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# First Finding of *Ostreopsis* cf. *ovata* Toxins in Marine Aerosols

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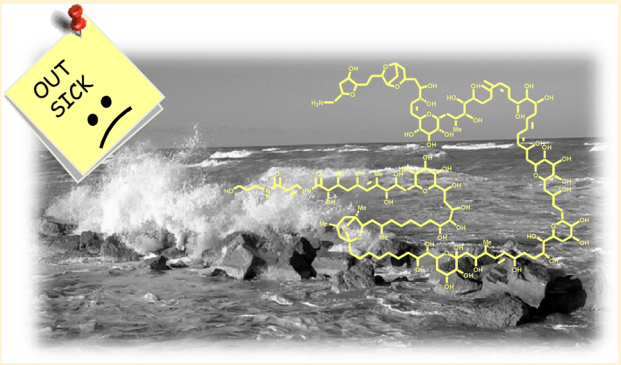
Supporting Information

**ABSTRACT:** Since the late 1990s, a respiratory syndrome has been repetitively observed in humans concomitant with *Ostreopsis* spp. blooms (mainly *O.* cf. *ovata*) in the Mediterranean area. Previous studies have demonstrated that *O.* cf. *ovata* produces analogues of palytoxin (ovatoxins and a putative palytoxin), one of the most potent marine toxins. On the basis of the observed association between *O.* cf. *ovata* blooms, respiratory illness in people, and detection of palytoxin complex in algal samples, toxic aerosols, containing *Ostreopsis* cells and/or the toxins they produce, were postulated to be the cause of human illness. A small scale monitoring study of marine aerosol carried out along the Tuscan coasts (Italy) in 2009 and 2010 is reported. Aerosols were collected concomitantly with *O.* cf. *ovata* blooms, and they were analyzed by both PCR assays and LC-HRMS. The results, besides confirming the presence of *O.* cf. *ovata* cells, demonstrated for the first time the occurrence of ovatoxins in the aerosol at levels of 2.4 pg of ovatoxins per liter of air. Given the lack of toxicological data on palytoxins by inhalation exposure, our results are only a first step toward a more comprehensive understanding of the *Ostreopsis*-related respiratory syndrome.




## INTRODUCTION

In the last decades, benthic dinoflagellates belonging to genus *Ostreopsis* (*O.* cf. *ovata* and less frequently *O.* cf *siamensis*) have been repeatedly blooming along the Mediterranean and the Atlantic coasts.[1] This phenomenon has caught the attention of public health protection authorities, as, during algal blooms, an unusual incidence of a respiratory syndrome was observed in people exposed to marine aerosols during their recreational or working activities. Several *Ostreopsis*-related toxic outbreaks have been documented so far,[2] not to mention anecdotal news reported yearly by media, Web sites, and blogs. Among toxic outbreaks, the first one occurred along the Tuscan coastline (Italy) in 1998, with about 100 people involved,[3] while the most alarming one, in terms of number of people involved and severity of symptoms, occurred along the Ligurian coasts (Italy) in 2005, when 209 people required medical attention following exposure to marine aerosols.[4] Symptoms included fever (≥38 °C), watery rhinorrhea, pharyngeal pain, dry or mildly productive cough, headache, nausea/vomiting, and broncho-constriction with mild dyspnea and wheezes; conjunctivitis and dermatitis were observed in some cases. Symptoms in humans peaked in association with *Ostreopsis* bloom climax and ended in association with bloom dissipation. Approximately 20% of the patients required hospitalization (24−72 h) and some of them in intensive care.

Based on these events, chemical studies have been carried out on Mediterranean/Atlantic strains of *O.* cf. *ovata* and *O.* cf. *siamensis* to investigate their toxin profiles: while *O.* cf. *siamensis* were devoid of any appreciable toxicity,[5] *O.* cf. *ovata* produced several congeners of palytoxin, named ovatoxins, and a putative palytoxin.[6−9]

Palytoxin and ovatoxins differ in small structural details (Figure S1, Supporting Information) and in biogenetic source. Palytoxin was originally isolated from zoanthids of the genus *Palythoa*.[10,11] Ovatoxin-a, the major component of most *O.* cf. *ovata* strains, has been recently isolated from algal cultures and structurally elucidated based on both NMR[12,13] and liquid chromatography high resolution mass spectrometry (LC-HRMS).[14] Ovatoxin-b, -c, -d, -e, and -f have not been isolated

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yet, and only their LC-HRMS data are currently known. (Table S1, Supporting Information).

Palytoxin itself is one of the most potent nonprotein marine toxins so far known with acute toxicity in mice strongly depending on administration route.[15] Besides high toxicity by intravenous ($LD_{50}$ = 0.15 μg/kg) and intraperitoneal (1.5 μg/kg) administration, Ito and Yasumoto recently demonstrated that palytoxin causes bleeding and alveolar destruction in lungs and death at 2 μg/kg by intratracheal administration.[16] Toxicological studies on ovatoxins are currently in progress; preliminary biochemical studies demonstrated that they significantly increased the levels of mRNAs encoding inflammation-related proteins in immune cells, i.e., monocyte-derived human macrophages.[17]

On the basis of the coincidence between *O.* cf. *ovata* blooms, respiratory illness in people, and detection of the palytoxin complex (palytoxin and ovatoxins) in algal samples, toxic aerosol, containing *Ostreopsis* cells and/or the toxins they produce, was postulated to be the cause of human illness. Indeed, algal cells, bacteria, and waterborne toxins may be transferred into the air through a bubble-bursting process with a wind-powered whitecapped wave mechanism. Within harmful algal bloom (HAB) toxins, this mechanism was proved to underlie aerosolization of brevetoxins from *Karenia brevis* during red tides episodes in the Gulf of Mexico[18] and of microcystins during cyanobacterial blooms of *Microcystis aeruginosa* in California lakes.[19,20] Inhalation exposure to aerosols containing either brevetoxins or mycrocistins has caused several respiratory problems in humans.[18,19]

*Ostreopsis*-related illness might be due to the palytoxin complex, or to an immunological reaction to cell pieces, or even to a combination of both. Indirect support to the toxin hypothesis has been recently provided by some case reports of palytoxin exposure from incidental contact with marine aquarium zoanthids. In these reports, marine aquarium hobbyists, in the attempt to remove infesting zoanthids from their home aquarium with boiling water, experienced a severe respiratory reaction following inhalation of steams. Palytoxin was found in the zoanthids by LC-MS and/or hemolysis assay.[21]

Because of the lack of toxicological studies on inhalation exposure to palytoxin and ovatoxins, characterization of the aerosol composition is necessary. A first study on the marine aerosol composition was reported by Casabianca et al. who succeeded in quantifying *O.* cf. *ovata* cells in marine aerosols collected along the Spanish Mediterranean coasts by a qPCR assay.[22] Palytoxins were detected in this study neither by hemolytic assay nor by LC with fluorescence detection, limits of detection of the methods being 0.5 pg and 750 pg, respectively. Herein, we report on a small scale monitoring study of marine aerosols carried out along the Tuscan coasts (Italy) in 2009 and 2010. Aerosols were collected concomitant with *Ostreopsis* spp. blooms, and they were analyzed both by PCR assay and LC-HRMS. The obtained results, besides confirming the presence of *O.* cf. *ovata* cells, demonstrated for the first time the presence of ovatoxins in the aerosols.

