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XIV CICLO

Interactions between the toxic benthic dinoflagellate *Ostreopsis* cf *ovata* and marine diatoms

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CHAPTER 1. General introduction and thesis objectives

1.1 Allelophatic interactions

Microalgae are photosynthetic unicellular organisms living in both fresh and marine ecosystems where play a key role in the carbon cycle transfering organic material to the top consumers such as zooplankton, zoobenthos and necton (Falkowski & Raven, 2007). As well as for terrestrial environment, in several aquatic species the prey–predator relationships and the competition for light and nutrients allowed the development of defense mechanisms involving the production of chemical compounds in order to achieve a competitive advantage over co-occurring competitors and prospective grazers (Demain and Fang, 2000; Gross, 2003; Ianora et al., 2012). In the second half of the 20th, Rice (1974) coined the term "allelopathy" that refers to direct and indirect biochemical interactions among several organisms and the involved molecules were called allelochemicals. Although allelophatic interactions are considered important drivers of ecology and evolution of aquatic ecosystems (Ianora et al., 2011), the interest in allelopathic compounds has grown only in the recent years (Leflaive and Ten-Hage, 2009), due the development of the chemical ecology that brought to light most of their chemical nature and biosynthetic pathways (Delory et al., 2016; MacIntyre et al., 2014). Allelopathic compounds are secondary metabolites, i.e., low-molecular weight organic not directly involved in the make up of basic life mechanisms and constitute a very small fraction of the total biomass of the producer organism (Ianora et al., 2011). Their production appears widespread in each of the six kingdoms of life and involves numerous groups such as plants (Gross, 2003), algae (Cabrita et al., 2010), cyanobacteria (Nunnery et al., 2010), bacteria (Teasdale et al., 2009), fungi and invertebrate animals (Sagar et al., 2010). In aquatic ecosystems, much attention has focused on chemical ecological interactions within and among invertebrate species (such as sponges, molluscs, echioderms and polychaetes) especially in the tropical environments, due to the high species diversity and resource competition (Ban et al., 1997; Juttner, 2005; Murphy, 2001; Paul et al., 2012). Allelochemical interactions include positive, negative and synergetic effects and are

implicated in several biological processes such as attractant and energy supply for growth (Ponhert, 2002), anti-predator defence mechanisms (Miralto et al., 1999), growth dynamics of harmful algal blooms (Fistarol et al., 2003; Uchida et al., 1999; Yamasaki et al., 2010), competitive inhibition, algal succession (Fistarol et al., 2004; Granéli & Hansen, 2006), intra-population infochemicals (Vardi et al., 2006) and antibacterial activity (Ribalet et al., 2008; Vidoudez and Pohnert, 2008). From a chemical point of view, allelopathic molecules include numerous groups, such as organic acids, phenolic substances, alkaloids terpenoids, nitrogen-containing compounds and so-called polyunsaturated aldehydes (PUAs) (Karappusamy, 2009). Also the release of biotoxins by plants and microrganisms in order to affect their potential competitors is interpreted as allelopathy (Rice, 1984). Even though the action mode of allelochemicals remains less clear in many cases, the interruption or stimulation of several essential functions in target organisms were observed (Gross, 2003; Varella et al., 2014). In particular, blistering/rupture of membranes, cell rounding, inhibition of nucleic acid synthesis cell lysis, ROS generation (Ribalet et al., 2007), damage to the extracellular matrix and cellular paralysis (Leflaive and Ten-Hage 2007) were described. Some studies reported a negative impact of macroalgal extract on the photosynthetic apparatus of several marine microalgae as for example, Ye and Zang (2013) that highlighted the blockage of the electron transport and oxygen-evolving complex of *Prorocentrum micans* when the cells were exposed to *Gracilaria tenuistipitata* (Rhodophyta) filtrate. However, abiotic (temperature, salinity, pH, nutrient limitation, light) and biotic factors (culture age, cell density) can affect the production and accumulation of allelochemicals within and among microalgal groups (Gross, 2003; Pohnert, 2002; Ribalet et al., 2007b; Vardi et al., 2002). Anderson et al. (1990) showed that the production of saxitoxin by the dinoflagellate *Alexandrium* increased under P-limitation and during the exponential growth phase. On the contrary, other authors observed higher concentrations of domoic acid during the stationary growth phase in several species belonging to the *Pseudo-nitzschia* genus (Pan et al., 1998).

