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Combining intact and oxidative LC-MS/MS methods to mitigate health risks from *Ostreopsis* cf. *ovata*

Chiara Melchiorre [a,b,*], Martina Carelli [a], Valeria Tegola [a], Samuela Capellacci [c], Silvia Casabianca [c], Antonella Penna [c], Carmela Dell'Aversano [a,b]

[a] University of Naples Federico II, School of Medicine and Surgery, Department of Pharmacy, via D. Montesano 49, 80131, Naples, Italy
[b] NBFC National Biodiversity Future Center, 90133, Palermo, Italy
[c] University of Urbino, Department of Biomolecular Sciences, Campus Enrico Mattei, Via Ca' le Suore, 2/4, 61029, Urbino, Italy

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ABSTRACT

Ostreopsis cf. *ovata* is a benthic marine dinoflagellate which produces highly potent neurotoxins named ovatoxins (OVTXs), with OVTX-a being the dominant variant in the Mediterranean strains. OVTXs are associated with respiratory issues via aerosol inhalation and skin/eye irritation from direct seawater contact. Recently, *FAO-IOC-IAEA guidelines* indicated potential health risk when *O.* cf. *ovata* concentrations exceed 3×10^4 cells L^{-1} in seawater and 5×10^5 cells g^{-1} in macroalgae. Routine monitoring programs focus only on cell counts and do not measure OVTX-levels, limiting the accuracy of health risk assessment. Herein, we detected and quantified ovatoxins in two of six samples collected along Campania coast by a combination of intact and oxidative sample preparation and Liquid Chromatography tandem Mass Spectrometry (LC–MS/MS). OVTXs concentration in seaweed wash seawater was in the range 1340–1497 ng/mL for Aragonese Castle-L sample and 60–107 ng/mL for Gaiola sample. Matrix effects ranged from –31 % to 2 %, and recovery yields from 41 % to 97 %. After correction for matrix effects and procedural losses, OVTX concentrations were estimated at 13.9 nM (Aragonese Castle-L) and 2.1 nM (Gaiola), values within the cellular toxicity range. However, the abundance of *O.* cf. *ovata* cells on macroalgae (6.4×10^4 and 1.6×10^4 cells g^{-1}) remained below the alert threshold. These results highlight the need to integrate toxin quantification into *O.* cf. *ovata* monitoring programs. Given the current lack of OVTX reference materials, the use of both intact and oxidative methods combined to LC-MS/MS is a valid strategy for effective risk assessment.

1. Introduction

Ostreopsis cf. *ovata* is a benthic dinoflagellate of tropical–subtropical origin that causes harmful algal blooms (HABs), which have progressively expanded across the Mediterranean Sea in the last decades [1–4]. Its expansion has been attributed not only to global warming but also to its ability to colonize a wide variety of natural (e.g. pebbles and macroalgae) and artificial substrates (e.g., rocky barriers, plastic debris, and ship material) [3,5–7]. *O.* cf. *ovata* produces potent neurotoxins structurally related to palytoxin (PLTX), known as ovatoxins (OVTXs) (Fig. 1), with OVTX-a being the dominant analogue in the Mediterranean strains. OVTXs have been associated also with toxic effects on marine organisms such as sea urchins and starfish [8–11].

OVTX-a can affect human health, primarily through inhalation of marine aerosols, dermal contact, or ingestion, leading to irritation of the eyes, skin, and respiratory mucosa, as well as gastrointestinal symptoms [11,15]. In Italy, *O.* cf. *ovata* and related OVTXs have been reported from several coastal regions including Liguria [16,17], Tuscany, Lazio, Campania, Apulia, Marche, Sardinia and Sicily [4,13,18–21]. To address the ecological and health risks associated with these toxins, a national program for the monitoring of harmful algae was launched in 2007 and later strengthened by the Ministerial Decree of March 30, 2010 [22], which set specific guidelines for the management of *O.* cf. *ovata* blooms in bathing waters. The surveillance plan defined three investigation levels: Routine, Alert, and Emergency based on cell abundance, with 1×10^4 cells L^{-1} established as the first threshold for risk management. In 2014, a multidisciplinary working group coordinated by the Ministry of Health, ISPRA, and the Istituto Superiore di Sanità published updated national guidelines (ISTISAN Report 14/19) [20,21], introducing an emergency phase triggered by concentrations above 3×10^4 cells L^{-1}

* Corresponding author at: University of Naples Federico II, School of Medicine and Surgery, Department of Pharmacy, via D. Montesano 49, 80131, Naples, Italy.
E-mail address: chiara.melchiorre@unina.it (C. Melchiorre).

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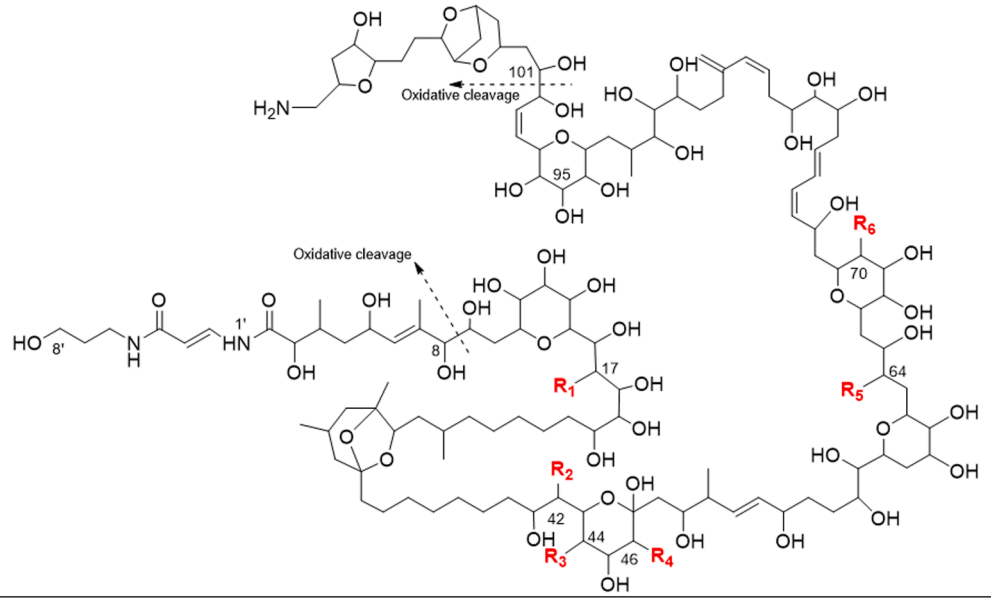
under favourable meteorological conditions. This threshold was officially adopted through the Ministerial Decree of April 19, 2018, and has been applied nationwide since the 2019 bathing season [23]. Recently joint FAO-IOC-IAEA technical guidelines confirmed the threshold of 3×10^4 cells L^{-1} of seawater and reported a threshold of 5×10^5 cells g^{-1} wet weight of macroalgae, above which *O.* cf. *ovata* may pose a risk to human health [24]. Nowadays, *Ostreopsis* blooms are increasingly widespread across several Italian regions, as documented by the extensive grey literature published in 2025 (Table S1).

