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Ostreopsis cf. *ovata* in the French Mediterranean coast: molecular characterisation and toxin profile

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Abstract – The presence of dinoflagellates of the genus *Ostreopsis* along Mediterranean coasts was first observed in 1972, in the bay of Villefranche-sur-Mer. However, over the past ten years, harmful events related to this benthic dinoflagellate have been reported in Italian, Spanish, Greek, French, Tunisian and Algerian coastal areas. In France, during a hot period in August 2006, cases of dermatitis and respiratory problems were registered in Marseille area. At that time, a link to the proliferation of *Ostreopsis* was highlighted for the first time in that area. A specific monitoring was designed and implemented in the summer 2007.
Two strains of *Ostreopsis* cf. *ovata*, collected in 2008 from Villefranche-sur-Mer and Morgiret coastal waters and grown in culture, were identified by molecular analysis and studied to characterise their growth and toxin profile. Liquid chromatography-mass spectrometry (LC-MS/MS) indicated that both strains produced ovatoxin-a (OVTX-a) as the major component (ca. 90%), and traces of palytoxin (PLTX). Toxin content was determined at the end of the exponential growth phase with highest concentration of 55 pg.cell^{-1} of OVTX-a and 2.5 pg.cell^{-1} of PLTX.

***Ostreopsis* cf. *ovata* / palytoxin / ovatoxin-a / Mediterranean sea**

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INTRODUCTION

Species of the family Ostreopsidaceae Lindeman(1928) are common members of benthic microalgal community in tropical and subtropical areas, but the world wide occurrence of this genus increased markedly in the last decade (Rhodes L., 2011) and was widely distributed in temperate waters of the Mediterranean sea (Tognetto *et al.,* 1995 ; Vila *et al.,* 2001 ; Penna *et al.,* 2005 ; Aligizaki *et al.,* 2008 ; Mangialajo *et al.,* 2008).

Since 1998, *Ostreopsis* has been repeatedly associated with toxic events, mainly human intoxications by inhalation (Gallitelli *et al.,* 2005) or irritation by contact (Kermarec *et al.,* 2008) and mass mortalities of invertebrates (Sansoni *et al.*, 2003). Chemical analysis of *Ostreopsis ovata* net samples along the Ligurian coast revealed the presence of putative palytoxin and ovatoxin-a, a new palytoxin-like molecule (Ciminiello *et al.,* 2006, 2008; Guerrini *et al.,* 2010).

In France, an *Ostreopsis* species (identified as *Ostreopsis siamensis*, the only described species of the genus at that time) was observed for the first time in 1972 in the bay of Villefranche-sur-Mer (Taylor, 1979). Then, it has been observed sporadically until the first sanitary report in 2006 when four divers suffered from mouth and throat irritation symptoms with fever after diving in Frioul Island (Morgiret) near Marseille where 38,000 cells of *Ostreopsis* sp. per litre were observed. The beaches were closed in that area and the consumption of seafood was banned until the end of august. A specific monitoring was designed and implemented in summer 2007; concentrations of *Ostreopsis* sp. cells of 4,000 cells per litre trigger palytoxin chemical analysis in shellfish.

The aim of this work was to characterize the toxin profiles of the two strains of *Ostreopsis* collected in 2008 on the Mediterranean eastern and western Provence coast of France and grown in culture. Molecular analyses were performed for taxonomic identification of the strains and LC-MS/MS analyses were carried out to identify the toxins produced in the cultured cells. The toxins profiles were compared with the ones determined from cells trapped in plankton nets and from strains of *Ostreopsis* cf. *ovata* isolated from the Tyrrhenian and Balearic seas and grown under the same conditions in the laboratory.

MATERIAL AND METHOD

Sampling

Field water samples were collected in two areas of the French Mediterranean coast in August 2008. One site was located near Marseille, on the Frioul Island at Morgiret creek (43° 16’ 56’’N, 05° 18’ 15’’E) which is located in the windest and coolest part of western Provence, and the second site is in the bay of Villefranche-sur-Mer (43° 41’ 35’’N, 07° 18’ 32’’E), in the eastern part of Liguria-Provence with warmer seas (Fig. 1). At Morgiret, the samples were collected in proximity of the seaweeds *Dictyota* sp. and *Haliptilon virgatum* while the dominant alga was *Halopteris scoparium* in the site located in the Bay of Villefranche-sur-Mer.