## MATERIALS AND METHODS

**Study Area.** The present study was carried out at two sites of the Tuscan coastline (Italy), Marina di Massa (MS) (44°00′00″N; 10°06′00″E) and Marina di Pisa (PI) (43°40′00″N; 10°16′00″E) (Figure S3, Supporting Information), during *Ostreopsis* spp. bloom events occurring in the summer of 2009 and 2010. Abundance of *Ostreopsis* spp. was measured in seawater and on macroalgae (*Rhodophyta* and *Ocrophyta*) in the period July 15 to August 30 of each year according to the Uthermöl method. Meteomarine conditions were measured in the sampling days through weather stations (Davis Instruments, Hayward, CA) that were placed next to the air samplers and worked continuously over a 24 h period.

**Aerosol Sampling.** Two types of portable air samplers were employed for aerosol collection: AirCube COM2 (Analitica Strumenti srl., Italy) equipped with 47 mm diameter glass/quartz microfiber filters (Whatman, Maidstone, UK) and SAS PCR (VWR International PBI, Italy) in which air and a collecting fluid flow together through a nozzle and are poured into a vessel with a coil; the collecting fluid is circulated continuously to prolong the contact time with aerosols and to enrich itself in toxins and/or cells. Both air samplers were located 10 m from the seashore and worked continuously for 1.5−6 h. Air flow speed was 10 L/min in 2009 and 30 L/min in 2010. In 2009, a total of 23 aerosol samples were collected at MS from July 31st to August 18th; among them, 16 samples were collected by the AirCube COM2 and 7 by the SAS PCR using distilled water (50 mL) as collecting fluid (Table S2, Supporting Information). In 2010, 10 aerosol samples were collected at MS from August 3 to August 6 and at PI from August 9 to August 12; among them, 4 samples were collected using the AirCube COM2 and 6 samples by SAS PCR using artificial saltwater as collecting fluid (50 mL) (Table S3, Supporting Information). The collecting fluids of the SAS PCR were stored at +4 °C until chemical analysis. The filters of AirCube COM2 were suspended in 50 mL of ethanol, and they were stored at +4 °C until molecular analysis.

**Epiphytic *Ostreopsis* spp. Sampling.** Molecular and chemical analyses were performed on epiphytic *O.* cf. *ovata* samples collected in 2010 at MS (on July 30 and August 9) and at PI (on August 9 and August 12). Macroalgae covered by a film of *O.* cf. *ovata* were harvested at a depth of 20−50 cm and closed underwater in sampling bags. The macroalgal samples were rinsed several times with seawater, according to Battocchi et al.,[23] obtaining a macroalgae wash water that was divided in two aliquots: one was stored at +4 °C until chemical analysis, and the other one was fixed with 0.8% formalin and used for molecular analyses. Counting was carried out on subsamples of the macroalgae wash seawater (1−5 mL) using Utermöhl chambers as described by Battocchi et al.[23] *Ostreopsis* spp. were identified under inverted light microscope (Axiovert 40 CFL) at 200× or 400× magnification according to Steidinger and Tangen.[24] Cell concentration, expressed as cells/mL, was used for the construction of the environmental standard curve in the qPCR assay applied to aerosol samples collected in 2010.

**PCR Based Assays for the Detection/Identification of *Ostreopsis*. spp.: DNA Extraction and Analysis.** The AirCube COM2 aerosol filters were placed in a tube with 50 mL of pure ethanol and shaken overnight at 60 osc/min to recover the marine aerosol particulate from the filter. Filters were then discharged, and the suspension was centrifuged at 4000 rpm for 10 min. The pellets were transferred to a 1.5 mL tube, centrifuged at 12 000 rpm for 10 min, and dried at room temperature for DNA extraction. In 2009, pellets were used for total DNA extraction and purification using the UltraClean Soil DNA Kit (MoBio Lab Inc., Solana Beach, CA) according to the manufacturer's instructions. PCR reactions were carried out in two steps: initially PCR using eukaryotic primers targeting the ITS-5.8S gene[25] was performed, and then a nested PCR


| # | Year[ref] | Site | People | # | Year[ref] | Site | People |
|---|---|---|---|---|---|---|---|
| 1 | 1972[27] | French Riviera (France) | - | 18 | 2004[28] | Barcellona (Spain) | 200 |
| 2 | 1979,[29] 2000-01[30] | Tripoli (Lebanon) | - | 19 | 2004[31,32] | Llavaneres (Spain) | 74 |
| 3 | 1994[33] | Civitavecchia (Italy) | - | 20 | 2005[5] | Taormina, Italy | - |
| 4 | 1997-98,[34] 2000,[35] 2007[1,23] | Catalan coasts (Spain) | - | 21 | 2005-2006[4,6] | Genoa, La Spezia (Italy) | 228 |
| 5 | 1998[34] | Corsica | - | 22 | 2005-2007[36] | Alexandria (Egypt) | - |
| 6 | 1998-2001[3, 37] | Massa Carrara (Italy) | >110 | 23 | 2006,[38] 2009[39] | Gulf of Trieste (Italy) | - |
| 7 | 1999[35] | Almeria (Spain) | - | 24 | 2006-2009[40] | French Riviera (France) | 47 |
| 8 | 2006[41] | Almeria (Spain) | 57 | 25 | 2007-2011[23] | Pesaro (Italy) | - |
| 9 | 2000[42] | Gulf of Gabès (Tunisia) | - | 26 | 2007,[23, 43] 2009[44] | Conero (Italy) | - |
| 10 | 2000[35] | Gioia Tauro (Italy) | - | 27 | 2007-2008[45] | Monaco | - |
| 11 | 2000[35] | Ganzirri (Italy) | - | 28 | 2008-10[46] | Sines, Cascais (Portugal) | - |
| 12 | 2000,[34] 2001[35] | Majorca (Spain) | - | 29 | 2009[1] | Algeria | >100 |
| 13 | 2001-2004[47,48] | Bari (Italy) | 28 | 30 | 2010[49] | Rovinj (Croazia) | undefined |
| 14 | 2002[50] | Otranto (Italy) | - | 31 | 2010[51] | Maratea, (Italy) | - |
| 15 | 2003-2006[52,53] | Aegean coasts (Greece) | - | 32 | 2011[54] | Palermo (Italy) | ~50 |
| 16 | 2004[55] | Gulf of Naples (Italy) | - | 33 | 2011[56] | Lagos coast (Portugal) | - |
| 17 | 2004[57] | Cape Ghir, (Marocco) | - | 34 | 2011[58] | Alghero, (Italy) | - |

**Figure 1.** Distribution of *Ostreopsis* spp. along the Mediterranean and Atlantic coasts. Empty triangles indicate sites where blooms occurred but no human illness was recorded. Filled dots indicate sites where a respiratory syndrome was recorded in humans concomitant with blooms.

reaction using species-specific primers[26] was carried out on the ribosomal gene for *O.* cf. *ovata* and *O.* cf. *siamensis* identification.[23] In 2010, pellets were resuspended in lysis buffer and processed by qPCR-based assay.[22] The samples containing total genomic DNA (crude extracts) were processed using 2 $\mu$L of undiluted, 1:10, and 1:100 diluted subsamples of crude extracts. Results from 10-fold dilutions with a Ct difference between 3.3 and 3.4 ($\Delta$Ct of 3.3 corresponds to an optimal efficiency of 100%) were accepted. The marine aerosol samples were analyzed by qPCR following a protocol previously reported.[23] A plasmid (pLSUO) standard curve was constructed by amplifying 10-fold scalar dilutions with the copy number ranging from $1 \times 10^6$ to $1 \times 10^2$ (two replicates), and from $1 \times 10^1$ to $2 \times 10^0$ (four replicates). A cellular standard curve of four mixed crude extracts of $5 \times 10^2$ cells from macroalgae wash seawater samples collected at MS and PI in 2010 was generated with dilutions from 8 to $8 \times 10^{-4}$ lysed cells. The rDNA copy number per cell of *O.* cf. *ovata* at the two sites was calculated, interpolating the Ct (threshold cycle) value corresponding to a single cell from environmental samples on the plasmid standard curve. The qPCR assay was performed in a final volume of 25 $\mu$L using the Hot-Rescue Real-Time PCR Kit-SG (Diatheva, Fano, Italy) in a StepOne Real-Time PCR System (Applied Biosystems, Foster City, CA). The thermal cycling conditions consisted of 10 min at 95 °C, followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min. No amplification of field samples was evaluated by spiking amounts of $1 \times 10^1$ and $1 \times 10^2$ copies of pLSUO, to exclude the presence of potential qPCR inhibitors. Acquisition and data analyses were performed using StepOne Software ver. 2.1. Values were accepted when the PCR efficiency was between 95 and 100% (slope: −3.44 and −3.32). The total *O.* cf. *ovata* rDNA copy number of field was obtained by interpolating the Ct on the plasmid standard.