In the Campania Region, the Regional Environmental Protection Agency (ARPAC) has conducted a systematic monitoring of *O.* cf. *ovata* since 2007 [20]. Publicly available datasets cover the period 2007–2024 [25] and include regular sampling from June to September on seawater and macroalgae, with additional testing on edible marine organisms such as mussels and sea urchins when cell densities exceeded the alert threshold of 1×10^4 cells L^{-1} of seawater or g^{-1} of substrate [21]. Seasonal trends in Campania are consistent with those observed in other Mediterranean regions, with a first proliferation peak typically occurring in mid-July, followed by a decline in August, and a second peak in September at some monitoring areas [21]. Such dynamics are favoured by high water temperature (> 25 °C) and low turbulence [21,26].

At the national scale, data collected by ISPRA and the regional ARPA network indicate that *O.* cf. *ovata* has been detected at least once in 12 out of the 15 Italian coastal regions. In 2024, it was reported in 11 regions, corresponding to 75 % of the monitored stations [26]. This widespread occurrence highlights the ecological and health relevance of *O.* cf. *ovata* proliferation and the need to strengthen monitoring protocols, particularly in bathing waters and shellfish harvesting areas, as well as to promote the development of robust analytical methods for direct detection and quantification of ovatoxins in environmental and biological matrices.

In this study, for the first time two distinct and complementary sample preparation approaches followed by liquid chromatography tandem mass spectrometry (LC-MS/MS) in Multiple Reaction Monitoring (MRM) mode were implemented to detect and quantify ovatoxins in samples from the Campania coast. The sampling sites included two marine protected areas (MPA), where waters were classified as safe for bathing and were not under alert phase at the time of sampling, according to the ISPRA 421/2025 report [25]. The first sample preparation approach (intact method) consisted in a simple cleanup procedure after toxin extraction from algal pellets [27]. The second approach (oxidative method) involved a microscale oxidation that generated two diagnostic oxidative cleavage products of the molecule [14], as highlighted in Fig. 1. This combined strategy was adopted to overcome limitations related to the lack of certified reference materials for OVTXs and to address the peculiar MS behavior of these toxins. Indeed, MS analysis of microalgal extracts containing ovatoxins is highly challenging, not only because of the structural complexity of these molecules but also because of their strong propensity to form adducts with metals such as calcium, magnesium, sodium and potassium [28]. In addition, the high number of vicinal hydroxyl groups contained in the molecules results in extensive water losses from the pseudo molecular ions at three charge states, namely $[M + mH - nH_2O]^{m+}$ (with $n \geq 1$; $m = 1,2,3$). Altogether, these factors result in extensive overlap among the ions formed in MS spectra, which can complicate spectral interpretation and pose significant challenges for absolute toxin quantification. For instance, in intact toxin analysis the most abundant adduct ion of OVTX-a is the calcium adduct $[M + H + Ca]^{3+}$ [29] corresponding to an exact m/z of 895.8197 with $z = 3$. This value overlaps with the magnesium adduct of OVTX-d $[M + H + Mg]^{3+}$ (m/z 895.8257, $z = 3$). Similarly, the magnesium adducts of



Toxin	Formula	R1	R2	R3	R4	R5	R6	Other structural details
Palytoxin	$C_{129}H_{223}N_3O_{54}$	OH	H	OH	OH	OH	OH	
Ovatoxin-a	$C_{129}H_{223}N_3O_{52}$	H	OH	H	OH	H	OH	
Ovatoxin-b	$C_{131}H_{227}N_3O_{53}$	H	OH	H	OH	H	OH	$+C_2H_4O$ in region N-C8'
Ovatoxin-c	$C_{131}H_{227}N_3O_{54}$	H	OH	OH	OH	H	OH	$+C_2H_4O$ in region N-C8'
Ovatoxin-d	$C_{129}H_{223}N_3O_{53}$	H	OH	OH	OH	H	OH	
Ovatoxin-e	$C_{129}H_{223}N_3O_{53}$	H	OH	H	OH	H	OH	+O in region C8-8'
Ovatoxin-f	$C_{131}H_{227}N_3O_{52}$	H	OH	H	OH	H	OH	$+C_2H_4$ in region C95-C102
Ovatoxin-g	$C_{129}H_{223}N_3O_{51}$	H	OH	H	OH	H	OH	
Isobaric palytoxin	$C_{129}H_{223}N_3O_{54}$	H	OH	OH	OH	H	H	+O in region C8-C8'

Fig. 1. Planar structure of palytoxin (PLTX) and of some of the most common ovatoxins (OVTXs) from the Mediterranean Sea. PLTX and OVTX-a structures were elucidated by NMR [12]. Structures of all the other congeners were proposed based on LC—$HRMS^n$ ($n = 1$, 2 and/or 3) data only [13]. The two oxidative cleavage sites following microscale oxidation [14] are highlighted.

OVTX-a $[M + H + Mg]^{3+}$ (m/z 890.4941, $z = 3$) fully overlaps with the calcium adduct of OVTX-g $[M + H + Ca]^{3+}$ (m/z 890.4870, $z = 3$). These examples illustrate only a fraction of the issue, as many additional overlapping masses are observed among different ovatoxin analogues, further increasing the difficulty of accurate identification and quantification of OVTXs [27]. In the oxidative approach, fragments generated through chemical oxidation (Fig. 1) are monitored, focusing the LC-MRM-MS analytical method on structural moieties produced by specific cleavages at the vicinal diols located at positions C8–C9 and C100–C101 [14]. These cleavages generate an acetamide specific fragment and an amine fragment common to all OVTXs (Table 1).

Although oxidative approach also presents some limitations – different ovatoxins generate identical oxidative fragments (e.g. acetamide-containing fragment ion at m/z 343 for OVTX-a, -d, -f, and -g) hampering identification of individual analogues – it nonetheless provides complementary information that strengthens both the identification and quantitation of OVTX analogues in complex matrices.

We chose to employ mass spectrometry in MRM mode, focusing specifically on the detection of target transition either reported in the literature or predicted using ChemDraw software V 22.2.0, as the objective of this study was confirmatory rather than exploratory. The results obtained here provide a basis for the development of alternative monitoring strategies for *O.* cf. *ovata* blooms, or the improvement of existing approaches, through the direct identification and quantification of OVTXs from macroalgal samples, thereby enhancing the reliability of risk assessments related to *O.* cf. *ovata* proliferation.

