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Co-Occurrence of Cyanotoxins and Phycotoxins in One of the Largest Southeast Asian Brackish Waterbodies: A Preliminary Study at the Tam Giang—Cau Hai Lagoon (Vietnam)

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Abstract: The Tam Giang-Cau Hai lagoon (TGCH) in Thua Thien Hue province (Vietnam) is a marsh/lagoon system and ranks among the largest waterbodies in Southeast Asia. It plays a significant role in terms of both socio-economic and environmental resources. However, anthropogenic stress, as well as the discharge of untreated domestic and industrial sewage with agricultural runoff from its three major tributaries, dramatically damages the water quality of the lagoon. Especially after heavy rain and flash floods, the continuous degradation of its water quality, followed by harmful algal and cyanobacterial bloom patterns (HABs), is more perceptible. In this study, several physicochemical factors, cyanotoxins (anatoxins (ATXs), saxitoxins (STXs), microcystins (MCs)), phycotoxins (STXs, okadaic acid (OA), and dinophysistoxins (DTXs)) were analyzed in water and shellfish samples from 13 stations in June 2023 from 13 stations, using enzyme-linked immunosorbent assay (ELISA) kits for the ATXs and STXs, and the serine/threonine phosphatase type 2A (PP2A) inhibition assay kit for the MCs, OA, and DTXs. The results showed for the first time the co-occurrence of freshwater cyanotoxins and marine phycotoxins in water and shellfish samples in this lagoon. Traces of ATXs and STXs were detected in the shellfish and the orders of magnitude were below the seafood safety action levels. However, toxins inhibiting the PP2A enzyme, such as MCs and nodularin (NODs), as well as OA and DTXs, were detected at higher concentrations (maximum: 130.4 µg equiv. MC-LR/kg shellfish meat wet weight), approaching the actionable level proposed for this class of toxin in shellfish (160 µg of OA equivalent per kg of edible bivalve mollusk meat). It is very important to note that due to the possible false positives produced by the ELISA test in complex matrices such as a crude shellfish extract, this preliminary and pilot research will be repeated with a more sophisticated method, such as liquid chromatography coupled with mass spectroscopy (LC-MS), in the upcoming research plan.

Keywords: cyanotoxins; Tam Giang-Cau Hai lagoon (Vietnam); microcystins; anatoxins; saxitoxins; okadaic acid; dinophysistoxins; contaminated shellfish

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1. Introduction

Eutrophication is an increasingly common issue in continental freshwater resources, including natural lakes, dams, and rivers, as well as brackish waters in lagoons and the coastal areas of oceans [1,2]. The anthropogenic loading of nutrients (particularly nitrogen

and phosphorus) derived from urban, industrial, and agricultural runoff are the primary causes of eutrophication in these aquatic ecosystems. Coupled with global warming, these eutrophic conditions provide ideal opportunities for certain toxic species of cyanobacteria and dinoflagellates to form significant seasonal blooms [3–9]. The problems associated with these harmful algal blooms (HABs) in different brackish and fresh waters are diverse, from hypoxia due to the excessive consumption of oxygen to purely aesthetic problems in recreational areas, when blooms appear in the form of colorful and often smelly scums on the water surface. Connected to these common problems, there is the production of various cyanotoxins and phycotoxins by certain species of cyanobacteria and dinoflagellates, respectively. These toxins can include cyanobacterial hepatotoxins, such as microcystins (MCs) and nodularin (NODs), neurotoxins (anatoxins (ATXs), gaunyolotoxin (GTX), and saxitoxins (STXs), as well as phycotoxins, such as okadaic acid (OA), dinophysistoxins (DTXs), yessotoxins, saxitoxins (STXs), and pectinotoxins [10,11]. These toxins have adverse effects on aquatic fauna and flora, as well as on animal and human health [12–19]. As a result, thousands of intoxication episodes and fatalities are annually recorded worldwide due to marine phycotoxins [20]. Additionally, the incidence of toxic outbreaks, associated with certain species of toxic cyanobacteria in marine and lagoon ecosystems, has recently increased in some regions [21].

The prevalence of cyanobacterial blooms in aquatic environments generally follows the hierarchy of water resources: freshwater > estuarine/brackish water > marine systems [22]. Cyanobacterial blooms in estuarine and marine environments are limited to only a few taxa, *Nodularia*, *Aphanizomenon*, *Dolichospermum*, *Trichodesmium*, *Lyngbya*, and *Schizothrix*, which are the most commonly observed taxa associated with health hazards [6,23–25]. For example, the pelagic genera *Nodularia* and *Aphanizomenon* dominate inshore low-salinity regions, such as the Baltic Sea and the Peel-Harvey estuary [26], and *Trichodesmium* forms extensive blooms in tropical and subtropical areas, in the Indian and Pacific oceans [6,27–29]. In general, low nitrogen concentrations and N/P ratios are assumed to be the main drivers favoring cyanobacteria dominance in the surface waters of the stratified Baltic Proper and Gulf of Finland [30–32]. Although similar conditions to these can occur in some coastal lagoons, no data on the blooms of cyanobacterial species in Vietnamese brackish water ecosystems have been reported. However, the diversity of cyanobacterial species and MCs, as well as their bioaccumulation in the visceral organs of aquatic organisms, have been outlined in some freshwater reservoirs in Vietnam [33–39]. Exposure to phycotoxins produced by harmful algal blooms through the consumption of shellfish was well recognized in the marine environment [40,41]. Among the toxin types involved, some are comparable to toxins produced by cyanobacteria (like saxitoxin derivatives, which cause paralytic shellfish poisoning (PSP)), and others are different but with the same molecular targets (like the inhibition of serine/threonine phosphatases by both okadaic acid and its derivatives, as well as MCs and NODs) [42]. Human poisoning by phycotoxins present in marine shellfish is caused by the biomagnification of these toxins in the food chain. This risk of biomagnification and the resulting human poisoning must also be assessed for cyanotoxins. Phycotoxins and cyanotoxins can also simultaneously co-occur in the same seafood samples to affect human health. For example, the co-occurrence of freshwater cyanotoxins, such as MCs, and marine phycotoxins, such as diarrheic shellfish toxins (DSTs), paralytic shellfish toxins (PSTs), and domoic acid (DA), have been reported in mussels [43]. Therefore, there is a need to better understand how the bioaccumulation of multiple toxins in shellfish, naturally grown or cultivated in the lagoon ecosystem may affect seafood security, which is still lacking awareness and policy in this country.

The Tam Giang-Cau Hai (TGCH) lagoon in Thua Thien Hue province (Vietnam) is among the largest marsh/lagoon systems in Southeast Asia, with an area of 22,000 hectares, spanning a length of 70 km and with a width varying from 0.6 to 1.4 km. The lagoon coastal area, which is of high ecological and socio-cultural importance, is very vulnerable due to the discharge of nutrient-laden water from three main related tributaries. Untreated domestic and industrial wastewater and agricultural runoff discharged into this lagoon

could thus trigger the proliferation of harmful algae, particularly cyanobacteria. Due to the increasing frequency, duration, and intensity of HABs in this lagoon, it is imperative to identify toxic dinoflagellates and cyanobacterial species and characterize their related toxins. Therefore, our international research program for this lagoon and HABs monitoring are established, for two reasons. Firstly, to seek the reactivation of the past linkages between Dalhousie (Canada) and Hue (Vietnam) Universities, which was started, from 2004–2009 by a CIDA-funded capacity-building project focused on ocean and coastal governance. Secondly, to a multidisciplinary approach integrating the various disciplines, including toxicology and ecotoxicology, ecology, analytical chemistry and public health. The aims of the research program are as follows: (1) To determine the correlations between HABs, their proliferation, and cyanotoxin and phycotoxin production; (2) to establish the transfer and bioaccumulation of cyanotoxins and phycotoxins in different shellfish species caught in the Tam Giang lagoon; and (3) to open new opportunities for research, in collaboration with local researchers in environmental sciences, biology and aquaculture, with a systematic monitoring research program for this lagoon. The overall objective of the current paper is to identify the co-occurrence of cyanotoxins and phycotoxins in the lagoon water and aquatic produce, such as fishes and shellfish, in order to improve water management and to harmonize capabilities for their risk management in terms of public health, from both conceptual (mechanistic) and empirical (quantitative) perspectives.

