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The *sxt* Gene and Paralytic Shellfish Poisoning Toxins as Markers for the Monitoring of Toxic *Alexandrium* Species Blooms

Antonella Penna,*[,†,‡] Federico Perini,[†,‡] Carmela Dell'Aversano,[§] Samuela Capellacci,[†] Luciana Tartaglione,[§] Maria Grazia Giacobbe,[∥] Silvia Casabianca,[†] Santiago Fraga,[⊥] Patrizia Ciminiello,[§] and Michele Scardi[#]

[†]Department of Biomolecular Sciences, University of Urbino, Pesaro, Italy

[‡]Institute of Marine Sciences, ISMAR - CNR, Ancona, Italy

[§]Department of Pharmacy, University of Federico II, Naples, Italy

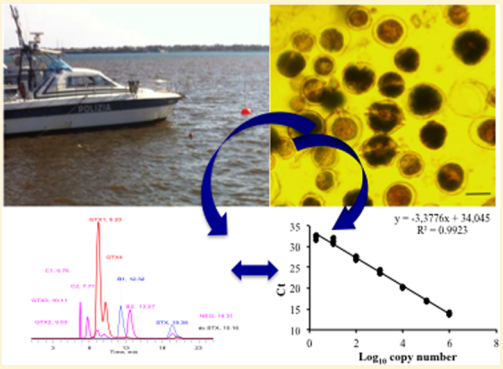
[∥]Institute for Coastal Marine Environment, IAMC - CNR, Messina, Italy

[⊥]Centro Oceanografico de Vigo, Instituto Espanol de Oceanografia (IEO), Vigo, Spain

[#]Department of Biology, University of Tor Vergata, Rome, Italy

Supporting Information

ABSTRACT: Paralytic shellfish poisoning (PSP) is a serious human illness caused by the ingestion of seafood contaminated with saxitoxin and its derivatives (STXs). These toxins are produced by some species of marine dinoflagellates within the genus *Alexandrium*. In the Mediterranean Sea, toxic *Alexandrium* spp. blooms, especially of *A. minutum*, are frequent and intense with negative impact to coastal ecosystem, aquaculture practices and other economic activities. We conducted a large scale study on the *sxt* gene and toxin distribution and content in toxic dinoflagellate *A. minutum* of the Mediterranean Sea using both quantitative PCR (qPCR) and HILIC-HRMS techniques. We developed a new qPCR assay for the estimation of the *sxtA1* gene copy number in seawater samples during a bloom event in Syracuse Bay (Mediterranean Sea) with an analytical sensitivity of 2.0 × 10° *sxtA1* gene copy number per reaction. The linear correlation between *sxtA1* gene copy number and microalgal abundance and between the *sxtA1* gene and STX content allowed us to rapidly determine the STX-producing cell concentrations of two *Alexandrium* species in environmental samples. In these samples, the amount of *sxtA1* gene was in the range of $1.38 \times 10^5 - 2.55 \times 10^8$ copies/L and the STX concentrations ranged from 41−201 nmol/L. This study described a potential PSP scenario in the Mediterranean Sea.



INTRODUCTION

Paralytic shellfish poisoning (PSP) is a syndrome caused by the consumption of seafood contaminated with saxitoxin and its derivatives (STXs). STXs can affect the human neuro-vegetative system leading to muscular paralysis and death. These toxins are a family of neurotoxins produced by some freshwater prokaryotic cyanobacteria and marine eukaryotic dinoflagellates. The STXs are alkaloids including carbamoyl toxins, namely saxitoxin (STX), neosaxitoxin (NEO), and gonyautoxins (GTX1 to GTX4), *N*-sulfocarbamoyl toxins (C1−C4, B1 and B2), and decarbamoyl toxins (dcSTX, dcNEO, and dcGTX1 to dcGTX4).[1] A number of minor derivatives of saxitoxin have reportedly been found in both microalgae[2] and seafood.[3] The biosynthetic pathway and genes responsible for STX synthesis have been recently identified in cyanobacteria and dinoflagellates (*Gymnodinium catenatum* and several *Alexandrium* species). In both eukaryotes and prokaryotes, STX is synthesized by similar processes.[4,5] The first two genes that start the biochemical synthesis of STX, also those best characterized, are the *sxtA* (coding for a *polyketide synthase*) and *sxtG* (coding for a *amidinotransferase*). The *sxtA* gene is the starting gene of STX synthesis.[6] It has a polyketide synthase (PKS)-like structure characterized by four catalytic domains with predicted activities of a *S*-adenosyl-methionine-(SAM) dependent methyltransferase (*sxtA1*), GCN5-related *N*-acetyltransferase (*sxtA2*), acyl carrier protein (*sxtA3*) and a class II aminotransferase (*sxtA4*). In dinoflagellates, the *sxtA* gene is present in multiple copies in the nuclear genome, the mRNA transcripts are monocistronic with polyadenilated tail.[7] The *sxtA* transcripts can belong to two families; the shorter transcripts encode the domains *sxtA1, sxtA2* and *sxtA3*, while the longer transcripts encodes all the 4 domains.[6] The other studied *sxtG* gene is not exclusively specific of the *Alexandrium*

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species reported producing STX. Furthermore, *Alexandrium* strains positive or negative for the STX genes may coexist within the same species.[7]

STXs mainly impact the marine trophic web, since they can accumulate in many organisms.[8] The accumulation of these compounds in seafood has a major economic impact on aquaculture industries. The annual economic loss due to harmful algal bloom events affecting aquaculture activities is estimated around US$895 million, with losses in Europe alone amounting to €300 million.[9,10]

Most of the dinoflagellate species producing STXs belong to the genus *Alexandrium*: *A. minutum*, *A. ostenfeldii*, *A. catenella*, *A. pacificum*, and *A. australiense*; other species that produce STXs include *Gymnodinium catenatum* and *Pyrodinium bahamense*.[11−16]

In the Mediterranean Sea, the increase in the frequency of toxic *A. minutum* outbreaks and the number of areas affected has coincided with the overdevelopment of coastlines, which increasingly offer confined nutrient enriched waters suitable for microalgal proliferation.[17−19] Despite the dinoflagellates' preference for settling in confined environments near shore, *A. minutum* has an enormous natural potential for dispersal because of its capacity to grow and produce resting cysts under a wide range of environmental conditions.[20−22]

Due to the increased risks posed to human health by PSP toxins worldwide, it is crucial to investigate the potential correlation between STX- production and *sxt* gene content in the Mediterranean Sea.

The *sxtA1* gene sequences appear to be strongly conserved within *Alexandrium* spp. This allowed us, in a previous research, to design *sxtA1* and *sxtG* gene specific primers for a sensitive qPCR assay to investigate gene expression modulation in Mediterranean *A. minutum*.[23]

Meanwhile, this study aimed to illustrate a potential scenario of STX producing harmful *A. minutum* and to investigate STX-related risk in the Mediterranean Sea. An innovative approach, based on both molecular qPCR and HILIC-HRMS (hydrophilic interaction liquid chromatography with high-resolution mass spectrometry) techniques, was developed and proposed for the monitoring of the PSP algal blooms. Different populations of *A. minutum,* genetically grouped according to their location in regional seas within the Mediterranean Sea,[24] were analyzed for *sxtA1* and *sxtG* gene presence and STX production. We used the qPCR assay to estimate toxic cell concentrations in seawater samples during a recurrent bloom of *A. minutum* co-occurring with *A. pacificum* in Syracuse Bay (Ionian Sea) together with the estimation of STX compounds in the same samples. The novelty of this molecular assay lies in its quantification of the *sxtA1* gene copy number from two toxic *Alexandrium* species in the seawater. Moreover, the relationship between the *sxtA1* gene and STX content provided a tool to estimate the abundance of the toxic *Alexandrium* cells in the environmental samples.

The potential scenario of STX-related risk in the Mediterranean Sea and the specific reproducible qPCR method developed in this study provide advanced strategies for the management of harmful algal blooms.