**Chemical Analyses: Extraction.** The collecting fluids of SAS PCR air sampler collected in 2009 (distilled water, 50 mL) were concentrated to 1 mL, added of 1 mL of methanol with 0.2% acetic acid, filtered through Ultrafree MC 0.45 $\mu$m centrifugal filter units (Millipore, Billerica, MA) and directly analyzed by LC-HRMS (5 $\mu$L injected). Collecting fluids of SAS PCR air sampler (saltwater, 50 mL) and epiphytic *O.* cf. *ovata* samples (macroalgae wash water, 50 mL) collected in 2010 were separately extracted five times with an equal volume of butanol. The butanol layer was evaporated to dryness, dissolved in 1 mL of methanol/water (1:1, v/v) with 0.2% acetic acid, filtered through Ultrafree MC 0.45 $\mu$m centrifugal filter units, and analyzed by LC-HRMS (5 $\mu$L injected). Recovery of the extraction procedure was 75%.[6]

**Liquid Chromatography-High Resolution Mass Spectrometry (LC-HRMS).** LC-HRMS experiments were carried out on a hybrid linear ion trap LTQ Orbitrap XL Fourier Transform MS (FTMS) equipped with an ESI ION MAX source (Thermo-Fisher, San Josè, CA) coupled to an Agilent 1100 LC binary system (Palo Alto, CA). LC conditions included the use of a 3 $\mu$m Gemini C18 (150 × 2.00 mm) column (Phenomenex, Torrance, CA) maintained at room temperature and eluted at 0.2 mL/min with water (eluent A) and 95% acetonitrile/water (eluent B), both containing 30 mM acetic acid. A fast gradient elution was used: 20−100% B over 10 min, hold for 5 min. Re-equilibration time was 8 min. Under this condition, most of the known ovatoxins and putative palytoxin coeluted.[7]

HR full MS experiments (positive ions) were carried out in the mass range $m/z$ 800−1400 at a resolving power of 60 000. Calibration was performed just before the analyses by using a mixture of caffeine, MRFA (L-methionyl-arginyl-phenylalanyl-alanine acetate·$H_2O$) and Ultramark 1621. The following source settings were used: a spray voltage of 4 kV, a capillary temperature of 290 °C, a capillary voltage of 22 V, a sheath gas and an auxiliary gas flow of 35 and 1 (arbitrary units). The tube lens voltage was set at 110 V. $HRMS^2$ data were acquired in collision-induced dissociation (CID) mode at a 30 000 resolving power by selecting the $[M + H + Ca]^{3+}$ ion of ovatoxin-a as precursor. A collision energy of 25%, an activation Q of 0.250, and an activation time of 30 ms were used. Calculation of elemental formulas of ions contained in HRMS spectra was performed by using the monoisotopic ion peak of each ion cluster. The isotopic pattern of each ion cluster was considered in assigning molecular formulas, and a mass tolerance of 5 ppm was used. Extracted ion chromatograms (XIC) for palytoxin and ovatoxins were obtained by selecting the most abundant ion peaks of both $[M + 2H - H_2O]^{2+}$ and $[M + H + Ca]^{3+}$ ion clusters.

A crude extract of *O.* cf. *ovata* containing ovatoxin-a to -e previously reported[8] was used as reference sample for qualitative identification of ovatoxins. A palytoxin standard (100 $\mu$g; lot STJ7776) from Wako Chemicals GmbH (Neuss, Germany) was dissolved in methanol/water (1:1, v/v) and used for quantitative analyses. It should be noted that this standard is not certified and may contain some minor contaminants besides palytoxin itself; qualiquantitative composition of the standard may vary within different lots. So, preliminarily to sample analyses, a 1:100 fold dilution of the standard was analyzed by LC-HRMS to determine its composition. The standard contained 83% of palytoxin itself, 5% of 42-hydroxypalytoxin, and 12% of contaminant(s) (Figure S2, Supporting Information). The identity of palytoxin and 42-hydroxypalytoxin was confirmed through $HRMS^n$,[14] while further studies would be needed to identify contaminant(s) as palytoxin-like compound(s) or not. Palytoxin standard was used to generate a five-level calibration curve (25, 12.5, 6.25, 3.13, and 1.6 ng/mL). At the highest point of the calibration curve, only palytoxin was detectable. Calibration points were the result of triplicate injection, and peak areas were used for plotting. The curve was used to quantify positive samples, assuming that ovatoxins present the same molar response as palytoxin. Blank samples (MeOH: water 1:1, v/v) were injected between aerosol samples and epiphytic *O.* cf. *ovata* extracts and standards to prevent cross contamination. Under the used instrumental conditions, measured limit of detection (LOD) and quantitation (LOQ) for palytoxin standard were 1.6 ng/mL and 3.13 ng/mL in 2009 and 3.13 and 6.25 ng/mL in 2010.

## RESULTS

Figure 1 shows Mediterranean and Atlantic sites where *Ostreopsis* spp. blooms have occurred and *Ostreopsis*-related illness has been recorded in humans so far. In Italy, *O.* cf. *ovata* blooms every year in most coastal regions, with the only exception of Veneto, Emilia Romagna, Abruzzo and Molise regions.[59] Due to potential risks to human health, regional monitoring programs of *Ostreopsis* spp. have been enacted along the entire coastline; none of them, however, provides collection and analyses of marine aerosols.

To verify the potential toxicity of marine aerosols, a small scale monitoring study of the aerosols has been carried out in the summer of 2009 and 2010 along the Tuscan coastline, at Marina di Massa (MS), the Italian site where *Ostreopsis*-related illness was first recorded in humans,[3] and at Marina di Pisa (PI), located about 60 km apart. Both sites are characterized by pebbles on the beach and by emerged and submerged breakwaters in the sea that have been built perpendicular and/or parallel to the coast to avoid coastal erosion (Figure S3, Supporting Information). Because *Ostreopsis* spp. are benthic algae that tend to form mats on hard substrata, such geomorphological conditions create environmental/hydrodinamism conditions most favorable to their proliferation. As a result, both sites are infested by *O.* cf. *ovata* every summer.

**Summer of 2009.** In the summer of 2009, aerosols were collected at MS concomitant with *O.* cf. *ovata* abundance in seawater of 5000−24 000 cells/L. It should be noted that, in the guidelines of the Italian Ministry of Health, *Ostreopsis* spp. cell abundance ≥10 000 cells/L seawater is indicator of an emergency level for the *Ostreopsis* phenomenon.[60] Sampling was done in the period ranging from the end of July to mid August for 2−3 h each sampling day. The air samplers were located 10 m from the seashore, and they operated at an airflow speed of 10 L/min, which is close to the human respiratory capacity in 24 h (10 $m^3$).[61] A continual monitoring of wind speed and direction was carried out during the air sampling. Weather conditions were quite stable with wind speed and temperature being in the range 0.05−2.6 m/s and 24−27 °C, respectively (Table S2, Supporting Information). No respiratory symptoms were reported at the local hospital during the monitoring period. However, this could be due to a combination of several factors, including the barely high cell abundance in seawater and the quite stable weather conditions.