2. Materials and methods

2.1. Sampling, collection, and pre-treatment

Sampling was conducted in July 2024 in the frame of BlueShellfish HORIZON-MSCA-2021-SE-01 project ID 101086234 at sampling sites located in the Gulf of Naples (Campania region, Italy), including the two MPA of Regno di Nettuno Zone B and C (July 5th and 6th 2024) and Gaiola underwater park Zone A (July 11th 2024) (Table 2, Fig. 2) [30]. The Regno di Nettuno MPA is divided into three main protection zones: Zone A (integral reserve), with maximum protection and only access for research, surveillance and rescue; Zone B (general reserve), where activities such as bathing, snorkelling, diving, small-scale fishing and limited recreational uses are permitted under authorization, with a more restrictive sub-zone B no-take; and Zone C (partial reserve), where navigation, recreational fishing and mooring are generally allowed still regulated (https://www.nettunoamp.it/mappa.php). The Gaiola Underwater Park is structured into two main protection zones: Zone A (integral reserve), where access and activities are strictly limited to scientific research and guided visits, and Zone B (general reserve), where controlled recreational activities (bathing, diving, snorkelling, non-motorized navigation) are allowed under regulation (https://www.areamarinaprotettagaiola.it/). Both sites are monitored by ARPAC periodically and were not under alert or emergency status at the time of the sampling exercise. At each site seaweeds were randomly collected from rocks and seawater column near the coastline, placed in approximately 500 mL of seawater in hermetic plastic bags, and shaken to dislodge benthic microalgal cells from seaweed surface. An aliquot of the resulting seawater was collected for counting *O.* cf. *ovata* cells and culturing *O.* cf. *ovata* strains, while the remaining seawater was separated from macroalgae and underwent further processing by intact and oxidized method with LC-MS/MS.

The wet seaweed weight (in grams) was recorded after drying under a fume hood for 20 min (Table 2 and S2). The seawater fraction was centrifuged at 2500 rpm for 30 min, yielding two pellets per sample; the supernatant was discarded.

2.2. Equipment

Centrifugation was carried out using a HERMLE Z326 centrifuge (©Hermle Labortechnik GmbH, Wehingen, Germany). Sonication was performed with a Bandelin UW 2200 sonicator equipped with Bandelin Sonoplus (BANDELIN Electronic GmbH & Co. KG, Berlin, Germany). Qualitative and quantitative LC-MRM-MS analyses were carried out on a Triple Quadrupole MS Agilent 6470 (SN: SG2050G208, Agilent, Santa Clara, CA 95051 USA) equipped with an ESI ION source and coupled with an ultra-high-performance liquid chromatography (UHPLC) Agilent 1290 Infinity II (SN: DEBAZ05437, Agilent, Santa Clara, CA 95051 USA). Target acquisition was carried out with Agilent MassHunter

Table 1
Oxidized ions of OVTX congeners were predicted using ChemDraw software (© 1998–2023 PerkinElmer Informatics, Inc.), based on structures reported in the literature by Selwood et al. [14] and Tartaglione et al. [13].

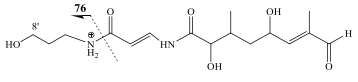
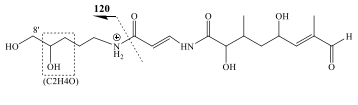
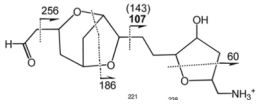
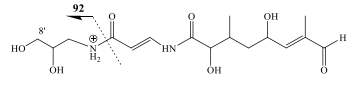
Acronym	Acetamide OX cleavage with relevant most probable MS fragments	Common Amine OX fragment [14]
PLTX, OVTX-a, -d, -f, -g	 Chemical Formula: $C_{16}H_{27}N_2O_6^+$ Exact Mass: 343,19	
OVTX-b, -c	 Chemical Formula: $C_{18}H_{31}N_2O_7^+$ Exact Mass: 387,21	 Chemical Formula: $C_{15}H_{26}NO_5^+$ Exact mass: 300,18
OVTX-e, -Isob-PLTX	 Chemical Formula: $C_{16}H_{27}N_2O_7^+$ Exact Mass: 359,18	

Table 2
Sampling site coordinate, wet weight (g) of the seaweed collected from each sampling site with relevant seawater volume (mL) and net wet weight of the obtained pellets (g).

Site n.	Site Coordinate	Site description	Sample name	Seaweed (g)	Seawater (mL)	Pellet 1 (g)	Pellet 2 (g)
1a	40°44′56.2″N 13°59′43.5″E	Vivara Island, Regno di Nettuno MPA - Zone B	Vivara	17.7	490	0.22	0.30
1b[1]	40°45′46.5″N 14°00′31.9″E	Pozzo vecchio, Procida Island, Regno di Nettuno MPA - Zone C	Pozzo Vecchio	33.3	294	1.72[1]	2.21[1]
2a	40°43′54.8″N 13°57′46.4″E	Left side of the Aragonese Castle, Ischia Island, Regno di Nettuno MPA - Zone C	Aragonese Castle-L	81.4	514	1.19	0.94
2b	40°43′52.7″N 13°57′48.3″E	Right side of the Aragonese Castle, Ischia Island, Regno di Nettuno MPA - Zone C	Aragonese Castle-R	110	355	0.78	0.90
2c	40°44′41.1″N 13°56′45.1″E	Ischia Porto, Island of Ischia, Regno di Nettuno MPA - Zone C	Ischia Porto	19.3	210	0.33	0.64
3a	40°47′32.0″N 14°11′13.8″E	Posillipo coast, Naples, Gaiola Underwater Park MPA - Zone A	Gaiola	59.9	500	0.48	0.40

[1] Pellets from the Pozzo Vecchio sample were not observed, and the net weight was calculated by considering the residual supernatant with suspended macroalgal particles.

Fig. 2. Site sampling spot along Regno di Nettuno MPA: Procida island 1a (Zone B) and 1b (Zone C), Ischia Island 2a, 2b and 2c (Zone C); and Gaiola Underwater Park MPA: Posillipo coastline 3a (Zona A), Naples, Italy. Background: modified data from Copernicus ESA Sentinel 2 data. Highlighted in yellow the protected Marine Area from *World Database on Protected Areas* (WDPA).

Qualitative and Quantitative Analysis software V10.0 (2006–2018). Chemical structures were drawn by ChemDraw software V 22.2.0.

2.3. Materials

MilliQ water (W) was obtained by double filtration by Sinergy UV LC-Pak Polisher (Lot. N° F3NB63201) purchased from Merck Millipore (Darmstadt, Germany). Methanol (MeOH), Acetonitrile (ACN) and 50 mL conic Polypropylene tubes were purchased from VWR International Srl (Milan, Italy). Periodic acid and Acetic Acid (AA) were purchased by Merck Sigma-Aldrich (Darmstadt, Germany). Solid phase extraction (SPE) cartridge Strata-X 33 μm polymeric reverse phase 60 mg /3 ml was purchased from Phenomenex (Torrance CA, USA). SPE vacuum manifold was purchased from Agela technologies (Torrance CA, USA) and connected to a VACUUBRAND™ Pumping Unit, MD 4C (VACUUBRAND GMBH + CO KG, Wertheim, Germany). PLTX standard 100 μg (Lot. N° WTR7399) was purchased from FUJIFILM Wako Chemicals Europe GmbH (Neuss, Germany) and stored at −20 °C until sample preparation.

