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Cell growth and toxins' content of *Ostreopsis* cf. *ovata* in presence and absence of associated bacteria

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Abstract – Bacteria associated to benthic dinoflagellate *Ostreopsis* cf. *ovata* cultures were removed to assess their effects on algal growth and toxins' production. Bacteria were removed using an antibiotic cocktail (streptomycin, ciprofloxacin, gentamicin and penicillin G). The actual axenic status of antibiotic treated cultures and bacterial growth in control cultures were assessed by epifluorescence microscopy using SYBR gold dye. The removal of bacteria unaffected algal growth, except conferring a higher cell number at mid stationary phase. Toxin profile and quantification of each toxin (PLTX, OVTX-a, -b, -c, -d, -e) were performed by HR LC-MS on both cell pellet and growth medium extracts. No changes in toxins' profile nor in cell and extra-cellular toxins' concentrations were found between bacteria-free and control cultures at the early stationary phase. Whereas, in late stationary phase axenic cultures showed significant lower cell toxins' concentrations and higher extra-cellular toxins' values, though not significantly (total cell toxins' concentrations: 39.3 and 24.9 pg $cell^{-1}$; total extra-cellular toxins' concentrations: 23.8 and 28.3 μgL^{-1} for control and bacteria-free cultures, respectively).

***Ostreopsis* cf. *ovata* / bacteria / palytoxin / axenic culture**

INTRODUCTION

In this last decade *Ostreopsis* cf. *ovata* Fukuyo 1981 outbreaks have been increasingly recorded along the Mediterranean coasts rising concern (Tichadou *et al*., 2010 and references therein; Mangialajo *et al*., 2011). *Ostreopsis* cf. *ovata* is an epiphytic/benthic dinoflagellate that produces palytoxin-like compounds (putative palytoxin and several ovatoxins) which are potent and complex nitrogen-containing toxins (reviewed in Ciminiello *et al*., 2011).

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In the past decade it has been speculated that the algal associated bacteria (*i.e.* harbouring the phycosphere as free living bacteria, or attached to the algal cell surface, or occurring as intracellular algal symbionts) may be implicated in algal toxin production or being toxin producers (reviewed by Kodama *et al*., 2006).

So far, most of the laboratory studies have been conducted on dinoflagellates producing paralytic shellfish toxins (PSTs) showing that bacteria-free dinoflagellate cultures produce toxins (Kodama *et al*., 2006). However, conflicting evidences have been presented on the effect of bacteria on dinoflagellate toxicity, showing either higher toxin levels in free-bacteria cultures (Hold *et al*., 2001*;* Wang *et al*., 2004) or in non-axenic ones (Uribe & Espejo, 2003), in other cases depending on growth stage and on specific toxin (e.g. *Alexandrium tamarense*; Hold *et al*., 2001). It is plausible that dinoflagellate toxin production may be modulated by an indirect effect of bacteria acting on algal cellular physiology by mechanisms such as nutrient competition, the production of inhibitory or stimulatory compounds, or removal of toxins (Donovan *et al*., 2009; Green *et al*., 2010 and references therein).

Focusing on benthic dinoflagellates, an early study on non-axenic cultured *Ostreopsis lenticularis* and *Gambierdiscus toxicus* clones showed high variability in toxicity degree, number and occurrence of associated cultivable bacterial genera which, however, did not show to produce toxins (Tosteson *et al*., 1989). More recently, Ashton *et al*. (2003) have reported the presence of non-culturable bacterial strains in association with *O. lenticularis* cultures, while Pérez-Guzmán *et al*., (2008) have shown that about 50% of bacteria associated to *O. lenticularis* clones belong to Cytophaga-Flavobacter-Bacteroides complex. The latter study, however, does not provide evidence for bacterial effect on algal toxin production nor on specific bacterium toxin producer. In sum, although some first data appeared to invoke the presence of some specific associated bacteria for *O. lenticularis* toxicity (González *et al.*, 1995) their revision has left open questions on the effect of bacteria on *O. lenticularis* growth and toxicity (Pérez-Guzmán *et al*., 2008 and references therein).

So far, information on the role of bacteria associated with *O.* cf. *ovata* are lacking. Thus, the aim of the present study was to obtain axenic culture of *O.* cf. *ovata* and to investigate on the effects of bacteria on algal growth, toxins' production and profile. The detection of the synoptic changes in cell algal size was also performed as they reflect biochemical and physiological status of the organism which in turn affects toxin production (Granéli & Flynn, 2006).