1.2 Polyunsaturated Aldehydes (PUAs)

Polyunsaturated aldehydes (PUAs) are highly reactive compounds belonging to oxilipins groups and produced from membrane bound lipids (Pohnert, 2000). They were identified for the first time by Miralto et al. (1999) in a study performed on *Thalassosira rotula, Skeletonema marinoi* and *Pseudo-nitzschia delicatissima* where clearly negative effects on the copepod embryogenesis were reported. These findings have been confirmed by subsequents studies carried out in field that highlighted a decrease of copepod hatching success during a diatom bloom in the northern Adriatic Sea (Ianora et al., 2004). Moreover, PUAs exert negative effect on other invertebrates such as echinoderms, polychaetes, ascidians and molluscs (Casotti et al., 2005; Kâ et al., 2014; Lavrentyev et al., 2015; Romano et al., 2010). Other important ecological functions were described for these compounds, cell-cell information (i.e. population synchronization and/or "signal of death", Gallina et al., 2014; Vardi et al., 2006) and antibacterial activity (Ribalet et al., 2008). The PUAs production appears widespread within diatoms and terrestrial plants and, albeit to a lesser extent, also in some macroalgal species (Guschina and Harwood 2006; Pohnert and Boland 2002; Bouarab et al. 2004). Wichard et al. (2005) observed that 36% of the 51 investigated diatom species released aldehydes showing a strain-specific production with a wide range of concentrations (Table 1).

Table 1. Overview of the isolates investigated by Solid Phase MicroEtraction and PFBHA-derivatisation "+" identifies a diatom as PUA-producer; "c" nnly traces of PUA were detected after PFBHA derivatisation; "k" acidic polyunsaturated aldehydes were detected: 12-oxo-dodeca-5,8,10-trienoic acid and 9-oxo-nona-5,7-dienoic acid (modified from Wichard et al., 2005).

Species	SPME/ PFBHA[a]	Detected PUA
Actinocyclus subtilis	•/•	–
Amphiphora paludosa	–/•	+[c]
Asterionella formosa	•/•	–[k]
Asterionellopsis glacialis	•/•	+
Chaetoceros calcitrans	•/•	–
Chaetoceros compressus	•/•	+
Chaetoceros muelleri	•/•	–
Coscinodiscus granii	•/•	–
Coscinodiscus sp.	–/•	–
Cyclotella meneghiniana	•/•	–
Ditylum brightwellii	•/•	–
Ditylum brightwellii	•/–	–
Ditylum brightwellii	–/•	–
Fragilaria capucina	•/•	–
Fragilaria sp.	•/•	+
Gomphonema parvulum	•/–	–[k]
Guinardia deliculata	–/•	+
Guinardia striata	–/•	–
Melosira nummuloides	•/•	+
Melosira sulcata	•/•	+
Navicula pelliculosa	•/•	–
Navicula sallinicola	–/•	–
Navicula sp.	•/•	–
Navicula sp.	•/–	–
Navicula transitans	•/•	–
Nitzschia sp.	•/•	–
Nitzschia closteridium	–/•	–
Nitzschia curvilineata	–/•	–
Nitzschia frustulum	–/•	–
Odontella regia	•/•	+[c]
Odontella sinensis	•/–	–
Paralia sulcata	•/•	–
Phaeodactylum tricornutum	•/•	–
Phaeodactylum tricornutum	•/•	–
Phaeodactylum tricornutum	–/•	–
Phaeodactylum tricornutum	•/•	–
Pleurosigma normanii	•/–	–
Pseudonitzschia sp.	•/•	–
Rhizosolenia setigera	–/•	–
Rhizosolenia setigera	–/•	–
Skeletonema costatum	•/•	+
Skeletonema costatum	–/•	+
Skeletonema costatum	•/–	+
Skeletonema costatum	•/–	+