The glass filters of the AirCube COM2, which had been proved suitable for molecular PCR analysis, were analyzed by molecular PCR amplifications using eukaryotic and species-specific primers for *Ostreopsis*. The PCR-amplified products from the genomic DNAs of natural samples provided positive results. A total of eight samples were positive for the *O.* cf. *ovata* presence (210 bp size of amplified product): among them, four samples were positive for the *O.* cf. *siamensis* (223 bp size of amplified product) (Table S2, Supporting Information). This confirmed the amplificability of the target genomic DNA by the nested PCR-based assay.

Considering that palytoxin is reported to bind plastic (at room light) and glass (at UV light) surfaces,[62] which could result in poor recoveries from filters of the AirCube COM2 air sampler, the collecting fluids of the SAS PCR air sampler were selected for chemical analysis. The fluids (distilled water, $V$ = 50 mL) were concentrated to 1 mL, 1 mL of methanol was


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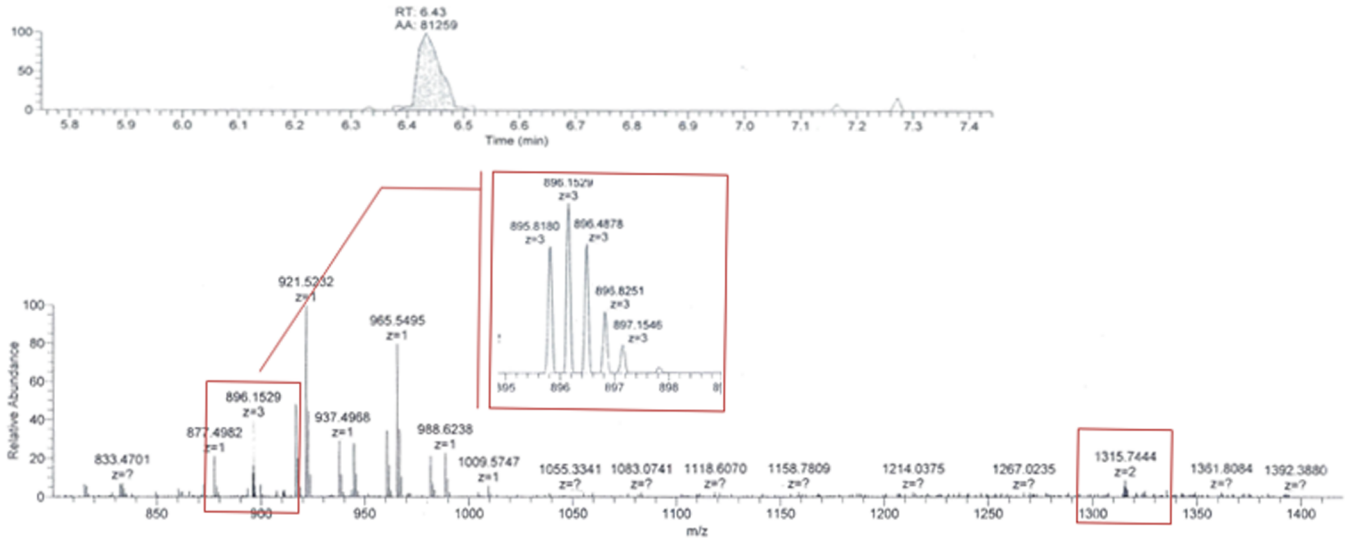


**Figure 2.** (Above) XIC of doubly and triply charged ions of ovatoxin-a in the aerosol sample of August 11, 2010 and (below) HR full MS spectrum associated to the peak at 6.43 min. The zoomed view of the isotopic ion cluster of $[M + H + Ca]^{3+}$ ion of ovatoxin-a is shown in the inset.

added, and the sample was directly analyzed by LC-HRMS in full MS mode versus a reference sample of *O.* cf. *ovata* containing all the ovatoxins so far known. No palytoxin-like compound was detected in the analyzed samples. Considering that the instrumental LOD of palytoxin in the day of analysis was 1.6 ng/mL and that the sample volume was 2 mL, the presence of palytoxin congeners in SAS PCR collecting fluids (50 mL) was excluded at levels above 64 pg/mL.

**Summer of 2010.** Preliminary results obtained by molecular and chemical analyses of the aerosol collected in 2009 prompted us to continue monitoring marine aerosols in 2010, enacting some strategies to enrich aerosol samples in cells and/or in toxins. In particular, the air flow speed of both air samplers (AirCube COM2 and SAS PCR) was increased to 30 L/min, 3 times higher than that from the year before. Furthermore, *Ostreopsis* spp. cells in seawater and on macroalgae were measured at three stations on the beach of Marina di Massa (MS1, MS2, and MS3; Figure S3, Supporting Information) and at Marina di Pisa (PI). Sites where the highest cell abundance was detected were selected for sampling of aerosols and epiphitic *O.* cf. *ovata* cells.

Among the sampling stations in Marina di Massa, the maximum cell concentration was recorded at MS1 (113 000 cells/L on July 30 and 285 000 cells/L on August 9), followed by MS2 (148 000 cells/L on August 9) and MS3 (28 500 cells/L on August 9); the concentration of *O.* cf. *ovata* on macroalgae was 25 000 cells/g (MS1), 21 000 cells/g (MS2), and 3000 cells/g (MS3). At PI station, the highest cell concentration in seawater was recorded on July 20 (185 000 cells/L) and decreased at the beginning of August, with levels of 14 000 cells/L and 11 000 cells/L being recorded on August 9 and August 12, respectively; the concentration of *O.* cf. *ovata* on macroalgae was 545 000 on July 20 and decreased to 12 000 and 30 000 cells/g on August 9 and August 12, respectively. At MS1 and PI stations, a rusty-brown mucilaginous film covered the macroalgae community (*Pterocladiella capillacea*) and was suspended as lumps in seawater throughout the sampling period; concomitantly, an extensive marine biocenosis suffering was observed.

No respiratory illness was recorded in humans during the sampling period. However, such as in 2009, the meteomarine conditions during the sampling were stable and unfavorable to massive formation of aerosols: wind speed and temperature were in the range of 0.3−1.9 m/s and 24−27 °C, respectively, and no sea-storm occurred (Table S3, Supporting Information).

LC-HRMS analyses of epiphytic *O.* cf. *ovata* collected at MS1 and PI indicated that the algal toxin profile was the same at both the stations, with ovatoxin-a (68%), -b (20%), -c (2%), -d +e (8%), and putative palytoxin (1%) being contained in the extracts. This toxin profile was similar to those reported for most of the field and cultured *O.* cf. *ovata* samples analyzed so far, in which ovatoxin-a is the dominant toxin.[63]

SAS PCR aerosol samples were extracted with butanol, and the obtained extracts (1 mL) were directly analyzed by LC-HRMS in full MS mode. Samples collected at PI on August 11 and August 12 contained palytoxin-like compounds. Quantifiable amounts of ovatoxin-a and trace levels of ovatoxin-b, ovatoxin-d+e, and putative palytoxin were detected in the sample of August 11. These findings were in good agreement with the toxin profile of epiphytic *O.* cf. *ovata* collected at the same site in which ovatoxin-a was the major component. Only trace levels of ovatoxin-a were detected in the sample of August 12. It should be noted, however, that when a sample (aerosol, seawater, or seafood) is slightly contaminated by *O.* cf. *ovata* toxins, ovatoxin-a is usually the only detectable component of the toxin profile.

