Genetic and toxinological characterization of North Atlantic strains of the dinoflagellate *Ostreopsis* and allelopathic interactions with toxic and non-toxic species from the genera *Prorocentrum, Coolia* and *Gambierdiscus*

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Abstract

The genus *Ostreopsis* includes several toxic species that can develop blooms in benthic ecosystems, with potential harmful consequences for human health and marine invertebrates. Despite of this, little is known about the allelopathic interactions between these organisms and other co-occurring microalgae that exploit similar spatial and nutrient resources in benthic ecosystems. The aim of this study was to follow these interactions in cultures of two *Ostreopsis* ribotypes with different toxin profiles (*O*. cf. *ovata* contained ovatoxins-a, b, c and e, while only ovatoxin-d was found in *O*.sp. "Lanzarote-type"), mixed with species of three benthic dinoflagellate genera (*Coolia*, *Prorocentrum* and *Gambierdiscus*), isolated from the same area (North East Atlantic, Canary Islands). In a first experiment, the potential allelopathic effects on growth rates were followed, in mixed cultures of *Coolia monotis* (a non toxic species) exposed to the clarified medium and to cells of *O*. sp. "Lanzarote-type" and *O*. cf. *ovata*. Growth delayed in *C. monotis* was observed specially in clarified medium, while the *O.* sp. "Lanzarote-type" strain attained much lower densities in mixed cultures. In a second experiment, we examined the potential effects of clarified media from *O.* sp. "Lanzarote-type" and *O*. cf. *ovata* on the adherence capacity in two toxic species (*Prorocentrum hoffmannianum* and *Gambierdiscus excentricus*). Contrasting effects were found: a significant increase of adherence capacity in *P. hoffmannianum vs* attachment decline in *G. excentricus*, that experienced also severe deleterious effects (cell lysis). Our results suggest the existence of weak to moderate allelopathic interactions between the studied organisms, although the outcome is dependent on the species involved.

Keywords: *Ostreopsis; Allelopathy; Ovatoxins; PTXs; Interespecific effects, Inhibitory effects; Adhesion.*

1. Introduction

Harmful algal blooms (HABs) are natural toxic events which are –at times- related with increases of phytoplankton biomass and that represent a potential threat for the public health and/or aquatic ecosystems (Smayda, 1997). Dinoflagellates are one of the most relevant HAB groups, and toxigenic species are sometimes, involved in this phenomenon (Liew et al., 2000).

In the recent decade, benthic and epiphytic dinoflagellates –which inhabit sediments or live epiphytic on macroalgae–, have attracted much attention given the apparent geographical expansion of some toxic genera like *Ostreopsis* and *Gambierdiscus* (Berdalet et al., 2012, Shears and Ross, 2009). Dinoflagellate blooms are correlated with environmental changes in the water column (Pitcher et al., 2010), even so, in the benthos, the processes that shape dinoflagellate populations and facilitate the blooming of certain species are poorly understood (Fraga et al., 2012).

The genus *Ostreopsis* produces palytoxins (PLTXs) and palytoxin-like compounds (Ciminiello et al., 2011), such as ovatoxins (OVTXs), which are implicated in poisonings through ingestion of some clupeoid fishes, known as clupeotoxism (Onuma et al., 1999). In the Mediterranean Sea the blooms of *Ostreopsis* have been associated with skin irritations (Hoffmann, 2008) and irritative processes after inhalation of marine aerosols in the Tuscan and Ligurian coasts in Italy (Ciminiello et al., 2008, Totti et al., 2010), Andalusian (García et al., 2008) and Catalan coast in Spain (Vila et al., 2012). One of the most relevant episodes occurred in 2005, when about 200 people suffered respiratory illnesses provoked by ovatoxins and cells transported through the wind as marine aerosols as recently described by Ciminiello et al. (2014).

Although HABs like those of *Ostreopsis* can be nearly monospecific (Fraga et al., 2012; Nascimento et al., 2012), benthic communities of dinoflagellates are usually constituted by diverse taxa and species in different world areas. For example, the most widespread assemblages in NW Mediterranean Sea are composed of *Ostreopsis* sp., *Coolia monotis*, *Prorocentrum lima*, also in East Malaysia there have been usually reported associations of *Coolia*, *Amphidinium*, *Gambierdiscus*, *Ostreopsis* and *Prorocentrum* spp. (Vila et al., 2001; Tan et al., 2013). The co-occurrence of microalgae is conditioned

by the balance between cell concentration (gains and losses), light and nutrient availability (Hutchinson, 1961). To overcome the low nutrients uptake, different adaptations have been proposed like nutrient retrieval through swimming, mixotrophy and allelopathic strategies (Smayda, 1997; Fraga et al., 2012).

The term "allelopathy" refers to the effect (positive or negative) produced in one population due to biochemicals secreted by a different one (Hulot et al., 2004), and it can be understood as another adaptation (Legrand et al., 2003) that involves the secretion of biochemicals and the action of secondary metabolites (Arzul and Gentien, 2006). Distinct responses and sensitivity to allelochemical substances (Tillman et al., 2008) could be mediated by specific donor/target combinations in each organism.

Toxic effects can be considered as allelopathic effects, but by no means are allelopathic effects restricted to toxins. There exist other molecules likely involved in allelopathy. Some alkaloids are able to inhibit RNA synthesis (Mendes et al., 2013) as well as other marine microorganisms can suppress β-glucosidase activity (Pandey et al., 2013) which is responsible for enzymatic activity. Allelopathy experiments are often carried out to test a hypothesis about interactions between target and donor species. Even though, other communities growing in non-axenic cultures (e.g. bacterial communities) could display feedback mechanisms and influence target/donor species behavior (Weissbach et al., 2010). Previous studies have documented different allelopathic interactions in planktonic dinoflagellates, such as cell death in *Karenia brevis* caused by *Skeletonema costatum* (Prince et al., 2008), growth inhibition in *Heterocapsa circularisquama* by *Karenia mikimotoi* (Uchida et al., 1999), cell lysis, morphological changes and motility loss in *Oxyrrhis marina* by *Alexandrium* spp. (Tillmann et al., 2002), same for *Gymnodinium catenatum* exposed to the raphidophyte *Chattonella marina* (Fernández-Herrera et al., 2016), and protein activity inhibition when using supernatant from *Prorocentrum lima* which contains okadaic acid (Sugg and VanDolah, 1999). Allelopathy could be also implicated in favouring the growth of certain algae (Suikkanen et al., 2011), in mechanisms of predatory behaviour (Park and Kim, 2010), and in the initiation of their blooms by reducing growth of

102 their competitors or affecting behavioural/physiological capabilities (Hakanen et al., 2014). In the case
103 of benthic dinoflagellates, specifically the genus *Ostreopsis*, only a few reports have described weak to
104 moderate allelopathic interactions between *O*. cf. *ovata* and other protists (Monti and Cecchin 2012;
105 Pezzolesi et al., 2011) or macroalgae (Accoroni et al., 2015).