Fig. 1. Map of average computed sea-surface temperature in the Mediterranean (from NOAA/ AVHRR imagery) over 2008 summer period.

Cultures

Two strains of *Ostreopsis* were isolated from the water samples collected in the two sites (strain IFR-OST-0.1M from Morgiret and strain IFR-OST-0.1V from Villefranche-sur-Mer) using a capillary pipette. After an initial growth in microplates, the cells were cultured in flask (Fig. 2) at 22°C under a 16:8 h L:D cycle. Cultures were established in filtered natural seawater, at salinity of 35 psu, adding nutrients at K or L1 concentration (Guillard, 1975). Two other strains of *Ostreopsis* cf. *ovata*, gracefully provided to our laboratory by Dr. M. G. Giacobbe, CNR-A1 (Gioia Tauro, Italy) and CNR-Z1 (La Fosca, Spain) were cultured under the same conditions.

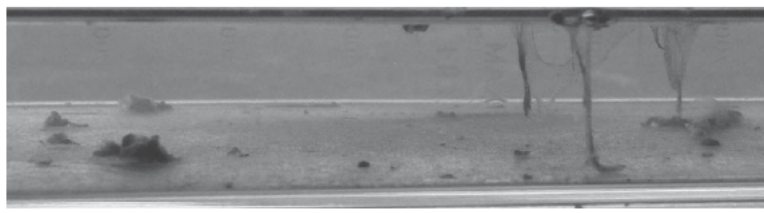

Fig. 2. Cell mucus aggregates in Villefranche-sur-Mer *Ostreopsis* cf. *ovata* cultures.

Taxonomic identification by molecular analysis

DNA amplification and sequencing

Approximately 15 mL of exponentially growing cultures IFR-OST-0.1V and IFR-OST-0.1M were harvested by centrifugation (4300 g, 10 min). DNA of pelleted cells was extracted using CTAB (N-cetyl-N,N,N-trimethylammoniumbromide) method (Doyle and Doyle 1987). The 5.8S rDNA and ITS regions (ITS1 and ITS2) were amplified by using oligonuclotide primers ITS-FW (5'-GTAGGTGAACCTGCGGAAGG-3'), and ITS-RV (5'-TCCTCTTGCT-TGATCTGAGATCCGG-3'). Genomic DNA was amplified in 25 µL PCR reaction containing 1 µL of extracted DNA, 6.5 µL of ultrapure water, 2.5 µL of each primer (10 µM) and 12.5 µL of PCR Master Mix 1X (Promega, Madison, WI, USA) which includes Taq polymerase, dNTPs, MgCl2 and reaction buffer. The polymerase chain reactions were performed in a Mastercycler Personal (Eppendorf, Hamburg, Germany) as follows: one initial denaturating step at 94°C for 2 min, followed by 45 cycles each consisting of 94°C for 30 sec, 54°C for 30 sec, and 72°C for 4 min, and a final elongation at 72°C for 5 min. The PCR products

were visualized on a 1% (w/v) agarose gel, excised, and purified with the Wizard SV Gel and PCR Clean-up system (Promega) according to the manufacturer's recommendations. Then, they were sequenced directly using the ABI PRISM BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, Carlsbad, CA, USA). Sequencing products were purified by exclusion chromatography using the Dye Terminator Removal Kit (Thermo Scientific) and the sequences were determined using an automated 3130 genetic analyser (Applied Biosystems).

Sequences alignment and phylogenetic analysis

The two sequences obtained were aligned with 71 sequences of *Ostreopsis* species and 7 sequences of *Coolia* species retrieved in Genbank, using MUSCLE software (Edgar, 2004). The alignment was refined by eye using with BioEdit version 7.0.0. (Hall, 1999).

