New *Ostreopsis* species record along Cyprus coast: toxic effect and preliminary characterization of chemical-molecular aspects

Valentina Giussani[1*], Demetris Kletou[2], Samuela Capellacci[3], Valentina Asnaghi[1], Antonella Penna[3], Patrizia Ciminiello[4], Carmela Dell'Aversano[4], Antonia Mazzeo[4], Luciana Tartaglione[4], Marco Faimali[5], Roberto Pronzato[1] and Mariachiara Chiantore[1]

[1.] DISTAV- University of Genoa, Corso Europa 26, 16132, Genoa, Italy valentina.giussani@edu.unige.it
[2.] Marine and Environmental Research (MER) Lab Ltd, 202 Amathountos Av., Marina Gardens Block B, Of. #: 13+14, 4533 Limassol, Cyprus
[3.] DISB - University of Urbino, Viale Trieste 296, 61121, Pesaro, Italy
[4.] Department of Pharmacy,. University Federico II, via Montesano 49, 80131, Naples, Italy
[5.] CNR –Institute of Marine Sciences (ISMAR), via De Marini 6, 16149, Genoa, Italy

Abstract

The genus *Ostreopsis* Schmidt includes harmful benthic species that have been reported worldwide in both tropical and temperate regions. To date, genetic studies confirmed the presence of two genotypes corresponding to the morphotypes of *O.* cf. *ovata* and *O.* cf. *siamensis* in the Mediterranean Sea; recently a new genotype of *Ostreopsis* sp. was found along Greece and Cyprus coasts.
Reliable data on harmful algal blooms and related intoxication cases in the eastern Mediterranean basin are still scarce. The present study describes, for the first time, toxic effects and chemical-molecular aspects of the Cypriot genotype of *Ostreopsis* sp.
Ecotoxicological bioassays were performed exposing *A. salina* nauplii to the following treatments of cultured *Ostreopsis* sp.: untreated culture, filtered and resuspended cells in fresh medium, resuspended and sonicated cells in fresh medium, growth medium devoid of algal cells by 6 µm (mucilage remains in the treatment) and 0.22 µm mesh size filtration. Our results show higher toxic effects (% of mortality) with the whole *Ostreopsis* sp. culture ($LC_{50\text{-}48h}$ = 45 cells/ml). Given these findings, the Cypriot strain seems to be less toxic than the most widespread species *O.* cf. *ovata* ($LC_{50\text{-}48h}$ < 4 cells/ml), though further studies are needed to better understand the toxicity of this new genotype.
Preliminary LC high resolution (HR) MS studies for the Cypriot Ostreopsis sp. strain reveal a peculiar toxic profile: no palytoxin and ovatoxins so far known were not detected, while the presence of new palytoxin-like compounds was highlighted, confirming the possibility of being considered a distinct species from Mediterranean *O.* cf. *ovata*.

Keywords: Ostreopsis, toxicology, HABs, benthic dinoflagellates, Cyprus, Mediterranean Sea.

Introduction

The genus *Ostreopsis* Schmidt includes harmful benthic species that have been reported worldwide, both in temperate and tropical coastal waters. Among this genus, some species are known to produce palytoxin (PLTX)-analogs complex and large amount of mucilage that can cover the sea bottom (Shears and Ross 2010). *Ostreopsis* spp. blooms have been associated with benthic marine organism mortalities and human health concerns and these events are increasing in the Mediterranean Sea.
Recently, as more isolates from Atlantic, Mediterranean basin and Pacific areas, have been sequenced, the identification and phylogeographical characterization of cryptic species belonging to this genus is starting to become clearer. To date, genetic studies confirmed that the Mediterranean Sea hosts two genotypes corresponding to the morphotypes of *O.* cf. *ovata* and *O.* cf. *siamensis.* A new genotype of *Ostreopsis* sp. has been recently found along Greece and Cyprus coasts (Parsons et al. 2012; Penna et al. 2014), but reliable data on harmful algal blooms and associated poisoning events in the eastern Mediterranean basin are still scarce.
This study describes, for the first time, toxic effects and chemical-molecular aspects of the Cypriot genotype of *Ostreopsis* sp. obtained by a sample collected during summer 2013 in Vasiliko Bay (south Cyprus), an area characterized by a

A. Lincoln MacKenzie [Ed]. *Marine and Freshwater Harmful Algae 2014*. Proceedings of the 16th International Conference on Harmful. Algae. Cawthron Institute, Nelson, New Zealand and the International Society for the Study of Harmful Algae (ISSHA)

bad ecological status, according to a macroalgal WFD index (Orfanidis *et al.*, 2001), assessed during the previous summer and spring .