2.4. Isolation, DNA extraction and PCR amplification of Ostreopsis cf. ovata strains

O. cf. *ovata* strains CBA41, CBA62, CBA50, and CBA54 were isolated from macroalgal wash water samples collected at Procida, Pozzo Vecchio, Naples, Italy (site 1b, Fig. 2). Clonal cultures were maintained in 1 L glass bottles containing 400 mL of sterilized F/4 medium without silicate (F/4 − Si) at 23 ± 1 °C, under cool-white fluorescent illumination at a photon flux density of 100 μE m^{-2} s^{-1} with a 14:10 h light:dark

photoperiod. Cell pellets of each strain, comprising approximately 1.0 × 10^6 cells, were harvested during the exponential growth phase for chemical analysis. Genomic DNA was extracted using the DNeasy Plant Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. Polymerase chain reaction (PCR) amplification of *O.* cf. *ovata* ribosomal genes was carried out as described by Battocchi et al. [31]. PCR products were loaded onto a 1.8 % (w/v) agarose gel prepared in 1 × TAE buffer and visualized using a Gel Doc System (Bio-Rad).

2.5. Extraction

Cell pellets from culture of *O.* cf. *ovata* strains CBA41, CBA62, CBA50, and CBA54 along with the pellets number 2 (Table 2) from seaweed samples pre-treatment were extracted by adding 6 mL of 80 % aqueous methanol solution (v/v) and vortexed for a few minutes to ensure homogenization. The resulting suspension was then divided into two equal aliquots (A and B, 3 mL each). Aliquots A along with the procedural blank-a sample (3 ml of 80 % aqueous methanol) were spiked by adding 30 µL of PLTX standard 50 µg/mL to a final concentration of 500 ng/mL. In parallel, aliquots B and procedural blank-b were left unspiked (Fig. 3).

All aliquots (both spiked and non-spiked, including procedural blanks) were subjected to the extraction procedure, as described by Miele et al. [29], namely sonication in ice-bath at 20 % AMP for 10 min in pulse mode (1 s stop-time), followed by centrifugation at 7100 g for 10 min at room temperature. The supernatants were collected and

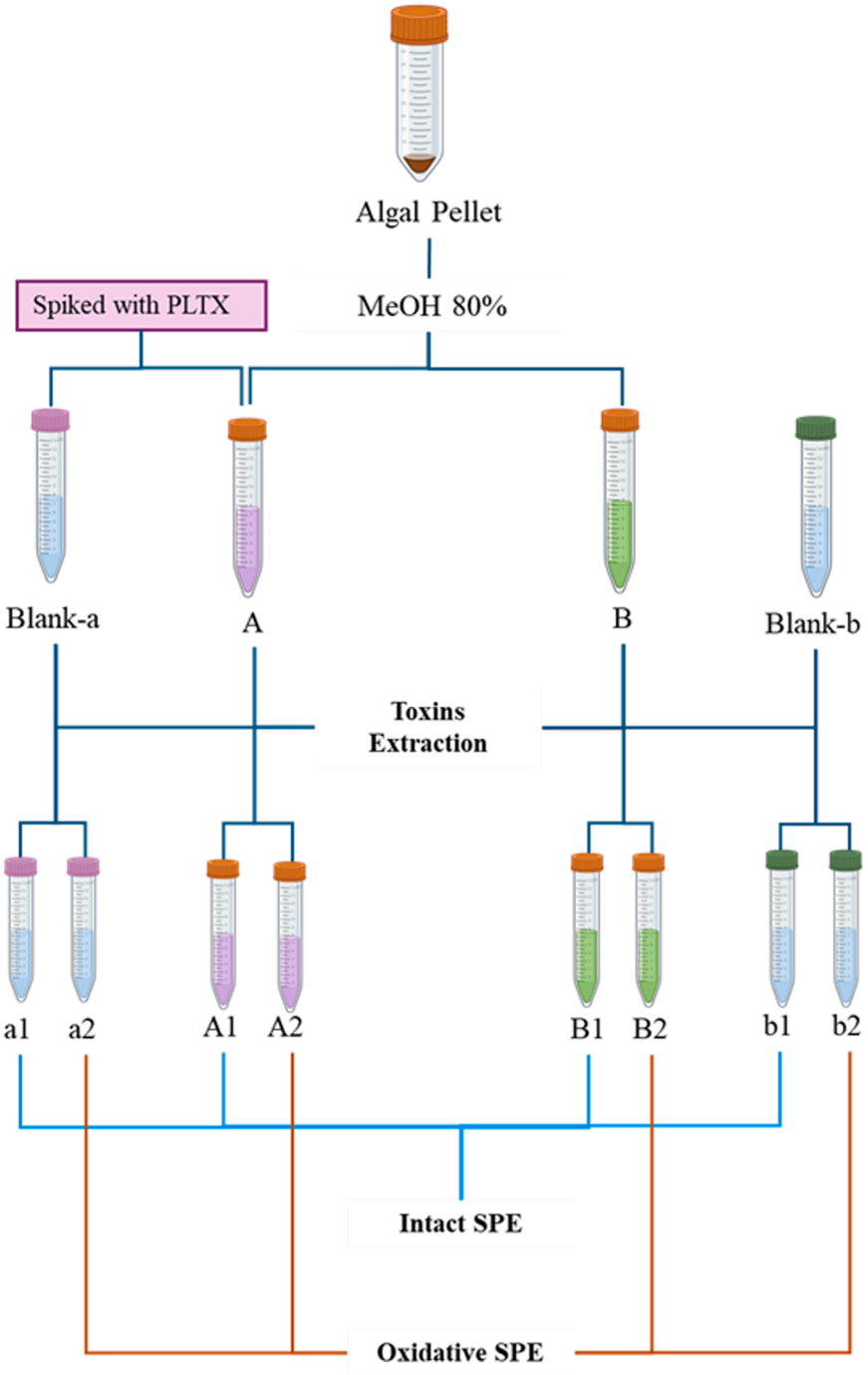


Fig. 3. Sample preparation workflow for solid phase extraction (SPE) clean-up intact and microscale oxidation.

diluted 1:4 by adding 1.8 mL of Milli-Q water and 7.2 mL of 50 % aqueous methanol, resulting in a final volume of 12 mL (crude extract). This dilution was performed to prepare the samples for intact solid phase extraction (SPE) clean-up and oxidative SPE.

At this stage, the supernatants were further divided into two 6 mL sub-aliquots: spiked sub-aliquots A1 (for intact method) and A2 (for oxidative method) and spiked procedural blanks a1 and a2; non-spiked sub-aliquots B1 (for intact method) and B2 (for oxidative method), along with corresponding non-spiked procedural blanks b1 and b2 (Fig. 3). From each crude extract samples spiked (125 ng/mL PLTX) and not spiked, 20 µL were taken for LC-MRM-MS analysis by the intact method described in paragraph 2.7.

2.6. Intact SPE clean-up and oxidative SPE

Aliquot A1 and B1 of each sample (6 mL) with relevant procedural blanks a1 and b1 were subjected to intact SPE clean-up procedure [27], while aliquots A2 and B2 with relevant procedural blanks a2 and b2 were subjected to on column oxidation [14] experiments (Fig. 3). Experimental procedures are reported in Table 3 as a slightly modified version of the Intact SPE clean-up procedure described by Ciminiello et al. [27] and Oxidative SPE reported by Selwood et al. [14].