Evolutionary models were examined with jModeltest version 0.1.1 Bayesian Inference (BI) analysis was run using Mr Bayes version 3.1.2 (Ronquist & Huelsenbeck, 2003). Initial Bayesian analyses were run with a GTR model (nst = 6) with rates set to invgamma and nucleotide frequencies set to equal. Each analysis was performed using four Markov chains (MCMC), with one million cycles for each chain. Trees were saved to a file every 100 cycles and the first 2000 trees were discarded. Therefore, a majority-rule consensus tree was created from the remaining 8000 trees in order to examine the posterior probabilities (pp) of each clade. Neighbor-joining (NJ) analysis was performed using MEGA software version 5.05 (Tamura *et al.*, 2011), with Maximum Composite Likelihood method. Bootstrap analysis (1000 pseudoreplicates) was used to assess the relative robustness of branches (Felsenstein, 1985).

Photosynthetic activity

Photosynthetic activity was measured using DW3 Oxylab unit with 2.5 mL chamber and 1 cm light path length (Hansatech, UK) fitted with a Clark electrode disc and a 36 red LED array LH36/2R (λ = 660 nm). After 1 min nitrogen flushing until almost 50% of O_2 saturation, oxygen production was measured with 1.3 mL of concentrated sample (110.10^3 cells) mixed with 0.055 mL of a 144 mmol.L^{-1} $NaHCO_3$ solution. 20 min light/10 min dark cycles were then applied for incident irradiance within the range 0 to 400 µmol photons·m^{-2}·s^{-1}.

Toxin Analysis: Extraction

Ten millilitres of each culture were sampled and centrifuged at 3000 g for 15 min. 1 mL of 90% aqueous methanol was added to each cell pellet before sonication for 40 min in pulse mode, while cooling in ice bath. 300 µL of supernatant was centrifuged on 0.2 µm filter at 8000 g for 5 min and analysed directly by LC-MS/MS (5 µL injected).

Toxin Analysis: Liquid Chromatography-Mass Spectrometry (LC-MS/MS)

Palytoxin analyses were carried out using an LC system (UFLC XR, Shimadzu) coupled to an hybrid triple quadrupole/ion trap mass spectrometer

(API-4000Qtrap, ABSCIEX) equipped with a turbo spray® interface, according to Ciminiello method (Ciminiello *et al.*, 2006). A 3 µm C18 Gemini column (150*2.0 mm, Phenomenex) was thermostated at 25°C with a flow rate set at 200 $\mu L \cdot min^{-1}$. Eluent A was 100% water and eluent B was 95% aqueous acetonitrile, both eluents containing 2 mM ammonium formiate and 50 mM formic acid. The gradient was raised from 20 to 100% B in 10 min and was held during 4 min before dropping down to the initial conditions. The instrument control, data processing and analysis were conducted using Analyst software (ABSCIEX).

Mass spectrometry detection was operated in positive mode and optimised from a palytoxin standard solution using Multiple Reaction Monitoring (MRM). The source settings were as follow: curtain gas set at 30 (arbitrary units), ion spray at 5500 V, a turbogas temperature of 450°C, gas 1 and 2 set at 30 and 40 respectively and an entrance potential of 10V. The collision energy was applied at 45 eV for bi-charged ions $[M+2H]^{2+}$, $[M+2H-H_2O]^{2+}$ and at 33 eV for tri-charged ion $[M+3H]^{3+}$ to gave the characteristic product ion at m/z 327. The following transitions: m/z 1340 $[M+2H]^{2+}$+>327 $[M+H\text{-B moiety-}H_2O]^{+}$ [declustering potential (DP) = 26V, cell exit potential (CXP) = 18V] and m/z 1332 $[M+2H-H_2O]^{2+}$+>327 $[M+H\text{-B moiety-}H_2O]^{+}$ (DP = 26V, CXP = 18V) for PLTX, m/z 1324 $[M+2H]^{2+}$ >327 $[M+H\text{-B moiety-}H_2O]^{+}$ (DP = 26V, CXP = 18V) and m/z 1315 $[M+2H-H_2O]^{2+}$ > 327 $[M+H\text{-B moiety-}H_2O]^{+}$ (DP = 26V, CXP = 18V) for OVTX-a, were monitored with a dwell time of 100 ms for each transition.