Figure 2 shows the extracted ion chromatogram (XIC) of doubly and triply charged ions of ovatoxin-a for the aerosol sample of August 11. The peak at 6.43 min eluted at the same retention time as ovatoxin-a contained in a reference sample of *O.* cf. *ovata* extract analyzed under the same conditions. In the associated HR full MS spectrum, although the background noise was quite high, the $[M + H + Ca]^{3+}$ and $[M + H - H_2O]^{2+}$ ions of ovatoxin-a were clearly visible, at $m/z$ 896.1529 (monoisotopic ion peak at $m/z$ 895.8180, $C_{129}H_{224}N_3O_{52}Ca$, $\Delta$ = −1.687 ppm) and at $m/z$ 1315.7444 (monoisotopic ion peak at $m/z$ 1315.2430, $C_{129}H_{223}N_3O_{51}$, $\Delta$ = −2.947 ppm), respectively. Further confirmation for this assignment was provided by the LC-HR CID $MS^2$ spectrum of the $m/z$ 896.1 ion. Although no diagnostic fragment ion of ovatoxin-a could be observed in this spectrum, due to its low concentration in the extract, the most intense fragment ions relevant to


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subsequent water losses from the precursor ion of ovatoxin-a were present.[14,63]

Quantitative analyses indicated a total amount of 13 ng of ovatoxin-a in the sample of August 11, while ovatoxin-b, -d+e, and putative palytoxins were present at levels below the LOQ of the method measured in the day of the analyses (LOQ = 6.25 ng/mL). So, the concentration of ovatoxin-a in the collecting fluid ($V$ = 50 mL) of the SAS PCR air sampler was estimated to be 260 pg/mL, with the other palytoxin analogues being below 125 pg/mL. Considering the flow speed (30 L/min) and the collection time (3 h), a concentration of 2.4 pg of ovatoxins per liter of air could be estimated on August 11. Trace levels of ovatoxin-a were contained in the aerosol collected on August 12.

No palytoxin-like compound was detected in samples collected at Marina di Pisa station on August 9 and August 10 and at Marina di Massa stations. Considering the LOD of the method (3.13 ng/mL), the presence of palytoxin congeners at levels above 63 pg/mL of SAS PCR collecting fluid was excluded.

The AirCube COM2 aerosol samples were analyzed by qPCR analyses. The rDNA copy number per cell of *O.* cf. *ovata* at the two sites MS1 and PI in 2010 was calculated, interpolating the Ct (threshold cycle) value corresponding to a single cell from environmental samples on the plasmid standard curve. The normalized copy per cell of *O.* cf. *ovata* was 1868 ± 179 and 2620 ± 145 from PI and MS1 samples, respectively. The only aerosol sample collected at MS1 on August 4 by AirCube COM2 was positive for *O.* cf. *ovata*. Its concentration was below one cell (putative cell fragments) corresponding to a Ct value of 34.87 ± 0.7 ($n$ = 3 replicates) > Ct value derived from two-copy plasmid dilution (32.21 ± 0.33). Spiking experiments showed no inhibitory activity of qPCR reactions, indicating that the negative amplification of aerosol samples was actually due to the absence of rDNA target.

## ■ DISCUSSION

The study of marine aerosols carried out in the frame of the 2009−2010 monitoring program achieved the double goal of (1) confirming the presence of *O.* cf. *ovata* cells in marine aerosols[22] and (2) detecting ovatoxins in the aerosols for the first time. Two air sampling devices (AirCube COM2 and SAS PCR) with different aerosol trapping mechanisms were employed, and two different detection methods (PCR assay and LC-HRMS technique) were used to characterize aerosol composition. In a methodological perspective, a SAS PCR air sampler proved effective to accumulate airborne toxins, and the LC-HRMS method for determination of palytoxins, originally developed for algal sample analysis, proved effective also for the analysis of the aerosols. On the other hand, PCR and qPCR molecular assays proved able to detect *Ostreopsis* spp. cell fragments on the fiber filters of the AirCube COM2. In 2009, the nested PCR approach (first step with eukaryotic primers; second step with species-specific primers) provided higher sensitivity in *Ostreopsis* spp. detection than the analytical method.

Studies on brevetoxins[18] and mycrocistins[19] have already demonstrated that both toxins and algae may be transferred into the air through a bubble-bursting mechanism: when bubbles formed by trapped air rise to the water surface, they burst and form jet and film drops; algal cells, bacteria, and waterborne toxins which are concentrated in water surface films are, thus, ejected with and carried into the air through such droplets. Likely, ovatoxins (high MW, highly polar, nonvolatile compounds) and *Ostreopsis* spp. cells are aerosolized through similar mechanisms. The natural follow-up of the present study would be testing the capability of the palytoxin complex and *O.* cf. *ovata* cells to be aerosolized from algal cultures in a laboratory setting. In addition, because aerosol particle size needs to be <5 $\mu$m to enter human lungs,[18] the particle size of the toxic aerosols should be measured in order to establish potential for respiratory effects in humans.

Toxins were detected in the aerosol collected in Marina di Pisa at the end of the *O.* cf. *ovata* bloom when the cell concentration in seawater was in the range 11 000−14 000 cells/L and levels on macroalgae were 12 000−30 000 cells/g. Likely, at the end of the blooming period when the dinoflagellate population is in the senescent phase, many cells break, resulting in a high amount of toxins released in seawater. A long-lasting bloom could enhance the toxin content in the water, resulting in toxic effects toward marine organisms or people inhaling the aerosol.

In this study, meteorological and wave conditions were unfavorable to massive aerosol formation. In addition, although environmental and hydrodinamism conditions at MS and PI were quite similar to those reported in the days immediately preceding the 2005 Ligurian outbreak,[4] *O.* cf. *ovata* cell abundance in seawater both in 2009 and 2010 did not reach levels recorded during the 2005 Ligurian outbreak ($1.8 \times 10^6$ cells/L). Likely, for all these reasons, toxin levels in the aerosols were low and none of the operators that collected aerosols nor beachgoers suffered from respiratory symptoms.

A combination of several factors must occur to cause an *Ostreopsis*-related outbreak. The presence and concentration of toxins/cells in marine aerosols strongly depends on wind speed and direction as well as on cell abundance in seawater. *O.* cf. *ovata* proliferation depends on the typology of the coastline and hydrodynamic conditions: small inlets with shallow and calm waters may favor *O.* cf. *ovata* bloom and high biomass density on soft or hard substrata.[1,3,4,51] The cell cycle can make short or long the blooming period. In addition, *O.* cf. *ovata* tends to produce trichocysts as fibrillar components of extracellular mucilage, which allows the formation of mucillagenous sheath embedding cells and avoiding their dispersion by hydrodynamic factors.[39] Wind and water turbulence is thus needed to disrupt surface accumulation and cause cell aggregates to break apart and, if the wind is strong enough and blows in-shore, to create toxic aerosols.

The finding of both cells and toxins in the aerosols suggests that *Ostreopsis*-related illness might be a reaction to ovatoxins or an allergic-like reaction to cell fragments or even a combination of both; in the lack of human cases, it is not possible to clarify this issue. Nonetheless, case reports of respiratory illness in aquarium hobbyists following contact with palytoxin-producing zoanthids provide indirect support to the toxin hypothesis.[21] Some symptoms (rhinorrhea, cough, breathing difficulty) reported in such case reports were similar to those recorded in people during *Ostreopsis* outbreaks.