Material and Methods

Ostreopsis spp. strains were isolated from seawater samples collected from Vasiliko bay, a heavily impacted coastline at south Cyprus in July 2013; sea water temperature on the sampling day was 25 °C and salinity equal to 39 PSU. Clonal cultures were established and maintained in F/4-Si medium (Guillard *et al.*, 1975) at temperature of 23 ± 1 °C, with a standard 14:10 h light – dark cycle (photon flux of 100 µE m^{-2} s^{-1}). Subsamples of cultures were collected at the exponential growth phase by centrifugation at 4000g for 15 min. The supernatant was removed and the pellets were immediately processed or stored at -80 °C until DNA extration. DNA extration, amplification and sequencing of ribosomal genes and phylogenetic analyses were carried out as described by Penna *et al.*, (2010). *Ostreopsis* sp. C1036 strain pellet was added of methanol:water 1:1 and sonicated in pulse mode under cooling in a ice bath. The mixture was centrifuged for 5 min at 4000g and the supernatant was analyzed by liquid chromatography-high resolution mass spectrometry (LC-HRMS) and MS^2 in Full MS^2 positive ion mode according to Ciminiello et al. (2012) to characterize its toxic profile. The ecotoxicological bioassay was performed using the same algal strain which has been cultured into sterilized marine water and F/2 medium (at a concentration of 1 ml l^{-1}) and maintained at 20 °C. *Artemia salina* nauplii were exposed to 4, 40, 400 cells/ml of the following treatments of C1036 culture collected during the stationary growth phase: a) whole culture, b) filtered and resuspended cells in fresh medium, c) resuspended and sonicated cells in fresh medium, d) growth medium free of algal cells (6 µm filter mesh) and e) growth medium containing mucilage but free of algal cells (0.22 µm filter mesh). Three replicates were prepared for each combination of treatments and cell concentrations, including a control (CTR; 0.22 µm Filtered Natural Sea Water); after 48 h, the number of dead nauplii was observed under a stereomicroscope. Two way ANOVA (Factors: Concentration, 3 levels; Treatments, 5 levels) and Student-Newman-Keuls tests were performed using R statistical software. The results of the ecotoxicological bioassay are compared with those obtained in a previous study (Giussani et al. 2015) of *O.* cf. *ovata*.

Results and Discussion

The ecotoxicological bioassay recorded stronger effects with the whole and the resuspended *Ostreopsis* sp. 2 culture treatments, having $LC_{50\text{-}48h}$ values of 45 cells/ml and 99 cells/ml respectivelly, while the other treatments showed $LC_{50\text{-}48}$ of 365 cells/ml (in GM 6µm) and above 400 cells/ml (with GM 0.22 µm and sonicated culture) (Table 1). These data describe a toxicity pattern similar to that previously observed in the most widespred species *Ostreopsis* cf. *ovata*. In fact both species caused higher lethal effects on model organisms when a direct contact with living cells occured (Faimali et al. 2012, Giussani et al. 2015). The Cypriot genotype also presented significantly high mortality values with treatments which did not contain algal cells (54 % in GM 6 µm and 41 % in GM 0.22 µm), but only at the highest concentration tested, suggesting a different mechanism for the toxins release compared to the one that occurs in *O.* cf. *ovata*.

Table 1. $LC_{50\text{-}48h}$ values obtained exposing nauplii of *A. salina* to several treatment of *Ostreopsis* sp.2 and *O.* cf. *ovata* cultures.

Treatments	*Ostreopsis sp.* 2 (Cyprus, C1036) **$LC_{50\text{-}48h}$**	*O.* cf. *ovata* (Genoa, CBA 292012) **$LC_{50\text{-}48h}$**
Culture	45 (31 – 63)	<4
Resuspended	99 (40 - 244)	15 (12 - 20)
Sonicated	>400	66 (51 - 84)
GM 6 µm	365 (n.c.)	-
GM 0.22 µm	>400	>400

These findings are supported by the LC-HRMS analysis, which from a qualitative stand point, reports a totally new toxic profile for the C1036 strain characterized by the presence of new palytoxin analogues, named ostreotoxin. As for the quantitative aspect, compared to *O.* cf. *ovata*, *Ostreopsis* sp.2 exihibits a lower toxin content (0.17 pg/cell) in agreement with the higher LC_{50} value.