In this study, to minimize time length since the original cell isolations, new *O.* cf. *ovata* isolates were obtained from fresh samples. During isolation by micropippetting a single algal cell from an environmental sample, bacteria present in the phycosphere and capable thereafter of growing under algal batch culture conditions were kept in association to the algae.

MATERIAL AND METHODS

Ostreopsis cf. *ovata* strain OOAN0918 was isolated by the capillary pipette method from water samples collected in September 2009 at Passetto site (43°30' N and 13°37' E; North-Western Adriatic Sea; Italy). After initial growth in sterile microplates, cells were cultured in 0.22 μm pre washed and

sterilized Erlenmeyer flasks sealed with cotton plugs at 20°C under a 16:8 h L:D (ca. 90 µmol m^{-2} s^{-1} by cool white lamp) in a thermostatic room. Cultures were established in 0.22 µm prefiltered and sterilized natural seawater adjusted to a salinity value of 36 and added with 0.22 µm prefiltered macronutrients at a five-fold diluted f/2 medium concentration plus selenium (Pezzolesi *et al.*, 2012).

Axenic cultures were obtained by cells from a dinoflagellate culture at mid exponential phase, firstly subjected to washing and subsequently treated with an antibiotic cocktail containing streptomycin (25 µg mL^{-1}), ciprofloxacin (46 µg mL^{-1}), gentamicin (240 µg mL^{-1}), and penicillin G (20 units mL^{-1}). For washing, aliquots of 100 mL of cell culture were filtered through a 8 µm-pore-size filters without using vacuum. The cells retained by the filter were then gently washed twice with 5 mL of 0.2 µm prefiltered and sterilized medium. The filtration procedure avoided damage to the cells caused by centrifugation. After this procedure, cells were suspended into 50 mL 0.2 µm prefiltered and sterilized medium containing the antibiotic cocktail and incubated for 12 days (Hold *et al.*, 2001 modified). Dinoflagellates were aseptically sub-cultured through three growth cycles to dilute out the antibiotics prior to assess their bacterial status, and their use for the successive experiment on algal growth, toxin profile and toxin production. Untreated cultures *i.e.* without addition of antibiotics, were maintained concurrently. All cultures were incubated under previously described conditions.

Experimental setting protocol and methods used for determining algal cell counts cell dimensions, and for toxins' extraction are reported in Vanucci *et al.* (2012). Briefly, experimental cultures were carried out in 2 litre Erlenmeyer flasks containing 1500 mL of medium. All batch cultures were grown under the same temperature, light, and salinity values as reported previously. For each condition (*i.e.* axenic and non-axenic) 2 series of batch cultures were set up in parallel; one series was used for determining cell growth and cell measurements (6 flasks, 3 for beginning and 3 for late stationary phase), the second one for toxin content (6 flasks, as reported above). Therefore the experiment was carried out in triplicate. Cell counts were performed every 2-3 days, whereas cell measurements were performed at day 9, 16, and 27. Algal cells were classified into two size classes, small cells and large cells, basing on the dorsoventral diameter (DV) for which a dimensional step at about 40 mm was observed in the culture cell populations. Moreover, a slight variation in the cytoplasmatic transparency was generally observed between the two sized cells in unfixed samples. When the DV of the cell exceeded the upper limit of class 1 (*i.e.* DV = 40 µm), the cell was considered as belonging to class 2.

The presence of bacteria and their growth in the algal cultures were assessed by direct bacteria counts using epifluorescence microscopy after staining with SYBR gold (Shibata *et al.*, 2006), a molecular technique that allows the detection of both cultivable and non-cultivable bacteria, and archea in marine samples.

For toxin analysis cells were collected at the beginning (day 9) and at late (day 27) stationary growth phase. Toxins' profile and concentrations were assessed in both cell pellet (intra-cellular) and growth medium (extra-cellular) extracts by HR LC-MS (Ciminiello *et al.*, 2011).

Differences in total, small and large cell abundances, biovolume, and in intra-cellular and extra-cellular concentrations of each toxin within and between treatments were tested by the analysis of variance (ANOVA).