106 The aim of the present work was to study the allelopathic interactions between *Ostreopsis* and several
107 dinoflagellates that commonly co-occur in benthic assemblages, including observations on adherence
108 capacity that have never been previously reported. For this purpose, a molecular and toxinological
109 characterization of several *Ostreopsis* strains from the Canary Islands was performed. Consequently,
110 two *Ostreopsis* ribotypes (*O*. cf. *ovata* and *O*. sp. "Lanzarote-type") with different toxin profiles were
111 selected to determine their effects (either by cell-to-cell contact or clarified medium) on cell viability,
112 growth and adherence capacity, on toxic and non-toxic species of the genera *Coolia* , *Prorocentrum*
113 and *Gambierdiscus* from the same geographical area.

114

115 **2. Material and methods**

116 2.1 *Culture conditions*

117 Experiments were conducted with clonal non-axenic strains (Table 1) from CCVIEO microalgal
118 culture collection of the Instituto Español de Oceanografía (Vigo, Galicia, Spain). Cultures for mixed
119 cells growth and supernatant experiments were grown in 250 ml Erlenmeyer flasks and cultures for
120 adherence experiments were grown on 6-well dish plates. L1 medium (Guillard and Hargraves, 1993)
121 adjusted to a salinity of 32 was used in all treatments with volumes adapted to experimental settings.
122 Flasks and multi-well plates were incubated at 25 ºC, 277 $\mu Em^{-2}s^{-1}$ light intensity under a 12:12 L:D
123 cycle.

124

125 2.2 *Mixed cells growth experiments*

Two *Ostreopsis* ribotypes differentiated by their rRNA gene sequences were used in this study. VGO1001, classified as *O*. cf. *ovata* (Penna et al., 2010) and VGO1000 which belongs to a distant clade (first characterized by Penna et al. (2010) using *Ostreopsis* sp. VGO881), that we label here as *O*. sp. "Lanzarote-type" ribotype following Parsons et al. (2012).

The experimental setup consisted of two culture treatments (150 ml total volume) where toxic (*O*. sp. "Lanzarote-type" VGO1000 and *O*. cf. *ovata*) and non-toxic *C. monotis* VGO858 (LC-FLD analysis did not shown the presence of YTXs; Fraga et al., 2008) species were seeded as follows: *C. monotis* was seeded at an initial concentration of 1,000 cells ml^{-1} together with 500 cells $ml^{-1}$of *O*. sp. "Lanzarote-type" (VGO1000). Same conditions were used with *O*. cf. *ovata*. Cell enumeration was carried out every two days throughout 14 days on Lugol's Acid Solution-fixed samples in a Sedgewick Rafter (S50) Chamber. The first sample was taken the same day of inoculation. pH range measurements were made each sampling day (pH meter Crison Basic 20, electrode Crison 52-02). Live observations were also made in each sampling day by means of a Zeiss Invertoscop D Microscope (Carl Zeiss AG, Germany). Control cultures were used to calculate growth rates and to establish comparisons with cultures exposed to the different treatments. For this purpose, nine 150 ml cultures were used as triplicate controls of *Ostreopsis* (VGO1000, VGO1001) and *C. monotis*. Cells of *C. monotis* were seeded at a concentration of 1,000 cells ml^{-1} with L1 medium. *O*. sp. "Lanzarote-type" (VGO1000) and *O*. cf. *ovata* were seeded with 500 cells ml^{-1} in L1 medium. A one-way analysis of variance (ANOVA) was carried out to evaluate the statistical significance of the cell densities reached in controls and treatments. Growth rates were expressed as instantaneous rate of increase (*r*; Gotelli, 1995), as given by (Wood et al., 2005):

$$r = (\ln (N_t) - \ln (N_0)) / \Delta t$$

Where N_t is the number of cells at the end of the time interval, N_0 is the number of cells at the beginning of the time interval and Δt is the length of the time interval ($t_t - t_0$). Units for *r* are given per unit time (t^{-1}).

152 2.3 *Ostreopsis supernatant experiments*

153 Exponentially growing cultures of *O*. sp. "Lanzarote-type" (VGO1000) and *O*. cf. *ovata* were diluted
154 to reach a concentration of 500 cells ml^{-1} and filtrated through glass microfiber filters with a diameter
155 of 47 mm size (MVF-5, Cde-Parmer Instrument, Filter-Lab, U.S.A.). This medium without cells
156 (hereinafter, clarified medium or supernatant) was distributed in six Erlenmeyer flasks, where *C.*
157 *monotis* was seeded at concentrations of 1,000 cells ml^{-1} (total volume of 150 ml). *Ostreopsis*
158 supernatants were added in proportions equivalent to 500 cells ml^{-1} for each *Ostreopsis* strain (23 ml
159 for VGO1000 and 22 ml for *O*. cf. *ovata*). Instantaneous rates of increase (*r*) and ANOVA were
160 calculated as described above. Cultures were monitored for 14 days to follow growth rates and
161 behavioural changes relative to control treatments and pH range measurements were made each
162 sampling day. The mixed growth experiments followed this same experimental regime.

