

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

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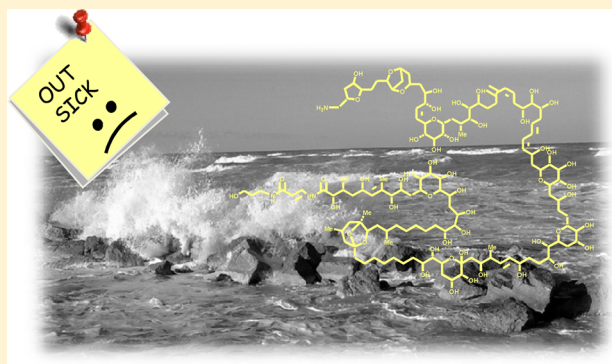
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S Supporting Information

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 bronchoconstriction 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 zoanths of the genus *Palythoa*.^{10,11} Ovatoin-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 Ovatoin-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 ($LD_{50} = 0.15 \mu\text{g}/\text{kg}$) and intraperitoneal ($1.5 \mu\text{g}/\text{kg}$) administration, Ito and Yasumoto recently demonstrated that palytoxin causes bleeding and alveolar destruction in lungs and death at $2 \mu\text{g}/\text{kg}$ 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 Mexico¹⁸ and of microcystins during cyanobacterial blooms of *Microcystis aeruginosa* in California lakes.^{19,20} Inhalation exposure to aerosols containing either brevetoxins or microcystins 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) ($44^{\circ}00'00''\text{N}$; $10^{\circ}06'00''\text{E}$) and Marina di Pisa (PI) ($43^{\circ}40'00''\text{N}$; $10^{\circ}16'00''\text{E}$) (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. Meteorological 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 (Analytica 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 $+4^{\circ}\text{C}$ until chemical analysis. The filters of AirCube COM2 were suspended in 50 mL of ethanol, and they were stored at $+4^{\circ}\text{C}$ 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 $+4^{\circ}\text{C}$ 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 Uthermöl chambers as described by Battocchi et al.²³ *Ostreopsis* spp. were identified under inverted light microscope (Axiovert 40 CFL) at 200 \times or 400 \times 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 gene²⁵ was performed, and then a nested PCR



#	Year ^{ref}	Site	People	#	Year ^{ref}	Site	People
1	1972 ²⁷	French Riviera (France)	-	18	2004 ²⁸	Barcelona (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 (Croatia)	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, (Morocco)	-	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 primers²⁶ 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 μ 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 (Δ 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 1×10^6 to 1×10^2 (two replicates), and from 1×10^1 to 2×10^0 (four replicates). A cellular standard curve of four mixed crude extracts of 5×10^2 cells from macroalgae wash seawater samples collected at MS and PI in 2010 was generated with dilutions from 8 to 8×10^{-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 μ L using the Hot-Rescue Real-Time PCR Kit-SG (Diateva, 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 1×10^1 and 1×10^2 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 μ m centrifugal filter units (Millipore, Billerica, MA) and directly analyzed by LC-HRMS (5 μ 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 μ m centrifugal filter units, and analyzed by LC-HRMS (5 μ 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 José, CA) coupled to an Agilent 1100 LC binary system (Palo Alto, CA). LC conditions included the use of a 3 μm Gemini C18 (150 \times 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·H₂O) 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. HRMS² 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 reported⁸ was used as reference sample for qualitative identification of ovatoxins. A palytoxin standard (100 μg ; 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; qualitative 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 HRMSⁿ,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/hydrodynamism 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 $\geq 10\,000$ 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 m³).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 amplifiability 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

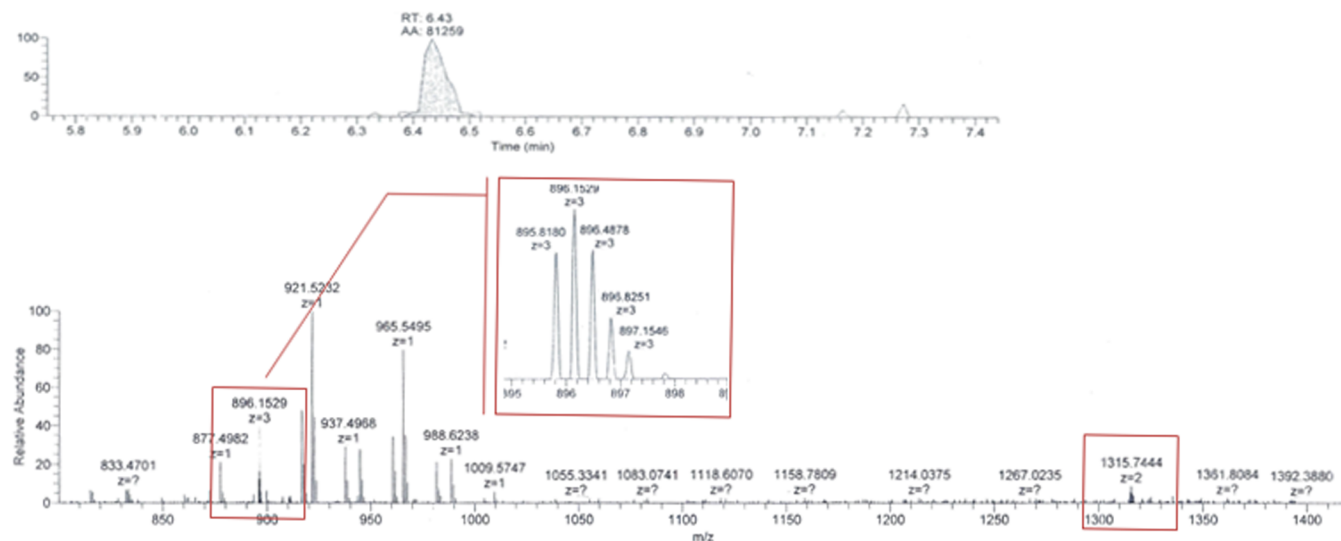


Figure 2. (Above) XIC of doubly and triply charged ions of ovatoxin-a in the aerosol sample of August 11, 2010 and (below) HR full MS spectrum associated to the peak at 6.43 min. The zoomed view of the isotopic ion cluster of $[M + H + Ca]^{3+}$ ion of ovatoxin-a is shown in the inset.

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 epiphytic *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 (*Pterocladia 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 meteorological

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 MS² 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

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 aerosols²² 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 brevetoxins¹⁸ and mycrocistins¹⁹ 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 $<5 \mu\text{m}$ 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 hydrodynamism 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 (1.8×10^6 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 zoanths 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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