2. Materials and Methods

Water samples from the lagoon were collected on 6th and 7th of June 2023 over thirteen sites (TC1 to TC13) (Figure 1). The TGCH system forms a unique brackish water ecosystem, with a diversity of aquatic species [44]. On its eastern side, the lagoon is separated from the Eastern Sea, Bien Dong (but named as the South China Sea on Google Maps), by sandy dunes with two openings, Thuan An and Tu Hien (Figure 1), while on the western side of the lagoon are rice fields and river estuaries. The average depth of the waterbody is 2 m, although along its length, there runs a channel 3 m to 4 m deep. The Thuan An estuary is the deepest, at than 7 m. The water merges with the sea through the two above-mentioned openings on the eastern side, and receives water from several rivers, namely Huong, Bo, Dai, O Lau, and Truoi [45]. These rivers receive untreated domestic and industrial wastewater and agricultural runoff, which directly discharges into the lagoon, particularly after heavy rains and flash floods, thus contributing to its eutrophication and the HAB apparition. The communities surrounding the coast exploit the natural resources of the lagoon by fishing and other aquacultural activities. The catch fisheries are based on fixed fishing gear groups, with larger capital investments, and mobile fishing gear groups, made up of poorer fishermen [46,47]. The unique topography and aquatic conditions of the lagoon make it favorable for the construction of aquaculture ponds and net enclosures in most parts of the lagoon [48]. Aquaculture methods involve ponds, net enclosures, and cages for the aquaculture production of shrimps, crabs, rabbit fish, grouper, and seaweed. The sampling sites were therefore selected based on the salinity gradient of the lagoon.

2.1. Field Handling Methods and Sample Collection

For each sampling site (TC1 to TC13), several abiotic variables, such as water temperature (°C), pH, dissolved oxygen (mg/L), turbidity (NTU), salinity (PSU), and conductivity (mS/m) were measured in situ at the subsurface (1 m depth) using a multi-parameter water-quality meter HORIBA U5000 (HORIBA, Ltd., Suppl. Head Office/Factory 2 Miyanohigashi, Kisshoin Minami-ku, Kyoto 601-8510, Japan). Water depth (m) and transparency (Secchi depth) were also measured in situ at each sampling site. Water samples from each sampling site were collected in a 0.5 L clean plastic bottle in duplicate and stored in a dark and fresh cooler to be transported to the laboratory for nutrient, algal pigment (chlorophyll-a and phycocyanin), and toxin (cyanotoxins and phycotoxins) analysis. For taxonomy and cell counts of phytoplankton, 15 mL water vials were collected and fixed with a Lugol acid solution. A quantity of 1 kg of samples of different batches (1 to 7) of shellfish (oysters,

clams, and mussels) was also obtained from different vendors at a local market located on the shore of the lagoon (Figure 2). Different shellfish samples were collected in the lagoon from the TC 13 site (Figure 1).

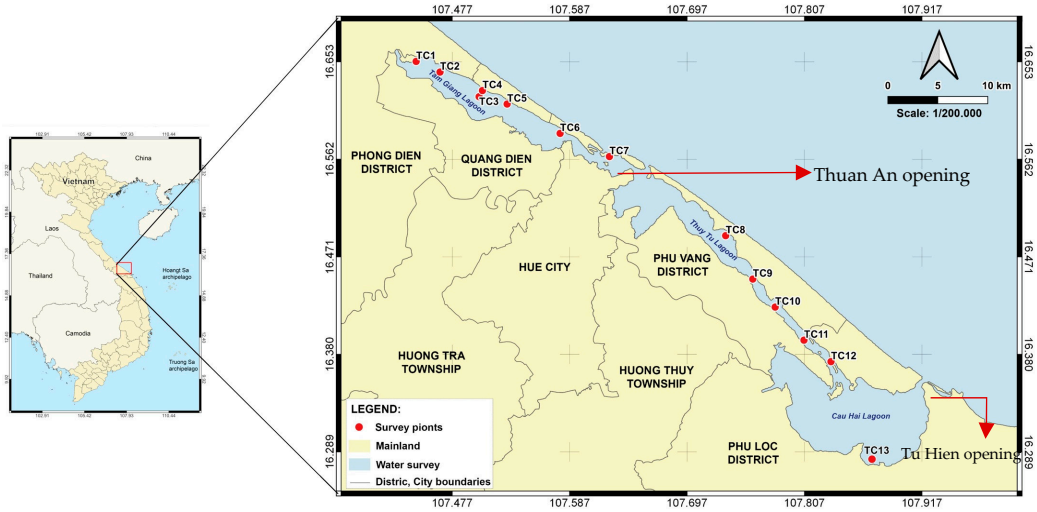

Figure 1. Sampling locations with a zoomed view of Tam Giang lagoon, Vietnam.

Figure 2. A local market in the fishery village at Tam Giang lagoon. Below: Seven different batches of shellfish samples collected from the lagoon at TC 13, including: (1) *Cyrenobatissa subsulcata*; (2) *Corbicula subsulcata*; (3) *Cristaria plicata*; (4) *Pila polita*; (5, 6) *Crasscostrea rivularis*; (7) *Perna viridis*.

2.2. Laboratory Analytical Methods

2.2.1. Nutrients and Chlorophyll-a and Phycocyanin Analysis

For each sampling site, several chemical variables, such as nitrate (NO_3-N), nitrite (NO_2-N), ammonium (NH_4-N), total phosphorus (TP), dissolved phosphorus (DP), chlorophyll-a, and phycocyanin were measured at the Environmental laboratory (Hue University of Science, Vietnam) following standard operating procedures. Ammonium (NH_4-N) was analyzed using an un-filtered water sample by the o-phenyl phenol method (OPP Method), as directed in the standard approach [49]. The analysis of nitrates + nitrites was conducted with the photometric method using N-(1-naphthyl)-ethylenediamine dihydrochloride, as directed by standard method [50]. Nitrate nitrogen was determined by applying the salicylate method, as directed by Rump and Krist (1988) [51]. Dissolved phosphorus (DP) and TP were measured in the filtered water by another method [52].

Ascorbic acid and persulfate methods were used as directed [52] to quantify dissolved phosphorus (DP) and total phosphorus (TP). The reaction of formation of molybdenum-blue complex in acid conditions is the basis of the phosphorus detection. TP was converted to phosphate–phosphorus using ammonium persulfate digestion in the presence of acid (sulfuric acid). The limit of detection for ammonium is 0.001 mg/L; for nitrates + nitrites, DP, and TP, it is 0.005 mg/L.

For pigments such as chlorophyll-a (Chl-a) and phycocyanin (PC), water samples (200 mL) were filtered on GF/C filters. The filters were then dried using an absorbent paper and placed in a 15 mL covered polypropylene centrifuge tube to avoid photodegradation. For the phycocyanin, filters were extracted for 16 h at 4 °C with 10 mL in a saline buffer and determined according to the method described by Yepremian et al. (2017) [53].

$$\text{Phycocyanin (mg/L)} = \frac{(\text{Abs}_{620} - 0.7 \times \text{Abs}_{650}) \times \text{Ve}}{7.38 \times \text{Vs} \times \text{I}} \quad (1)$$

where the following apply:

- Ve = volume of buffer extract in mL.
- Vs = volume of water sample in Liters.
- I = path length of cuvette in cm.