MATERIALS AND METHODS

Microalgal Cultures. The Mediterranean *Alexandrium minutum* strains (Supporting Information (SI) Table S1) were isolated from different regional seas, namely the Adriatic, Ionian, Tyrrhenian and Catalan (see SI Figure S1). The *A. pacificum* strains (CNR-ACATA1, CNR-ACATA2, CNR-ACAT02, CNR-ACATSRA4, CNR-ACAT15) were isolated from Syracuse Bay Ionian Sea (Italy) in the period 2012−2014 during recurrent spring blooms. The cultured strains were grown in F/2 medium[25] at 20 ± 1 °C, under a 14:10 light dark cycle, and photon irradiance of 100 μE $m^{-2}s^{-1}$. The cultured strains were harvested at two concentrations (1.0×10^5 and 6.0×10^6 cells) during exponential growth phase by centrifugation at 4000 rpm, for 15 min and then freezing pellets at −20 °C until molecular and toxin analyses, respectively.

Environmental Samples. Phytoplankton samples were collected at several stations in Syracuse Bay (Ionian Sea, Italy) on May 5th, 6th, and 8th 2014 during an *Alexandrium* spp. bloom event. A total of 10 surface seawater samples were collected in 500 mL polyethylene bottles for both microscopic and molecular analyses (see SI Table S2). The samples were immediately fixed with Lugol's acid solution (1% final concentration). Amounts of 1−10 mL of each fixed Lugol subsample were taken for molecular analyses. A total of other 13 surface seawater samples (0.8−2.5 L) were collected for STX analysis at Station 1, Station 2, Station 5, and Station 6. All field samples containing 1.0×10^5−1.0×10^6, and 1.0×10^6 cells were centrifuged at 4000 rpm for 15 min and frozen at −20 °C until molecular and chemical analyses, respectively.

Microscopy Analyses. Counting was carried out on subsamples of surface seawater (2−10 mL) or cultured strains (2 mL) according to the Utermöhl method[26] or on the entire sedimentation chamber. *Alexandrium* spp. were identified under an inverted microscope (Zeiss Axiovert 40 CFL) equipped with phase contrast, at 200× or 400× magnification according to Steidinger and Tangen.[27] *Alexandrium* spp. abundance in seawater and cultured strain samples was expressed as cells/L.

DNA Extraction and qPCR *sxt* Gene Analysis. Genomic DNA was extracted and purified using the DNeasy Plant Mini Kit (Qiagen, Valencia, CA). DNA was quantified using a Qubit fluorometer with a Quant-iT dsDNA HS Assay Kit (Invitrogen, Carlsbard, CA). The *sxtA1* and *sxtG* gene fragments of each strain were amplified using the *sxtA1* and *sxtG* primers from Perini et al.[23] The following qPCR protocol was used: tubes contained 25 μL of the following mixture: 600 nM of each primer, 1× Hot-Rescue Real-Time PCR Kit-SG Mix containing Sybr Green (Diatheva, Fano, Italy), 0.5 U Taq Polymerase (Diatheva, Italy) and 5−10 ng of DNA template. The qPCR assay was carried out using a StepOne Real-Time PCR System (Applied Biosystems, CA). The thermal cycling conditions consisted of 10 min at 95 °C, followed by 40 cycles at 95 °C for 15 s and at 60 °C for 1 min. The PCRs were followed by a dissociation protocol ranging from 60−95 °C and melting curve analysis. Several amplified products (n = 10) were purified using the DNeasy gel extraction Kit (Qiagen, Valencia, CA), and the products were directly sequenced (Eurofins MWG Operon, Ebersberg, Germany) to confirm the *sxtA1* and *sxtG* gene identities.

The species-specific qualitative PCR amplification assay for *A. minutum* and *A. pacificum* was carried out on all fixed seawater samples (10 mL) from Syracuse Bay to confirm the taxonomic identification made by microscopic analysis. Briefly, seawater samples containing co-occurring species, such as *A. minutum* and *A. pacificum,* were analyzed. DNA extraction and PCR amplification protocols were carried out according to Penna et al.[28] In the PCR assay the primers[28] are different respect to those used in the qPCR assay described above.

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Quantitative PCR Analysis. *Environmental and Cultured Sample Processing and DNA Extraction.* A total of 43 *A. minutum* cultured strains and 10 surface seawater samples were analyzed. Cell pellets were carefully washed with 1 mL sterile artificial seawater, centrifuged at 10 000 rpm for 15 min, and stored at −80 °C or immediately processed. Pellets of cultures and field samples were resuspended in 0.8 mL of lysis buffer[29] containing 0.5 mm zirconia-silica beads (500 mg) for each tube. The suspension was shaken 30 min at room temperature, and incubated at 55 °C for 1h with vortexing every 20 min. Finally, the samples were incubated at 100 °C for 5 min to inactivate proteinase K. After centrifugation at 10 000 rpm for 2 min at room temperature to precipitate cell debris, the supernatants containing total DNA were transferred into new tubes and diluted at 1:10 for the qPCR assay.

Alexandrium spp. *Quantification.* Environmental and cultured samples were analyzed by qPCR following the protocol described above. The standard curves were constructed from a six point 10-fold dilution series of purified *sxtA1* PCR products (from 2.0 × 10 to 1.0 × 10^6 copies) generated from the DNA of *A. minutum* CBA53. The PCR product was purified with the MinElute Gel Extraction Kit (Qiagen, Hilden, Germany) and quantified with a Qubit (Invitrogen, Life-Technologies, Carlsbad, CA). The amplification efficiency of the qPCR assays was estimated from standard curves using the StepOne software ver. 2.3 (Applied Biosystems, LifeTechnologies, Carlsbad, CA). The same standard curve was used for the qPCR analysis of the copy number of *A. pacificum* strains. All samples were run in three technical replicates. Standard curves for *sxtA1* were included in each PCR run.

Quantification of A. minutum Using The sxta1 Standard Curve. Three strains of *A. minutum* (CNR-AMIB5, CBA53, and CNR-AMISY1) were used for the qPCR method validation. Aliquots of different *A. minutum* cells of each strain corresponding to 2.0 × 10^3, 4.0 × 10^3, 1.0 × 10^4, 2.5 × 10^4, 5.0 × 10^4, 1.0 x10^5, 2.5 × 10^5, 5.0 × 10^5 were centrifuged at 4000 rpm for 15 min at room temperature and resuspended in 50 mL of seawater samples not containing *A. minutum* cells (checked by microscopy) to simulate the environmental samples. These samples were lysed and qPCRs were performed as described above. The Ct values were plotted against *sxtA1* standard curve and *sxtA1* gene copy number/L was obtained. A correlation test between the *A. minutum* abundance (cells/L) against *sxtA1* gene copy number/L was carried out.

Data Analyses. Acquisition of qPCR data and subsequent analyses were carried out using StepOne software v. 2.3. The automatically generated standard curves were accepted when the slope was between −3.27 and −3.47 (94−100% efficiency) and the value of the linear correlation coefficient was not smaller than 0.99. *Alexandrium* spp. abundance in surface seawater was normalized to cells/L. Data analyses were performed with nonparametric Mann−Whitney and Kruskal−Wallis tests. Spearman's rank correlation was computed in place of linear correlation when monotonic relationships were expected. Major axis regression was used in place of ordinary linear regression when the two variables involved were both affected by a similar level of error. All statistical calculations were performed using PAST ver. 2.17[30] with a p-value < 0.05 determining significance.

Chemical Analyses. *Chemicals.* Water and acetonitrile (HPLC grade), glacial acetic acid, formic acid, and ammonium formate (Laboratory grade) were purchased from Sigma-Aldrich Corporation (St. Louis, MO). Standard solutions of PSP toxins were obtained from the NRC Certified Reference Materials (CRM) Program (Institute for Marine Biosciences, Halifax, NS, Canada).

Extraction. Pellets from 43 *Alexandrium* strains were separately extracted with 1 mL of 0.1 M acetic acid in water, while being sonicated for 10 min in pulse mode in an ice bath. The mixture was centrifuged at 5000 rpm for 10 min and the supernatant was decanted. The extract (1 mL) was directly analyzed by HILIC-HRMS^n.[31] Planktonic samples from Syracuse Bay containing *A. minutum* and *A. pacificum* were extracted as described above. The extracts were diluted 1−5 with water 0.1 M acetic acid before HILIC-HRMS^n analysis.