163

164 2.4 *Adherence capacity experiments*

165 With the purpose of studying the allelopathic effects of clarified medium from *Ostreopsis* on
166 adherence capacity of other co-occurring benthic species, two toxic strains of *Prorocentrum*
167 *hoffmannianum* (VGO1031; OA producer, P. Riobó, *pers. comm*.) and *Gambierdiscus excentricus*
168 (VGO 791, MTX and CTX producer according to Neuro-2a CBA analysis; Fraga et al., 2011), were
169 used. These strains were seeded in triplicate for controls and treatments (8 ml total volume). Controls
170 of *P. hoffmannianum* and *G. excentricus* were seeded at 1,000 and 500 cells ml^{-1}respectively and filled
171 up to 8 ml with L1 medium. Experimental treatments included 1,000 cells ml^{-1} of *Prorocentrum,* 500
172 cell ml^{-1} of *Gambierdiscus* and clarified medium of *O*. sp. "Lanzarote-type" (VGO999) and *O*. cf.
173 *ovata,* inoculated in concentrations equivalent for1:2 and1:4 ratios for *Prorocentrum:Ostreopsis* and
174 *Gambierdiscus:Ostreopsis* (8 ml total volume). Controls and treatments were prepared same day
175 corresponding to *Ostreopsis* exponential growth phase and pH measurements were made each
176 sampling day (pH meter Crison Basic 20,electrode Hamilton MiniTrode). The ratios of *Prorocentrum*,
177 *Ostreopsis* filtrate, and L1 fresh medium added were 1:2:5 and 1:4:3 for 1:2 and 1:4 treatments

178 respectively, for both *Ostreopsis* strains. L1 medium was adjusted in *Gambierdiscus* treatments and
179 controls due to lower growth rates achieved in lab cultures for this species. In this case, ratios were
180 4:2:3 and 4:4:1 for 1:2 and 1:4 treatments, respectively.

181 Cell enumeration was carried out on the days 3 and 6 in triplicate, by gently picking a coverslip placed
182 into the bottom of a well using a steel lab tweezer and soaking it three times in a 100 ml beaker with
183 90 ml of fresh L1 medium to remove the unattached or loosely attached cells and counting the attached
184 cells with a 10X eyepiece reticle with a10mm grid (100 x 100) coupled with an inverted microscope
185 (Nikon Eclipse, TE2000-S). The adherence capacity was estimated by comparing the number of cells
186 stuck to the coverslip among control and treatment wells. An analysis of variance (ANOVA) was
187 carried out at the end of the experiments to evaluate the statistical significance of results.

188

189 2.5 *Nutrient analysis*

190 Triplicate samples of 10 ml from mixed cells growth and *Ostreopsis* supernatant experiments (sections
191 2.2 and 2.3, respectively) were collected for nutrient analysis on days 0, 6 and 14. With the same
192 purpose, triplicate samples of 8 ml from wells used for adherence experiments were collected on days
193 0, 3 and 6. Nutrient contents were determined by segmented flow analysis with Futura-Alliance
194 autoanalyzers following Hansen and Grassoff (1983). The analytical error for nitrate was ± 0.06 μM, ±
195 0.01 μM for nitrite and ± 0.005 μM for phosphate.

196

197 2.6 *Toxins extraction*

198 Sample aliquots (1.5 ml) from each culture were fixed with Lugol's and cell counts were made using a
199 Sedgewick Rafter Counting Slide. Cell pellets from total culture volumes of 260 ml, 430 ml and 460
200 ml, containing $1.21x10^6$ cells, $1.23x10^6$ cells and $1.74x10^6$ cells (*O*. cf. *ovata*, *O*. sp. "Lanzarote-type"
201 (VGO1000 and VGO999), respectively) were collected during exponential phase by filtration through
202 glass microfiber filters with a diameter of 47 mm size (MVF-5, Cde-Parmer Instrument, Filter-Lab,

U.S.A.). The filters with cells were re-suspended in MeOH and sonicated to homogenize the suspension using Ultrasonic Homogenizer (Cole-Palmer, Chicago, IL. USA) sonication probe 4710. Next, samples were centrifugated at 4,620 g for 20 min at10ºC. The supernatant was removed and the pellet was extracted again with MeOH, following the same procedure. Both supernatants were combined and the total volume was adjusted to 3 ml. Extracts were stored at -20º until their analysis by LC-HRMS, at which time they were tempered and filtered through 0.45 µm syringe filters.

2.7 *Toxins analyses: LC-HRMS.*

Mass spectrometry analyses (Ciminiello et al., 2010) were performed using a Thermo Scientific Dionex LC coupled to an Exactive mass spectrometer, equipped with an Orbitrap mass analyzer. The instrument was mass calibrated for positive and negative modes, and the capillary and tube lens voltages were also optimized, using the automated script within the Extractive acquisition. HRMS experiments were carried out without and with all ion fragmentation (AIF) (HCD 35 eV). The mass range for both, full MS and AIF mode, ranged from 200 to 3,000 *m/z* at a resolving power of 140,000 and 70,000 respectively.

The following conditions were used (Garcia-Altares et al., 2014): a column of 2.7 µm XBRIDGE BEH-C18, 50×2.10 mm (Waters), was kept at 20 ºC and eluted at 0.2 ml min^{-1} with water (eluent A) and 95 % acetonitrile/water (eluent B), both containing 30 mM acetic acid. The following gradient elution was used: 28 % B at t=0, 29 % B at t=10, 30 % B at t=20, 100 % B at t=21, and hold for 10 min; reequilibration time was 13 min. For toxins identification an analytical standard of Palytoxin from *Palythoa tuberculosa* containing 5 ng μL^{-1} was purchased from Wako chemicals GmbH (Neuss, Germany).

2.8 *DNA extraction, PCR amplification and sequencing*

Samples for DNA extraction were processed from *Ostreopsis* strains listed in Table 1 as follows: 1.5 ml from each culture were picked up into Eppendorf-tubes (1.5 ml) and centrifuged (Centrifuge 5424 R, Eppendorf AG, New York, USA) at 15,871 g for 2 min, washed with sterile MQ water and centrifuged again (15,871 g, 2 min). The resulting pellets were resuspended in 100 µL of 10% Chelex 100 (BioRad, Hercules, CA, USA), centrifuged (15,871 g, 2 min), boiled at 95ºC in an Eppendorf Mastercycler EP5345 thermocycler (Eppendorf AG, New York, USA) for 10 min, then vortexed. The boiling and vortex steps were done twice and samples were centrifuged (15,871 g, 2 min) three times. The supernatants were transferred to clean 200 µL tubes. Genomic DNA was quantified and checked for its purity in a Nanodrop Lite spectrophotometer (ThermoScientific, Waltham, MA, USA). In some cases (*Ostreopsis* VGO999, VGO1000, VGO1001 and VGO1187), single cells were picked up manually for direct amplification with the aid of a capillary glass micropipette under a Zeiss Invertoscop D Microscope (Carl Zeiss AG, Germany), rinsed into three drops of sterile distilled water over a microscope drilled slide and transferred to 200 µL tubes.