Species	SPME/ PFBHA[a]	Detected PUA
Skeletonema costatum	•/–	+
Skeletonema pseudocostatum	•/•	+
Skeletonema subsalsum	–/•	+
Stephanodiscus hantzschii	•/•	–
Stephanodiscus minutulus	•/•	–
Stephanophyxis turris	•/•	–[k]
Thalassionema nitzschioides	–/•	–
Thalassiosira aestivalis	•/•	+
Thalassiosira anguste-lineata	•/•	+
Thalassiosira eccentrica	•/•	–
Thalassiosira minima	•/•	+
Thalassiosira nordenskioeldii	•/•	+
Thalassiosira pacifica	•/•	+
Thalassiosira pseudonana	•/•	–
Thalassiosira pseudonana	•/•	–
Thalassiosira pseudonana	–/•	–
Thalassiosira punctigera	•/–	–
Thalassiosira rotula	•/•	+
Thalassiosira rotula	•/•	+
Thalassiosira rotula	•/•	+
Thalassiosira rotula	–/•	+
Thalassiosira rotula	–/•	+
Thalassiosira rotula	•/–	+
Thalassiosira sp.	•/–	+
Thalassiosira weissflogii	•/•	–
Thalassiosira weissflogii	–/•	–
Thalassiosira weissflogii	–/•	–
Prorocentrum micans[i]	•/–	–
Prorocentrum minimum[i]	•/•	–

Moreover, quantification and composition profile of PUAs have been investigated also in field, especially during and after intensive diatom blooms (Bartual et al., 2014; Ribalet et al., 2014). Vidoudez et al. (2011) monitored PUAs concentrations during a spring bloom of *Skeletonema marinoi* in the Adriatic Sea, showing a patchy distribution in the surface and a decrease of these compounds with depth. PUAs concentrations observed in this survey not exceed 0.1 nM and are lower compared to the values reached in *S. marinoi* cultures (Wichard et al., 2005).

A wide PUAs range was indentified during the last decade: 2E,4E-decadienal, 2E,4E/Z,7Z-decatrienal, 2E,4E/Z-heptadienal, 2E,4E/Z-octadienal, and 2E,4E/Z,7Z-octatrienal (d'Ippolito et al.,

2004, 2006; Dittami et al., 2010; Pohnert, 2000). Among them, 2E,4E-decadienal was the compound largely used as a model aldehyde in the toxicological assays (Ribalet et al., 2007).

The biological mechanism that lead to the PUAs production in marine microalgae is rather similar to those of higher terrestrial plants, including a enzyme cascade that starts from the oxidative degradation of polyunsaturated fatty acids (PUFAs) (Ianora et al., 2012). These precursors are liberated from plasma and thylakoid membranes by galacto- and phospholipase that are associated to the loss of membrane integrity (Fontana et al., 2007) and typically occurs after wounding during predation (Pohnert, 2000).

These PUFAs are then rapidly transformed into unstable hydroperoxides (FAHs) by a lipoxygenase (LOX) that in turn are transformed into PUAs by lipolytic acyl hydrolases (HPLs) (d'Ippolito et al., 2004) (Figure 1).

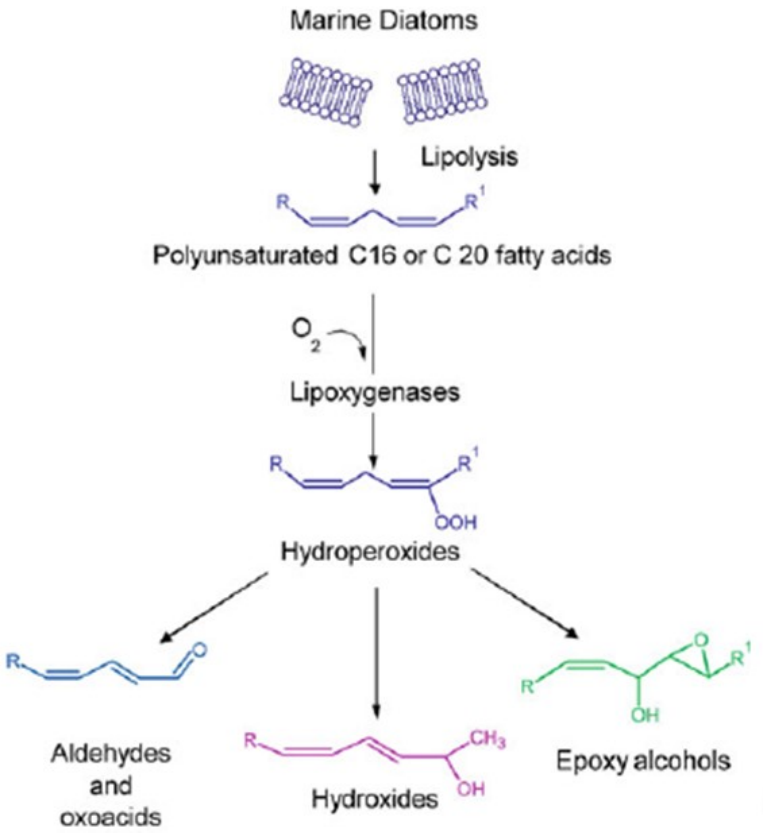

Figure 1. Oxidative metabolism of fatty acids in marine diatoms. Starting mainly from eicosapentaenoic (C20) and exadecatrienoic (C16) fatty acids that are oxygenated by oxygen addition through lipoxygenase activity (modified from Ianora et al., 2012).