Liquid Chromatograpy-High Resolution Multiple Stage Mass Spectrometry (LC-HRMS^n, n = 1,2). LC-HRMS^n experiments were performed using a hybrid linear ion trap LTQ Orbitrap XL Fourier transform mass spectrometer (FTMS), equipped with an ESI ION MAX source (Thermo-Fisher, San Jose, CA) and coupled to an Agilent 1100 HPLC system (Palo Alto, CA), equipped with solvent reservoir, in-line degasser, binary pump, and refrigerated autosampler.

Separations were performed on a 250 × 2.0 mm, 5 μm TSK gel Amide-80 column (Tosoh Bioscience LLC, PA), according to Dell'Aversano et al.[31] Injection volume was 5 μL. Complete HRMS experiments were acquired in the mass range m/z 200−500 using source settings reported by Perini et al.[23] Full HRMS^2 experiments were performed in collision induced dissociation (CID) mode in the mass range m/z 70−350, using collision energies in the range 20−25%.[23] In all experiments, a resolving power of 30 000 was used (FWHM at m/z 400).

An extracted ion chromatogram (XIC) was obtained for each toxin by selecting diagnostic product ions (see SI Table S3). The average of triplicate measurements was used to plot a calibration curve for each PSP toxin standard. The limit of detection (LOD, μg/mL) for each toxin was extrapolated from the related calibration curve using the Formula 3.3 × (SD/S), where SD was the standard deviation of the response and S was the slope of the calibration curve; the SD of the response was determined from the standard deviation of y intercepts of the regression lines. Experimental LODs were also measured and they corresponded to the lowest concentration level that could be determined at an S/N ratio >3 over three replicates. Quantitation of the toxins contained in the extracts was accomplished by direct comparison of XIC areas of individual toxins to relevant CRM solutions at similar concentrations injected under the same experimental conditions. When CRM of B2 was lacking, quantitation of B2 was performed using CRM GTX2 assuming that GTX2 and B2 have the same molar response. The results were expressed on a per cell basis (amol/cell and fmol/cell). LOD was calculated on a per cell basis taking into account the number of cells and the extract volume.

RESULTS

The *sxtA1* and *sxtG* Gene Amplification. The specificity of the two primer sets designed to target the *sxtA1* and *sxtG* genes in *Alexandrium* spp. had already been tested by Perini et al.[23] The genomic DNAs from a total of 117 Mediterranean *A. minutum* strains were analyzed for the presence of *sxtA1* and *sxtG* genes by qPCR amplification. The amplified *sxtA1* gene fragment length was 75 bp in 105 strains, and the amplified *sxtG* gene fragment was 62 bp in 88 strains. No *sxtA1* were amplified in 12 and no *sxtG* products were amplified in 29 strains. In particular, a total of 24 and 9 Adriatic strains resulted positive for *sxtA1* and *sxtG* fragment PCR amplification,

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respectively, whereas, the remaining Adriatic strains were negative for *sxtA1* and *sxtG* qPCR amplification (n = 8 and n = 23, respectively). A total of 81 and 79 *A. minutum* strains of the other regional seas (Ionian, Tyrrhenian and Catalan) showed positive amplification for the presence of both the *sxtA1* and *sxtG* gene, respectively, with few exceptions for the Tyrrhenian and Catalan areas (see SI Table S1).

Standard Curve and Copy Number Quantification. The *sxtA1* curve showed a PCR efficiency of 98% ($y = -3.37x + 34.04$), a linear relationship over a range of 6 orders of magnitude ($r^2 = 0.99$) and a quantification limit of two copies per PCR reaction. The reproducibility analyzed as CV_{Ct} mean interassay variability was 1.4% (range from 10^6 to two copies). The *sxtA1* gene copy number per cell of the 43 Mediterranean *A. minutum* cultured strains from the four regional seas was calculated by plotting the Ct values obtained against the standard curve, taking into account the dilution factors of the crude extracts. Median copy numbers per cell ranged from 1.8 to 2.5, with 2.2 as the overall median value (see SI Figure S2). The Kruskall-Wallis test showed that there were no differences in *sxtA1* gene median copy number per cell among the four Mediterranean *A. minutum* populations (H = 5.467, p = 0.1406).

The *sxtA1* gene copy number/cell of the five Mediterranean *A. pacificum* cultured strains from the Syracuse Bay was calculated as described above for *A. minutum*. The mean copy number/cell was 13.18 ± 0.46.

Toxin Content in *A. minutum* Strains. A total of 43 cultured strains of *A. minutum* from the NW Adriatic, Ionian, Tyrrhenian and Catalan seas were extracted and analyzed by HILIC-HRMS^n. In this study, the absolute limit of detection (LOD) of the analytical method for each toxin (STX, GTX1 to 4, B1, B2, C1, C2, NEO, dcSTX, dcGTX2-3, and dcNEO) was in the range of 13−363 pmol/mL. Considering sample preparation (6.0×10^6 cells/mL of extracting solvent), LOD was converted into amol/cell and was in the range 2.2−61 amol/cell (see SI Table S3). Most of the extracts contained only GTX1 and GTX4, some contained primarily GTX1 and GTX4 together with lower amounts of GTX2 and GTX3, and only a few extracts did not contain any toxin in detectable amounts. It has been reported[32] that an isolated GTX stereoisomer is gradually converted into another stereoisomer in aqueous solution to reach equilibrium between α and β isomers. As a consequence, although the epimeric pairs of GTX1/4 and GTX2/3, were analytically separated (data not shown), their content was stated as total GTX1+GTX4 and GTX2+GTX3 because of facile epimerization.

The presence of the major gonyautoxins (GTX1 + GTX4) was detected in 88% of the cultured strains with toxin content in the range of 0.003−3.45 fmol/cell (see SI Figure S3). A total of seven *A. minutum* strains contained GTX1+GTX4 together with small amounts of GTX2+GTX3 (3.6−38 amol/cell). Very few Mediterranean strains (n = 5) did not produce any toxin in detectable amounts. The total GTX content did not show any significant difference among strains from the different Mediterranean areas (H = 3.096, p = 0.377).

Correlation between *A. minutum* Abundance and *sxtA1* Gene Copy Number. The total *sxtA1* gene copy number of cultured *A. minutum* strains determined by qPCR assay was plotted against cellular serial dilutions. The major axis regression between cell concentration (determined by microscopy) and *sxtA1* gene copies/L (assessed by qPCR) showed a highly significant linear correlation (r = 0.972, $p \ll 0.001$) and a slope (b = 2.45) that is consistent with the expected number of copies/cell (Figure 1). The intercept was not far from the origin (1.53×10^6 *sxtA1* gene copy number/L), although the latter was not within its confidence interval.

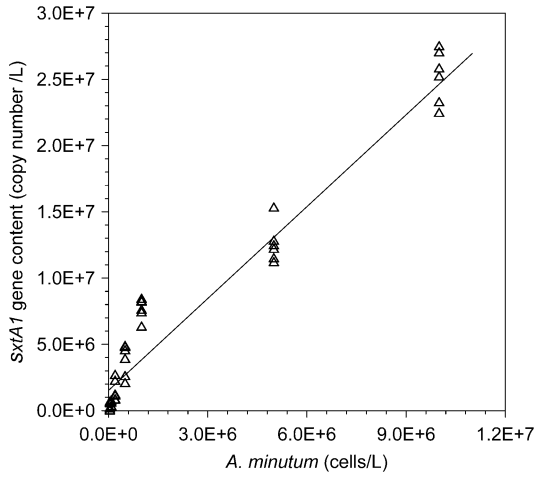

Figure 1. Linear relationship between *A. minutum* abundance (cells/L) and *sxtA1* gene content (copy number/L) based on qPCR assay (*sxtA1* copy number/L = 1.53×10^6 + 2.45·cells/L, r = 0.972, $p \ll 0.001$). Three cultured strains of *A. minutum* (CNR-AMIB5, CNR-AMISY1, and CBA53) were used for cellular serial dilution calculation and *sxtA1* gene copy number determination.