For the Chl-a, filters were extracted with 10 mL of 90% acetone and were then centrifuged. The concentration in the supernatant was calculated using the following equation [54]:

$$\text{Chl-a} = \frac{[11.64(\text{Abs}_{663}) - 2.16(\text{Abs}_{645}) + 0.10(\text{Abs}_{630})]\text{E(F)}}{\text{V(L)}} \quad (2)$$

where the following apply:

- F = Dilution factor (i.e., if the 663 Abs is >0.99 with the 1 cm cell, diluting, re-analyzing, and inserting the dilution factor in the equation).
- E = The volume of acetone used for the extraction (mL).
- V = The volume of water filtered (L).
- L = The cell path length (cm).

The limit of detection for Chl-a is 0.05 µg/L, and for PC, it is 0.04 µg/L.

2.2.2. Phytoplankton Diversity

The taxonomic identification of phytoplankton in all sampling sites was performed by direct light microscopy analysis of the Lugol-preserved samples [55] at the Environmental laboratory (Hue University of Science, Vietnam). The identification was carried out at the lowest possible taxonomic level using morphological criteria.

2.2.3. Toxins Analysis in Water and Shellfish Samples

The presence of cyanotoxins (anatoxins, saxitoxins, and microcystins) and phycotoxins (saxitoxins and okadaic acid and its derivatives) in water and shellfish samples was analyzed in duplicate. Enzyme-linked immunosorbent assay (ELISA) kits were used for anatoxins and saxitoxins analysis and the serine/threonine phosphatase type 2A (PP2A) inhibition assay kit was used for MCs and okadaic-acid-like toxins analysis according to the manufacturer's provided protocol. Briefly, for analysis of different classes of toxins in water samples, an aliquot of 200 mL of water samples was filtered through a glass microfiber filter (GF/C, Whatman from Avantor, Mount Royal, QC, Canada) to separate out the toxins dissolved in water (dissolved toxins) and those associated with cyanobacterial cells (0.5–60 µm) and/or adsorbed on particles (particulate toxins). The filters were extracted with 10 mL of 75% (v/v) methanol/water and were then centrifuged. The toxin fractions in each filtrate and in each methanol filter extract were then analyzed using the PP2A inhibition assay kit for microcystin-like toxins and ELISA kits for anatoxins and saxitoxins. For analysis of toxins in each dead shellfish sample, the whole flesh tissue

was removed from the shell and rinsed with freshwater, dried with filter paper, and then cut into small pieces using scissors. An aliquot (5 g) was then extracted with 5 mL of 75% (*v*/*v*) methanol/water, centrifuged, and used for analysis of the different classes of toxins by ELISA kits for ATXs and STXs and PP2A kits for microcystin-like toxins (MCs, NODs, OA, and DTXs). The kits were purchased from Gold Standard Diagnostics Horsham Inc.(Warminster, PA, USA) (formerly Eurofins Abraxis) and were stored at 4 °C before analyzing, which were conducted before expiration dates. A standard calibration curve was constructed with microcystin-LR (MC-LR) standard, saxitoxin (STX) standard, and anatoxin-a (ATX-a) standard for each cyanotoxin class, and results were fit to the curve. The control analysis for saxitoxin and anatoxin-a was within the accepted ranges indicated by the manufacturer of kits (e.g., for saxitoxin, these were 0.075 µg/L certified, and 0.061 µg/L calculated, and for anatoxin-a, these were 0.75 µg/L certified, and 0.77 µg/L calculated). For toxins inhibiting PP2A (microcystins and okadaic acid-like toxins), saxitoxins, and anatoxins, results were expressed as MC-LR equivalent/L or kg, STX equivalent/L or kg, and ATX-a/L of kg, respectively. The detection limits for the PP2A assay and ELISA kit values for STXs and ATXs were 0.1, 0.005, and 0.1 ng/mL, respectively.

3. Results

3.1. Physical Chemical Parameters

Figure 3A shows that moving from station 1 towards station 13, the water becomes more turbid, i.e., less transparent. The salinity of the water is indicated in Figure 3B, which is in concurrence with our sampling sites, implying an increase in the salinity as we traverse towards the marine region in the lagoon from fresh water. However, the pH remained uniformly level throughout the stations, i.e., neutral to slightly basic (7.4–8.45).

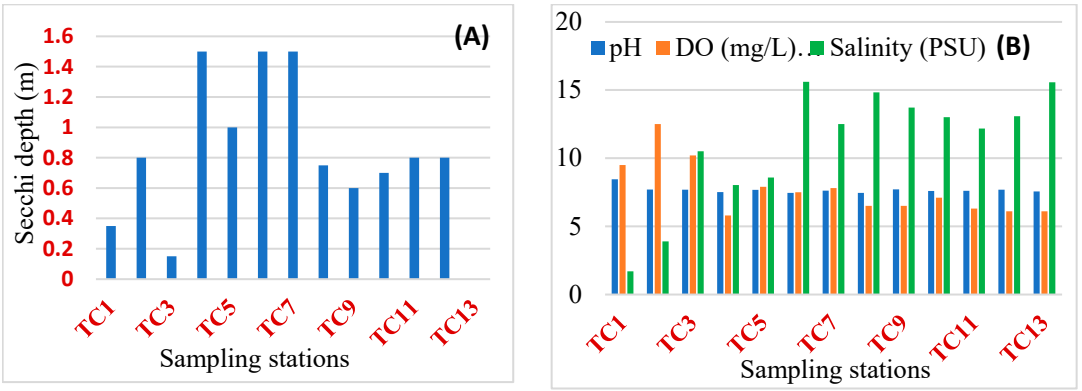

Figure 3. (**A**) Secchi depth from Stations TC1 to TC13; (**B**) trend of dissolved oxygen (DO), pH, and Salinity in TG-CH Lagoon.

Table A1 (in Appendix A.1) shows some of the physical and chemical parameters of thirteen collected samples. It was observed that our sampling locations have a positive gradient of salinity from low (TC1) to high, with the highest at TC13, which means that our sampling trend is from the quasi-freshwater inland to the brackish water in the coastal area. The water temperature at all points was above 30 °C. The dissolved oxygen (DO) values, ranging from 5.8 to 12.5 mg/L, were quite high at just three locations, which are more inland (TC1, TC2 and TC3), but very moderate and even low at many others, with the exception at TC4, where the DO was very low, 5.8 mg/L. The Chl-a values of six different locations (TC1, TC2, TC4, TC6, TC7, and TC10), which were very close to or above 10 mg/L, indicate that there was a possibility of algal bloom exposure at these sites (although we did not actually observe any actual blooms during our field trips.)

Based on the total phosphorus concentration (TP) of the water, according to OECD (1982), it is classified as eutrophic [56]. The TP content varied from 0.042 to 0.106 mg/L, with an average value of 0.069 mg/L. According to Carlson's trophic state index (CSTI) [57],

which is a longstanding measure to assess and classify the trophic systems of water bodies based on their transparency (see Appendix A.2), total phosphorus, and Chl-a contents, most of the sampling stations are eutrophic, with CSTI values between 60 and 70 (Table A2 in Appendix A.2).

