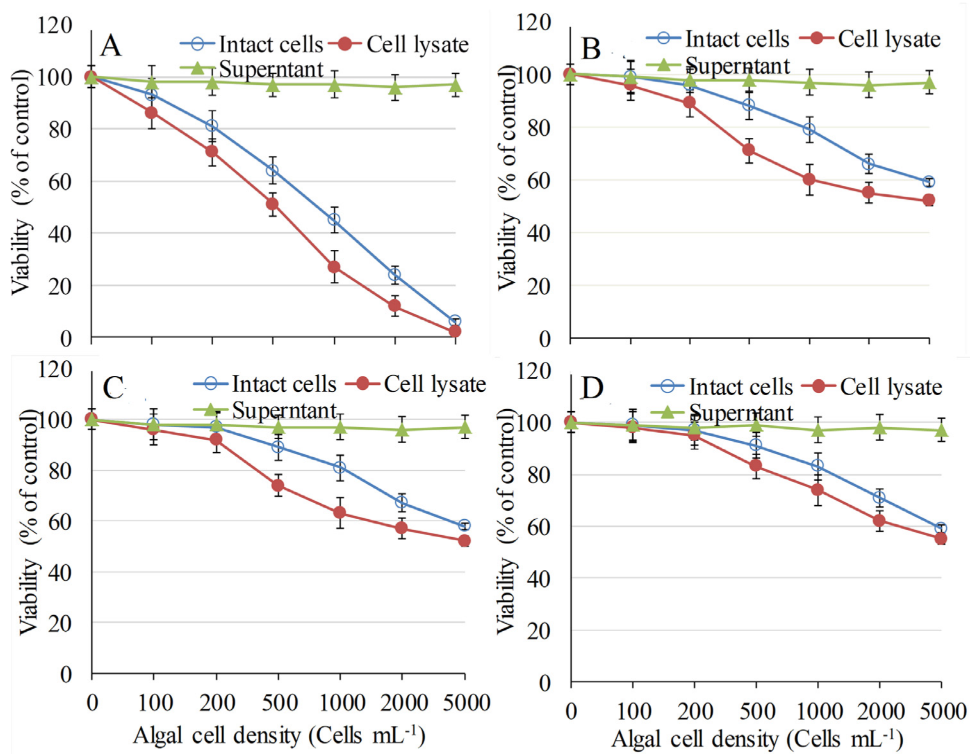

Fig. 3. Ichthyotoxicity to fish gill cells (% of viability) by cultured dinoflagellates species isolated from Lake Qarun: (A) *Protoperidinium quinquecorne*, (B) *Prorocentrum aporum*, (C) *Gymnodinium simplex*, and (D) *Prorocentrum gracile*.

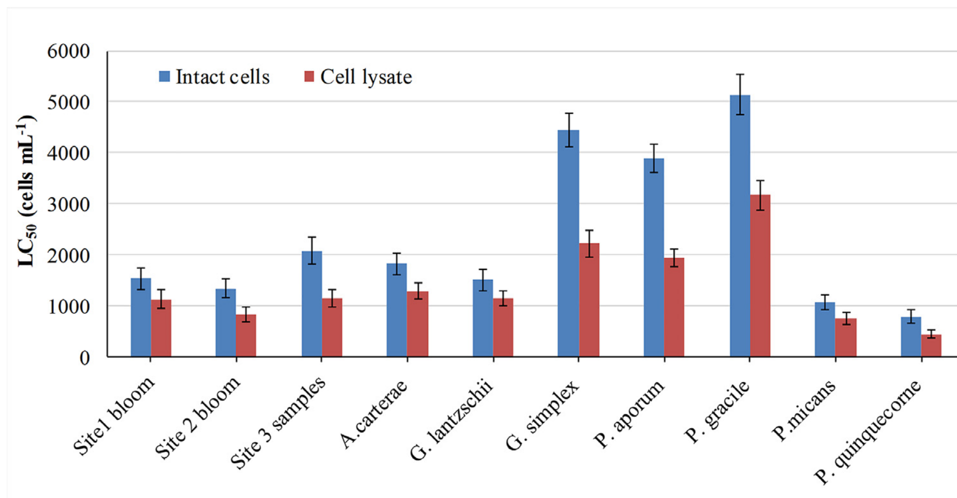

Fig. 4. Ichthyotoxicity to fish gill cells (LC_{50}=cells mL^{-1}) by intact cells and cell lysate of cultured dinoflagellates species isolated from Lake Qarun bloom during the present study.