The D1-D2 regions of the LSU rRNA gene were amplified from culture extracts and single cells using the pairs of primers D1R/D2C (5'-ACCCGCTGAATTTAAGCATA-3'/5'-ACGAACGATTTGCACGTCAG-3') (Lenaers et al., 1989). The amplification reaction mixtures (25 µL) contained 2.5 µL reaction buffer, 2 mM $MgCl_2$, 0.25 pmol of each primer, 2 mM of dNTPs, 0.65 units Taq DNA polymerase (Qiagen, CA, USA), 2 µL of DNA (or the isolated cells, as described before). The DNA was amplified in an Eppendorf Mastercycler EP5345 (Eppendorf AG, New York, USA) under the following conditions: initial denaturation at 95 ºC for 1 min, followed by 40 cycles of denaturation at 54 ºC for 1 min, annealing at 72 ºC for 3 min, extension at 72 ºC for 3 min, and a final extension cycle at 72 ºC for 10 min. A 10 µL aliquot of each PCR reaction was checked by agarose gel electrophoresis (1% TAE, 50 V) and SYBR Safe DNA gel staining (Invitrogen, CA, USA). The PCR products were purified with ExoSAP–IT (USB Corp., Cleveland, OH, USA), sequenced using Big Dye Terminator v3.1 (Applied Biosystems, Foster City, CA, USA) and migrated in an AB 3130 Sequencer (Applied Biosystems) at the CACTI Sequencing Facilities (Universidad de Vigo, Spain).

253

254 2.9 *Phylogenetic analyses*

255 The sequences of the studied *Ostreopsis* strains were compared with 37 other *O.* spp. strains obtained
256 from Genbank. *Coolia monotis* (AM902747) was used as outgroup to root the tree. LSU sequences
257 were aligned using BioEdit v.7.2.5. The final alignment for the LSU phylogeny included 544 sites
258 from 52 sequences. Phylogenetic model selection was performed on MEGA 6. The model that best fits
259 the tree was Tamura 3 Parameter with the following likelihood settings: base frequencies of *A* and
260 *T*= 0.3029 and for *C* and *G* = 0.1971, 1.3965 as estimated value of transition/transversion and gamma
261 distribution shape (*G* = 0.89). Bootstrap ML analysis was performed with 1,000 pseudo-replicates and
262 the consensus tree was computed using a freely available French bioinformatic platform
263 (http://atgc.lirmm.fr/phyml/) using a Maximum Likelihood (ML) method. Additionally, a phylogenetic
264 reconstruction was carried out in MEGA 6 using a Neighbor-joining (NJ) method. The reconstructed
265 topologies were very similar with both methods and the phylogenetic tree was represented using the
266 ML results, with bootstrap values from ML and NJ method (n=1000). Net average distance between
267 different groups of taxa was calculated on MEGA 6 according to the following formula:

268 $d_A = d_{XY} - ((d_X + d_Y)/2)$

269 Where d_{XY} is the average distance groups X and Y, and d_X and d_Y are the mean within-groups
270 distances.

271

272 **3. Results**

273 3.1 *Phylogenetic analysis*

274 Sequences of LSU rDNA (D1-D2) from 14 *Ostreopsis* strains isolated in the Canary Islands (listed in
275 Table 1) and those of 37 *Ostreopsis* strains obtained from GenBank database were used to build the
276 ML phylogenetic tree in Figure 1. The LSU-based phylogeny showed two groups of *O.* cf. *ovata* (the
277 Mediterranean-Atlantic-Pacific and Indo-Pacific subclades), as previously described in the literature

(Penna et al., 2010; Tawong et al., 2014). Topologies from ML and NJ methods were very similar, there were slight differences just regarding the branching pattern of *O.* sp. 1 and *O.* sp. 2 from Japanese waters. The latter two species were split by NJ in different groups with low bootstrap support (<50), while ML placed *O.* sp. 2 and two sequences from Russian waters in the same branch. Net average distances between *Ostreopsis* cf. *ovata vs Ostreopsis* sp. 1 and *O*. sp. 2 were very similar (Table 2).

The LSU phylogenetic tree revealed a clade of *Ostreopsis* clearly differentiated from *O*. cf. *ovata* Mediterranean-Atlantic-Pacific (d_A=1.044) and Indo-Pacific (d_A=1.069) subclades (Table 2). This clade included six strains from the Canary Islands sequenced in our study, termed previously (Parsons et al., 2012) as *Ostreopsis* sp. "Lanzarote-type". Among the rest of *Ostreopsis* sequences obtained in the present study, eight strains from the Canary Islands were identified as *O.* cf. *ovata*, and one strain from the Southern Iberian Peninsula (VGO1187) belonged to the *O.* cf. *siamensis* clade.

3.2 *Toxins profile*

Toxin profiles of the three *Ostreopsis* strains shown different patterns. The gradient system allowed a chromatographic separation of the major components of the extracts (Fig. 2). Even though all *Ostreopsis* analysed belong to the same location -Canary Islands- relevant differences were found between species. Ovatoxin-a (OVTX-a) was by far the major component of *O.* cf. *ovata* (VGO1001) toxins profile representing 54% of the total toxins content, followed by ovatoxin-b (OVTX-b) with 28.6%, ovatoxin-c (OVTX-c) with 11.3% and ovatoxin-e (OVTX-e) with a 6.1%. On the other hand, only ovatoxin-d (OVTX-d) was found in the other two *Ostreopsis* strains (VGO1000 and VGO999), designated as *Ostreopsis* sp. *"*Lanzarote-type*"*. The amount of OVTX-d for VGO1000 was 9.85 pg PLTX equivalents $cell^{-1}$ and 8.55 pg PLTX equivalents $cell^{-1}$ for VGO999. HR full MS spectra (Fig. 3) were acquired in the range *m/z* 890-920, *m/z* 1,330-1,380 and *m/z* 2,590-2,710 for OVTX-a, *m/z* 890-920, *m/z* 1330-1380 and *m/z* 2,600-2,710for OVTX-b, -c, -e contained in *O*. cf. *ovata* (VGO1001) culture extract. Moreover, *m/z* 890-920, *m/z* 1,330-1,380 and *m/z* 2,600-2,710 ranges were also