Moreover, although the production and the persistence of these oxilipins is strongly modulated by physiological conditions (nutrient and stress conditions, temperature, cell density) (Bartual and Ortega, 2013; Ribalet et al., 2007b, 2009), these enzymes can remain active in seawater over several minutes leading to a locally high concentration of PUAs (Pohnert, 2002; Ribalet et al., 2007a). The bioactivity of PUAs depends on the presence of an α, β, γ, δ -unsaturated aldehyde group, which is a potent Michael acceptor able to bind nucleic acids and to create numerous covalent adducts (Carvalho et al., 2000). Casotti et al. (2005) reported an irreversible blockage of cells in G1-phase of the cell cycle in *Thalassiosira weissflogii* exposed to 2*E*,4*E*-decadienal, preventing the DNA transcription necessary for the synthesis of structural components (Figure). Moreover a strong positive relationship between length and cytotoxic effect of PUAs molecules has been highlighted in both bioassays performed using synthesized molecules and toxicological studies conducted on phytoplankton and invertebrate species (Adolph et al., 2004; Ribalet et al., 2007a; Wichard et al., 2005).

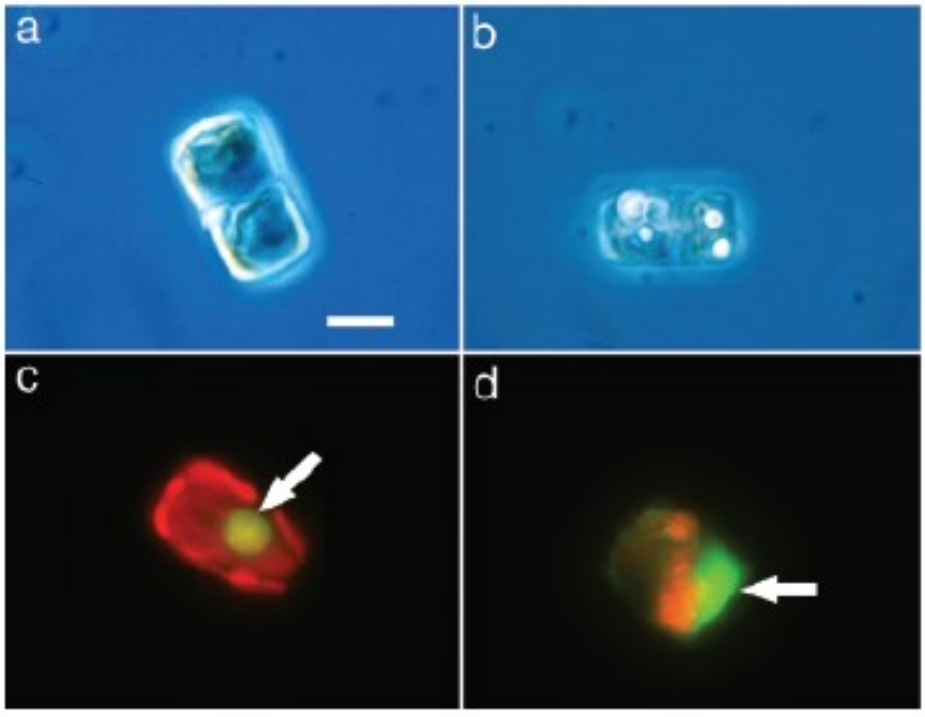

Figure 2. Morphology of *Thalassiosira weissflogii* upon aldehyde addition. (a) Control cells shortly after cell division, unfixed. (b) Cells exposed to 1mgL–1 of 2,4-decadienal for 24 h, unfixed, showing vesicles and refractive bodies. (c) Control cells fixed and stained with SYTOX Green after exposure to A3 ($1mgL^{-1}$) for 24 h. Note the DNA dispersal in the cytoplasm. Scale bar, 5 mm. Arrows indicate the nucleus in c and d (from Casotti et al., 2005).