PUFA, the lowest the LC_{50} to RTgill-W1 cells (r=-0.7). More specifically, LC_{50} of cell lysate of the tested species correlated (r=-0.9) with the percentages of octadecatetraenoic (18:4ω3, OTA), eicosapentaenoic (20:5ω3, EPA), and docosahexaenoic (DHA, 22:6ω3), indicating the role of these fatty acids in ichthyotoxicity of these species (Table 5). For instance, the most ichthyotoxic (i.e., the lowest LC_{50}) species to gill cells, *P. quinquecorne* had the highest percentages (18.2, 8.1 and 21.6% respectively) of these fatty acids (Table 4). On the other hand, the least toxic (i.e., highest LC_{50}) species, *P. gracile* contained the lowest percentages (4.6, 4.5, and 4.1%, respectively) of these fatty acids. Similar to toxicity towards RTgill-W1 cells, the toxicity of dinoflagellate species to *Artemia* also correlated with the percentage of PUFA (r=-0.7), and OTA (r= -0.9), EPA (r= -0.8), and DHA (r=-0.9) (Table 5). Based on ELISA, nei-

Table 4
Fatty acid composition (as % of total fatty acids) in dinoflagellate species constituting Lake Qarun bloom during the present study.

	Fatty acids (%)						
	A. carterae	*G. lantzschii*	*G. simplex*	*P. aporum*	*P. gracile*	*P. micans*	*P. quinquecorne*
SFA							
14:0	7.2	5.9	9.7	8.1	9.5	5.1	6.1
16:0, PA	21.2	21	20.2	20.8	22.2	20.2	22.3
18:0	1.4	1.7	2.7	1.5	1.7	1.6	1.4
MUFA							
18:1ω7c	1.2	1.4	0	0	0	1.2	-
18:1ω9c	5.3	4.9	4.7	6.9	5.3	4.1	4.3
PUFA							
18:2ω6 LA	5.8	1.2	12.4	10.9	15.6	1.1	2.4
18:3ω3 ALA	8.7	4.9	12.9	10.4	14.8	4.9	3.9
18:4ω3 OTA	11.2	15.2	6.8	8.1	4.6	16.1	18.2
18:5ω3 OPA	8.8	5.4	14.1	13.6	14.6	6.5	8.3
20:5ω3 EPA	7.3	6.7	5.2	6.9	4.5	7.3	8.1
22:5ω3 DPA	0.9	0	2.6	1.7	2.4	0.7	0.8
22:5ω6 DPA	0.7	0	0.9	0.9	0.7	0.5	0.6
22:6ω3 DHA	14.6	19.1	7.8	9.6	4.1	20.1	21.6
Sum SFA	29.8	28.6	32.6	30.4	33.4	26.9	29.8
Sum MUFA	6.5	6.3	4.7	6.9	5.3	5.3	4.3
Sum PUFA	63.7	65.1	62.7	62.1	61.3	67.8	63.9

PUFA, polyunsaturated fatty acids; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PA, palmitic acid; OTA, octadecatetraenoic acid; LA, linoleic acid; ALA, α-linolenic acid; OPA, octadecapentaenoic acid; EPA, eicosapentaenoic acid; DPA, docosapentaenoic acid; DHA, docosahexaenoic acid.

ther STXs nor okadaic acid and its analogues of DTXs were detectable in bloom samples or cultured species isolated from these blooms (therefore data are not shown).