observed for OVTX-d contained in both *O*. sp. "Lanzarote-type" (VGO1000 and VGO999) culture extracts. In the former range (*m/z* 890-920) ovatoxins presented characteristic tri-charged ion peaks due to $[M+2H+K]^{3+}$ and $[M+2H+Na]^{3+}$ in addition to bi-charged ion peaks presented in the range *m/z* 1,330-1,380 from $[M+2H+K]^{2+}$ and $[M+2H+Na]^{2+}$. In the ultimate range (*m/z* 2,590-2,690) mono-charged ion peaks are represented by $[M+H]^+$, $[M+H-H_2O]^+$, $[M+H-2H_2O]^+$ and $[M+H-3H_2O]^+$ in OVTX-a and only $[M+H]^+$, $[M+H-H_2O]^+$ were identified in the range of *m/z* 2,600-2,710 for the remaining OVTXs. Water losses represented by $[M+H]^{2+}$ and $[M+3H]^{3+}$ were also present in these three ranges as described in Ciminiello et al. (2010). Characteristic fragment ions were revealed by HRMS at *m/z* 327 for OVTX-a, -d, *m/z* 371 for OVTX-b, -c and *m/z*343 for OVTX-e. HRMS data of palytoxin (PLTX) and ovatoxin-a, -b, -c and –d obtained from full MS spectra are presented in Table 3.

3.3 *Mixed cells cultures*

In this experiment, *Ostreopsis* sp. "Lanzarote-type" (VGO1000) grown with *C. monotis* cells (Fig. 4A) exhibited a short exponential growth phase, significantly lower maximum densities than in controls ($F(89.23) > 7.71$; $p < 0.05$), and entered into stationary phase two days earlier (Fig. 5A). In turn, *O*. cf. *ovata* followed the same exponential growth than control treatments, but exhibited lower densities in stationary phase and a sharp decline towards the end of the experiment (Fig. 5B). Maximum growth rates in both *Ostreopsis* species were similar in controls and mixed cultures, while in *C. monotis* maximum rates were higher when coexisting with *Ostreopsis* sp. "Lanzarote-type" (VGO1000) (r = 0.52 d^{-1}) than in controls (r = 0.34 d^{-1}). Even though, a growth decline was observed in *C. monotis* cells until day 6 in both *Ostreopsis* cell treatments (Fig. 5C). Such effect was stronger with *O*. cf. *ovata* and lasted until day 10. Evolution of pH range in these experiments varied from 8 to 8.8, these maximum values being observed in bi-algal cultures of *O.* sp. "Lanzarote-type" (VGO1000). Nitrates and phosphates were not limiting at the end of the experiment, the lowest values (513.8±40.3 µM and

329 6.2±1.4 µM for nitrates and phosphates respectively; nearly 50% and 10% of initial nutrient
330 concentrations) being registered in *O.* cf. *ovata* and *C. monotis* bi-algal cultures.

331

332 3.4 *Ostreopsis supernatant cultures*

333 The addition of cell-free filtrate from *Ostreopsis* sp. "Lanzarote-type" (VGO1000) strain did not affect
334 the maximum cell densities reached by *Coolia monotis* (Fig. 5D). This is not the case of *O.* cf. *ovata*
335 filtrate treatment in which *C. monotis* highest cell numbers (~$15.5x10^3$ cells ml^{-1}) were significantly
336 lower ($F(15.48) > 7.71$; $p < 0.05$) than in the controls (~$22.8x10^3$ cells ml^{-1}). No morphological
337 alterations or deleterious effects were observed in *Coolia* (Fig. 4B) but a delay of *C. monotis* growth
338 was observed during the first eight days. Growth rates of *C. monotis* were slightly higher in controls (r
339 = 0.34 d^{-1}) than in supernatant treatments (r = 0.22 d^{-1} in *O.* sp. "Lanzarote-type" (VGO1000) and r =
340 0.27 d^{-1} in *O.* cf. *ovata*). Moreover, we noticed reduced motility in *C. monotis* exposed to *O.* sp.
341 "Lanzarote-type" (VGO1000) supernatant in the short-term, 4 days, but not at the end of the
342 experiment. The treatment with *O.* cf. *ovata* did not exhibit this effect. A series of short videos was
343 recorded to illustrate this observation comparing *C. monotis* controls (Videos 1A, 1B) and *Ostreopsis*
344 sp. "Lanzarote-type" (VGO1000) filtrate treatments (Videos 2A, 2B, 2C, 2D) on day 4. Maximum pH
345 values in supernatant experiments were slightly lower (8.6) than in controls (8.7). Nitrates and
346 phosphates were not limiting at the end of the experiments, and the lowest values (617±72 µM and
347 7.9±1.6 µM for nitrates and phosphates respectively; nearly 50% and 12% of initial nutrient
348 concentrations) were recorded in *O.* cf. *ovata* supernatant treatment.

349

350 3.5 *Adherence capacity experiments*

351 A short-term experiment (6 days, sampling days 3 and 6) to examine the effect of adding *Ostreopsis*
352 supernatant over adherence capacity was assayed using two species: *Gambierdiscus excentricus* (Fig.
353 6A) and *Prorocentrum hoffmannianum* (Fig. 6B). Deleterious effects on *G. excentricus* cells treated

with *Ostreopsis* filtrate were observed, including lysis and shedding of the theca. These effects were apparent through inverted microscope on day 6 and stronger with *O*. sp "Lanzarote-type" (VGO999) (Fig. 6C) and *O*. cf. *ovata* (Fig. 6D) treatments at 1:4 ratios. Concomitantly, *G. excentricus* cells exhibited a significant decrease in the number of attached cells ($F(26.70) > 7.71$; $p < 0.05$) after exposure to *O*. sp. "Lanzarote-type" (VGO999) supernatant at 1:4 ratio on day 3 (Fig. 7A). Even though, *O*. cf. *ovata* treatments at both ratios yielded significant lower numbers of attached cells on day 6 ($F(21.48; 25.86) > 7.71$; $p < 0.05$, for ratios 1:2 and 1:4 respectively).