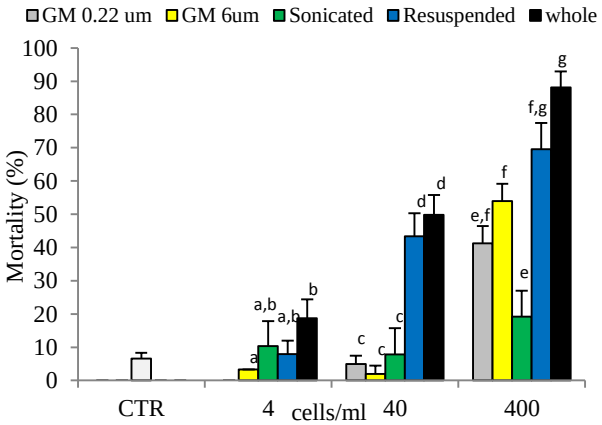

Fig. 1. Mortality (avg ± SE, N = 3) of *Artemia salina* after 48 h of exposure to 0.22 µm filtered growth medium, 6 µm filtered growth medium, sonicated *Ostreopsis* sp. 2 culture, resuspended *O*streopsis sp. 2 culture and whole *Ostreopsis* sp. 2 culture during late stationary phase of the growth curve. CTR (white bar): control in filtered natural seawater. Different letters (a-g) represent significant differences (SNK test results).

Table 2. Mono-isotopic ion peaks (*m/z*). Percentage (%) and amount (pg/cell) of new Ostreotoxins produced by the C1036 Cypriot *Ostreopsis* sp. 2 strain.

Name	$[M+H+Ca]^{3+}$ (*m/z*)	pg/cell	%
Ostreotoxin-a	909.8246	0.100	58
Ostreotoxin-b	915.1553	0.070	40
Ostreotoxin-c	920.4868	0.004	2

Phylogenetic results shows that the Cypriot strain is included in the *Ostreopsis* sp. 2 clade (David et al. 2013).

Ostreopsis sp. 2 can be considered a separate species from *O.* cf. *ovata*. Nowadays, in the Mediterranean Sea three different genotypes within the genus *Ostreopsis*, as *O.* cf. *ovata*, *O.* cf. *siamensis* and *Ostreopsis* sp. *2*. are recognised. Further interdisciplinary studies are ongoing to assess and characterize the genus *Ostreopsis* in the eastern Mediterranean area.

Acknowledgements

This publication has been produced with the financial assistance of the European Union under the ENPI CBC Mediterranean Sea Basin Programme (M3-HABs project). Authors are thankful to ISSHA for travel expenses support.

References

Ciminiello, P., Dell'Aversano, C., Dello Iacovo, E., et al. (2012). J. Am. Soc. Mass. Spectrom. 23(5): 952-963.

David, H., Laza-Martinez, A., Miguel, I., Orive, E. (2013). Harmful Algae 30: 44-55.

Faimali M., Giussani V., Piazza V. et al. (2012). Mar. Environ. Research, 76: 97-107.

Giussani, V., Sbrana, F., Faimali, M. et al. (2015). Harmful Algae.

Guillard, R.R.L. (1975). In: Smith W.L., Chanley M.H. (eds). Culture of phytoplankton for feeding marine invertebrates . Culture of Marine Animals. Plenum Press, New York, pp.26-60.

Orfanidis, S., Panayotidis, P., Stamatis, N., (2001). Mediterranean Marine Science 2.2: 45-66.

Parsons, M.L., Aligizaki, K., Bottein, M.-Y.D., et al (2012). Harmful Algae 14: 107–129.

Penna A., Fraga S., Battocchi C. et al. (2010). J. Biogeogr. 37: 830-841.

Penna A., Battocchi C., Capellacci S. et al. (2014). Harmful Algae 40: 40-50.

Shears, N.T., Ross, P.M. (2010). Ecol. Lett. 13: 1149–1159.