Variability of toxin profile and content of *Ostreopsis* cf. *ovata* from the Mediterranean Sea

Luciana Tartaglione (1), Carmela Dell'Aversano (1), Emma Dello Iacovo (1), Patrizia Ciminiello (1), Silvia Casabianca (2), Antonella Penna (2)

(1) Department of Pharmacy, University of Naples Federico II, Naples, Italy. Presenter contact details: luciana.tartaglione@unina.it, Phone: +39 081 678133
(2) Department of Biomolecular Sciences, University of Urbino, Urbino, Italy

Summary
Blooms of *Ostreopsis* spp., once confined to tropical and subtropical areas, have recently spread to more temperate regions such as the Mediterranean and the Southern-Atlantic coasts of Europe. In the last decade, *O*. confronta (cf.) *ovata* has become increasingly frequent with massive blooms with consequent relevant negative impacts on benthic communities and on human health through skin contact and toxic aerosols. Following the Mediterranean *Ostreopsis*-related outbreaks we developed a liquid chromatography tandem mass spectrometry (LC-MS) method to be employed in the investigation of both field and cultured algal samples of *O*. cf. *ovata*. Successively, our LC-high resolution MS (LC-HRMS) studies characterized *O*. cf. *ovata* as the producer of minute amounts of a putative palytoxin, one of the most potent marine toxins so far known, and of much higher amounts of several palytoxin congeners, ovatoxins. LC-HRMS analyses carried out on several samples of *O*. cf. *ovata* from several geographical origin revealed that different *O*. cf. *ovata* strains could have different toxin profiles both in a qualitative and quantitative perspective. Among the analyzed strains, the most widespread toxin profile presents ovatoxin-a as major component.

Introduction
Currently, the benthic dinoflagellate *Ostreopsis* cf. *ovata* represents a serious concern to human health in the whole Mediterranean basin: LC-HRMS studies on *O*. cf. *ovata* demonstrated it to produce several congeners of palytoxin (PLTX, $C_{129}H_{223}N_3O_{54}$), one of the most potent nonprotein marine toxins known so far (Deeds and Schwartz 2010) namely a putative palytoxin and ovatoxins (OVTX-a, -b,-c, -d/-e, -f), slightly different in their elemental composition in comparison to PLTX (Ciminiello *et al.* 2010, 2012 a). The most extensive sanitary events associated with *Ostreopsis* blooms occurred in Italy 2005, in Spain in 2004 and 2006, and in France in the period 2006–2009 (Ciminiello *et al.* 2014). In these occasions, people exposed to marine aerosols during recreational or working activities required medical attention due to symptoms of respiratory distress. OVTX-a has been recently isolated and structurally elucidated, while OVTX-b, -c, -d, -e, and -f have not been isolated yet (Ciminiello *et al.* 2012 b). Our recent research has been focused on characterization of toxin profiles of *O.* cf. *ovata* strains of different geographical origin that resulted to be quite different both qualitatively and quantivatively.

Materials and Methods
O. cf. *ovata* strains, collected from different areas of Mediterranean basin, namely Ancona, Alghero, Genova, Pisa, Porto Romano, Trieste, Taormina (Italy) and Villefrance sur Mer (France), were grown in culture and used for chemical analyses. Cell pellet (1-3 x 10^6 cells) of each strain was extracted with a methanol/water (1:1) solution and 0.2% acetic acid and sonicating for 30 min in pulse mode, while cooling in an ice bath, centrifuged at 3000g for 30 min. The supernatant was decanted and the pellet was washed twice more with methanol/water (1:1, v/v) and 0.2% acetic acid. The extracts were combined and directly analyzed by LC-HRMS on a hybrid linear ion trap LTQ Orbitrap XL Fourier transform mass spectrometer equipped with an ESI ION MAX source coupled to a LC binary system

operating in HR full MS mode (positive ions) in the range *m/z* 800−1400 at resolution of 60.000. LC and MS conditions are those reported in Ciminiello *et al.* (2012 a). Calculation of elemental formulas was performed by using the monoisotopic ion peak of each ion cluster. Extracted ion chromatograms were obtained from the HR full MS spectra by selecting the most abundant and the monoisotopic ion peaks of the $[M+2H-H_2O]^{2+}$ and $[M+H+Ca]^{3+}$ ion clusters of each toxin (5 ppm mass tolerance). The chromatographic peaks were identified by comparing their retention times and associated HR full MS spectra to those of OVTXs and putative PLTX contained in a reference *O.* cf. *ovata* extract previously characterized and analyzed under the same experimental conditions. In quantitative analyses, because of the lack of standards for OVTXs, their molar responses were assumed to be the same as that of PLTX, on the basis of structural similarities between PLTX and OVTXs. Peak areas were measured and interpolated within the calibration curve of PLTX standard.

Results and Discussion

Over 40 cultured strains of *O.* cf. *ovata* collected along the Italian and the French Mediterranean coasts were extracted and analyzed by LC-HRMS identifying basically 4 kinds of toxin profiles (Figure 1). In most of the strains (34/45 strains) ovatoxin-a dominates toxin profiles accounting for the 54% of the total toxin content followed by OVTX-b (27%), OVTX-d/-e (12%), OVTX-c (6%) and putative PLTX (< 1%). About 20% of the analyzed strains were found to produce only OVTX-a (80%), -d/-e (12%) and putative PLTX (< 1%). Unique toxin profiles were found in a strain from Ancona (Marche, Italy) in which OVTX-f was the dominant toxin accounting for 50% of the total toxin profile followed by OVTX-a,-b, -d/-e, -c and putative PLTX, listed in decreasing order of abundance, and a strain from Villefrance sur Mer (France) that did not produce OVTX-a, but just OVTX-b (89%) and –c (11%). In most cases, total toxin contents (pg/cell) ranged from 5 to 60 pg/cell. The most productive strains were found in the area of Genoa and Villefrance sur Mer exhibiting a toxin per cell production even of 230 pg/cell. These data trigger some questions on the reasons why toxin profiles and toxin contents are so different as well as on toxins that need to be determined in monitoring programs of *O.* cf. *ovata* toxins in environmental and seafood sample.

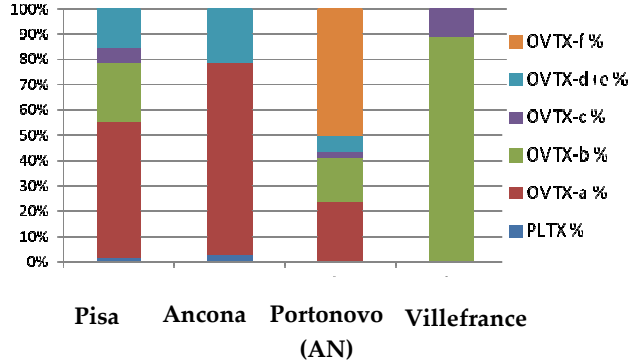

Figure 1: Percentages (%) of putative palytoxin (PLTX) and ovatoxins (OVTX-a, -b, -c, -d + -e, and -f) contained in *O.* cf. *ovata* analyzed strains.

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