In the case of *P. hoffmannianum* (Figs. 7B), higher numbers of attached cells were found in *Ostreopsis* cell-free filtrate compared with controls. ANOVA results showed that 3 days after inoculation the number of attached cells were significantly higher in *O*. sp. "Lanzarote-type" (VGO999) treatments ($F(134.96; 35.98) > 7.71$; $p < 0.05$, for concentrations 1:2 and 1:4 respectively). All treatments showed significant differences in cell numbers according to ANOVA after six days. Largest differences were found in *O*. cf. *ovata* treatments at 1:4 ratio ($F(164.85 >> 7.71$; $p << 0.05$).

Maximum pH values in cultures was always below 8.2. Nutrients analysis indicated that nitrate and phosphate were not limiting. In *G. excentricus* the lowest values for nitrate (738±25 µM; nearly 72% of initial nutrient concentrations) where observed in controls whereas the final lowest phosphate concentration (9.6±4.7 µM; nearly 30% of initial nutrient concentrations) was found with *O*. cf. *ovata* treatment at 1:4 ratio. In *P. hoffmannianum* lowest values for both nitrate and phosphate (538±43 µM and 4.3±0.1 µM; nearly 81% and 20% of initial nutrient concentrations of nitrate and phosphate, respectively) were observed in *O*. sp. "Lanzarote-type" (VGO999) treatment at 1:4 ratio.

4. Discussion

4.1 *Molecular analysis and toxin profiles of the studied Ostreopsis strains*

In the last few years, several phylogenetic studies in *Ostreopsis* have become available at global and regional scales (e.g. Penna et al., 2010; Sato et al., 2011; Tawong et al., 2014). These surveys, based

379 on LSU and ITS analyses, yield an increasingly complex picture. This includes several
380 biogeographical groups in *Ostreopsis* cf. *ovata*, together with *O*. cf. *siamensis* and putative *O.*
381 *lenticularis/O. labens* groups, and an increasing number of molecular clades (and subclades), that may
382 represent cryptic species labeled, at present, as *Ostreopsis* sp. 1-7 (e.g. Tawong et al., 2014).
383 Unfortunately, genetic results were not available in the original descriptions of *Ostreopsis* species and
384 this leads to an increasing gap between the number of genetic clades and the accepted taxonomic
385 entities. The LSU sequence from Canary Islands (VGO881, Acc. No. FM994895) was included in one
386 of these genetic surveys (Penna et al., 2010), that emerged as a sister clade to *O*. cf. *siamensis* from the
387 Mediterranean Sea. This sequence was designated as *Ostreopsis* sp. "Lanzarote-type" (Parsons et al.,
388 2012), and only two other strains belonging to this molecular group have been found elsewhere in
389 Eastern Mediterranean Sea (Fraga, Aligizaki and Penna, *pers. comm.*; Parsons et al. 2012). In this
390 study, we reported the partial LSU rDNA (D1-D2 domains) analyses of six additional *Ostreopsis* sp.
391 strains –VGO999, VGO1000, VGO1014, VGO1015, VGO1016 and VGO1150- that matched the
392 VGO881 sequence and built up a robust *Ostreopsis* sp. "Lanzarote-type" clade. All these strains
393 originated from the Canary Islands and LSU analyses indicated they are closer to *O*. sp. 5 and 6 from
394 Asiatic areas (Japan and Thailand; Sato et al., 2011; Tawong et al., 2014). Genetic distance results
395 suggest that *Ostreopsis* sp. "Lanzarote-type" ribotype would likely represent a new species. Even
396 though, the similar morphological traits among *Ostreopsis* species and the lack of molecular
397 information on individuals from the type localities (excepting *O. lenticularis* and *O. labens*; Parsons et
398 al., 2012), not allow the discarding of the possibility that *O*. sp. "Lanzarote-type" could belong to some
399 formerly described species.

400 Regarding toxin profiles, it must be stressed that their variability in *Ostreopsis*, which could be related
401 to methodological issues and/or true intraspecific differences between *Ostreopsis* strains (Ciminiello et
402 al., 2013). Moreover, it has also been proposed that different sampling periods could also explain
403 variable intraspecific toxin profiles (Honsell et al., 2013), besides the fact that toxin amount is linked
404 with the evolution of cell numbers and toxin contents in distinct growth phases of *Ostreopsis*

405 (Vidyarathna et al., 2012). In the case of the *O.* cf. *ovata* clade VGO1001 its toxin profile was
406 quantitatively dominated by ovatoxin-a, ovatoxin-b and ovatoxin-c, in agreement with previous
407 references (Table 4), such as three *O.* cf. *ovata* strains, two from the Adriatic Sea (Ciminiello et al.,
408 2012; Pezzolesi et al., 2014), and one from NW Mediterranean Sea (Brissard et al., 2014). In the light
409 of these results, it is noteworthy that the two *Ostreopsis* sp. "Lanzarote-type" analyzed in this study
410 displayed an identical toxin composition, different to other profiles reported in the literature (Table 4).
411 Such a characteristic toxin profile would help to support the fact that they actually represent a distinct
412 species. Nevertheless, its formal characterization as a new species was not the objective of the present
413 study and additional data, including morphological and toxinological data, should be accumulated and
414 compared against currently accepted species to describe *O.* sp. "Lanzarote-type" as a new species.

415

416 4.2 *Allelopathy experiments with Ostreopsis: effects of mixed cultures and cell-free filtrate on growth*
417 *rates.*

418 Our principal aim was to describe the allelopathic effects between *Ostreopsis* cells or their cell-free
419 filtrate and other benthic dinoflagellate genera that commonly share the same habitat. The hypothesis
420 was that *Ostreopsis* could display some inhibitory effects on its competitors that provide an adaptative
421 advantage to this bloom-forming toxic genus. Allelopathic studies on benthic dinoflagellates are
422 limited (e.g. Wang and Tang, 2008; Cai et al., 2014). Most of the available research deals with
423 planktonic organisms and indicate a variety of deleterious effects (mortality, reduced growth and/or
424 swimming speed, etc), associated with direct cellular contact or mediated by dissolved toxins in cell-
425 free filtrates. For example *Prymnesium parvum* and *Karlodinium* spp. are known to release toxins in
426 cell-to-cell contact while toxin-assisted micropredation is taking place (Berge et al., 2012; Place et al.,
427 2012; Remmel et al., 2012), and *Alexandrium ostenfeldii* filtrate triggers short-term species-specific
428 effects (lysis, shedding of theca and encystment) on other dinoflagellates (Hakanen et al., 2014).