The SPE vacuum manifold was connected to a vacuum pump operating at 750 millibar, with the manometer maintained between −5 and −10 inHg.

Intact eluted samples, both spiked (250 ng/mL PLTX) and non-spiked, were analysed by LC-MRM-MS intact method as described in paragraph 2.7. Oxidated eluted samples, both spiked (250 ng/mL PLTX) and non-spiked, were analysed by LC-MRM-MS Oxidative method as described in paragraph 2.8.

2.7. LC-MRM-MS intact method

Optimal ion source, acquisition parameters and chromatographic settings were as described in Melchiorre et al. [32]. The LC autosampler (Agilent, Santa Clara, CA 95051 USA) was kept at the operating temperatures of 25 °C during the experiments. Experiments were carried out in MRM mode (positive ions), scanning two transitions precursor ion → product ion for each target analyte (Table 4). Dwell time was set at 100 ms for each transition. The $[M + H + Ca]^{3+}$ ion of each OVTX was selected as precursor ion of the transitions to the following product ions $[M + H - B\ moiety - H_2O]^{+}$ and $[M + Ca - A\ moiety - 2H_2O]^{2+}$ with the former used as Quantifier transition. These transitions were selected based on previous data proving that calcium adducts is the most prominent ion formed under the used conditions [28,32] and included about all the known OVTX congeners known so far [29].

Semi-quantitative analysis was performed using multiple level external calibration approach: a five-point calibration curve of PLTX standard from Wako (not certified material) diluted in MeOH 50 % at 1000, 500, 250, 125, 62.5 and 31.25 ng/mL ($y = 0.5619\,x + 1.8634$; $R^2 = 0.9954$) was employed, assuming a comparable molar response between PLTX and OVTXs. Although this assumption is reasonable based on their structural similarity (Fig. 1), it cannot be considered as conclusive. Accurate absolute quantification can only be achieved using certified reference materials for each individual PLTX analogues, which are currently not commercially available. Consequently, the production of certified OVTX-a reference material should be considered as a priority. The limits of detection (LOD) and quantitation (LOQ) were measured empirically over two instrumental replicates at 31 and 62 ng/mL (ppb) (Figure S1), respectively.

Table 3
Procedural SPE steps in both Intact and Oxidative sample preparation approaches.

Intact SPE	Oxidative SPE
Step 1- Conditioning with 3 ml of MeOH	**Step 1-** Conditioning with 3 ml of MeOH
Step 2- Conditioning with 3 mL of milliQ water	**Step 2-** Conditioning with 3 mL of milliQ water
Step 3- Loading of 6 ml extract in 50 % MeOH	**Step 3-** Loading of 6 ml extract in 50 % MeOH
Step 4- Washing with 3 mL of 40 % MeOH	**Step 4-** Washing with 3 mL of 40 % MeOH
Step 5- Elute with 3 mL of 80 % MeOH 0.1 % AA	**Step 5-** Washing with 3 mL of milliQ water
	Step 6- Oxidation with 2 mL of 50 mM periodic acid
	Step 7- Wash with 2 mL of milliQ water
	Step 8- Elute with 3 mL of 60 % MeOH 0.1 % AA

Table 4
Monitored transition in LC-MRM-MS Intact acquisition method.

PLTX-Like compounds		Precursor Ion	Product ion 1 Quantifier transition	Product Ion 2 Qualifier transition
Name	Acronym	$[M + H + Ca]^{3+}$	$[M - B\ moiety + H - H_2O]^{+}$	$[M - A\ moiety + Ca - 2H_2O]^{2+}$
Palytoxin	PLTX	906.8	327.2	1169.1
Ovatoxin-a	OVTX-a	896.2	327.2	1153.1
Ovatoxin-b	OVTX-b	910.8	371.2	1153.1
Ovatoxin-c	OVTX-c	916.2	371.2	1161.1
Ovatoxin-d	OVTX-d	901.5	327.2	1161.1
Ovatoxin-e	OVTX-e	901.5	343.2	1153.1
Ovatoxin-f	OVTX-f	905.5	327.2	1167.1
Ovatoxin-g	OVTX-g	890.8	327.2	1159.1
Ovatoxin-h	OVTX-h	891.5	327.2	1146.1
Ovatoxin-i	OVTX-i	910.2	327.2	1174.1
Ovatoxin-j1	OVTX-j1	915.5	327.2	1182.1
Ovatoxin-k	OVTX-k	920.8	327.2	1190.1
Isobaric palytoxin	isobPLTX	906.8	343.2	1161.1

2.8. LC-MRM-MS oxidative method

Optimal ion source parameters were Gas Temperature (°C) 250, Gas Flow (L/min) 11, Nebulizer pressure (psi) 40, Sheath Gas Temperature (°C) 350, Sheath Gas flow (L/min) 12, Capillary current (nA) 4500 and Nozzle Voltage 1000 (V). The LC autosampler was kept at the operating temperatures of 25 °C during the experiments. Experiments were carried out in MRM mode (positive ions), scanning one transitions precursor ion → product ion specific to the group of target analyte and one common transition to all target OVTX congeners (Table 5). The transitions were predicted for each analyte based on literature data available for OVTX-a and PLTX [13,14] and further elaborated using ChemDraw software (v.22.2.0; Table 1). Dwell time was set at 100 ms, Fragmentor at 135 (V), Collision Energy at 30 (V), Cell accelerator voltage at 5 (V) for each transition. Chromatographic column and mobile phases were the same as those used within the intact method [32]. The elution gradient increased from 10 % to 80 % eluent B (ACN aqueous solution 0.1 %

Table 5
Monitored transition in LC-MRM-MS oxidative acquisition method.

PLTX-like compound	Acronym	Precursor Ion	Transition Quantitative	Transition Qualitative
Palytoxin, Ovatoxin-a, -d, -f, -g	PLTX, OVTX-a, -d, -f, -g	343.2	76.0	135.1
Ovatoxin-b, -c	OVTX-b, -c	387.2	119.5	-
Ovatoxin-e, Isobaric Palytoxin	OVTX-e, IsobPLTX	359.2	92.07	-
Common Amine fragment	R-NH_2 frag.	300.1	-	107.0

Acetic Acid) over 5 min, then from 80 % to 100 % B over the next 5 min, followed by a 5-minute hold at 100 % B. The gradient was then returned to the initial condition of 10 % B within 2 min. The total run-time was 26 min. Quantitation was performed using single level external calibration approach using oxidized procedural blank spiked with PLTX std at a final concentration of 250 ng/mL. Since oxidized product standards are not available, recovery could not be determined. Therefore, based on Selwood et al. [14], a complete on-column oxidation for all PLTX-like compounds as well as a recovery > 90 % for the SPE procedure were assumed. However, variations in matrix composition and experimental conditions may influence both oxidation efficiency and extraction recovery. Therefore, the reported concentrations should be interpreted as estimates under the applied conditions rather than fully recovery-corrected absolute values.