Abundance of *Alexandrium* Species and Toxin Content in Environmental Samples. The spring bloom in Syracuse Bay was characterized by the presence of both *A. minutum* and *A. pacificum*. The identification and abundance of the two species in surface water samples were determined by microscopy, whereas molecular taxonomic identification was also assessed by PCR using species-specific primers. The abundance of *A. pacificum* and *A. minutum* was in the range of 10^4−10^7 and 10^4−10^6 cells/L, respectively. The greatest abundance of both *Alexandrium* species was found at Station 1, Porto Grande Cantiere Di Benedetto, on 8 May, with $2.67 \times 10^7 \pm 2.79 \times 10^6$ cells/L; minimum abundance was found at Station 2, Porto Grande Sanità Marittima, on 5 May, with $4.21 \times 10^4 \pm 1.3 \times 10^3$ cells/L.

The total *sxtA1* gene copy number/L of both *Alexandrium* species was determined in the seawater samples by qPCR assay during the bloom period. Maximum *sxtA1* gene content was found at Station 1, with $2.55 \times 10^8 \pm 3.08 \times 10^7$ copies/L on 8 May, and minimum *sxtA1* gene content was found at Station 2 with of $1.38 \times 10^5 \pm 1.80 \times 10^3$ copies/L on 5 May (see SI, Table S2).

Furthermore, a positive correlation was found between cell densities determined by microscopy and *sxtA1* gene copies by qPCR in surface water (n = 29, Pearson's r = 0.978 $p \ll 0.0001$). In order to verify if the qPCR assay could be suitable for environmental samples, the expected *sxtA1* gene copy number/L was also estimated. Specifically, the abundance of *A. minutum* and *A. pacificum* obtained by microscopy was multiplied by the *sxtA1* gene copy number/cell of the two species (2.2 and 13 copy number/cell, respectively) and the values were added. These computed data were compared with the *sxtA1* gene copy number/L determined by qPCR. The overall relationship between observed and expected copy number of the *sxtA1* gene is shown in Figure 2. Most points

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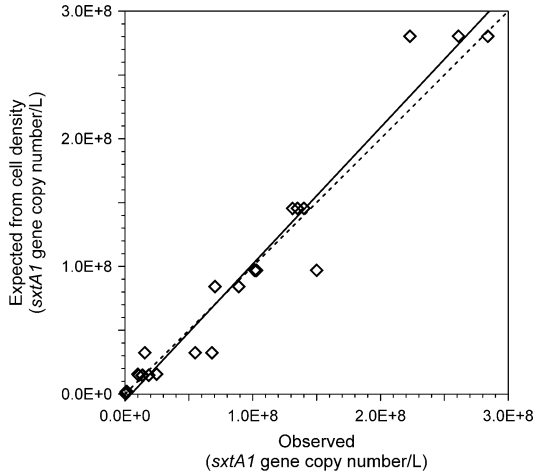

Figure 2. Expected and observed copy number of *sxtA1* gene in all environmental samples containing bloom of co-occurring species *A. minutum* and *A. pacificum* in Syracuse Bay (spring 2014). The abundance of both species in seawater samples ranged from 10^4 to 10^7 cells/L. The major axis (solid line) is very close to the unit slope line (dashed line) and shows a good agreement between expected and observed *sxtA1* gene copy number/L values ($r = 0.978$, $p \ll 0.001$). In fact, the unit slope lies within the 95% confidence interval of the major axis slope (0.954, 1.192) and the axes origin within that of its intercept (-1.11×10^7, 2.13×10^6).

are very close to the unit slope line, while those that are a little farther away are located on both sides of the line, thus showing that errors were not systematic. It is not surprising, therefore, that the major axis that interpolates the data set, taking into account errors affecting both observed and expected data, had a slope that was not significantly different from unit ($b = 1.07$) and an intercept that was not significantly different from zero ($a = -5.18 \times 10^6$). This result shows that the relationship between observed and expected gene copy numbers was accurate and unbiased.

Environmental samples, containing a mixture of *A. minutum* and *A. pacificum,* were extracted and analyzed for the STX content by LC-HRMS^n ($n = 1, 2$). The extracts showed the same toxin profile from a qualitative standpoint, in containing the *N*-sulfocarbamoyl derivatives C1, C2, B1, and B2, the carbamoyl toxins STX, NEO, GTX1, GTX4, GTX2, GTX3, and the decarbamoyl STX. The GTX1+GTX4 provided the highest contribution (from 40 to 49% of the total toxin content) (Table 1).

The linear correlation obtained between the *sxtA1* gene copy number by qPCR assay and STX content in seawater samples observed during the spring bloom in Syracuse Bay was strong ($r = 0.809$) and significant ($r = 0.809$, $p < 0.001$, $n = 13$) as shown in Figure 3.

DISCUSSION

The dramatic increase of HAB events and changes in their frequency and intensity over the last few decades is an urgent issue that needs to be managed.[33] These harmful algal bloom events, including those due to *Alexandrium* spp., seem to be partially a consequence of human mediated actions, such as eutrophication, habitat change, and alien and/or invasive species.[34,35] The Mediterranean Sea harbors a large number of invasive toxic *Alexandrium* species. *A. minutum* is reported to be the most harmful and widespread species along Mediterranean coastal areas forming high density blooms in

Table 1. STX Content in Seawater Samples Determined by HILIC-MS[2] Collected at Syracuse Bay in Spring 2014[a]

		sampling stations			
		St. 1[b]	St. 2[c]	St. 5[d]	St. 6[e]
GTX1+GTX4	nM	81	95	43	16
	fmol/cell	6.2	5.8	3.4	8.4
	%[f]	49	47	41	40
GTX2+GTX3	nM	10	11	5	2
	fmol/cell	0.79	0.7	0.39	0.93
	%[f]	6.3	5	4.7	4.4
C1+C2	nM	32	37	21	10
	fmol/cell	2.4	2.3	1.6	5.3
	%[f]	19	18	20	25
B1	nM	29	42	27	9.5
	fmol/cell	2.2	2.6	2.1	4.8
	%[e]	18	21	26	23
B2	nM	5.7	10	4.4	1.8
	fmol/cell	0.44	0.63	0.35	0.93
	%[f]	3.5	5.1	4.2	4.4
STX	nM	3.9	3.6	1.4	0.7
	fmol/cell	0.30	0.22	0.11	0.35
	%[f]	2.4	1.8	1.3	1.7
NEO	nM	1.1	1.4	2.0	0.3
	fmol/cell	0.09	0.09	0.16	0.17
	%[f]	0.70	0.70	1.88	0.79
dcSTX	nM	0.4	1.4	0.8	0.4
	fmol/cell	0.03	0.08	0.06	0.20
	%[f]	0.2	0.70	0.8	1.0
Total STXs	nM	163	201	104	41
	fmol/cell	13	12	8	21

[a]Instrumental variation coefficient was in the range 0.7−18%. [b]Porto Grande Cantiere Di Benedetto, 6th May. [c]Porto Grande Sanità Marittima, 6th May. [d]Porto Grande Canale Grimaldi, 6th May. [e]Porto Piccolo Circolo Ribellino, 8th May. [f]Relative abundance expressed as percentage (%).

confined sites and consequent resting cyst deposition after blooms.[36] Hence, there is a continuous risk of recurrent blooming events.[17,37]

In this study, the *sxtA* gene content, *sxtG* presence, as well as toxin profile and content, were investigated over a wide range of *A. minutum* strains isolated from various localities. In particular, a qPCR assay was applied to quantify the *sxtA1* gene copy number in Mediterranean strains in relation to STX production and rapidly quantify the abundance of *sxtA1* gene in field samples. Further, *sxtA1* gene content was correlated with toxin presence in environmental samples to provide an indication of PSP risk during a bloom.