RESULTS

Algal and bacterial growth

Growth curves of *Ostreopsis* cf. *ovata* under control and antibiotic-treated conditions are shown in figure 1; cultures' initial cell densities were on average, 270 and 140 cells mL^{-1} in control and treated cultures, respectively. A steep increase in cell concentration was observed in axenic cultures over the first few days compared to control cultures; however, the exponential growth phase ended within the first 6 days under both conditions. At the beginning of the stationary phase (*i.e.* day 9), mean cell yield showed no significant difference between the two conditions (ANOVA, $p > 0.05$). Interestingly, at mid stationary phase (*i.e.* day 16) cell concentrations were significantly higher in axenic cultures than in control ones (mean values: 3.27×10^3 and 4.79×10^3 cell mL^{-1}, control and axenic cultures, respectively; ANOVA, $p < 0.05$) onwards, in late stationary phase (*i.e.* day 27) cell numbers were very similar between the two conditions. The presence of bacteria and their growth pattern were assessed synoptically throughout the *O.* cf. *ovata* growth in both axenic and non-axenic conditions, and no bacteria were observed in the treated cultures all over the algal growth. In the control cultures (bacterial initial densities, on average: 1.69×10^6 cell mL^{-1}), the bacterial exponential growth phase was coupled to the early stationary phase of *O.* cf. *ovata* when the highest cell algal abundances were observed, while the highest bacterial cell densities were recorded coupled to the late algal stationary phase, reaching values up to 9.33×10^6 cell mL^{-1} (Fig. 1).

Under both conditions, over the growth cycle, *O.* cf. *ovata* cultures were composed by small and large cells not overlapping in size (small cells: ≤ 40 µm; large cells: > 40 µm). As a general trend, both kinds of cells showed an increasing volume from early to late stationary phase (data not shown). The relative contribution of the two size classes to total cell abundance showed different temporal trend under the two conditions (Fig. 2). Particularly, in bacteria-free condition small cells increased significantly from early to late stationary phase

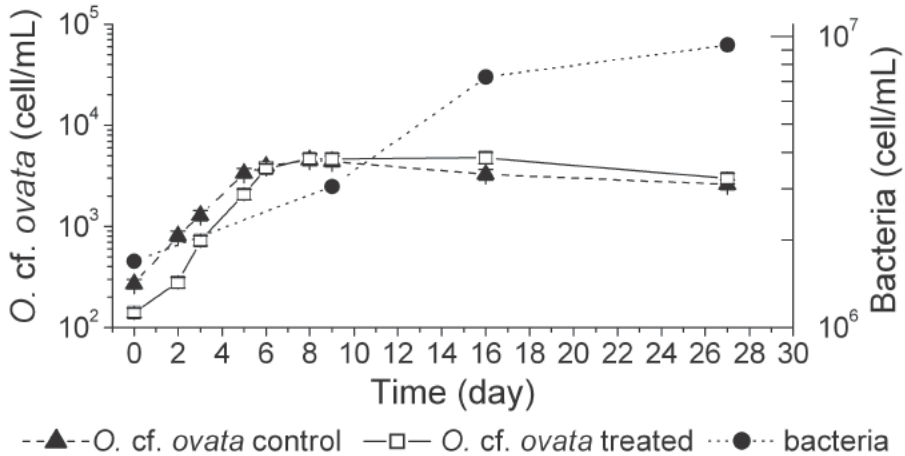

Fig. 1. Growth curve of *O.* cf. *ovata* (cell mL^{-1}) in axenic and control cultures. The growth of bacteria (cell mL^{-1}) in the control cultures is also shown.