3. Results and discussion

Macroalgal and seawater samples were collected in July 2024 in the Gulf of Naples (Fig. 2) at Marine Protected Areas Regno di Nettuno and Gaiola underwater park. The sampling sites are in diverse geomorphological settings shaped by the Phlegraean volcanic system and influenced by bradyseism and hydrothermal activity. Vivara (Zone B, Regno di Nettuno MPA) features steep rocky coasts of an ancient volcanic crater with pyroclastic deposits. Pozzo Vecchio (Zone C, Regno di Nettuno MPA) has cliffs of consolidated tuff and surge deposits alternating with small sandy pockets. Aragonese Castle (Zone C, Regno di Nettuno MPA) includes both left (western) and right (eastern) sides of a trachytic dome. Both sites are characterized by rocky substrates plunging steeply into the sea but show different exposures: the left side (Aragonese Castle-L) is more directly impacted by wave action and hosts several active CO_2 vents that generate localized acidified conditions and specific microhabitats for flora and fauna, whereas the right side (Aragonese Castle-R) is more sheltered, with higher sediment accumulation and distinct benthic cover. Ischia Porto (Zone C, Regno di Nettuno MPA) is anthropogenically modified but underlain by volcanic deposits, with shallow seabed dominated by fine sediments and debris. Gaiola (Zone A, Gaiola Underwater Park) features high cliffs, submerged caves, and partially sunken Roman archaeological remains, with rocky seabed influenced by hydrothermal CO_2 emissions which cause localized water acidification and impacts on marine biodiversity.

Samples were processed using two distinct and complementary chemical approaches after toxin extraction: SPE clean-up of the intact toxins and oxidative SPE, both followed by LC-MRM-MS analyses to determine the OVTX concentration in the collected samples (Table 6). For each approach, each sample was divided into two aliquots (Fig. 3), one of which was spiked with a known amount of PLTX (500 ng/mL) prior to performing both the entire experimental procedures (Fig. 3). By comparing the measured PLTX concentrations in the spiked intact sample aliquots with those in the spiked procedural blank, we evaluated the impact of the sample matrix on ionization, which caused signal suppression/enhancement ranging from +2 % to –31 % depending on the sample (Table 7). In addition, we quantified the overall recovery of the sample treatments by comparing PLTX concentrations in spiked samples before and after SPE, obtaining values ranging from 41 % to 97 % (Table 7).

In all sampling sites (except Ischia Porto), the presence of *O.* cf. *ovata* was confirmed by cell identification in seaweed wash seawater. Results showed that *O.* cf. *ovata* cells varied across all samples. Aragonese Castle-L and Gaiola yielded the highest cell abundance, expressed as number of *O.* cf. *ovata* cells per gram of macroalgae (Table 6). Interestingly, both sampling sites are impacted by hydrothermal activity, with reported evidence of CO_2 emissions, which result in localized acidification and consequently create a distinctive marine habitat [33].

O. cf. *ovata* cells abundance on macroalgal samples were consistent with OVTXs quantitative results (Table 6, Table S3 and S4). LC-MRM-MS analysis using the intact method (paragraph 2.7) revealed a total OVTXs concentrations of 1497 ng/mL and 107 ng/mL in the Aragonese Castle-L and Gaiola eluate samples, respectively. With the oxidative method (paragraph 2.8), the concentrations were 1340 ng/mL and 60 ng/mL (Table 6). Fig. 4a and Fig. 4b show the extracted MRM transitions of all the monitored ovatoxins using the intact LC-MRM-MS method. The Aragonese Castle-L sample exhibited a toxin profile characteristic of Mediterranean strains of *O.* cf. *ovata* (Fig. 4c), whereas in the Gaiola sample only the major component of the toxin profile, OVTX-a, was detectable, as its concentration was near the estimated LOQ of the intact method (Fig. 4d).

Fig. 5a and Fig. 5b present the extracted MRM transitions obtained using the LC-MRM-MS oxidative method. As discussed above, this method does not allow differentiation between the main component of the toxin profile, OVTX-a, and the other three analogues (OVTX-d, -f, and -g) as they share the same oxidized ions (Table 1). Nevertheless, the oxidized method improved overall detection, enabling the identification of OVTX-b and -c in the Gaiola sample, whereas these toxins could not be detected using the intact method.

It cannot be excluded that ovatoxins were also present in the other samples, but their concentrations were likely below the LOD of the LC-MRM-MS intact and oxidative methods.

Four distinct *O.* cf. *ovata* strains were isolated from Procida Pozzo Vecchio sample, despite the field sample collected at this same site had tested negative for OVTXs by both intact and oxidative methods. Strains were subsequently cultured following the procedure described in paragraph 2.4.

Ovatoxins were identified in all the isolated strains (Fig. 6a). The most productive strain was CBA 41 (Fig. 6a) whose toxin profile resulted

Table 6
O. cf. *ovata* cells abundance per gram of fresh macroalgae and OVTXs concentration in SPE eluate obtained by both intact and oxidative LC-MRM-MS methods for each sample.

Sample name	*O.* cf. ovata cells per g of macroalgae (Cells g^{-1})	OVTXs concentration (ng/mL) Intact method	OVTXs concentration (ng/mL) Oxidation method
Vivara	35	<LOD	<LOD
Pozzo Vecchio	893	<LOD	<LOD
Aragonese Castle-L	64177	1497	1340
Aragonese Castle-R	120	<LOD	<LOD
Ischia Porto	NA	<LOD	<LOD
Gaiola	16035	107	60

NA: not acquired due to the degradation of the sample.

Table 7
Matrix effects (%) were calculated for Aragonese Castle-L, Gaiola and Aragonese Castle-R samples. Values represent the influence of the sample matrix on the detection of the target compound PLTX, determined by comparing the analytical response of a procedural blank extract in 80 % MeOH spiked with 500 ng/mL PLTX to that of the corresponding samples spiked at the same level.

Sample name	Peak area PLTX 906.8→ 327.2	PLTX Conc. (ng/mL)	Matrix effect (%)	SPE Recovery (%)
Procedural blank Crude Extract_sp 500ppb	314.37	500		45
Aragonese Castle-L Crude Extract_sp 500ppb	239.87	382	−24	84
Gaiola Crude Extract_sp 500ppb	319.50	508	2	41
Aragonese Castle-R Crude Extract_sp 500ppb	215.84	343	−31	97