The quantitative determination of palytoxin and ovatoxin-a was carried out using the calibration of the palytoxin standard (Wako chemicals GmbH (Neuss, Germany) at six concentration levels ranging from 0.05 to 1 µg/mL. Because of the lack of standard for ovatoxin-a, ovatoxin-a was assumed to have the same molar response as palytoxin (Ciminiello *et al.*, 2008).

RESULTS

Molecular analysis and identification

The PCR amplifications of 5.8S and ITS regions produced a single fragment of 341 bp for both cultures. The 5.8S fragment was 160 bp while ITS1 was 95 bp and ITS2 86 bp. The sequences obtained in this study have been deposited in GenBank under the accession numbers FJ905896 and FJ905897. In the phylogenetic tree (Fig. 3), the two sequences cluster with other sequences of *Ostreopsis* cf. *ovata* from various areas of the Mediterranean Sea and Atlantic Ocean, from Greece to Brazil. These sequences are almost identical, but they differ from sequences of strains of *O.* cf. *ovata* collected in the Pacific Ocean which form a sister clade. Within the *Ostreopsis* lineage, *O. labens*, *O. lenticularis* and *O. siamensis* (including *O.* cf. *siamensis*) appear to be the most basal taxa.

Potential photosynthetic activity

The photosynthetic activities of either the benthic cells or the floating clumps of cells for the two strains, from Morgiret and Villefranche-sur-Mer are represented as a function of irradiance on figure 4. The photosynthetic activity of