429 In the case of *Ostreopsis* several recent studies addressed the allelopathic interactions (of cells and
430 cell-free medium) including invertebrates, protists and macroalgae.

In the case of invertebrates (Giussanni et al., 2015; Privitera et al., 2012), toxic effects have been described on sea urchins and crustaceans associated with intact *Ostreopsis* cells, but not with cell-free filtrate (Giussanni et al., 2015, and references therein). Also, the implication of mucilaginous matrix devoid of *Ostreopsis* cells in toxic effects on *Artemia salina* was recently demonstrated (Giussanni et al., 2015), together with the presence of palytoxins in the mucous matrix. These authors also found that cell-free filtrate from *Ostreopsis* cf. *ovata* did not contain any toxins, and that sonicated cultures had much lower toxic effects than intact cells. Regarding allelopathic effects on *Ostreopsis*, Accoroni et al. (2015) observed negative effects on the growth of *Ostreopsis* cf. *ovata* by three macroalgae. Despite complete algicidal effects were not detected, most *O*. cf. *ovata* cells exposed to powder of macroalgae were stressed and encysted. In the case of our mixed cell experiments we did not observe morphological changes or encystment in *Ostreopsis* exposed to *C. monotis*. Rather, mixed cultures of two *Ostreopsis* ribotypes with different toxin profile did not show up strong allelopathic effects on growth rates of *Coolia monotis* and do not support the idea that OVTXs produced by *Ostreopsis* sp. can be held liable for growth inhibition. In fact, slightly stronger effects namely growth delay were observed in clarified medium of *Ostreopsis* which would not contain toxins as previously stated (Giussanni et al., 2015).

Monti and Cecchin (2012) also found weak reductions in the growth of *C. monotis* and *Prorocentrum minimum* exposed to *Ostreopsis* cells, but not at all on the diatom *Coscinodiscus granii*. In the same study, cell-free filtrate effects were screened but these did not induce any observable allelopathic effect. Our observation of reduced growth rates in Ostreopsis in mixed cultures with *C. monotis* was also reported by Pezzolesi et al. (2011). From our data, it was concluded that strongest negative effects on growth rates were observed in *O*. sp "Lanzarote-type" by cell-to-cell contact with *C. monotis*, whereas neutral effects on growth occurred in *O*. cf. *ovata*. In this sense, nutrient analyses in our study showed that nitrates and phosphates were still abundant by the end of every experiment, ruling out that differential reduction in growth rates were due to nutrient depletion. It is known that an increase in pH to ~9 could affect the growth of phototrophs (Pedersen and Hansen, 2003). Although pH tolerance

457 could be variable among the studied species, culture pH values in our experiments were always below
458 <9 and both nutrients and pH were similar in bi-algal cultures and monospecific controls.

459 Allelopathic potency can be variable among *Ostreopsis* strains (Hattenrath-Lehmann et al., 2011), and
460 in that sense, clarified medium of both *Ostreopsis* affected *C. monotis* growth in the first days, with a
461 stronger reduction by *O*. cf. *ovata* supernatant than *O*. sp. "Lanzarote-type" (VGO1000). Reduced
462 motility in *Coolia monotis* was observed only with *O*. sp. "Lanzarote-type" (VGO1000) cell-free
463 filtrate, and similar effects have been also reported in other interactions between dinoflagellates such
464 as *Fragilidium duplocampanaeforme* and some *Dinophysis* spp. (e.g. Park and Kim, 2010; Rodríguez
465 et al., 2014).

466

467 4.3 *Adherence capacity*

468 Attachment of benthic algae to the substrate is an ecological adaptation to survive turbulence reducing
469 the likelihood of being dispersed by waves or desiccation at low tide, as well as to colonize living and
470 inert substrates (Smayda 2002; Fraga et al., 2012; Hoppenrath et al., 2014). Given that their attachment
471 to the substrate is not rigid the alternative term of "metaphyton" has been also proposed to describe the
472 unicellular free-living algae that can be found amongst the microscopic algae and macrophytes
473 (Round, 1984; Carnicer et al., 2015). Benthic dinoflagellates attach to sediments or macroalgae by
474 different mechanisms, using stalks, flagella or mucus like some *Prorocentrum*, *Coolia* and *Ostreopsis*
475 species (Hoppenrath et al., 2014). For these reasons, the development of strategies to counteract the
476 adhesion/colonization of other organisms could be a competitive advantage for some benthic
477 microalgae. Most available studies on this issue are related with anti-biofouling methods associated
478 with bacteria, e.g. the release of extracellular products acting as chemosensory inhibitory signals
479 (Silva-Aciares and Riquelme, 2008), and repressive effects on diatoms adhesion capacity from fatty
480 acids in bacterial crude extracts (Gao et al., 2014). Similar studies on micro- and macroalgal induced
481 effects on adhesion have been rarely explored. To our knowledge, only Allen and Leflaive (2014)

482 reported inhibition of adhesion of a freshwater diatom (*Nitzschia palea*) after exposure to cell-free
483 filtrate from the filamentous green algae *Uronema confervicul*um.

484 Our work revealed that cell-free filtrate of *Ostreopsis* caused opposite effects among the tested species:
485 it favoured cell attachment in *Prorocentrum hoffmannianum* with a positive dose-dependent
486 relationship, whilst the reverse was true in *G. excentricus*. Even though, in *Gambierdiscus* is not
487 possible to determine if adhesion capacity was negatively affected because deleterious effects (cell
488 lysis) were observed at the same time. The results in *P. hoffmannianum* were unexpected and
489 contradict the simple idea of an extracellular release of allelochemicals exerting negative effects on
490 adhesion to eliminate competitors of a bloom-forming organism like *Ostreopsis*. Notwithstanding,
491 given that this nature of allelopathic interaction has never been reported in benthic dinoflagellates we
492 believe that the extent and intensity of these changes in adhesion capacity should be explored in the
493 future.

494 Given that activity associated with alleged allelochemicals persists in cell-free filtrates but in some
495 cases seems degraded after a certain length of time, length of these effects needs to be monitored over
496 time and eventually new filtrate additions could be necessary (Wang et al., 2006; Fistarol et al., 2003).
497 Finally, physical variables like turbulence could significantly change the final results of adherence
498 experiments in the laboratory, and in the natural environment this factor is expected to induce a faster
499 washing out of the allelochemicals as well as resuspension and lower attachment capacities of the
500 organisms (Totti et al., 2010).