Detection of ovatoxins in marine aerosols represents the first hint that toxins produced by *O.* cf. *ovata* may be accumulated in the aerosol, thus constituting the missing link between *O.* cf. *ovata* blooms and human respiratory syndrome. Our results are only a first step toward a more comprehensive understanding of the *Ostreopsis*-related respiratory syndrome, and further experiments are actually necessary. The aerosol monitoring should be repeated at the sites mostly affected by the *Ostreopsis*

phenomenon, when meteomarine conditions are favorable to aerosol formation and possibly in concomitance with a syndromic surveillance aimed at ascertaining respiratory distress in humans. Most importantly, a toxicological evaluation aimed at determining the acute reference dose of palytoxin and ovatoxins by inhalation exposure should be carried out. In the lack of these data, a final conclusion cannot be drawn.

## ■ ASSOCIATED CONTENT

### Supporting Information

Figure S1: Structure of palytoxin and ovatoxin-a. Figure S2: LC-HRMS of palytoxin standard. Figure S3: View of the sampling sites. Table S1: Elemental formulas and HRMS data of palytoxin and ovatoxins. Table S2: Environmental data and aerosol analyses in 2009. Table S3: Environmental data and aerosol analyses in 2010. This material is available free of charge via the Internet at http://pubs.acs.org.

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### Notes

The authors declare no competing financial interest

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## ■ REFERENCES

(1) Mangialajo, L.; Ganzin, N.; Accoroni, S.; Asnaghi, V.; Blanfuné, A.; Cabrini, M.; Cattaneo-Vietti, R.; Chavanon, F.; Chiantore, M.; Cohu, S.; Costa, E.; Fornasaro, D.; Grossel, H.; Marco-Miralles, F.; Masó, M.; Reñé, A.; Rossi, A. M.; Montserrat Sala, M.; Thibaut, T.; Totti, C.; Vila, M.; Lemée, R. Trends in *Ostreopsis* proliferation along the Northern Mediterranean coasts. *Toxicon* **2011**, *57*, 408−420.

(2) Tubaro, A.; Durando, P.; Del Favero, G.; Ansaldi, F.; Icardi, G.; Deeds, J. R.; Sosa, S. Case definitions for human poisonings postulated to palytoxins exposure. *Toxicon* **2011**, *57*, 478−495.

(3) Sansoni, G.; Borghini, B.; Camici, G.; Casotti, M.; Righini, P.; Rustighi, C. Fioriture algali di*Ostreopsis ovata* (Gonyaulacales: Dinophyceae): un problema emergente. *Biologia Ambientale* **2003**, *17*, 17−23.

(4) Durando, P.; Ansaldi, F.; Oreste, P.; Moscatelli, P.; Marensi, L.; Grillo, C.; Gasparini, R.; Icardi, G. *Ostreopsis ovata* and human health: epidemiological and clinical features of respiratory syndrome outbreaks from a two year syndromic surveillance, 2005 − 2006, in northwest Italy. *Euro Surveill.* **2007**, *12* (23). http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=3212 (accessed March 7, 2014).

(5) Ciminiello, P.; Dell'Aversano, C.; Dello Iacovo, E.; Fattorusso, E.; Forino, M.; Tartaglione, L.; Yasumoto, T.; Battocchi, C.; Giacobbe, M.; Amorim, A.; Penna, A. Investigation of toxin profile of Mediterranean and Atlantic strains of *Ostreopsis* cf. *siamensis* (Dinophyceae) by liquid chromatography−high resolution mass spectrometry. *Harmful Algae* **2013**, *23*, 19−27.

(6) Ciminiello, P.; Dell'Aversano, C.; Fattorusso, E.; Forino, M.; Magno, G. S.; Tartaglione, L.; Grillo, C.; Melchiorre, N. The Genoa 2005 outbreak. Determination of putative palytoxin in Mediterranean *Ostreopsis ovata* by a new liquid chromatography tandem mass spectrometry method. *Anal. Chem.* **2006**, *78*, 6153−6159.

(7) Ciminiello, P.; Dell'Aversano, C.; Fattorusso, E.; Forino, M.; Tartaglione, L.; Grillo, C.; Melchiorre, N. Putative palytoxin and its new analogue, ovatoxin-a, in *Ostreopsis ovata* collected along the Ligurian coasts during the 2006 toxic outbreak. *J. Am. Soc. Mass Spectrom.* **2008**, *19*, 111−120.

(8) Ciminiello, P.; Dell'Aversano, C.; Dello Iacovo, E.; Fattorusso, E.; Forino, M.; Grauso, L.; Tartaglione, L.; Guerrini, F.; Pistocchi, R. Complex palytoxin-like profile of *Ostreopsis ovata*. Identification of four new ovatoxins by high-resolution liquid chromatography/mass spectrometry. *Rapid Commun. Mass Spectrom.* **2010**, *24*, 2735−2744.

(9) Ciminiello, P.; Dell'Aversano, C.; Dello Iacovo, E.; Fattorusso, E.; Forino, M.; Tartaglione, L.; Battocchi, C.; Crinelli, R.; Carloni, E.; Magnani, M.; Penna, A. Unique toxin profile of a Mediterranean *Ostreopsis* cf. *ovata* strain: HR LC-$MS^n$ characterization of ovatoxin-f, a new palytoxin congener. *Chem. Res. Toxicol.* **2012**, *25*, 1243−1252.

(10) Uemura, D.; Ueda, K.; Hirata, Y.; Naoki, H.; Iwashita, T. Further studies on palytoxin. II. Structure of palytoxin. *Tetrahedron Lett.* **1981**, *22*, 2781−2784.

(11) Moore, R. E.; Bartolini, G. Structure of palytoxin. *J. Am. Chem. Soc.* **1981**, *103*, 2491−2494.

(12) Ciminiello, P.; Dell'Aversano, C.; Dello Iacovo, E.; Fattorusso, E.; Forino, M.; Grauso, L.; Tartaglione, L.; Guerrini, F.; Pezzolesi, L.; Pistocchi, R.; Vanucci, S. Isolation and structure elucidation of ovatoxin-a, the major toxin produced by *Ostreopsis ovata*. *J. Am. Chem. Soc.* **2012**, *134* (3), 1869−1875.

(13) Ciminiello, P.; Dell'Aversano, C.; Dello Iacovo, E.; Fattorusso, E.; Forino, M.; Grauso, L.; Tartaglione, L. Stereochemical studies on ovatoxin-a. *Chem.—Eur. J.* **2012**, *18* (52), 16836−16843.

(14) Ciminiello, P.; Dell'Aversano, C.; Dello Iacovo, E.; Fattorusso, E.; Forino, M.; Grauso, L. Tartaglione. High resolution LC-$MS^n$ fragmentation pattern of palytoxin as template to gain new insights into ovatoxin-a structure. The key role of calcium in MS behavior of palytoxins. *J. Am. Soc. Mass. Spectrom.* **2012**, *23* (5), 952−963.

(15) Munday, R. Palytoxin toxicology: Animal studies. *Toxicon* **2011**, *57*, 470−477.

(16) Ito, E.; Yasumoto, T. Toxicological evaluation on palytoxin and ostreocin-D administered to mice by three different routes. *Toxicon* **2009**, *54*, 244−251.

(17) Crinelli, R.; Carloni, E.; Giacomini, E.; Penna, A.; Dominici, S.; Battocchi, C.; Ciminiello, P.; Dell'Aversano, C.; Fattorusso, E.; Forino, M.; Tartaglione, L.; Magnani, M. Palytoxin and an *Ostreopsis* toxin extract increase the levels of mRNAs encoding inflammation-related proteins in human macrophages via p38 MAPK and NF-κB. *PLoS One* **2012**, *7* (6), e38139.