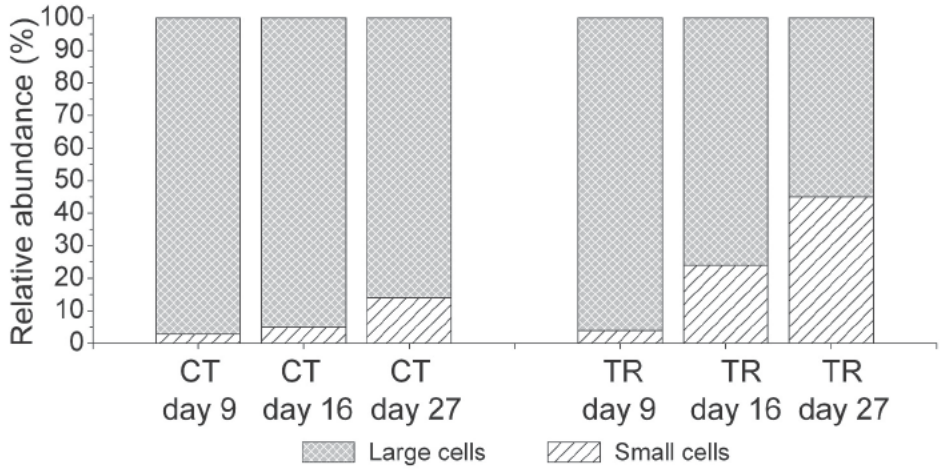

Fig. 2. Relative contribution (%) of the two cell size classes of *O.* cf. *ovata* to total cell abundance in control (CT) and antibiotic treated cultures (TR), in early (day 9), mid (day 16), and late (day 27) stationary growth phase.

(ANOVA, $p < 0.05$), accounting for more than 45% of the total cell abundance in the latter stage. So that, on the whole, the recorded changes in cell size composition and biovolume (data not shown) resulted into: i) a significant higher small cell concentrations in bacteria-free condition than in control at late stationary phase (ANOVA, $p < 0.05$); ii) a significant smaller mean cell volume under axenic condition than under control condition at late stationary phase (ANOVA, $p < 0.05$; 31% lower than in control).

Toxin concentrations: comparison between control and bacteria-free condition

Under both control and free-bacteria conditions *O.* cf. *ovata* showed the same qualitative toxin profile producing putative palytoxin (pPLTX) and all the ovatoxins so far known (OVTX-a, -b, -c, -d, and -e) with OVTX-a and OVTX-b as major contributors (accounting on average for 49% and 28%, respectively) and pPLTX as the minor one (1.1%). Moreover, under both conditions, toxins' concentrations showed higher values in late than in early stationary phase, although differences were not significant in bacteria-free cultures (ANOVA, all toxins, $p < 0.01$ for control, and $p > 0.05$ treated condition, respectively).

More in detail, in early stationary phase all toxins' concentrations on a cell basis (*i.e.* intracellular concentration) were slightly higher in bacteria-free condition than in control one, and differences were not significant (ANOVA, all, $p > 0.05$); on average, total toxin concentration (*i.e.* the sum of all toxins) was 17.6 and 19.8 pg cell^{-1} in control and axenic cultures, respectively (Fig. 3). Conversely, at day 27 concentrations of all toxins were significantly lower in axenic cultures than in control ones (ANOVA, all, $p < 0.01$) with mean total toxin values up to 24.9 and 39.3 pg cell^{-1} in axenic and control condition, respectively, corresponding to a decrease of about 37% with respect to control.

Extracellular toxins, showed the same qualitative profile and quantitative temporal trend (data not shown) found for toxins in the algal cells with significant higher concentrations in late than in early stationary phase (ANOVA, $p < 0.01$);

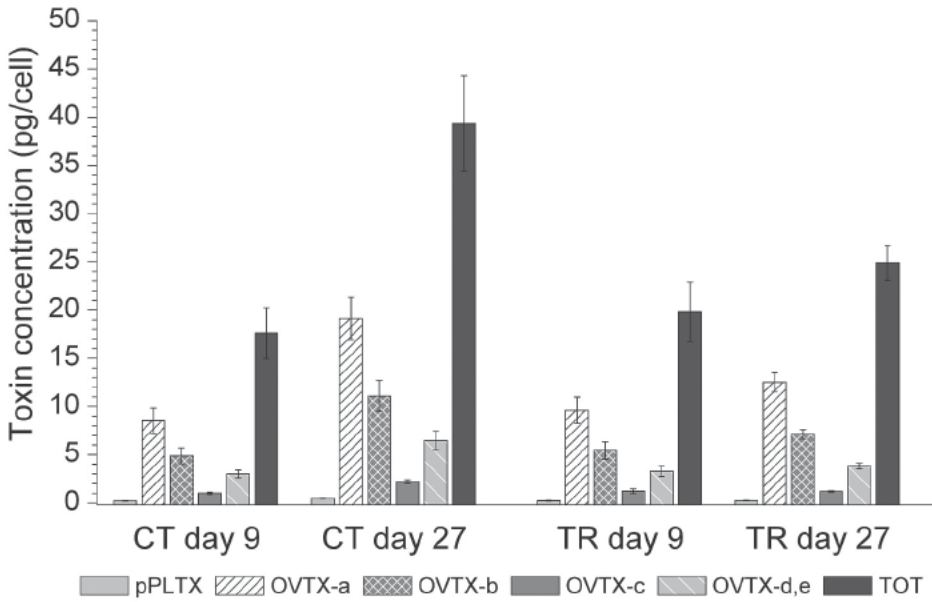

Fig. 3. Total and individual toxin contents of putative palytoxin (pPLTX), ovatoxin (OVTX)-a, -b, -c, and -d plus -e in *O.* cf. *ovata* cells grown under control (CT) and antibiotic treated (TR) conditions in early (day 9) and late (day 27) stationary phase, expressed on a cell basis (pg cell^{-1}).