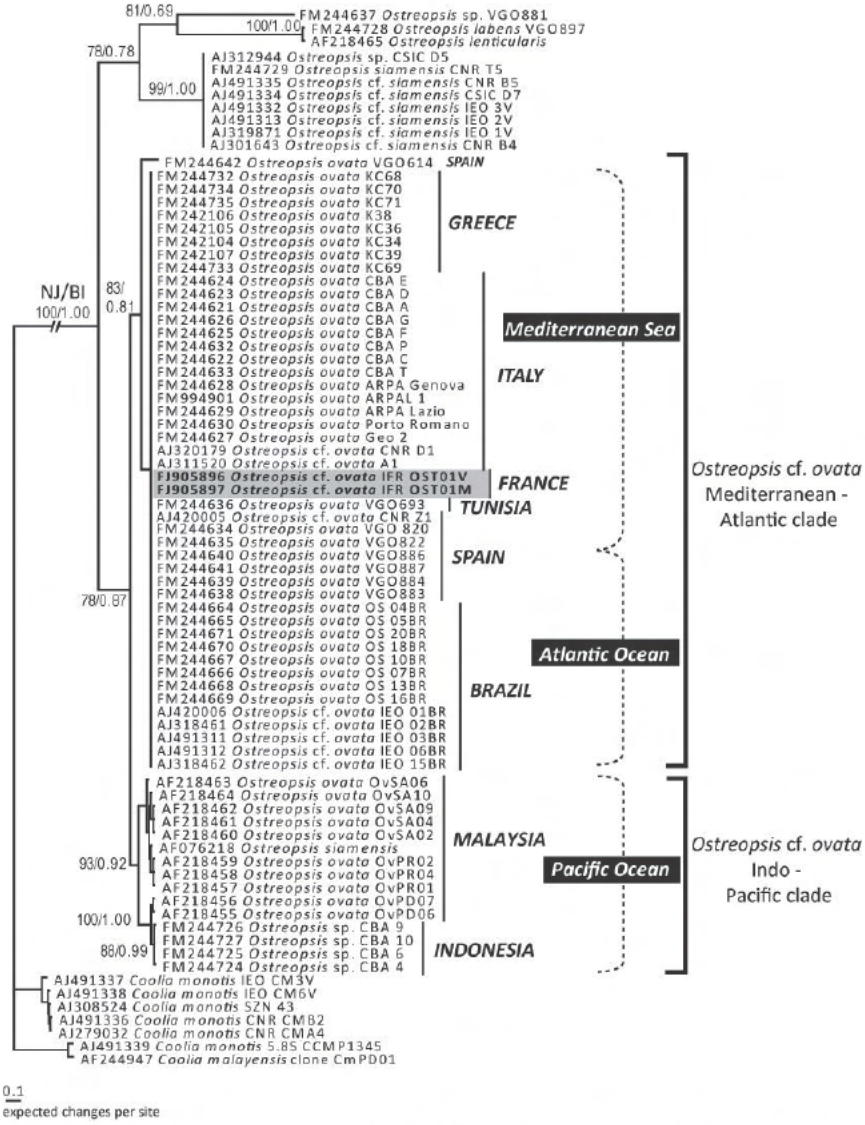

Fig. 3. Phylogenetic tree (NJ tree) of the genus *Ostreopsis* based on the ITS region and 5.8S sequences. Numbers on the nodes represent bootstrap values (NJ) (1000 pseudoreplicates) and posterior probabilities (BI). The trees were rooted using *Coolia* sequences.

the benthic cells (IFR-OST-0.1V) reached a maximum value of 0.284 µmol O_2 cell^{-1} min^{-1} for an irradiance of 300 µmol m^{-2} s^{-1} before light saturation at 400 µmol m^{-2} s^{-1}. The saturation light was not reached at this level for the cells included in mucous-filamentous clumps. The compensation point was measured between 90 and 100 µmol·m^{-2}s^{-1} for benthic cells. These results highlighted the high level of irradiance needed for optimal photosynthetic activity, an irradiance of 460 µmol·m^{-2}s^{-1} was then selected for algal cultures.

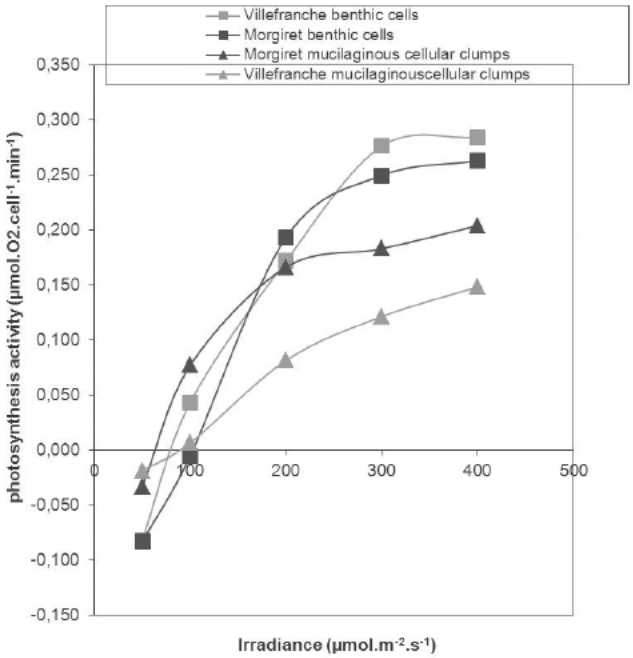

Fig. 4. Photosynthesis activity as a function of the irradiance for Morgiret (IFR-OST-0.1M) and Villefranche-sur-Mer (IFR-OST-0.1V) *Ostreopsis* cf. *ovata* cultures.

Comparison of growth and toxin production in various culture media

Once we established the level of light saturation, we tested the influence of culture media on growth and toxin production. Growth was enhanced using L1 with soil extract (Fig. 5, right) which also promoted the toxin production in the cultures of *Ostreopsis* cf. *ovata* up to a maximum level of 0.8 µg eq. PLTX per mL (Fig. 5, left).

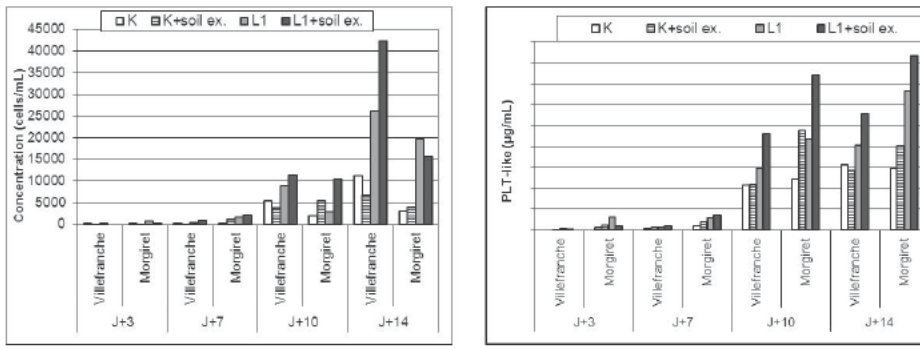

Fig. 5. Comparison of growth (left) and toxin production (right) in cultures of Villefranche-sur-Mer and Morgiret strains in various K or L1 media supplemented or not with soil extract.