4. Discussion

4.1. Dinoflagellate blooms and associated environmental factors

In the present study, the dinoflagellate bloom has occurred in Lake Qarun at site 1 and site 2 (bloom sites), which are located near El-Bats drain and El-Wadi drain, but was not observed at site 3 (the non-bloom site), which lies at the western side of the lake and not exposed to agricultural discharge. This bloom was dominated by *Gymnodinium lantzschii, Prorocentrum aporum* and *Prorocentrum micans* with cell density more than 10^7 cells L^{-1}. These species have also been previously reported as the main forming-bloom species in Lake Qarun (Zaher and Ibrahim, 2018, Ibrahim et al., 2021). In addition to these dominant species, we also recorded other dinoflagellate species participating in the species composition of this bloom (Table 2) with lower cell densities ($< 10^4$ cells L^{-1}). The occurrence of this bloom in site 1 and site 2 was associating with the high concentrations of nutrients (NH_4, NO_3, PO_4 and silicate) and low salinity (29 PSU) compared to lower nutrient concentrations and high salinity (39 PSU) in the non-bloom-site. Such eutrophic conditions could have favored the formation of dinoflagellate bloom at site 1 and 2, in line with previous studies demonstrating that HABs have often been associated with or promoted by waste and agricultural water discharge (Mohamed and Al-Sheri, 2012; Díaz et al., 2016; Ibrahim et al., 2021; Sathishkumar et al., 2021). It seemed that decreased salinity in site 1 and 2 in Lake Qarun stemming from increased agricultural runoff from El-Bats drain and El-Wadi drains favored the dinofalgellate proliferation and blooms. This agrees with previous studies, which have reported that low salinity of water stimulates the formation of dinoflagellates blooms in marine and brackish waters (Accoroni and Totti, 2016; Sathishkumar et al., 2021).

4.2. Ichthyotoxicity of dinoflagellate bloom and cultured species

In this study, both intact and cell lysate of dinoflagellate bloom and cultured species, but not cell-free supernatants, were toxic to the brine shrimp *A. salina*, with higher toxicity incurred by lysed cells. The significant increase in toxicity when using lysed algal cells indicates that toxic substances are released upon cell lysis (Mooney et al., 2010; Mardones et al., 2015). Previously, *Artemia* spp. have been widely used in ecotoxicological studies for the determination of general toxicity of harmful microalgae including *H. akashiwo* (Mohamed and Al-Shehri, 2012), *Karlodinium veneficum* and *Ostreopsis* (Yang et al., 2018) and *Amphidinium operculatum* (Mejía-Camacho et al., 2021).

Besides toxicity to *A. salina*, both dinoflagellate bloom and cultured species of Lake Qarun had various ichthyotoxicity to fish gill cells. Gill cells showed lower viability (i.e., high toxicity) upon exposure to algal cell lysate than intact cells. These results agree with several studies reporting that ichthyotoxic microalgae such as the raphidophytes *Chattonella marina, Fibrocapsa japonica* and *Heterosigma akashiwo*, and the dinoflagellates *Alexandrium catenella, Karenia mikimotoi,* and *Karlodinium veneficum, P. micans* were more toxic to fish gill cells after cell lysis than whole living cells (Dorantes-Aranda et al., 2015; Mardones et al., 2020). On the other hand, most cultured species were more toxic towards fish gill cells than natural bloom samples. This could be due to that the natural bloom is composed of various species with different cell densities and different quantities of ichthyotoxins, compared to pure cultures containing only one single species, which produces a fixed amount of ichthyotoxins . However, mesocosm experiments using a coculture of algal species

Table 5
Correlations between fatty acid composition and ichthyotoxicity (expressed as LC_{50}) to *Artemia salina* and fish gill cells during the present study. The number of data points for each parameter is 7.