(18) Fleming, L. E.; Kirkpatrick, B.; Backer, L. C.; Walsh, C. J.; Nierenberg, K.; Clark, J.; Reich, A.; Hollenbeck, J.; Benson, J.; Cheng, Y. S.; Naar, J.; Pierce, R.; Bourdelais, A. J.; Abraham, W. M.; Kirkpatrick, G.; Zaias, J.; Wanner, A.; Mendes, E.; Shalat, S.; Hoagland, P.; Stephan, W.; Bean, J.; Watkins, S.; Clarke, T.; Byrne, M.; Baden, D. G. Review of Florida red tide and human health effects. *Harmful Algae* **2011**, *10*, 224−233.

(19) Backer, L. C.; McNeel, S. V.; Barber, T.; Kirkpatrick, B.; Williams, C.; Irvin, M.; Zhou, Y.; Johnson, T. B.; Nierenberg, K.; Aubel, M.; LePrell, R.; Chapman, A.; Foss, A.; Corum, S.; Hill, V. R.; Kieszak, S. M.; Cheng, Y. S. Recreational exposure to microcystins during algal blooms in two California lakes. *Toxicon* **2010**, *55*, 909−921.

(20) Cheng, Y. S.; Zhou, Y.; Irvin, C. M.; Kirkpatrick, B.; Backer, L. C. Characterization of aerosols containing microcystin. *Mar. Drugs* **2007**, *5*, 136−50.

(21) Deeds, J. R.; Schwartz, M. D. Human risk associated with palytoxin exposure. *Toxicon* **2010**, *56* (2), 150−162.

(22) Casabianca, S.; Casabianca, A.; Riobó, P.; Franco, J.; Vila, M.; Penna, A. Quantification of the toxic dinoflagellate *Ostreopsis* spp. by qPCR assay in marine aerosol. *Environ. Sci. Technol.* **2013**, *47*, 3788−3795.

(23) Battocchi, C.; Totti, C.; Vila, M.; Masò, M.; Capellacci, S.; Accoroni, S.; René, A.; Scardi, M.; Penna, A. Monitoring toxic microalgae *Ostreopsis* (dinoflagellate) species in coastal waters of the Mediterranean Sea using molecular PCR-based assay combined with light microscopy. *Mar. Pollut. Bull.* **2010**, *60* (7), 1074−1084.

(24) Steidinger, K.; Tangen, K. Dinoflagellates. In *Identifying Marine Phytoplankton*; Tomas, C., Ed.; Academic Press: New York, 1997; pp 387−584.
(25) Adachi, M.; Sako, Y.; Ishida, Y. Restriction fragment length polymorphism of ribosomal DNA internal transcribed spacer and 5.8S regions in Japanese *Alexandrium* species (Dinophyceae). *J. Phycol.* **1994**, *30*, 857−863.
(26) Penna, A.; Bertozzini, E.; Battocchi, C.; Giacobbe, M. G.; Galluzzi, L.; Garces, E.; Vila, M.; Lugliè, A.; Magnani, M. Monitoring of HAB species in the Mediterranean Sea through molecular techniques. *J. Plank. Res.* **2007**, *29*, 19−38.
(27) Taylor, F. J. R. A description of the benthic dinoflagellate associated with maitotoxin and ciguatoxin, including observations on Hawaiian material. In *Toxic Dinoflagellate Blooms*; Taylor, D. L., Seliger, H. H., Eds.; Elsevier/North-Holland: New York, 1979; pp 71−76.
(28) Kermarec, F.; Dor, F.; Armengaud, A.; Charlet, F.; Kantin, R.; Sauzade, D.; de Haro, L. Health risks related to *Ostreopsis ovata* in recreational waters. *Environnement, Risques & Santé* **2008**, *7*, 357−363.
(29) Abboud-Abi Saab, M. Les dinoflagellés des eaux côtières libanaises. Espèces rares ou nouvelles du phytoplancton marin. *Leban. Sci. Bull.* **1989**, *5* (2), 5−16.
(30) Abboud-Abi Saab, M.; Fakhri, M.; Kassab, M. T.; Matar, N. Seasonal and spatial variations of the dinoflagellate *Ostreopsis siamensis* in the Lebanese coastal waters (Eastern Mediterranean). *Cryptogam.: Algol.* **2013**, *34* (1), 57−67 2013..
(31) Vila, M.; Masó, M.; Sampedro, N.; Illoul, H.; Arín, L.; Garcés, E.; Giacobbe, M. G.; Alvarez, J.; Camp, J. The genus Ostreopsis in recreational waters of the Catalan coast and Balearic Islands (NW Mediterranean sea): Is this the origin of human respiratory difficulties? In *Proceedings of the 12th International Conference on Harmful Algae*; Moestrup, Ø. et al., Eds.; International Society for the Study of Harmful Algae and Intergovernmental Oceanographic Commission of UNESCO: Copenhagen, 2008; pp 334−336.
(32) Vila, M.; Arin, L.; Battocchi, C.; Bravo, I.; Fraga, S.; Penna, A.; Reñé, A.; Riobó, P.; Rodriguez, F.; Sala, M.; Camp, J.; de Torres, M.; Franco, J. M. Management of *Ostreopsis* blooms in recreational waters along the Catalan coast (NW Mediterranean Sea): cooperation between a research project and a monitoring program. *Cryptogam.: Algol.* **2012**, *33*, 143−152.
(33) Tognetto, L.; Bellato, S.; Moro, I.; Andreoli, C. Occurrence of *Ostreopsis ovata* (Dinophyceae) in the Tyrrhenian sea during Summer 1994. *Bot. Mar.* **1995**, *38*, 291−295.
(34) Vila, M.; Garcés, E.; Masó, M. Potentially toxic epiphytic dinoflagellates assemblages on macroalgae in the NW Mediterranean. *Aquat. Microb. Ecol.* **2001**, *26*, 51−60.
(35) Penna, A.; Vila, M.; Fraga, S.; Giacobbe, M. G.; Andreoni, F.; Riobó, P.; Vernesi, C. Characterization of *Ostreopsis* and *Coolia* (Dinophyceae) isolates in the Western Mediterranean Sea based on morphology, toxicity and internal transcribed spacer 5.8S rDNA sequences. *J. Phycol.* **2005**, *41*, 212−225.
(36) Ismael, A.; Halim, Y. Potentially harmful *Ostreopsis* spp. in the coastal waters of Alexandria − Egypt. *Medit. Mar. Sci.* **2012**, *13* (2), 208−212.
(37) Rustighi, C.; Casotti, M. Fioriture tossiche di *Ostreopsis ovata* sul litorale Apuano. *Rapp. ISTISAN* **2005**, *05/29*, 118−122.
(38) Monti, M.; Minocci, M.; Beran, A.; Ives, L. First record of *Ostreopsis* cfr. *ovata* on macroalgae in the Northern Adriatic Sea. *Mar. Pollut. Bull.* **2007**, *54*, 598−601.
(39) Honsell, G.; Bonifacio, A.; De Bortoli, M.; Penna, A.; Battocchi, C.; Ciminiello, P.; Dell'Aversano, C.; Fattorusso, E.; Sosa, S.; Yasumoto, T.; Tubaro, A. New insights on cytological and metabolic features of *Ostreopsis* cf. *ovata* Fukuyo (*Dinophyceae*): A multi-disciplinary approach. *PLoS One* **2013**, *8* (2), e57291.
(40) Tichadou, L.; Glaizal, M .; Armengaud, A.; Grossel, H.; Lemée, R.; Kantin, R.; Lasalle, J. L.; Drouet, G.; Rambaud, L.; Malfait, P.; de Haro, L. Health impact of unicellular algae of the *Ostreopsis* genus blooms in the M editerranean Sea: Experience of the French Mediterranean coast surveillance network from 2006 to 2009. *Clin. Toxicol.* **2010**, *48* (8), 839−844.
(41) Barroso García, P.; Rueda de la Puerta, P.; Parrón Carreño, T.; Marín Martínez, P.; GuillénEnríquez, J. Brote con síntomas respiratorios en la provincia de Almería por una posible exposición a microalgas tóxicas. *Gac. Sanit.* **2008**, *22*, 578−584.
(42) Turki, S.; Harzallah, A.; Sammari, C. Occurrence of harmful dinoflagellates in two different Tunisian ecosystems: the lake of Bizerte and the Gulf of Gabes. *Cah. Biol. Mar.* **2006**, *47*, 253−259.
(43) Totti, C.; Accoroni, S.; Cerino, F.; Cucchiari, E.; Romagnoli, T. *Ostreopsis ovata* bloom along the Conero Riviera (northern Adriatic Sea): relationships with environmental conditions and substrata. *Harmful Algae* **2010**, *9*, 233−239.
(44) Accoroni, S.; Romagnoli, T.; Colombo, F.; Pennesi, C.; Di Camillo, C. G.; Marini, M.; Battocchi, C.; Ciminiello, P.; Dell'Aversano, C.; Dello Iacovo, E.; Fattorusso, E.; Tartaglione, L.; Penna, A.; Totti, C. *Ostreopsis* cf. *ovata* bloom in the northern Adriatic Sea during summer 2009: Ecology, molecular characterization and toxin profile. *Mar. Pollut. Bull.* **2011**, *62* (11), 2512−9.
(45) Cohu, S.; Thibaut, T.; Mangialajo, L.; Labat, J. P.; Passafiume, O.; Blanfuné, A.; Simon, N.; Cottalorda, J. M.; Lemée, R. Occurrence of the toxic dinoflagellate *Ostreopsis* cf. *ovata* in relation with environmental factors in Monaco (NW Mediterranean). *Mar. Pollut. Bull.* **2011**, *62*, 2681−2691.
(46) Amorim, A.; Veloso, V.; Penna, A. First detection of *Ostreopsis* cf. *siamensis* in Portuguese coastal waters. *Harmful Algal News* **2010**, *42*, 6−7.
(47) Casavola, N.; Troncone, A.; Rizzi, E.; Favale, M. G.; Bello, G. Microalghe marine tossiche nella provincia di Bari:danni ambientali, ittiofaunistici, evidenze epidemiologiche. In *Diffusione delle fioriture algali tossiche nelle acque italiane: gestione del rischio ed evidenze epidemiologiche*; Mattei, D., Melchiorre, S., Messineo, V., Bruno, M., Eds.; Rapporti Istisan 05/29, Istituto Superiore di Sanità: Rome, 2005; pp 92−97.
(48) Gallitelli, M.; Ungaro, N.; Addante, L. M.; Gentiloni Silver, N.; Sabba, C. Respiratory illness as a reaction to tropical algal blooms occurring in a temperate climate. *JAMA, J. Am. Med. Assoc.* **2005**, *293*, 2599−2600.
(49) Pfannkuchen, M.; Godrijan, J.; Pfannkuchen, D. M.; Iveša, L.; Kružić, P.; Ciminiello, P.; Dell'Aversano, C.; Dello Iacovo, E.; Fattorusso, E.; Forino, M.; Tartaglione, L.; Godrijan, M. Toxin-producing *Ostreopsis* cf. *ovata* are likely to bloom undetected along coastal areas. *Environ. Sci. Technol.* **2012**, *46* (10), 5574−82.
(50) Bottalico, A.; Milella, P.; Felicini, G. P. Fioritura di Ostreopsis spp. (Dinophyta) nel porto di Otranto. In *Abstract Book of the Riunione Scientifica Annuale del Gruppo di Lavoro per l'Algologia — Società Botanica Italiana*, Chioggia (VE), Italy, 2002, p 21.
(51) *Ostreopsis ovata*, Coolia monotis e Prorocentrum lima lungo le coste della Regione Basilicata; Monitoraggio di *Ostreopsis ovata* e altre microalghe potenzialmente tossiche tossiche lungo le aree marino costiere italiane. Anno 2010. ISPRA, Rapporti 148/2011. http://www.isprambiente.gov.it/contentfiles/00010300/10382-rapporto-148-2011.pdf/view (accessed March 7, 2014).
(52) Aligizaki, K.; Nikolaidis, G. The presence of the potentially toxic genera *Ostreopsis* and *Coolia* (Dinophyceae) in the North Aegean Sea, Greece. *Harmful Algae* **2006**, *5*, 717−730.
(53) Aligizaki, K.; Katikoub, P.; Nikolaidisa, G.; Panou, A. First episode of shellfish contamination by palytoxin-like compounds from *Ostreopsis* species (Aegean Sea, Greece). *Toxicon* **2008**, *51*, 418−427.
(54) Tavolo Tecnico Regionale sulle Acque. http://www.artasicilia.eu/old_site/web/servizio_7_dra/site/3acque/Documenti/Tavolo_tecnico_Scheda%20tecnica%20Ostreopsis.pdf (accessed March 7, 2014).
(55) Zingone, A.; Siano, R.; D'Alelio, D.; Sarno, D. Potentially toxic and harmful microalgae from coastal waters of the Campania region (Tyrrhenian Sea, Mediterranean Sea). *Harmful Algae* **2006**, *5*, 321−337.