3.2. Abundance of Phytoplankton Species

A total of 27 phytoplankton taxa were identified in samples from 13 stations in the Tam Giang lagoon in June 2023. Figure 4 represents the relative abundance of the six phytoplankton phyla, including Bacillariophyta, Cyanophyta, Dinophyta, Chlorophyta, Ochrophyta, and Euglenophyta, observed in this study. Table 1, below, shows a list of the identified genera for each phylum. Bacillariophyta (diatoms) was the most abundant taxa recorded (16 taxa), indicating an abundance of 68% in the water samples, followed by Cyanophyta (13%), with four taxa. Dinophyta (8%) and Chlorophyta (5%) were represented by two and three taxa, respectively. Ochrophyta (3%) and Euglenophyta (3%) were represented by only one taxon. For Cyanophyta, four potentially toxigenic genera (*Dolichospermum* sp., *Raphidiopsis raciborskii*, *Pseudanabaena* sp., and *Oscillatoria* sp.), which can produce different classes of cyanotoxins, such as microcystins (MCs), anatoxins (ATXs), saxitoxins (STXs), and Cylindrospermopsin (CYNs), were identified in the water samples of this lagoon. However, only one potentially toxigenic species (*Dinophysis* sp.) was identified for the phylum Dinophyta. This species is known to produce diarrheic toxins, such as okadaic acid (OA) and dinophysistoxins (DTXs), in brackish and marine environments.

Table 1. Dominant phytoplankton genera observed at the 13 sites in the Tam Giang lagoon (Vietnam) during June 2023.

Phylum	Genera
Bacillariophyta (diatoms)	*Amphiphrora* sp., *Aulacoseira granulata*, *Bacillaria paxillifera*, *Chaetoceros* sp., *Coscinodiscus radiates*, *Cylindrotheca closterium*, *Cymbella* sp., *Guinardia flaccida*, *Leptocylindrus* sp., *Melosira* sp., *Navicula* sp., *Nitzschia* sp., *Pleurosigma angulatum*, *Rhizosolenia* sp., *Tabellaria* sp., *Thalassionema frauenfeldii*
Cyanophyta	*Anabaena* (actually *Dolichospermum*) sp., *Raphidiopsis raciborskii*, *Pseudanabaena* sp., *Oscillatoria* sp.
Dinophyta	*Protoperidinium* sp., *Dinophysis* sp.
Chlorophyta	*Chlamydomonas* sp., *Closterium* sp., *Desmidium baileyi*
Ochrophyta	*Dinobryon sertularia*
Euglenophyta	*Euglena viridis*

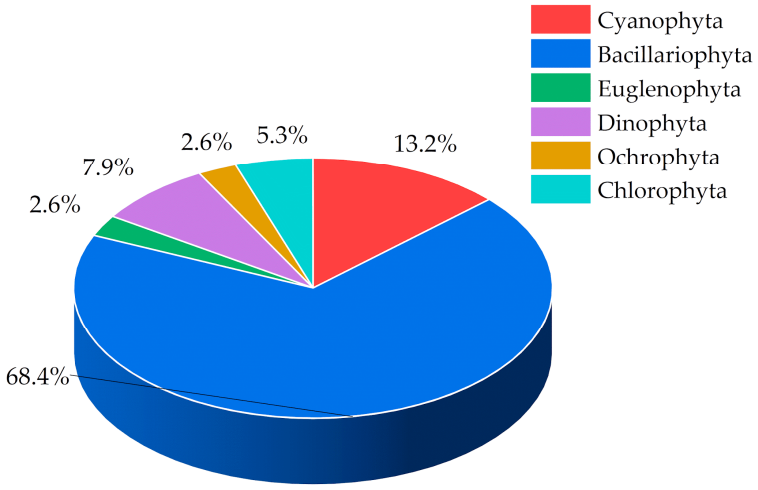

Figure 4. Relative abundances of phytoplankton from all phyla identified at 13 sites in the Tam Giang lagoon (Vietnam) during June 2023.

3.3. Toxin Concentrations in Water Samples

Lagoon water samples from thirteen sites (TC1 to TC13) were analyzed for the presence of neurotoxins, such as ATXs and STXs, and for toxins inhibiting the PP2A, such as MCs, NODs, OA, and DTXs. For each class of toxins, measurable concentrations (dissolved + particulate) were detected at these 13 sampling sites. As indicated in Figure 5, the total (dissolved + particulate) concentrations of STXs ranged from 0.056 (TC7) to 1.76 µg/L (TC2), with the highest concentrations observed at sites TC2 (1.76 µg/L) and TC12 (1.74 µg/L). The dissolved STX concentrations were always lower than the particulate concentrations, with the proportion in the surface water samples at all the sites never exceeding 50% of the total concentrations, except for at sites TC7 (53.2%), TC10 (53.5%), and TC13 (58.8%). The total (dissolved + particulate) concentration of the specific cyanobacterial ATXs at all the sampling sites was higher than those of the STXs, ranging from 1.85 (TC6) to 6.25 (TC4) µg/L (Figure 5). The highest total concentration was observed at site TC4 (6.25 µg/L), which was forty six times higher than the corresponding STXs concentration. Figure 6 indicates that the dissolved ATX concentrations were always lower than the particulate concentrations, with the proportions in the surface water samples from all the sites never exceeding 6.5% (TC5) of the total concentration. For the toxins inhibiting the PP2A (Figure 7), the total (dissolved + particulate) concentration ranged from 0.85 (TC4) to 1.39 (TC11) µg/L. However, the dissolved concentrations of this class of toxins were higher than those of the two other toxin classes, ranging from 26.8 (TC9) to 50.7% (TC5).

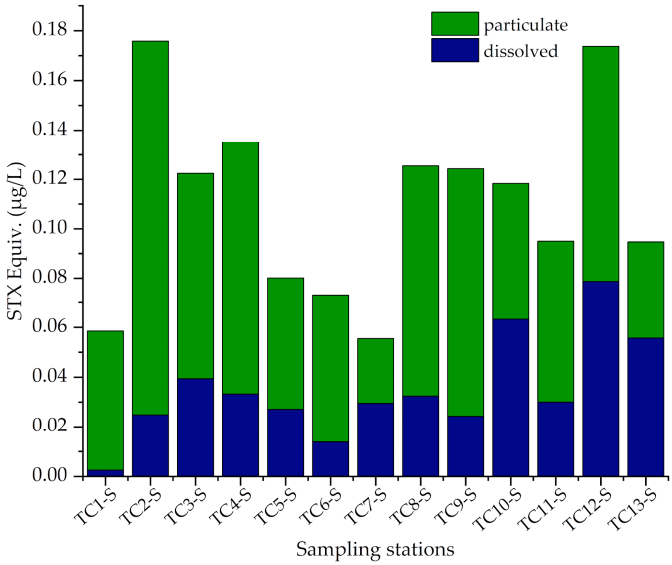

Figure 5. Concentrations (µg/L) of paralytic shellfish toxins (PSTs) expressed as saxitoxin (STX) equivalent in water samples collected from the different sites (TC1 to TC13) in the Tam Giang lagoon, Vietnam. The regulatory guidance level for saxitoxin is 3 µg/L in drinking water and 30 µg/L in recreational water.

3.4. Toxin Levels in Shellfish Samples

Seven different batches of shellfish (two of mussels, two of oysters, and three of clams) samples (Figure 2, lower panel) were collected from the TGCH lagoon and analyzed to detect cyanobacterial anatoxins (ATXs), cyanobacterial or/and dinoflagellates, paralytic shellfish toxins, such as saxitoxins (STXs),e cyanotoxins (MCs and NODs), or/and phycotoxins (OA and DTXs), which inhibit the serine/threonine protein phosphatase type 2A (PP2A). Each sample was analyzed without a hydrolysis step to determine the level of free toxins only. The levels of each class of toxins are shown in Figures 8–10. The analysis of all the shellfish samples revealed that they contained quantifiable levels of saxitoxins, ranging from 0.0496 to 0.308 µg/kg (Figure 8), anatoxins, ranging from 4.48 to 8.09 µg/kg

(Figure 9), and cyanotoxins or/and phycotoxins inhibiting the PP2A, ranging from 112.68 to 130.4 µg/kg (Figure 10).