	18:2ω6, LA	18:3ω3, ALA	18:4ω3, OTA	18:5ω3, OPA	20:5ω3, EPA	22:5ω3, DPA	22:5ω6, DPA	22:6ω3, DHA	Sum SFA	Sum MUFA	Sum PUFA	Fish gill LC_{50}	Fish gill LC_{50}	Artemia LC_{50}	Artemia LC_{50}
18:2ω6, LA	1														
18:3ω3, ALA	0.97907	1													
18:4ω3, OTA	-0.96398	-0.98823	1												
18:5ω3, OPA	0.97201	0.91825	-0.89942	1											
20:5ω3, EPA	-0.81882	-0.87209	0.84703	-0.70299	1										
22:5ω3, DPA	0.93837	0.91338	-0.86724	0.96136	-0.76416	1									
22:5ω6, DPA	0.69606	0.63907	-0.6087	0.80971	-0.25434	0.80154	1								
22:6ω3,DHA	-0.98406	-0.99283	0.99437	-0.92680	0.86299	-0.89613	-0.61870	1							
Sum SFA	0.91552	0.88311	-0.83263	0.88294	-0.78633	0.86845	0.57048	-0.86936	1						
Sum MUFA	0.00680	0.05317	-0.19478	-0.06470	0.11100	-0.25385	-0.11776	-0.11998	-0.21782	1					
Sum PUFA	-0.85523	-0.78210	0.77510	-0.84637	0.56326	-0.71993	-0.57081	0.80286	-0.90798	-0.07770	1				
Fish gill LC_{50}-I	0.94634	0.96715	-0.94027	0.87331	-0.95684	0.90066	0.50268	-0.95907	0.90146	-0.09627	-0.73557	1			
Fish gill LC_{50}-L	0.89319	0.93539	-0.91110	0.77298	-0.96741	0.79502	0.35817	-0.92588	0.85282	-0.02476	-0.68928	0.97178	1		
Artemia LC_{50}-I	0.86223	0.83057	-0.79173	0.88552	-0.81862	0.93701	0.60725	-0.82982	0.82484	-0.32190	-0.64241	0.89264	0.77200	1	
Artemia LC_{50}-L	0.89613	0.85532	-0.85222	0.93226	-0.75306	0.92462	0.67892	-0.87521	0.79378	-0.08765	-0.70795	0.86568	0.73422	0.962635	1

Fish gill LC_{50}-I, means LC50 of intact algal cells to fish gill cells; Fish gill LC_{50}-L, means LC50 of algal cell lysate cells to fish gill cells; Artemia LC_{50}-I, means LC_{50} of intact algal cells *Artemia salina*, Artem ia LC_{50}-L, means LC_{50} of algal cell lysate cells to *Artemia salina*. All abbreviations of fatty acids are listed in Table 3.

with fish is needed to test whether fish can induce the algal cells to produce such potent ichthyotoxins. Ichthyotoxicity of microalgae to fish gill cells could be attributed to the production of polyunsaturated fatty acids (PUFA) (Mooney et al., 2010), reactive oxygen species (ROS) (Marshall et al., 2003), chemically defined phycotoxins (Van Deventer et al., 2012) or synergistic effect of PUFA and ROS (Dorantes-Aranda et al., 2015; Mardones et al., 2015, 2021). As the most common phycotoxins including diarrhetic shellfish toxins (e.g., OA and DTXs) and paralytic shellfish toxins (e.g., saxitoxins) were not detectable by ELISA in dinoflagellate bloom or cultured species of Lake Qarun, the ichthyotoxicity of these microalgae could be attributed to uncharacterized ichthyotoxins. Similarly, previous studies demonstrated that some harmful microalgae such as *Alexandrium catenella* and *Prorocentrum concavum,* which could not produce saxitoxins or okadaic acid (OA) had severe ichthyotoxic effects against *A. salina* and fish gill cells (Mardones et al., 2015; Zou et al., 2020). Therefore, those authors suggested the involvement of uncertain substances rather than such known phycotoxins in the ichthyotoxiicty of these harmful microalgae.