Determination of toxin content by LC-MS/MS

The presence of PLTX and OVTX-a was demonstrated in all the analysed samples, not only in the cells grown in cultures independently of their geographic origin, Gulf of Lion, Provence-Liguria, Tyrrhenian or Balearic seas but also in cells from natural plankton nets collected in 2008 (Fig. 6). At the end of the exponential phase, the strain IFR-OST-0.1V from Villefranche-sur-Mer was found to produce 55 pg·cell^{-1} of OVTX-a and 2.5 pg cell^{-1} of PLTX while the strain from Morgiret (IFR-OST-0.1M) produced 50 pg cell^{-1} and 3.7 pg cell^{-1} of OVTX-a and PLTX respectively.

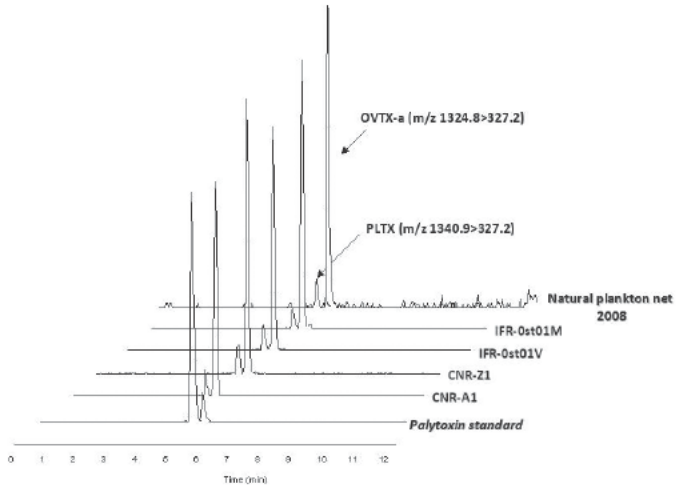

Fig. 6. LC-MS/MS analysis acquired on API 4000Qtrap in positive MRM mode, the selected transitions m/z 1340.9/327.2 and 1324.8/327.2 permit to detect respectively PLTX and OVTX-a in *Ostreopsis* cf. *ovata* samples.

DISCUSSION

In regards to environmental factors, the main insight was the high level of irradiance required for the development of *Ostreopsis* cf. *ovata* which might play a major role in the control of the depth distribution in the environment; the maximum of cells abundance has been found at 0.5 m depth in Villefranche bay (Cohu & Lemée, *comm. pers.*). In the laboratory a temperature of 22°C was the threshold for the onset of filamentous-mucous cell aggregates and the maximum of growth and toxicity was reached for a salinity of 40 psu.

The identification of the two strains isolated from two different localities of South of France confirms that they belong to the same taxon. In addition, the ITS – 5.8S sequence is identical to all the other sequences acquired from cells collected in bordering areas such as Italy, and Spain, but also from all the Mediterranean Sea and Atlantic Ocean as previously shown by Penna *et al.*, (2010). Since no sequence was previously available for strains from the French

Mediterranean coast, this result fills a gap and confirms that this strain of *O.* cf. *ovata* is widespread in a large geographic area.

The high OVTX-a content per cell found in the strain from Villefranche-sur-Mer and Morgiret raises the question of the fate of the toxins in the seafood products and the risk for human health. In fact, intoxications by ingestion have not been reported in France yet, even if PLTX and OVTX-a have already been found (Amzil *et al*., 2009); the highest level of OVTX-a and putative PLTX was found in sea-urchins during the summer 2008 (450 µg eq. PLT/kg of flesh) and significant levels were dosed in artificially immersed mussels in Morgiret (217 µg eq. PLT/kg of flesh), exceeding by far the European Food Safety Authority (EFSA, 2009) recommended threshold value of 30 µg eq. PLTX/kg of flesh.

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