however, while at day 9 values were similar in the two conditions (mean total toxin concentration: 3.37 and 3.03 μgL^{-1}, for control and treated condition, respectively) at day 27 bacteria-free medium showed a total toxin value higher (16%) than in control (mean values: 23.8 and 28.3 μgL^{-1}, for control and treated condition, respectively), although not significantly different (ANOVA, $p > 0.05$).

DISCUSSION

To our knowledge, the current study represents the first report on effects of associated bacteria on *O.* cf. *ovata* growth and toxin production.

This investigation shows that the removal of bacteria associated to *O.* cf. *ovata* unaffected cell yield algal cultures, while it appears to confer a higher cell number at mid stationary growth phase. This finding is in agreement with previous results reported for other toxic dinoflagellates (e.g. Uribe & Espejo, 2003; Green *et al.*, 2010 and references therein), and it is reasonably due to the lack of bacteria-algal competition for nutrients, together with the absence of algal degradation by bacteria. Moreover, the bacterial growth pattern in the untreated cultures clearly indicates a prompt bacterial response to the release of organic compounds produced in large amounts by *O.* cf. *ovata* throughout the entire growth cycle (Guerrini *et al.*, 2010 and references therein), and that their availability will be increasing as *O.* cf. *ovata* numbers become higher.

The removal of bacteria did not show any effect on *O.* cf. *ovata* toxin qualitative profile nor on toxins' quantitative temporal trend; however, in late stationary phase it affected cell toxins' content in axenic cultures with a reduction of more than one-third with respect to non-axenic ones. A similar behaviour has been observed in some PSP producing dinoflagellates (e.g. Hold *et al*., 2001; Uribe & Espejo, 2003). The removal of bacteria appears also to allow toxins' accumulation in the algal growth medium, possibly due to the lack of toxins' degradation by bacteria, as suggested by the higher extracellular toxin values found in axenic cultures than in non-axenic ones, although not significantly. It has also to be taken into account that the significant lower cell toxin content found in axenic than in non-axenix cultures, and consequently a less toxin amount released into the medium, might have biased the statistical result. Therefore, further studies focusing on this aspect are needed. The removal of toxins would, in fact, play an important role in the environmental toxin dynamics (Uribe & Espejo, 2003; Donovan *et al*. 2009; Green *et al*., 2010).

Effects on *O.* cf. *ovata* physiology due to axenity have been shown by the reduction in size of a significant proportion of cells in bacteria-free cultures from mid stationary phase onwards. Interestingly, a significant high proportion of these small cells, with respect to control, was also found in *O.* cf. *ovata* cultures under nitrogen limitation (but not under control condition or phosphorus limitation) throughout the growth cycle with increasing numbers in late stationary phase; this state was accompanied to a reduced toxins' cell amount all over the growth with respect to balanced nutrient conditions (Vanucci *et al*., 2012). The eco-physiological role of these small cells, which differ from vegetative resting forms (Pearce *et al*., 2001; Barone & Pranzato, 2006), is still to be clarified and likely linked to intracellular N-declining status (Reguera, 2002). So that, we speculate that the removal of bacteria affects, among other algal-bacteria interactions, nitrogen availability by preventing bacterial nitrogen-remineralisation. This condition could affect toxin production, especially when nutrients are nearly exhausted, as ovatoxins and p-PLTX are complex N-containing polyketides.

In conclusion, on the balance of our data, bacteria appear to interfere indirectly with algal growth and toxins' production via their effect on algal physiology and likely on removal of toxins. Thus, bacteria as an environmental variable could have marked effects on *O*. cf. *ovata* growing in a bloom and/or on its toxicity. In the awareness that the current results deal with a selected group of bacteria further investigation is needed, also on the assessment of eventual algal bacterial endosymbionts.

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