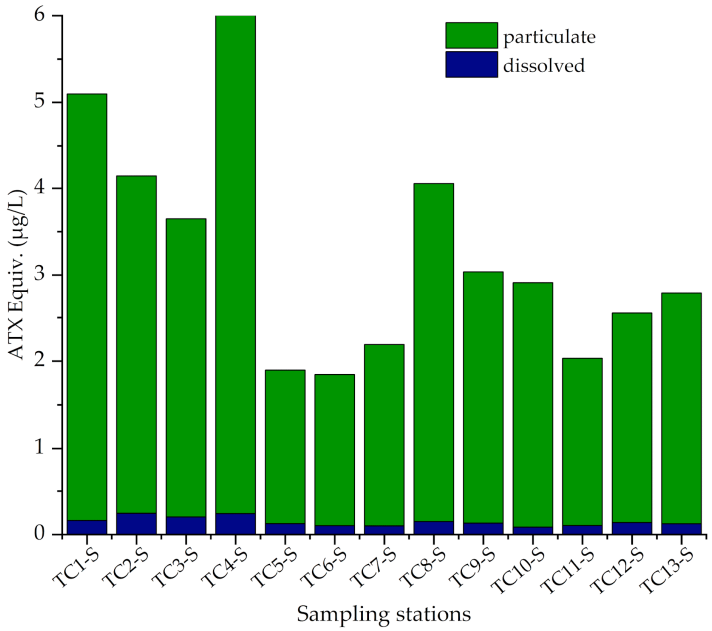

Figure 6. Concentrations (µg/L) of anatoxins (ATXs) expressed as anatoxin-a (ATX-a) equivalent in water samples collected from the different sites (TC1 to TC13) in the Tam Giang lagoon, Vietnam. The regulatory guidance level for ATX-a is 30 µg/L in drinking water and 60 µg/L in recreational water.

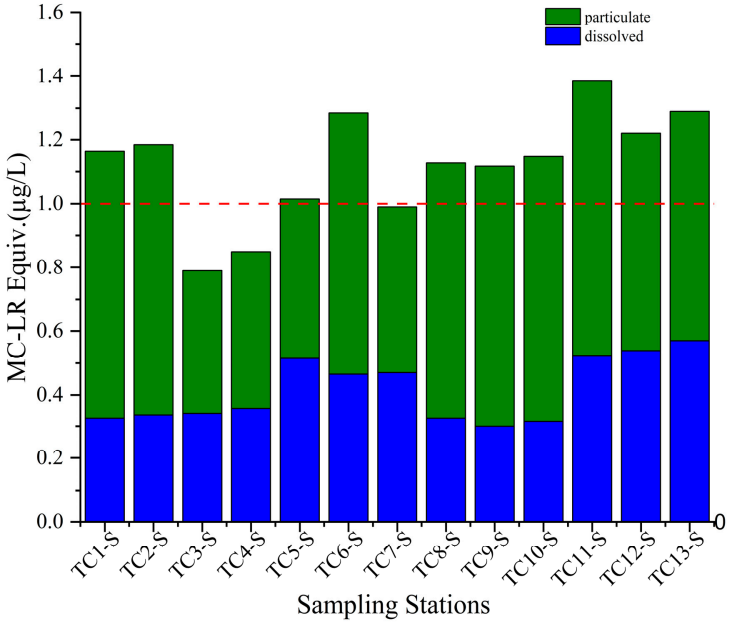

Figure 7. Concentrations (µg/L) of toxins inhibiting the PP2A, such as microcystins (MCs), nodularin (NODs), okadaic acid (OA), and dinophysistoxins (DTXs), expressed as microcystin-LR (MC-LR) in water samples collected from the different sites (TC1 to TC13) in the Tam Giang lagoon, Vietnam. The regulatory guidance level for MC-LR is 1 µg/L in drinking water (dotted red line) and 24 µg/L in recreational water.

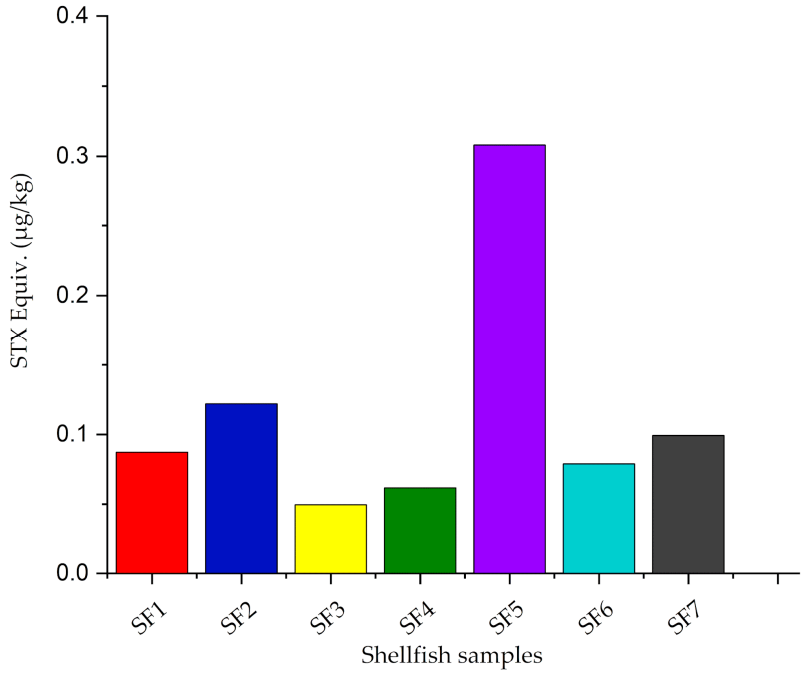

Figure 8. Levels (µg/kg) of paralytic shellfish toxins (PSTs) expressed as saxitoxin (STX) equivalent in shellfish samples collected from the Tam Giang lagoon, Vietnam. The regulatory guidance level is 800 µg STX equivalent/kg.

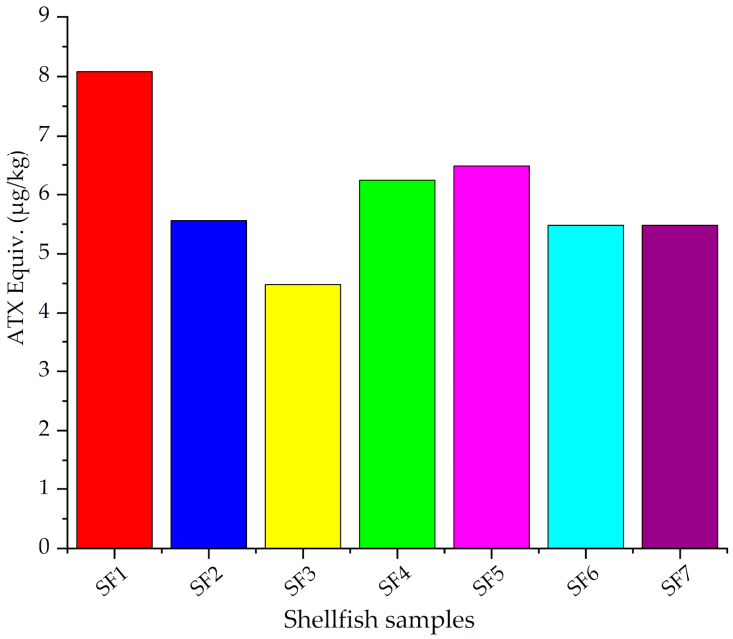

Figure 9. Levels (µg/kg) of anatoxins (ATXs) expressed as anatoxin-a (ATX-a) equivalent in shellfish samples collected from the Tam Giang lagoon, Vietnam. No regulatory guidelines are mentioned for ATX in shellfish samples.