Regarding ichthyotoxic substances, several studies have recognized the role of PUFA as a known source of ichthyotoxicity of harmful microalgae (Mardones et al., 2015; Dorantes-Aranda et al., 2015; Mardones, 2020; Zou et al., 2020). In our study, ichthyotoxicity of bloom and cultured dinoflagellates isolated from Lake Qarun correlated markedly with the total percentage of PUFA in these algae. In particular, this ichthyotoxicity showed greater correlation with the percentages of the fatty acids; OTA, EPA and DHA in microalgae, indicating the role of these fatty acids in ichthyotoxicity of these algal species to *Artemia* and fish gill cells. These three fatty acids have been previously reported as the major contributors to PUFA produced by ichthyotoxic dinoflagellates and raphidophytes (Dalsgaard et al., 2003; Evjemo et al., 2008; Hyun et al., 2016; Dorantes-Aranda et al., 2013, Mooney et al., 2011, Mardones et al., 2021). Although, the exact mechanism of ichthyotoxicity of PUFA is still fully unclear, the synergistic reaction between ROS and PUFA has been suggested as a primary ichthyotoxic mechanism (Marshall et al., 2003; Mardones et al., 2015). Due to their high degree of unsaturation (Else and Kraffe, 2015), PUFA can be readily oxidized in the presence of high levels of ROS producing other toxic compounds via lipid peroxidation (e.g., aldehydes and lipid radicals), which can cause physiological changes in gill cells by increasing superoxide dismutase activity, and damaging gill cell membranes (Dorantes-Aranda et al., 2015). However, it has been found that PUFA (e.g., EPA) on its own can cause fish mortality, but its combination with ROS accelerated fish mortality (Marshall et al., 2003; Shikata et al., 2021). On the other hand, ROS on their own exhibited weak ichthyotoxicity to fish (Marshall et al., 2003; Mooney et al., 2011).

5. Conclusions and recommendations

The present study has documented the occurrence of dinoflagellate bloom in Lake Qarun and confirmed its ichthyotoxicity. The bloom was confined to two sites

nearby agricultural drains rather than the site not-exposed to any discharge. The intensity of the bloom correlated with high nutrient concentrations and low salinity in bloom sites due to agricultural water from close drains.

Both bloom and cultured species of dioflagellates exhibited severe toxicity to *A. salina* and fish gill cells with varying extents. No phycotoxin such as saxitoxin and okadaic acid was detected in these algae. Instead, the ichthyotoxicity of these microalgae were highly correlated with the contents of PUFAs, particularly OTA, EPA and DHA. As a similar dinoflagellate bloom was observed in 2018 (Ibrahim et al., 2021), it is reasonable to assume that Lake Qarun will also show dinoflagellate blooms in the future. Taken into account that climate change would have an effect on rainfall and thus increasing nutrient input (Trainer et al., 2020), agricultural discharge to the lake from nearby drains should receive a high priority as a bloom management strategy for Lake Qarun. Also, early warning and monitoring program for phytoplankton abundance in Lake Qarun should also include: (1) determination of known phycotoxins by biochemical and analytical techniques (e.g., ELISA and HPLC), and (2) evaluating the ichthyotoxicity of dinoflagellate and other potential HAB species using the fish gill cell line assay, as it has proved to be a promising new tool for ichthyotoxic assessment of fish killing microalgae (Mardones et al., 2020). This program could assist in understanding the relationship between ichthyotoxic HABs and fish mortality in the lake and generation of scientific knowledge to better prevent, control and mitigate HAB occurrence and maintain fisheries and safeguarding seafood safety.

Authors Contributions

All authors participated in experimental design and relevant analysis. All authors also took part in data analysis and interpretation, read and approved the final manuscript.

Ethical Statement

The authors confirm that neither the manuscript nor any parts of its content are currently under consideration or published in another journal

Declaration of Competing Interest

The authors declare that there is no conflict of interest.

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