The median *sxtA1* gene copy number/cell was 2.2, with a range of values that spanned from 1.0 to 6.17 gene copies/cell indicating that the *sxtA1* gene is not tandemly repeated up to thousands of copies like the rDNA in dinoflagellates.[29,38] Furthermore, no difference in the *sxtA1* gene copy number was found among Mediterranean *A. minutum* strains. Almost all the

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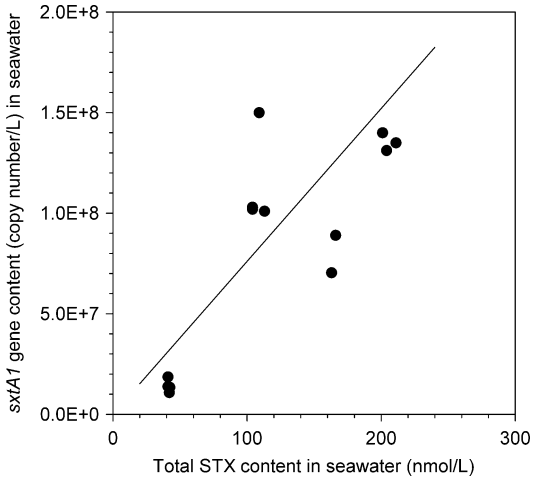

Figure 3. Relationship between total STX and *sxtA1* gene content in seawater samples. The linear relationship between *sxtA1* gene content and total STX was summarized by a major axis regression with zero intercept (*sxtA1* = 760160·total STX). Linear correlation between total STX and *sxtA1* gene content was high and significant (r = 0.809, p < 0.001). The seawater samples were collected at Station 1, Station 2, Station 5, and Station 6 in Syracuse Bay (Ionian Sea, Italy) during the spring bloom (see SI Table S2).

Mediterranean *A. minutum* strains contained the *sxtA1* gene that codifies for the SAM-dependent methyltransferase,[39] one of the main enzymes involved in STX synthesis and they were toxic. Only about 11% of the analyzed strains (five strains) with the gene were not producing STXs, as already observed for other species.[6] Thus, we are confident that in the Mediterranean *A. minutum* strains the *sxtA1* gene can be considered a consistent molecular marker to trace the potential toxicity of strains and phytoplankton cells in seawater samples. However, the absence of a correspondence between the *sxtA1* gene and STX production in a few strains could be due to the possible regulation of PSP toxin biosynthesis enzymes by transcriptional and post-translational mechanisms.[7,39] In addition, it can be hypothesized that the *sxtA1* gene can be represented by pseudogenes with no transcription. This evolutionary scenario, in fact, has already been demonstrated for the *sxtG* gene in dinoflagellates.[7] It is likely that the evolutionary mechanism of *sxt* genes from cyanobacteria to dinoflagellates went through multiple horizontal transfer with loss or duplication mechanisms.[6,39]

In this study, the *sxtG* gene was absent in 21% of the analyzed *A. minutum* strains (nine strains) including strains producing STXs, as already described by Perini et al.[23] Furthermore, *sxtG* is not exclusively of the toxic *Alexandrium* species.[7] In fact, *sxtG* is present in other *Alexandrium* species (*A. affine*, *A. andersoni*, *A. insuetum*, *A. tamarense* CCMP1771) that do not produce STXs. Therefore, this gene may be involved in other biochemical pathways or may have been lost during evolutionary history.

The quali-quantitative determination of the toxin profile was made by modifying the HILIC-MS/MS method for STXs[31] through the use of high resolution MS^n apex detection (n = 1,2). This allowed us to confidently detect STXs in algal extracts with a high degree of selectivity at levels as low as amol/cell. Most of the analyzed *A. minutum* produced different GTXs, as primarily GTX1 + GTX4. The absence of significant differences in toxin content among different geographical strains suggests that STX content of *A. minutum* is not strain- or population-specific. The toxin profile of *A. minutum* strains was similar to that observed in other Mediterranean areas[40,41] and other regions.[42,43] The levels of STXs detected in the Mediterranean *A. minutum* were on the fmol/cell or amol/cell order, lower than levels reported for strains from other areas worldwide, but, in agreement with previous observations by Bechemin et al.[44] and Yang et al.[45]

The presence of toxic *A. minutum* in the Mediterranean Sea suggests that the monitoring of this species is important for the management of HAB species to provide an early and/or accurate warning of shellfish contamination. Phytoplankton and harmful species monitoring is not always included in routine STX determination in shellfish at many sites of the Mediterranean Sea, as such monitoring is costly and time-consuming. Nonconventional methods (i.e., molecular qPCR) have been developed and validated[46,47] to meet the urgent need for specific, sensitive and rapid analyses of harmful phytoplankton species.

The selectively amplified *sxtA1* specific primers target *sxtA1* gene fragments of all *Alexandrium* species producing STXs.[23] In this study, first, the *sxtA1* gene was used as a molecular target to develop a qPCR assay for the enumeration of toxic *A. minutum* cells in simulated seawater conditions. The strong correlation obtained between *A. minutum* abundance and *sxtA1* gene content suggested that the method could be applied to environmental samples.

Since 2011, *A. pacificum* has been sporadically detected only at low density at Syracuse Bay, with *A. minutum* in bloom events (10^4–10^7 cells/L) (Giacobbe M.G. Pers. comm). Nevertheless, the seawater samples collected during the spring bloom of 2014 contained this species in bloom proportions. To date, *A. pacificum* has been reported to be restricted to northwestern Mediterranean areas only.[48] In this case, the amount of total *sxtA1* gene copies in environmental samples came from both toxic *A. minutum* and *A. pacificum*, which contributed differently in terms of gene copy number and then, in abundance. But, the accuracy and reliability of the qPCR assay developed for the estimation of abundance in monospecific STX - producing *Alexandrium* spp. blooms was confirmed by the significant correlation between the expected and observed *sxtA1* gene copy number in the environmental samples collected in Syracuse Bay in 2014.

The different copy number/cell made the single species-specific estimation of harmful cells unfeasible. However, we demonstrated that a bispecific *Alexandrium* assemblage can be partly decomposed thanks to the linear relationship that links the *sxtA1* gene copy number/cell in the two species to the overall copy number, which can be easily obtained from the qPCR assay. The correspondence is not biunivocal, however, and given the observed overall copy number it is not possible to define the exact density of the two species, but only the range within which they are expected to fall (see SI Figure S4).

Furthermore, environmental samples were also analyzed for the STX content. The total toxin concentration ranged from 41 to 201 nmol/L, with a content of 8–21 fmol/cell on a per cell basis. The toxin profile exhibited by Mediterranean *A. minutum* cultured strains was dominated by GTX1 and GTX4, with GTX2 and GTX3 present at low levels in very few samples (16%). This was in good agreement with toxin profiles reported for *A. minutum* of different geographical origin, which contained only GTX 1 to GTX 4[40,42] and quite occasionally STX[43,49] Moreover, most studies on the toxin profile of *A. catenella*[50,51] have found it to be a producer of all the toxins found in the

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planktonic samples (including GTX-1/4 and GTX2/3). Hence, our results suggest that both species, *A. minutum* and *A. pacificum*, contributed to GTX1+GTX4 and GTX2+GTX3 content, and that only *A. pacificum* is responsible for the presence of all the other STXs.

In this specific bloom of mixed HAB species, we demonstrated that the determination of the total *sxt* gene copy number could be an indicator of the toxin content in the water samples due to the significant correlation among *sxtA1* gene copy number and toxin content.

Based on the average toxin content of 14 ± 5.3 fmol/cell, corresponding to $1.4 \times 10^{-6} \pm 0.5 \times 10^{-6}$ μg/cell, we can hypothesize that at least 5.71×10^{8} toxic cells would have to be accumulated in 1 kg of shellfish to reach the maximum permitted level (MPL) for PSP toxins (MPL = 800 μg/kg). In Station 2 of Syracuse Bay, characterized by the lowest toxic cell concentration, based on the clearance rate (3.8 L/h), absorption efficiency (44%)[52] and mean weight of a mussel (1 g of 60 mm length), 1 kg of mussels would have to be exposed to toxic algal cells for at least 2.5 h in order to exceed the MPL of STX equivalent. Hence, there may be a potential risk of STX accumulation in seafood in the investigated Mediterranean area. Obviously, this is a prudent estimate because it does not take into account the fact that all derivatives of saxitoxin do not have the same toxicity, nor does it consider either of the toxin transformation processes that may occur inside the shellfish.[53]

This *sxtA1* gene qPCR based assay has the advantage of quantifying the potential STX- producing cell during bloom events. The *sxtA1* gene qPCR assay can support the analytical methods for STX determination in seawater and shellfish, especially at the early warning stage of toxic blooms (at 10^3 cells/L) as the sensitivity and specific detection.