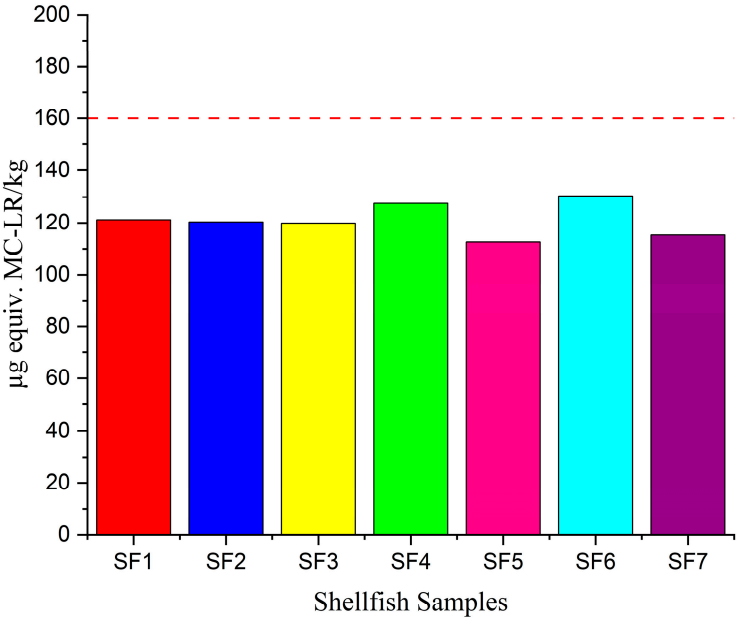

Figure 10. Levels (µg/kg) of toxins inhibiting the PP2A, such as microcystins (MCs), nodularin (NODs), okadaic acid (OA), and dinophysistoxins (DTXs), expressed as microcystin-LR (MC-LR) equivalent in shellfish samples collected from the Tam Giang lagoon, Vietnam. The regulatory guidance level (dotted red line) for diarrheic toxins in shellfish is 160 µg of OA equivalent per kg of edible bivalve mollusk meat, by total amounts of OA, DTXs, and pectenotoxins.

4. Discussion

This is the first-ever comprehensive examination of phycotoxin and cyanotoxin concentrations and their co-occurrence in water and shellfish samples in the TGCH Lagoon (Vietnam). Shellfish consumption in coastal regions of Vietnam is high compared to consumption levels in other countries. The mean consumption rates for bivalves, crustaceans, gastropods, cephalopods, echinoderms, and all shellfish combined were 39.3, 20.9, 16.4, 11.2, 0.3, and 88.1 g/person/day, respectively [58]. These shellfish, growing in different lagoons throughout the coastal areas of Vietnam, from North to South, can be easily purchased at markets or from vendors, and they are mostly consumed all year round in Vietnam. Shellfish, like various samples we purchased during our field trip for this study from a local market on the shore of the Tam Giang lagoon (Figure 2), are unfortunately often sold without being checked for their quality and contamination status regarding toxins such as phycotoxins and cyanotoxins. Water from lagoons is presently well-known worldwide as being affected by the presence of HAB and cyanobacterial blooms due to the increasing eutrophication from various anthropogenic activities, as well as the inflow of nutrients from surrounding watersheds. This issue is further exacerbated by climate change and global warming effects. Therefore, our current study is indispensable for the TGCH communities and for the entire Thua Thien Hue province. It can help to create awareness among the population of cyanobacteria and dinoflagellates diversity, as well as their toxin profiles in the water at distinct locations mainly used for aquaculture. This study, when it reaches a wider audience, will increase the knowledge of communities about the bioaccumulation of these toxins in their aquatic products and food consumption. The preliminary results serve to create a basis for making recommendations to manage water quality, its monitoring, and the risks associated with cyanotoxins and phycotoxins in shellfish produced from this lagoon.

The eutrophic statuses of all the sampling locations mentioned above are associated with high water temperatures (all above 30 °C at the sampling sites—see Table A1), which can favor the quick growth of cyanobacteria and dinoflagellates, which are the main contributors to the levels of toxins, including cyanotoxins and phycotoxins, which are lethal to

humans and other aquatic organisms. Based on microscopic observations, diatoms dominated the phytoplankton community in the TGCH lagoon, comprising 68.42% of the total population, followed by the cyanobacteria and dinoflagellates, with 13.2 and 7.9% of the total population, respectively (Figure 4). Among the dinoflagellates, only the genus *Dinophysis* sp., which is known to produce diarrheic shellfish toxins like OA and DTXs [41], was observed at all the sampling sites during this study. However, many potentially toxigenic genera of cyanobacteria were identified during the analyses, such as *Pseudanabaena* sp., *Anabaena* (actually *Dolichospermum*) sp., *Raphidiopsis raciborskii,* and *Oscillatoria* sp. The species *Raphidiopsis raciborskii* is known to produce cylindrospermopsins (CYNs), which are classified as cytotoxic and hepatotoxic toxins, and *Oscillatoria* and *Dolichospermum* are known to produce microcystins (MCs) [10]. The species *Raphidiopsis raciborskii* might also be responsible for the production of MCs [59]. These genera, along with *Pseudanabaena*, have also been reported to produce anatoxins (ATXs) [10]. Some freshwater cyanobacteria, including *Dolichospermum* sp. and *Raphidiopsis raciborskii*, were identified in this lagoon, and marine dinoflagellates including *Pyrodinium* sp., *Gymnodinium* sp., and *Alexandria* sp., have been reported to produce STXs [10].

The influx of fresh water from precipitation, surface runoff, and river flow led to a substantial reduction in salinity, with a gradient from the North to the main tributary mouths in the South, with values ranging from 1.7×10^{-10} PSU (TC1) to 15.56×10^{-9} PSU (TC13). This low range of salinity with warm temperatures (above 30 °C), combined with eutrophication, is often favorable for the development of certain species of dinoflagellates and cyanobacteria. For example, field research has shown salinity levels from 3.8 PSU to 11.5 PSU to be a principal factor in the spatial distribution of cyanobacterial bloom in the Baltic Sea [60]. *Microcystis* blooms typically occur in freshwater environments. However, several studies have shown that *Microcystis* can tolerate a wide variation in salinity (0 to 18 PSU), indicating that it can survive and grow at the higher salinities commonly encountered in lagoons and estuaries [61–63]. Tanabe et al. (2018) reported that *M. aeruginosa* could acquire salt-tolerance genes (sucrose genes) via horizontal gene transfer, leading to its invasion and proliferation in brackish waters [62]. Recently, Tarafdar et al. (2023) provided insight into changes in phytoplankton co-occurrence patterns and the environmental factors driving the dominance of the *M. aeruginosa* bloom in a tropical coastal lagoon, with similar conditions to those we observed in the TGCH lagoon [63]. According to these authors, a calm water surface combined with rising water temperature, high dissolved inorganic nitrogenous loading, strong phosphorus limitation, and low salinity during the late monsoon, triggered a *Microcystis* bloom. Other studies have shown that *Microcystis* strains can persist [64] and produce toxins with salinities as high as 17.5 g/L [65].