501

502 **5. Conclusions**

503 Competition with other benthic/metaphytic species plays an important role in determining the
504 formation of harmful algal blooms (Carnicer et al., 2015). In this sense, allelopathic interactions have
505 been only explored in recent years in benthic dinoflagellates, and weak to moderate effects between
506 the bloom-forming genus *Ostreopsis* and other organisms have been documented. In the present work
507 we reported diverse allelopathic interactions through cell-to-cell contact or exposure to *Ostreopsis* cell-

508 free extracts in benthic dinoflagellates. Short-time effects (reduced motility, growth delay) to – for the
509 first time- severe deleterious effects (cell lysis) and adhesion capacity changes were observed. In
510 addition, some negative effects involved *Ostreopsis*, i.e. lower growth in *Ostreopsis* sp. "Lanzarote-
511 type" exposed to *Coolia monotis*, but not in *O.* cf. *ovata*. Together, these observations point out that
512 the characterization of allelopathic interactions, and the allelochemical substances involved (Granéli et
513 al., 2012), could represent an interesting line of research to assess the role of biotic factors on the
514 bloom dynamics in benthic marine ecosystems.

515

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522

523 **Author Contributions**

524 M.G-P. participated in the experimental design, performed allelopathic experiments, molecular
525 analyses and wrote the manuscript. P.R. and J.-M.F. performed LC-HRMS and co-wrote the
526 manuscript. R. M. B. F. carried out nutrient analysis and F.R. designed the study and co-wrote the
527 manuscript.

528

529 **Conflicts of Interest**

530 The authors declare no conflict of interest.

531

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734 Figure captions

735 Table 1

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737 Figure 1. Maximun likelihood (ML) phylogenetic tree of genus *Ostreopsis* based on the D1-D2
738 domains of the LSU gene sequence. Numbers on the major nodes represent, from left to right, ML
739 (1,000 psudoreplicates) and NJ (1,000 pseudoreplicates) bootstrap values. Only bootstrap values >
740 60% are shown. Asterisks indicate major differences between methods. The tree was rooted using
741 *Coolia monotis* (AM902747). Med: Mediterranean. Atl: Atlantic. Pac: Pacific. Indo: Indonesian.

742

743 Table 2

744

745 Figure 2. Total ion chromatogram (TIC) of (A) *Ostreopsis* cf. *ovata* (VGO1001) culture extract
746 containing OVTX-a, -b, -c and –e. (B) *Ostreopsis* sp. "Lanzarote-type" (VGO1000) and (C)
747 *Ostreopsis* sp. "Lanzarote-type" (VGO999) culture extracts both containing OVTX-d.

748

749 Figure 3. HR Full MS spectra (positive ions) in the mass range m/z 890-2710 of OVTX-a, -b, -c, -e
750 contained in *Ostreopsis* cf. *ovata* (VGO1001) culture extract and OVTX-d contained in *Ostreopsis* sp.
751 "Lanzarote-type"(VGO1000 and VGO999). Clusters from left to right indicate tri-, bi- and mono-
752 charged ion peaks.

753

754 Table 3

755

756 Figure 4. Micrographs of *Coolia monotis* cells in (A) bi-algal culture with *Ostreopsis* sp. "Lanzarote-
757 type" (VGO1000) and (B) exposed to *Ostreopsis* cf. *ovata* supernatant. Scale bars (A) 20 µm and (B)
758 10 µm.

759

760 Figure 5. Abundances in mixed cell cultures. (A) *Ostreopsis* sp. "Lanzarote-type" (VGO1000) in
761 controls and mixed with *Coolia monotis* cells. (B) *Ostreopsis* cf. *ovata* in control and mixed with
762 *Coolia monotis* cells. (C) *Coolia monotis* in control and in mixed cultures with *Ostreopsis* sp.
763 "Lanzarote-type" (VGO1000) and *Ostreopsis* cf. *ovata* cells. (D) *Coolia monotis* in control and in
764 cultures with *Ostreopsis* sp. "Lanzarote-type" (VGO1000) and *Ostreopsis* cf. *ovata* filtrates. The error
765 bar is the standard deviation of triplicate cultures.

766

767 Video 1. (A) Single cell from controls of *Coolia monotis* on day 4. (B) Group of cells from one of the
768 controls of *Coolia monotis* on day 4.

769 Video 2. (A) *Coolia monotis* single cell with *Ostreopsis* sp. "Lanzarote-type" (VGO1000) filtrate on
770 day 4. (B) *Coolia monotis* single cell with *Ostreopsis* sp. "Lanzarote-type" (VGO1000) filtrate on day
771 4. (C) *Coolia monotis* single cell with *Ostreopsis* sp. "Lanzarote-type" (VGO1000) filtrate on day 4.
772 (D) *Coolia monotis* cells group with *Ostreopsis* sp. "Lanzarote-type" (VGO1000) filtrate on day 4.

773

774 Figure 6. Micrographs of (A) *Gambierdiscus excentricus* and (B) *Prorocentrum hoffmannianum* cells
775 in controls during adherence short-term experiments. Micrographs of *Ostreopsis* sp. treatment effects
776 on *Gambierdiscus excentricus* after 6 days showing (C) ecdysis (arrow) with *Ostreopsis* sp.
777 "Lanzarote-type" (VGO999) treatment at 1:4 ratio and (D) deleterious effects (left arrow) and ecdysis

778 (right arrow) with *Ostreopsis* cf. *ovata* treatment at 1:4 ratio. Scale bars (A-C-D) 60 µm and (B) 10
779 µm.

780

781 Figure 7. Adherence experiment. (A) *Gambierdiscus excentricus* (B) *Prorocentrum hoffmannianum*
782 cell numbers (attached to coverslips) under *Ostreopsis* sp. "Lanzarote-type" (VGO999) and *Ostreopsis*
783 cf. *ovata* cell-free filtrate treatments, at 1:4 and 1:2 equivalent cellular ratios
784 (*Gambierdiscus:Ostreopsis* and *Prorocentrum:Ostreopsis*). X and Y axis represent sampling days after
785 exposure and number (N) of attached cells, respectively.

786

787 Table 4