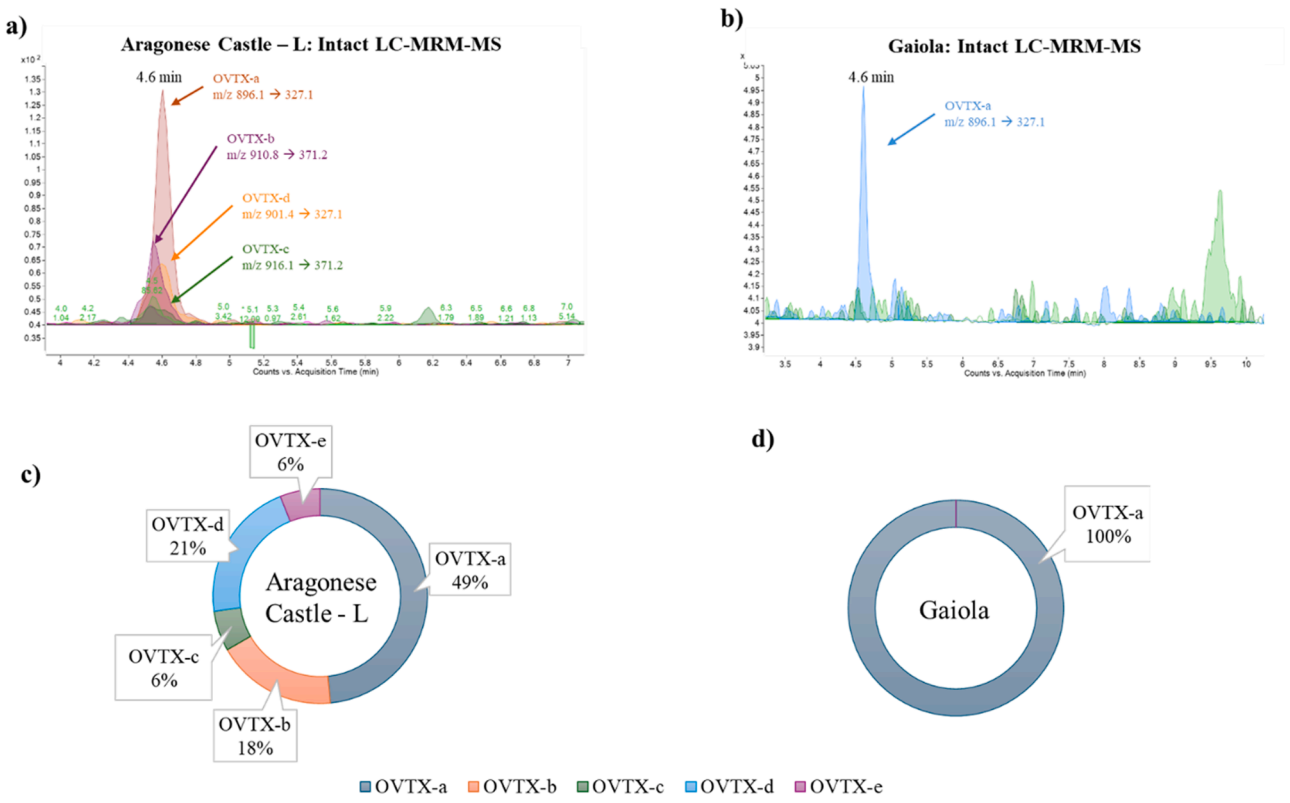


Fig. 4. Extracted MRM transitions from the total ion chromatogram (TIC) of LC-MRM-MS intact analyses of a) Aragonese Castle-L and b) Gaiola samples, with assigned targeted OVTXs monitored transitions. Panels c) and d) show the corresponding ovatoxin profiles for Aragonese Castle-L and Gaiola samples, respectively.

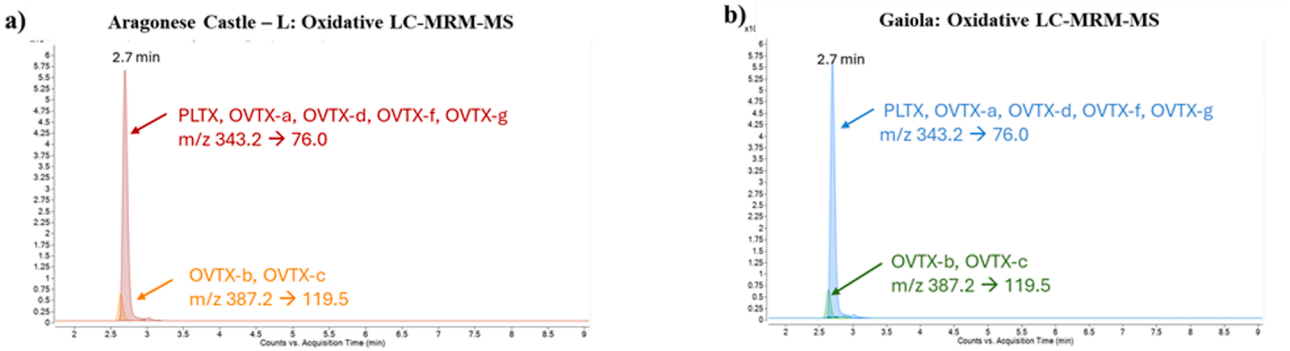


Fig. 5. Extracted MRM transitions from the total ion chromatogram (TIC) of LC-MRM-MS oxidative analyses of a) Aragonese Castle-L and b) Gaiola samples, with assigned targeted OVTXs oxidated ions monitored transitions.

in containing 37 % OVTX-a, 36 % OVTX-b, 10 % OVTX-c, 2 % OVTX-d, and 15 % OVTX-e (Fig. 6b), a quite typical Mediterranean *O.* cf. *ovata* toxin profile [13]. Further studies are ongoing on structural elucidation of all the OVTXs identified in the strains.

This finding implies that the absence of detectable toxins in some environmental samples (e.g., Procida Pozzo Vecchio, Vivara, Aragonese Castle-R and Ischia Porto) does not necessarily exclude the presence of toxic strains in those areas. Rather, it highlights the need for continuous monitoring, as even apparently "toxin-free" samples may harbour populations capable of producing toxins under suitable environmental conditions. Moreover, although some correlation was observed between higher cell abundances and detectable ovatoxins, toxin concentrations were not directly proportional to cell density. The Aragonese Castle-L sample exhibited toxin concentrations approximately 14 times higher (~1500 ppb) than those measured at Gaiola (~110 ppb), despite both sites being among the richest in terms of *O.* cf. *ovata* cell abundance, with Aragonese Castle-L showing only about a fourfold higher number of cells per gram of macroalgae than Gaiola sample. This disproportionate relationship confirms that cell counts alone cannot reliably predict toxin abundance, suggesting that strain-specific factors and environmental conditions play a critical role in regulating OVTX biosynthesis.

Comparison of the quantitative results obtained using the intact and oxidative methods demonstrated that the two approaches are complementary and generally yield consistent results (Tables S3 and S4). In the absence of certified reference material for OVTXs, the application of both methods reinforces confidence in toxin detection. Observed discrepancies between the two quantitative methods can be partially attributed to matrix effects, which were assessed in the intact method by analysing the two OVTX-richest samples, Aragonese Castle-L and Gaiola, alongside a blank (toxin-free) sample, namely Aragonese Castle-R, spiked with a known amount of palytoxin. These results indicate that matrix effects vary among different samples (Table 7).