In conclusion, an initial scenario of the toxic *A. minutum* distribution in the Mediterranean Sea based on the specific *sxtA1* gene and STX content was developed. This scenario illustrates the potential risk of real PSP occurrences, even though the toxin amount on a per cell basis was lower than in other areas worldwide. In any case, in high biomass blooming events, the potential risk of toxin accumulation in shellfish is likely. The qPCR based assay developed in this study is a specific quantification method for the effective toxic assessment of *A. minutum* or STX-producing *Alexandrium* species in environmental samples collected from bloom events.

Efficient monitoring strategies by combined molecular and chemical methodologies could play a fundamental role in preventing and managing health and economic risks related to STX-producing *Alexandrium* spp. blooms in coastal and marine farm areas.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.est.5b03298.

Table S1: List of the Mediterranean *Alexandrium minutum* strains, geographical locations, sxtA1 and sxtG gene presence (+ positive amplification) or absence (- negative amplification) determined by qPCR amplification, and STX production (PDF)

Table S2: Determinations of *Alexandrium* spp. abundance by microscopy and total *sxtA1* gene content in seawater samples by qPCR assay collected at several stations in Syracuse Bay during spring 2014 (PDF)

Table S3: Precursor and product ions used in the LC-$HRMS^n$ analyses and measured limits of detection (LOD) of individual STX toxins (PDF)

Figure S1: Map of sampling sites of *A. minutum* strains used in this study in the Mediterranean Sea. Trieste, Adriatic Sea, Italy; Marotta, Adriatic Sea, Italy; Taranto, Ionian Sea, Italy; Syracuse, Ionian Sea, Italy; Oliveri, Tyrrhenian Sea, Italy; Olbia, Tyrrhenian Sea, Italy; Arenys, Catalan Sea, Spain; Mallorca, Balearic Sea, Spain (PDF)

Figure S2: The *sxtA1* gene copy number of the Mediterranean *A. minutum* populations.[24] Boxes show the two central quartiles of the distribution, while the whiskers show minimum and maximum. Adriatic Sea, n = 9 strains; Ionian Sea, n = 12 strains; Tyrrhenian Sea, n = 11 strains; Catalan Sea, n = 11 strains (PDF)

Figure S3: Total GTX content of the four Mediterranean *A. minutum* populations.[24] Boxes show the two central quartiles of the GTX distribution, while the whiskers show minimum and maximum per geographical area: Adriatic Sea, n = 9 strains; Ionian Sea, n = 12 strains; Tyrrhenian Sea, n = 11 strains; Catalan Sea, n = 11 strains (PDF)

Figure S4: The *sxtA1* gene copy number isopleths showing how any given copy number of *sxtA1* gene may correspond to an infinite set of coupled *A. minutum* and *A. pacificum* abundance. Once the copy number has been determined, the range that contained the two *Alexandrium* species true densities can be easily defined (PDF)

■ AUTHOR INFORMATION

Corresponding Author

*Phone: 0039 0722 304908; fax: 0039 0722 304902; e-mail: antonella.penna@uniurb.it.

Notes

The authors declare no competing financial interest.

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Table S1. List of the Mediterranean *Alexandrium minutum* strains, geographical locations, *sxtA1* and *sxtG* gene presence (+ positive amplification) or absence (- negative amplification) determined by qPCR amplification, and STX production.

Strains	Regional Sea	Locality	*sxtA1* gene amplified	*sxtG* gene amplified	STX production
AMITA	NW Adriatic	Trieste	+	+	**yes**
AL1T	NW Adriatic	Trieste	-	-	n.a.[a]
AL3T	NW Adriatic	Trieste	+	-	n.a.[a]
AL4T	NW Adriatic	Trieste	+	+	n.a.[a]
AL5T	NW Adriatic	Trieste	-	-	n.a.
AL7T	NW Adriatic	Trieste	+	+	**yes**
AL8T	NW Adriatic	Trieste	+	+	n.a.[a]
AL9T	NW Adriatic	Trieste	+	+	n.a.[a]
LAC27	NW Adriatic	Trieste	+	+	n.a.[a]
CBA2	NW Adriatic	Marotta	+	+	n.a.[a]
CBA3	NW Adriatic	Marotta	+	+	n.a.[a]
CBA4	NW Adriatic	Marotta	-	-	n.a.[a]
CBA5	NW Adriatic	Marotta	-	-	n.a.
CBA6	NW Adriatic	Marotta	-	-	n.a.
CBA37	NW Adriatic	Marotta	-	-	n.a.
CBA38	NW Adriatic	Marotta	-	-	n.a.[a]
CBA39	NW Adriatic	Marotta	+	-	n.a.[a]
CBA40	NW Adriatic	Marotta	+	-	n.a.[a]
CBA41	NW Adriatic	Marotta	+	-	**yes**
CBA42	NW Adriatic	Marotta	+	-	no
CBA43	NW Adriatic	Marotta	+	-	n.a.[a]
CBA44	NW Adriatic	Marotta	+	-	**yes**
CBA47	NW Adriatic	Marotta	+	-	n.a.[a]
CBA48	NW Adriatic	Marotta	+	-	n.a.[a]
CBA49	NW Adriatic	Marotta	-	-	n.a.[a]
CBA52	NW Adriatic	Marotta	+	-	**yes**
CBA53	NW Adriatic	Marotta	+	-	**yes**
CBA54	NW Adriatic	Marotta	+	+	n.a.[a]
CBA55	NW Adriatic	Marotta	+	-	**yes**
CBA56	NW Adriatic	Marotta	+	-	n.a.[a]
CBA57	NW Adriatic	Marotta	+	-	**yes**
CBA60	NW Adriatic	Marotta	+	-	n.a.[a]
CNR-AMIA1	Ionian	Syracuse	+	+	**yes**
CNR-AMIA4	Ionian	Syracuse	+	-	**yes**
CNR-AMIA5	Ionian	Syracuse	+	+	**yes**
CNR-AMIB5	Ionian	Taranto	+	+	**yes**
CBA29	Ionian	Syracuse	+	+	n.a.[a]
CBA30	Ionian	Syracuse	+	+	**yes**
CBA31	Ionian	Syracuse	+	+	n.a.[a]
CBA32	Ionian	Syracuse	+	+	**yes**
CBA33	Ionian	Syracuse	+	+	**yes**
CBA34	Ionian	Syracuse	+	+	n.a.[a]
CBA35	Ionian	Syracuse	+	+	n.a.[a]
CBA36	Ionian	Syracuse	+	+	n.a.[a]
CBA45	Ionian	Syracuse	+	+	n.a.[a]
CBA46	Ionian	Syracuse	+	+	**yes**
CBA50	Ionian	Syracuse	+	+	n.a.[a]