(56) David, H.; Moita, M. T.; Laza-Martínez, A.; Silva, A.; Mateus, M.; de Pablo, H.; Orive, E. First bloom of *Ostreopsis* cf. *ovata* in the continental Portuguese coast. *Harmful Algal News* **2012**, *45*, 12−13.
(57) Bennouna, A.; El Attar, J.; Abouabdellah, R.; Palma, S.; Penna, A.; Moita, T. First records of *Ostreopsis* cf. *siamensis* in Moroccan Atlantic upwelling waters. *Harmful Algal News* **2010**, *42*, 1−3.
(58) Monitoraggio di Ostreopsis ovata lungo le coste della provincia di Sassari nell'estate 2011. *Ostreopsis* cf. *ovata* lungo le coste italiane: monitoraggio 2011. ISPRA, Rapporto 173/2012. http://www.isprambiente.gov.it/files/pubblicazioni/rapporti/Rapporto%20173%20_2012.pdf (accessed March 7, 2014).
(59) Monitoraggio di Ostreopsis ovata e altre microalghe potenzialmente tossiche lungo le aree marino costiere Italiane. Anno 2010. ISPRA, Rapporti 148/2011. http://www.isprambiente.gov.it/contentfiles/00010300/10388-rapporto-148-2011.pdf/view (accessed March 7, 2014).
(60) Linee guida gestione del rischio associato alle fioriture di *Ostreopsis ovata* nelle coste italiane; approved by Consiglio Superiore di Sanità: Rome, May 24, 2007. http://www.salute.gov.it/imgs/C_17_pubblicazioni_641_allegato.pdf (accessed March 7, 2014).
(61) Grönkvist, M. J.; Emery, M. J.; Gustafsson, P. M. Mechanisms of ventilation inhomogeneity during vital capacity breaths standing and supine. *Respir. Physiol.* **2002**, *129*, 345−355.
(62) Taylor, T. J.; Parker, G. W.; Fajer, A. B.; Mereish, K. A. Non-specific binding of palytoxin to plastic surfaces. *Toxicol. Lett.* **1991**, *57* (3), 291−296.
(63) Ciminiello, P.; Dell'Aversano, C.; Dello Iacovo, E.; Fattorusso, E.; Forino, M.; Tartaglione, L. LC-MS of palytoxin and its analogues: state of the art and future perspectives. *Toxicon* **2011**, *57*, 376−389.