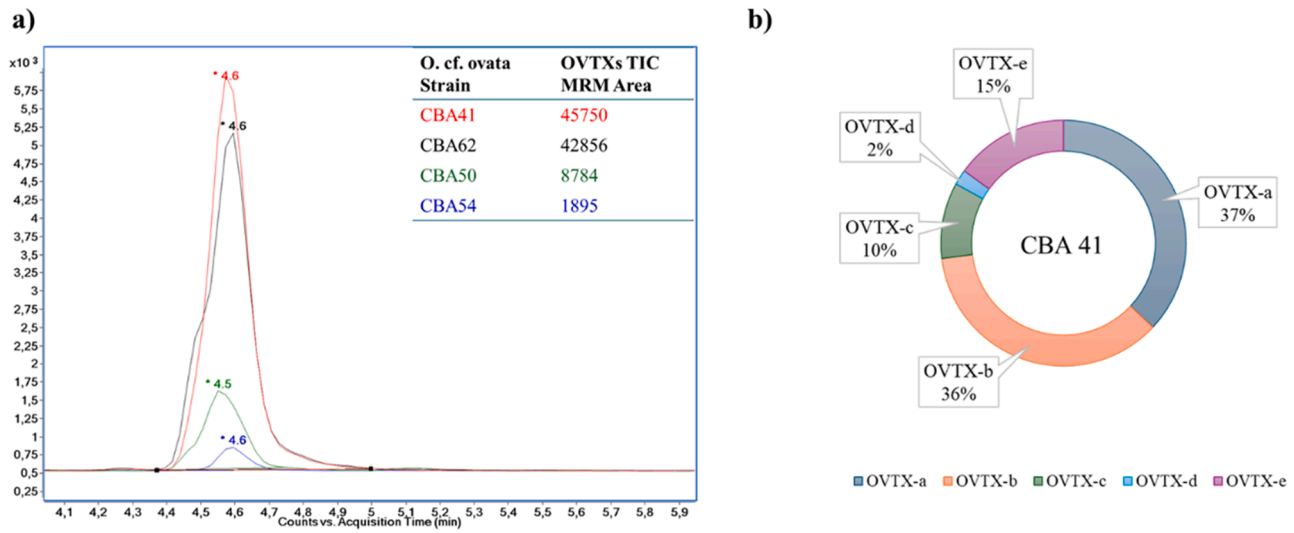


Fig. 6. a) Total Ion chromatogram (TIC) MRM of LC-MRM-MS intact analysis of crude extract of strains CBA 41 (in red), CBA62 (in black), CBA50 (in green), CBA 54 (in blue) with relative peak integration area. b) Ovatoxin profile of CBA 41 strain isolated from Procida Pozzo Vecchio culture.

Additional sources of variability may arise from the experimental procedure itself, particularly the solid-phase extraction (SPE) step, which can result in partial sample loss (Table 7). In the intact method, for example, excellent recoveries were observed for Aragonese Castle-L and -R samples, whereas the Gaiola sample and the procedural blank showed recoveries of only 41 and 45 % respectively. Considering procedural losses, the final OVTXs concentration in the seaweed wash seawaters of the Aragonese Castle-L and Gaiola samples were estimated to be 13.9 nM and 2.1 nM, respectively (Table S5). Such recovery correction, however, cannot be performed for the microscale oxidation method due to the lack of oxidized product standards. Thus, for the latter method were assumed both complete oxidation reaction and SPE recovery >90 % for all PLTX-like compounds, in agreement with Selwood et al. [14]. These limitations introduce uncertainty in quantitation of oxidative method, potentially contributing to the observed differences in OVTXs quantitation between methods.

It should be noted that recent in vitro studies on human skin keratinocytes have established that cytotoxicity of OVTX-a falls in the nanomolar range [34]. This level of concentration is comparable to those observed in seaweed wash seawater of Aragonese Castle-L and Gaiola samples (13.9 and 2.1 nM respectively, Table S5), indicating a potential risk of adverse effects following dermal exposure during the sampling activities at these sites. Conversely, the abundance of *O.* cf. *ovata* cells per gram of macroalgae in Aragonese Castle-L and Gaiola samples (6.4×10^4 and 1.6×10^4 cells g^{-1} respectively) fall well below the skin-effect alert threshold (5×10^5 cells g^{-1}) above which human health effects have been reported, as described in FAO-IOC-IAEA guidelines [20,24]. Our findings indicate that dermal toxicity might occur even at *O.* cf. *ovata* cell densities below the alert thresholds. This, in turn, suggests that mild or moderate OVTX-related skin irritations may have been underreported or misattributed, given the similarity of symptoms to more common conditions such as solar erythema. From a monitoring perspective, these findings raise significant concerns, as the Italian regulatory framework currently relies exclusively on cell-density thresholds to trigger health alerts. This approach risks underestimating exposure when relatively low cell densities are associated with disproportionately high toxin production. Consequently, a shift toward toxin-focused surveillance, supported by standardized LC-MS/MS protocols and the development of certified reference materials, is essential to provide a more accurate assessment of public health risks associated with *O.*cf. *ovata* blooms.

4. Conclusion

This study demonstrates that the potential health risks associated with toxins produced by *O.* cf. *ovata* cannot be reliably inferred from cell abundance alone. In the context of the climate change driven expansion of harmful algal blooms, the persistence of monitoring frameworks based solely on cell counts, without complementary toxin quantification, represents a critical regulatory gap that risks underestimating human exposure to ovatoxins via inhalation of contaminated aerosols, dermal contact, or consumption of contaminated seafood. The underreporting of OVTX-related syndromes, often misdiagnosed as viral or flu-like illnesses, further complicates surveillance and epidemiological assessment along the Italian coastline. Both intact and oxidative LC-MRM-MS methods used in this study proved effective for the detection of ovatoxins in seaweed wash seawater, although small discrepancies in quantification were observed between the two approaches. This observation fully reflects the inherent challenges of mass spectrometric analysis of ovatoxins, resulting from matrix effects, the formation of ion adducts, and the difficulty in distinguishing isobaric ions. Further investigations involving a larger number of samples from diverse sources are therefore needed to strengthen method validation. The production of certified OVTX reference materials is urgently needed to enhance inter-laboratory comparability, improve reproducibility, and increase confidence in quantitative measurements. Establishing such standards would strengthen LC-MS-based monitoring frameworks, thereby enabling more accurate risk assessment and ensuring effective protection of public health in areas affected by *O.* cf. *ovata* blooms. Meanwhile, the analytical strategy proposed herein allows maximization of sensitivity in ovatoxin detection without compromising selectivity.

Declaration of generative AI and AI-assisted technologies in the manuscript preparation process

During the preparation of this work the authors used ChatGPT Edu in order check the English grammar of some sentences in the paper. After using this tool, the authors reviewed and edited the content as needed and take full responsibility for the content of the published article.

CRediT authorship contribution statement

Chiara Melchiorre: Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Resources, Methodology, Investigation, Conceptualization. **Martina Carelli:** Visualization,

Software, Investigation, Formal analysis, Data curation. **Valeria Tegola:** Software, Formal analysis, Data curation. **Samuela Capellacci:** Writing – review & editing, Formal analysis, Data curation. **Silvia Casabianca:** Writing – review & editing, Resources, Formal analysis, Data curation. **Antonella Penna:** Writing – review & editing, Supervision, Resources, Conceptualization. **Carmela Dell'Aversano:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

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

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2026.466915.

Data availability

Data will be made available on request.

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