CBA51	Ionian	Syracuse	+	+	n.a.[a]
CBA58	Ionian	Syracuse	+	+	n.a.[a]
CNR-AMIA2	Ionian	Taranto	+	+	n.a.[a]
E9A12	Ionian	Taranto	+	+	n.a.[a]
B8A12	Ionian	Taranto	+	+	n.a.[a]
CNR-AMISY1	Ionian	Syracuse	+	+	no
CNR-AMISY2	Ionian	Syracuse	+	+	**yes**
CNR-AMID5	Ionian	Syracuse	+	+	**yes**
CNR-AMID6	Ionian	Syracuse	+	+	**yes**
CNR-AMI2OL	Tyrrhenian	Olbia	+	+	**yes**
CNR-AMI3OL	Tyrrhenian	Olbia	+	+	**yes**
CNR-AMI5OL	Tyrrhenian	Olbia	+	-	**yes**
CNR-AMI4PT	Tyrrhenian	Olbia	+	+	**yes**
CNR-AMI2PT	Tyrrhenian	Olbia	+	+	**yes**
CNR-AMIV1	Tyrrhenian	Oliveri	+	+	no
CNR-AMIV2	Tyrrhenian	Oliveri	+	+	**yes**
CBA7	Tyrrhenian	Olbia	+	+	**yes**
CBA8	Tyrrhenian	Olbia	+	+	n.a.[a]
CBA10	Tyrrhenian	Olbia	+	+	n.a.[a]
CBA11	Tyrrhenian	Olbia	+	+	**yes**
CBA12	Tyrrhenian	Olbia	+	+	n.a.[a]
CBA13	Tyrrhenian	Olbia	+	+	n.a.[a]
CBA14	Tyrrhenian	Olbia	+	+	n.a.[a]
CBA15	Tyrrhenian	Olbia	+	+	n.a.[a]
CBA16	Tyrrhenian	Olbia	+	+	**yes**
CBA17	Tyrrhenian	Olbia	+	+	n.a.[a]
CBA18	Tyrrhenian	Olbia	+	+	n.a.[a]
CBA19	Tyrrhenian	Olbia	+	+	n.a.[a]
CBA21	Tyrrhenian	Olbia	+	+	n.a.[a]
CBA22	Tyrrhenian	Olbia	+	+	n.a.[a]
CBA23	Tyrrhenian	Olbia	+	+	n.a.[a]
CBA24	Tyrrhenian	Olbia	+	+	n.a.[a]
CBA25	Tyrrhenian	Olbia	+	+	n.a.[a]
CBA27	Tyrrhenian	Olbia	+	+	**yes**
CBA28	Tyrrhenian	Olbia	+	+	n.a.[a]
VGO663	Tyrrhenian	Olbia	-	-	n.a.[a]
Palmira1	Catalan	Majorca	+	+	**yes**
Palmira2	Catalan	Majorca	+	+	n.a.[a]
Palmira3	Catalan	Majorca	+	+	n.a.[a]
Palmira4	Catalan	Majorca	+	+	n.a.[a]
Palmira5	Catalan	Majorca	+	+	no
CBA61	Catalan	Majorca	+	+	**yes**
CBA62	Catalan	Majorca	+	+	**yes**
CBA63	Catalan	Majorca	+	+	**yes**
CBA64	Catalan	Majorca	+	+	n.a.[a]
AMP4	Catalan	Majorca	+	+	n.a.[a]
AMP10	Catalan	Majorca	+	+	n.a.[a]
AMP13	Catalan	Majorca	+	+	**yes**
CSIC-D1	Catalan	Arenys	-	-	n.a.[a]
AL8C	Catalan	Arenys	+	+	**yes**
AL10C	Catalan	Estartit	+	+	n.a.[a]
AL11C	Catalan	Estartit	+	+	n.a.[a]
Min1	Catalan	Arenys	+	+	n.a.[a]
Min2	Catalan	Arenys	+	+	no
Min3	Catalan	Arenys	+	+	**yes**
Min4	Catalan	Arenys	+	+	n.a.[a]

Min5	Catalan	Arenys	+	+	**yes**
Min6	Catalan	Arenys	+	+	n.a.[a]
Min7	Catalan	Arenys	+	+	n.a.[a]
Min8	Catalan	Arenys	-	-	n.a.[a]
Min9	Catalan	Arenys	+	+	n.a.[a]
Min10	Catalan	Arenys	+	+	n.a.[a]
Min11	Catalan	Arenys	+	+	n.a.[a]
Min16	Catalan	Arenys	+	+	n.a.[a]
Min17	Catalan	Arenys	-	-	n.a.[a]
Min18	Catalan	Arenys	+	+	n.a.[a]
Min19	Catalan	Arenys	+	+	n.a.[a]
Min21	Catalan	Arenys	+	+	**yes**
Min22	Catalan	Arenys	+	+	n.a.[a]
Min23	Catalan	Arenys	+	+	n.a.[a]

[a]not analysed

Table S2. Determinations of *Alexandrium* spp. abundance by microscopy and total *sxtA1* gene content in seawater samples by qPCR assay collected at several stations in Syracuse Bay during spring 2014.

Sampling Stations and date	*A. minutum* abundance by microscopy (cells/L)[a]	*A. pacificum* abundance by microscopy (cells/L)[a]	Total *sxtA1* gene content (copies/L)[b]
St. 1 - Porto Grande Cantiere Di Benedetto 5 May	$7.68 \times 10^4 \pm 2.22 \times 10^4$	$3.47 \times 10^4 \pm 1.0 \times 10^4$	$4.44 \times 10^5 \pm 2.87 \times 10^5$
St. 2 - Porto Grande Sanità Marittima 5 May	$2.16 \times 10^4 \pm 7.03 \times 10^2$	$2.05 \times 10^4 \pm 6.68 \times 10^2$	$1.38 \times 10^5 \pm 1.80 \times 10^3$
St. 1 - Porto Grande Cantiere Di Benedetto 6 May	$6.67 \times 10^6 \pm 4.39 \times 10^5$	$5.0 \times 10^6 \pm 3.29 \times 10^5$	$7.97 \times 10^7 \pm 1.32 \times 10^7$
St. 2 - Porto Grande Sanità Marittima 6 May	$7.22 \times 10^6 \pm 1.31 \times 10^6$	$7.15 \times 10^6 \pm 1.29 \times 10^6$	$1.30 \times 10^8 \pm 4.42 \times 10^6$
St. 3 - Porto Grande Fonte Aretusa 6th May	$1.18 \times 10^5 \pm 4.27 \times 10^4$	$7.34 \times 10^4 \pm 2.65 \times 10^4$	$1.22 \times 10^6 \pm 2.97 \times 10^5$
St. 4 - Porto Grande Capitaneria di Porto 6 May	$1.25 \times 10^6 \pm 6.99 \times 10^4$	$2.34 \times 10^6 \pm 1.31 \times 10^5$	$4.61 \times 10^7 \pm 2.73 \times 10^7$
St. 5 - Porto Grande Canale Grimaldi 6 May	$7.10 \times 10^6 \pm 2.92 \times 10^5$	$6.48 \times 10^6 \pm 2.67 \times 10^5$	$1.14 \times 10^8 \pm 2.39 \times 10^7$
St. 1 - Porto Grande Cantiere Di Benedetto 8 May	$9.14 \times 10^6 \pm 9.56 \times 10^5$	$1.76 \times 10^7 \pm 1.84 \times 10^6$	$2.55 \times 10^8 \pm 3.08 \times 10^7$
St. 2 - Porto Grande Sanità Marittima 8 May	$1.04 \times 10^6 \pm 7.12 \times 10^4$	$9.15 \times 10^5 \pm 6.27 \times 10^4$	$1.98 \times 10^7 \pm 8.43 \times 10^6$
St. 6 - Porto Piccolo Circolo Ribellino 8 May	$1.01 \times 10^6 \pm 3.82 \times 10^4$	$9.80 \times 10^5 \pm 3.70 \times 10^4$	$1.41 \times 10^7 \pm 3.25 \times 10^6$

[a]Mean values measured in eight replicates with standard deviation ± SD.
[b]Mean values measured in three replicates with standard deviation ± SD.

Table S3. Precursor and product ions used in the LC-HRMS^n analyses and measured limits of detection (LOD) of individual SXT toxins.

Toxin	Precursor ion (*m/z*)	Formula	Product ion (*m/z*)	LOD (pmol/mL)	LOD (amol/cell)
GTX1	332.1	$[M + H - SO_3]^+$	314.1204	15	2.4
GTX4			253.1041	275	46
[a] STX	300.1	$[M + H]^+$	282.1311 221.1143 204.0877	13	2.2
B1	300.1	$[M + H-SO_3]^+$	282.1311 221.1143 204.0877	47	7.9
B2	316.1	$[M + H-SO_3]^+$	298.1254 220.0824	n.a.[a]	n.a.[a]
dcSTX	257.1	$[M + H]^+$	239.1255 222.0984 180.0765	105	18
GTX2	316.1	$[M + H-SO_3]^+$	298.1254	121	20
GTX3			220.0824	51	8.4
C1	316.1	$[M + H-2SO_3]^+$	298.1254	46	7.7
C2				231	39
NEO	316.1	$[M + H]^+$	298.1254 220.0824	19	3.2
dcGTX2	273.1	$[M + H-SO_3]^+$	255.1201	68	11
dcGTX3			238.0933	363	61
dcNEO	273.1	$[M + H]^+$	255.1201	203	34

[a]CRM B2 was not available at the time of the analysis.