All the water and shellfish samples collected in June 2023 in the TGCH lagoon and analyzed by the ELISA kits for anatoxins (ATXs) and saxitoxins (STXs), as well as the PP2A inhibition test for microcystin-like toxins, contained, simultaneously, three categories of toxins with levels above the detection limits of the kits (see in Section 2.2.3). However, the concentrations of these toxins separately analyzed in shellfish were well below the action levels set to protect public health, according to Ortero and Silva (2022) [11], in the first known report of anatoxins (ATXs) and specific cyanobacterial toxins detected in shellfish from brackish waters. Therefore, they do not pose a health risk to the population. The highest concentration of ATXs detected in the water samples was 6.25 µg equivalent ATX-a/L (TC4). The current study found that the oysters, mussels, and clams in the TGCH lagoon can accumulate low amounts of ATXs, ranging from 4.48 µg equivalent ATX-a/kg in mussel samples (SF3, Figure 4) to 8.08 µg equivalent ATX-a/kg in clam samples (SF1, Figure 4). There is no standard set of regulations yet for this class of cyanobacterial toxins in shellfish, but the findings can provide a necessary justification for a regional plan of cyanotoxin monitoring and lagoon ecosystem management. However, our results are preliminary and need to be confirmed by liquid chromatography coupled with mass spectrometry, because the ELISA test used for the detection of ATXs in a complex matrix such as a crude shellfish extract could give false positives. Compared to ATXs, the STX

concentrations in water samples and in shellfish meat were low, ranging from 0.176 μg/L to 0.308 μg/kg, respectively. The highest level of STXs detected in shellfish was at least a thousand times below the action level for STXs in shellfish (800 μg of STX equivalent per kg of edible shellfish meat [11]. It can be assumed that there is no current risk associated with consuming aquatic food, but further estimations can be provided for regional monitoring action plans for phycotoxins, in both marine and brackish waters.

The PP2A-inhibiting toxins, traditionally freshwater cyanotoxins (MCs and NODs) and marine phycotoxins (OA and DTXs), were also detected in our water samples at all the sites, ranging from 0.79 (TC3) to 1.45 μg equivalent MC-LR/L (TC11). It can be observed in Figure 7 that, at most of the sampling sites, the concentrations of MC-like toxins found are above the guideline level for drinking water (1 μg MC-LR/L) [66]. However, in shellfish, the highest level detected in oyster samples (130.4 μg equivalent MC-LR/kg, Figure 10) was slightly below the guideline level for diarrheic toxins in shellfish (160 μg of OA equivalent per kg of edible bivalve mollusk meat, as total amounts of OA, DTXs and pectinotoxins) [11], suggesting that there is no current risk to seafood safety. Note that the current study may underestimate MC-type toxins in shellfish, as only free MCs were quantified, since the extraction methods used in this study excluded the covalently bound MCs and esterified forms of DSTs [67,68]. Although the PP2A test is very sensitive and quantitative, it does not allow discrimination between MC-type cyanotoxins and OA-type phycotoxins and derivatives. Therefore, high-performance liquid chromatography coupled with mass spectrometry will be necessary to identify the nature of the toxins present. The dinoflagellate species producing OAs and DTXs, *Dinophysis* sp., was identified in the water samples from the Tam Giang lagoon. Therefore, shellfish in Vietnamese coastal areas can be exposed to OAs and DTXs and accumulate these toxins in their bodies. Indeed, an earlier study [69] confirmed the presence of *Dinophysis caudata*, a dinoflagellate species capable of producing OA group toxins, in Vietnamese coastal waters. Furthermore, a large-scale survey at 15 mollusk production areas in 2011 using a mouse bioassay showed that 185 out of 1277 samples were positive with lipophilic diarrheic phycotoxins, but the exact nature of the accumulated toxins was not specified [70]. Recently, bivalve mollusk samples consisting of oysters (*Crassostrea rivularis*), clams (*Anadara subrenata*, *Tegillarca granosa*, *Meretrix lyrata*), and green mussels (*Perna viridis*) were collected in a period, from April 2016 to April 2017, from the main seafood markets in six coastal provinces in Vietnam: Nam Dinh (ND), Thanh Hoa (TH), Da Nang (DN), Binh Dinh (BD), Phu Yen (PY), and Ba Ria–Vung Tau (VT). The determination of OA, DTX-1 and DTX-2 in 194 samples of bivalve mollusks collected during this one-year period (April 2016–April 2017) confirmed the presence of these toxins in 23/194 samples (11.8%), at levels ranging from 1 to 11.3 μg/kg [68]. MCs, traditionally freshwater cyanotoxins, in addition to their bioaccumulation in the visceral organs of aquatic organisms, have been outlined in some freshwater reservoirs in Vietnam [33–39,58]. However, this is the first known report of MCs in shellfish in the brackish water of the TGCH lagoon in Vietnam. The study adds to a growing body of research on MCs detected in estuarine and marine environments [43,71,72]. For example, a recent study [73] reported that eastern oysters can readily bioaccumulate MCs to levels of seafood safety concern and depurate them slowly, making eastern oysters potential vectors for hepatotoxic MC shellfish poisoning (HSP) in estuaries. Regarding diarrheic shellfish poisoning (DSP), an earlier study [64] demonstrated that shellfish have been found to bio-magnify MCs to concentrations more than 100 times higher than those detected in water.

This study showed for the first time that shellfish in the TGCH lagoon (Vietnam) can co-accumulate multiple cyanotoxins and phycotoxins that threaten seafood safety. Indeed, the co-occurrence of freshwater cyanotoxins and marine phycotoxins in shellfish has been reported in mussels for DSTs, PSTs, DA, and MCs [43]. Recently, Pease et al. (2023) [74] reported the co-occurrence of marine phycotoxins such as azaspiracids-1 and -2 (AZA1, AZA2), domoic acid (DA), okadaic acid (OA), and dinophysistoxin-1 (DTX1), as well as three variants of freshwater MCs (MC-LR, MC-YR and MC-RR) in oysters (*C. virginica*) collected from the Virginia portion of the Chesapeake Bay (USA). Phycotoxins were detected

at low concentrations below seafood safety action levels. However, MCs were detected at higher levels, with maximum found in the variant MC-RR at 7.12 μg/kg of wet weight of shellfish meat, providing justification for the monitoring of these cyanotoxins and for including them as a potential shellfish safety concern in marine and brackish ecosystems. While recreational and drinking water guideline levels exist for all cyanotoxin groups in freshwater ecosystems [75], MCs are currently not regulated in shellfish. For example, the California Environmental Protection Agency [76] has proposed an action level for MCs of 10 μg/kg wet weight for sport fish and shellfish, while the Victorian Department of Health in Australia has calculated an MCs health guideline value (for people aged 16 years and younger) of 51 μg/kg in mollusks [77]. In Vietnam, the mean consumption rate for shellfish was slightly higher in the age group of 30–54 years than in the younger (18–29 years) and older (55 years and above) age groups. However, the rate was not estimated for even younger groups (aged less than 18 years) [78]. This high consumption of shellfish could thus expose consumers in Vietnam to further rates in excess of the guideline values, especially during blooms of cyanobacteria and dinoflagellates.

While the highest concentrations of both neurotoxins (ATXs and STXs) and MC-type toxins detected in the current study were below the range of existing guidance values, the co-occurrence of both phycotoxins and cyanotoxins in this lagoon suggests that more data should be acquired on shellfish, mainly in areas with intense fishing and shellfish farming activity, and especially during HABs, when the concentrations of these toxins could exceed the threshold values. Therefore, these findings support the need for further research on chronic, sub-acute exposure to these toxins, as well as a risk assessment of the combined effects of phycotoxins and cyanotoxins for seafood safety. According to several epidemiological and experimental studies, chronic exposure to phytoplankton toxins in humans has been linked to carcinogenesis, particularly in the skin, lungs, nasopharynx, pancreas, kidneys, breast, prostate, urinary bladder, and hematological systems [12]. Indeed, MCs, NODs, OA, and DTXs are considered as powerful tumor promoters in experimental animals [79]. Furthermore, MC-LR has been classified as "possibly carcinogenic to humans" (group 2B), and NOD-R is listed as "not classifiable as to their carcinogenicity" (group 3) [14,80,81].