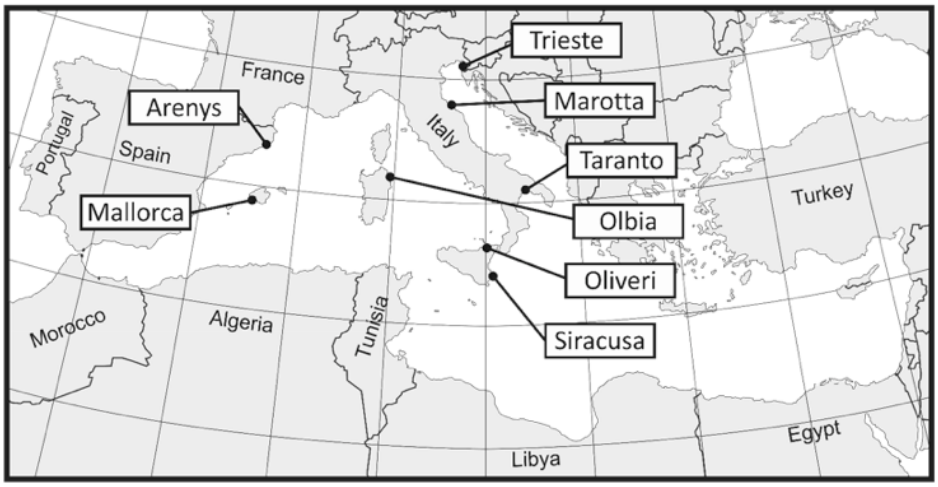

Fig. S1. Map of sampling sites of *A. minutum* strains used in this study in the Mediterranean Sea. Trieste, Adriatic Sea, Italy; Marotta, Adriatic Sea, Italy; Taranto, Ionian Sea, Italy; Syracuse, Ionian Sea, Italy; Oliveri, Tyrrhenian Sea, Italy; Olbia, Tyrrhenian Sea, Italy; Arenys, Catalan Sea, Spain; Mallorca, Balearic Sea, Spain.

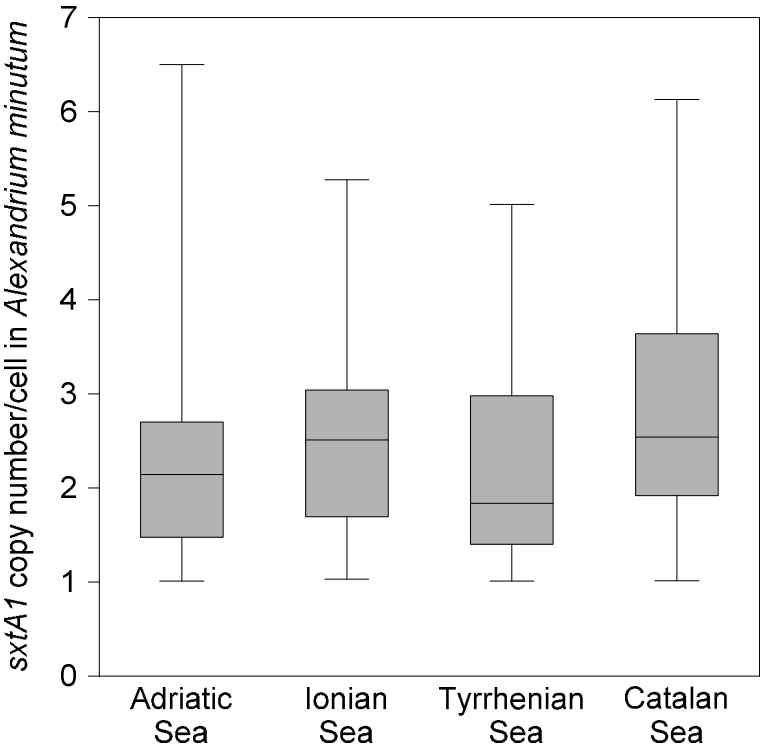

Fig. S2. The *sxtA1* gene copy number of the Mediterranean *A. minutum* populations.[24] Boxes show the two central quartiles of the distribution, while the whiskers show minimum and maximum. Adriatic Sea, n = 9 strains; Ionian Sea, n= 12 strains; Tyrrhenian Sea, n = 11 strains; Catalan Sea, n = 11 strains.

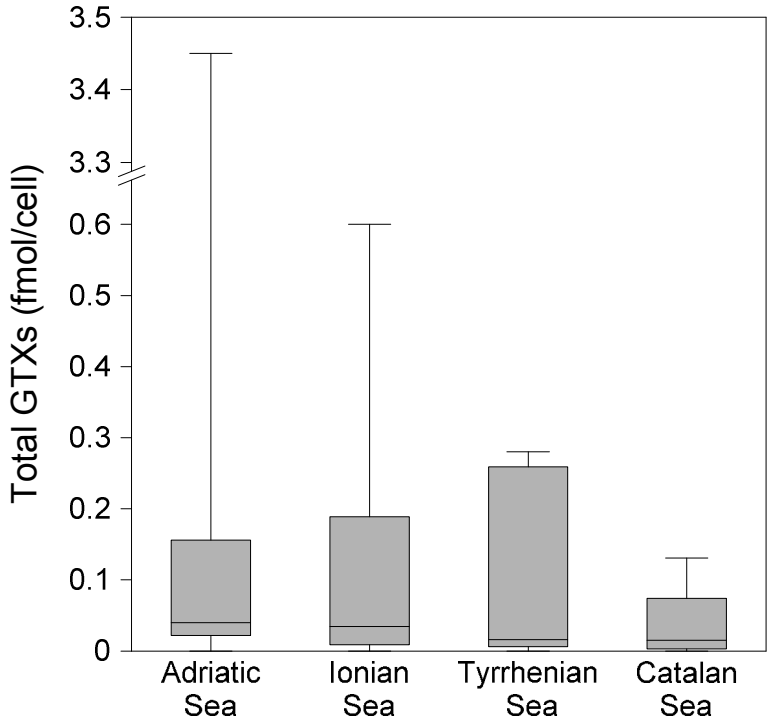

Fig. S3. Total GTX content of the four Mediterranean *A. minutum* populations.[24] Boxes show the two central quartiles of the GTX distribution, while the whiskers show minimum and maximum per geographical area: Adriatic Sea, n = 9 strains; Ionian Sea, n= 12 strains; Tyrrhenian Sea, n = 11 strains; Catalan Sea, n = 11 strains.

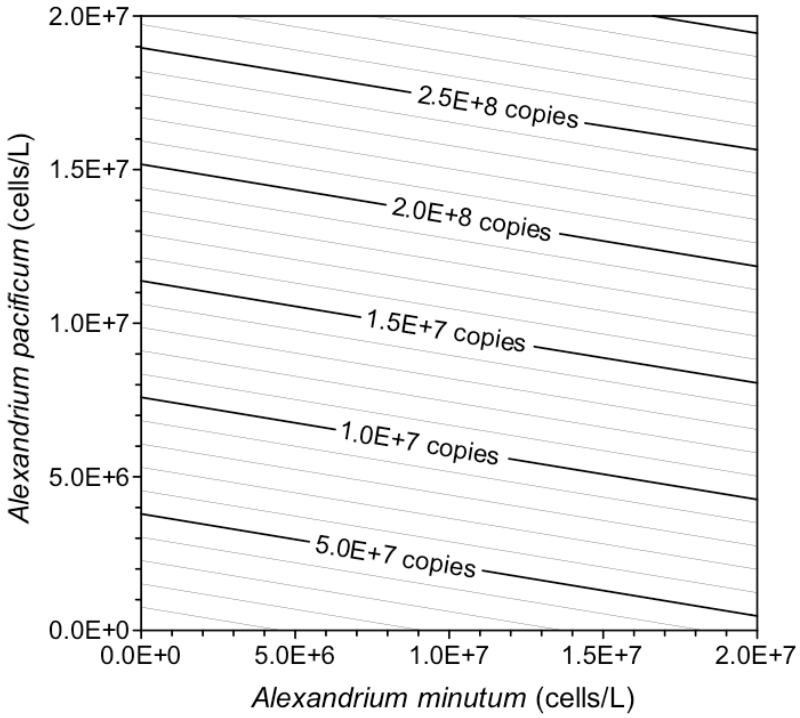

Fig. S4. The *sxtA1* gene copy number isopleths showing how any given copy number of *sxtA1* gene may correspond to an infinite set of coupled *A. minutum* and *A. pacificum* abundance. Once the copy number has been determined, the range that contained the two *Alexandrium* species true densities can be easily defined.