5. Conclusions

This is the first comprehensive examination of phycotoxins and cyanotoxins in the TGCH lagoon (Vietnam), and the first known report of the co-occurrence of these two classes of toxins in water and shellfish samples in this lagoon. Trace to low concentrations of neurotoxins such as ATXs (cyanobacteria) and STXs (cyanobacteria and dinoflagellates) were detected in shellfish, orders of magnitude below seafood safety action levels. However, toxins inhibiting the PP2A enzyme, such as MCs and NODs (cyanobacteria) and OA and DTXs (dinoflagellates,) were also found in shellfish, at higher concentrations (maximum: 130.4 μg equiv. MC-LR/kg shellfish meat wet weight), approaching the action level proposed for this class of toxin in shellfish (160 μg of OA equivalent per kg of edible bivalve mollusk meat). The presence of potentially toxigenic cyanobacterial and dinoflagellate species in this lagoon clearly emphasizes the need for regular monitoring of the water quality, the microorganisms present, and their toxins in shellfish. Such bloom incidents are proof that the concentrations of toxins could exceed threshold values and cause health and economic problems.

The findings of this project improved the knowledge on the factors and processes favoring the development of cyanobacteria and the production of toxins at different sites in the TGCH Lagoon. The knowledge about the dynamics of cyanobacteria/cyanotoxins leads to more options for intervention strategies, including more cost-effective monitoring, early-warning prediction systems, and the use of physical, chemical, or biological interventions to eliminate or reduce the effects of these HABs. The results of our initial findings also provide an enhanced understanding of the bioaccumulation of cyanotoxins in aquatic food webs that affect human consumers through the ingestion of contaminated edible tissues of

fish and shellfish. However, we emphasize the potential for the provision of false positives by our ELISA tests in complex matrices. Therefore, this pilot study will be validated with liquid chromatography coupled with mass spectroscopy (LC-MS) in our next research plan. This first step in the research program is also a good opportunity for the training and mobility of young researchers between the different teams to learn techniques for the identification of cyanobacteria and the analysis of cyanotoxins.

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Data Availability Statement: The data presented in this study are available on request.

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Appendix A

Appendix A.1. Physical Chemical Parameters from Each Sampling Site on Tam Giang Lagoon (Vietnam)

Table A1. Physical and chemical parameters at each sampling site in Tam Giang lagoon (Vietnam).

Site	Coordinates	Depth (m)	Secchi Disk (m)	D.O (mg/L)	Temp (°C)	pH	Turbidity (NTU)	Salinity (PSU)	NH_4 (mg/L)	NO_2 (mg/L)	NO_3 (mg/L)	TN (mg/L)	PO_4 (mg/L)	TP (mg/L)	Chl-a (mg/L)	PC (mg/L)
TC1	N 16°33′52.7″ E 107°37′26.6″	0.5	0.35	9.5	34.75	8.45	45	0.17	0.12	0.001	0.09	0.582	LOD *	0.062	30.71	0.08
TC2	N 16°35′10.4″ E 107°34′40.0″	2	0.8	12.5	33.6	7.7	34.7	3.9	0.07	0.0040	0.14	0.819	LOD	0.075	20.03	0.06
TC3	N 16°36′49.1″ E 107°31′41.9″	~2.3	0.15	10.16	33.2	7.69	2	10.5	0.08	0.003	0.80	1.220	LOD	0.076	0.00	0.01
TC4	N 16°37′34.4″ E 107°30′18.6″	~1.5	1.5	5.8	34.3	7.51	50	8.03	0.07	0.0097	0.57	1.487	LOD	0.106	42.72	0.01
TC5	N 16°37′13.8″ E 107°30′7.4″	4.1	1	7.9	32.63	7.68	12.2	8.58	0.10	0.0067	0.79	1.012	LOD	0.060	0.00	0.02
TC6	N 16°38′36.5″ E 107°27′55.5″	3.40	1.5	7.5	33.34	7.45	13.5	15.6	0.08	0.006	0.43	1.060	LOD	0.060	9.35	0.01
TC7	N 16°39′12.3″ E 107°26′35″	12.5	1.5	7.8	31.6	7.62	8.2	12.5	0.06	0.002	1.11	1.100	LOD	0.042	9.35	0.02
TC8	N 16°29′26.8″ E 107°43′59.0″	14.8	0.75	6.5	33.5	6.5	23.6	14.82	0.11	0.002	1.14	0.774	LOD	0.071	6.23	0.05
TC9	N 16°27′01.0″E 107°45′30.4″	13.7	0.6	6.5	34.2	6.5	16.8	13.71	0.10	0.002	0.95	0.622	LOD	0.067	4.45	0.02
TC10	N 16°25′26.9″ E 107°46′46.4″	13	0.7	7.1	34.5	7.1	16.2	13.01	0.10	0.001	0.94	0.523	LOD	0.067	11.57	0.02
TC11	N 16°23′35.7″ E 107°48′23.1″	12.2	0.8	6.3	34.3	6.3	15	12.17	0.10	0.002	0.99	0.678	LOD	0.069	4.45	0.03
TC12	N 16°22′23.6″ E 107°49′54.2″	13.1	0.8	6.1	34.3	6.1	17.9	13.07	0.11	0.001	0.99	0.795	LOD	0.071	8.01	0.01
TC13	N 16°39′41″ E 107°26′43″	15.6	-	6.1	34.7	6.1	11	15.56	0.14	0.002	1.07	0.974	LOD	0.064	0.00	0.02

* LOD = 0.15–1.3 mg/L.

Appendix A.2. Formula Used to Calculate Carlson's Trophic State Index

TSI for Chlorophyll-a TSI = 9.81 [ln Chlorophyll-a (in μg/L)] +30.6

TSI for Secchi depth TSI = 60 − 14.41 [ln Secchi depth (in meters)]

TSI for Total phosphorus TSI = 14.42 [ln Total phosphorous (in μg/L)] + 4.15

Carlson's trophic state index (CTSI) = 1/3 [TSI (TP) + TSI(CA) + TSI(SD)].

Table A2. Categorization of all the sampling stations on the basis of Carlson's trophic state index.

POINTS	CTSI (SECHHI)	CSTI (TP)	CSTI (CHL-A)	CSTI	Attributes
TC 1	75.12793681	63.59996901	64.19361423	67.64051	Eutrophic dominance of blue-green algae, algal scum
TC 2	63.21549857	66.35498552	60.00038845	63.19029	Eutrophic dominance of blue-green algae, algal scum
TC 3	87.33749898	66.665105	0	51.3342	Eutrophic, decreased-transparency, warm-water fisheries only
TC 4	54.15724779	71.34064102	67.43328518	64.31039	Eutrophic dominance of blue-green algae, algal scum
TC 5	60	63.21541289	0	41.0718	Mesotrophic, moderately clear, but increasing probability of anoxia during the summer
TC 6	54.15724779	63.21541289	52.52379454	56.63215	Eutrophic, decreased transparency, warm-water fisheries only
TC 7	54.15724779	58.12772866	53.8337375	55.3729	Eutrophic, decreased transparency, warm-water fisheries only
TC 8	64.14549866	65.56663295	0	43.23738	Mesotrophic water moderately clear, probability of anoxia during the summer
TC 9	67.36099724	64.85614442	48.54618182	60.25444	Eutrophic, decreased transparency, warm-water fisheries only
TC 10	65.13968594	64.85614442	45.24538918	58.41374	Eutrophic, decreased transparency, warm-water fisheries only
TC 11	63.21549857	65.21576405	54.61895646	61.01674	Eutrophic, algal scum probable, extensive macrophyte problems
TC 12	63.21549857	65.56663295	47.03396366	58.60537	Eutrophic, decreased transparency, warm-water fisheries only
TC 13	83.19200032	64.10882694	51.01157637	66.10413	Eutrophi, algal scum probable, extensive macrophyte problems

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