1.3 *Ostreopsis* cf. *ovata*

One of the main reasons of the interest in allelopathic interactions is the key role that can play these biological compounds to promote the dominance harmful algal bloom (HABs)-forming species over other co-occurring species (Granéli and Hansen, 2006; Gross, 2003). HABs are a serious ecological and socioeconomics as able to produce secondary metabolites, which can be affect zooplankton grazers, fish, shellfish and other microrganisms (Bownik, 2016; Shears and Ross, 2009). Fistarol et al. (2004) reported a clear negative effect on the phytoplankton community, in terms of abundance and structure, by the HAB-species *Alexandrium tamarense.* The same authors affirmed that the compounds released by *A. tamarense* are able to provoke cells lysis of several competitors and suggested a probable involvement of these allelopatic molecules in algal succession of the phytoplankton community.

Ostreopsis cf. *ovata* is a harmful benthic dinoflagellate responsible for intensive blooms both in tropical and temperate regions (Accoroni and Totti, 2016; Mangialajo et al., 2011; Rhodes, 2011). It is able to colonize both abiotic substrata (soft sediments and rocks) (Totti et al., 2010) and biotic (macrophytes, seagrasses and invertebrates) (Accoroni et al., 2015b; Vila et al., 2001). In concomitance with *Ostreopsis* blooms, mass mortalities of benthic marine organisms and human intossications have been observed, due to the production of palytoxin-like compounds (Scalco et al., 2012; Uchida et al., 2013). Although most of studies performed on *Ostreopsis* bloom dynamics concerned the influence of environmental factors such as temperature, nutrient concentrations, hydrodinamics (Accoroni et al., 2015a; Mangialajo et al., 2011; Pistocchi et al., 2011; Vanucci et al., 2012), recently also the relationships of this dinoflagellate with macroalgae and microphytobenthos have been investigated. In fact, Monti and Cecchin (2012) described a weak allelopathic effect of *O. ovata* filtrate on the growth of *Coscinodiscus granii*, *Prorocentrum minimum* and *Coolia monotis*. On the other hand, Accoroni et al. (2016) carried out a study on the relationship between the *O.* cf. *ovata* bloom and the microphytobenthos communities of the Conero Riviera (northern Adriatic Sea) evaluating their composition and temporal variation in the course of an annual cycle. The results

highlighted that the proliferation of *Ostreopsis* affected the structure of the benthic diatom community with a microphytobenthos diversity significantly lower observed during the *O.* cf. *ovata* bloom than during the rest of the year.

1.4 Aims of the thesis

In the last decade numerous studies were carried out on the PUAs production by diatoms and the deleterious impact of diatom PUAs-producers towards co-occurring organisms has been deeply investigated (Carotenuto et al., 2006; Gallina et al., 2014; Hansen et al., 2004; Ianora and Miralto, 2010). However, there are still very few information about the effect of these reactive biological compounds on the microphytobenthic communities (Ianora et al., 2003). Studies concerning the ecological role of PUAs have primarily focused on planktonic organisms highlighting the functionality consequences limited to this ecosystem.

The aims of this thesis were to investigate the possible effect of benthic diatoms on the bloom dynamics by:

(i) examining the possible PUAs production in three of the most common benthic diatoms (*Tabularia affinis*, *Proschkinia complanatoides*, *Navicula* sp.) belonging to the *Ostreopsis* community of the Conero Riviera area (northen Adriatic Sea),

(ii) studying of the effect of three commercial PUAs (2E,4E-decadienal, 2E,4E-octadienal and 2E,4E-heptadienal) on growth, cell morphology and cytological features of *Ostreopsis* cf. *ovata*;

(iii) describing the direct effect of two planktonic (*Thalassiosira* sp., *Skeletonema marinoi*) and three benthic (*Tabularia affinis*, *Proschkinia complanatoides*, *Navicula* sp.) diatoms species, known as PUAs-producers, on the *O.* cf. *ovata* growth and cytosolic features.

1.5 References

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CHAPTER 2. First report of PUAs production by three benthic diatom species

2.1 Introduction

Aquatic organisms have been shown to produce several chemical compounds responsibles of the regulation of numerous biological relationships (Gross, 2003; Ianora and Miralto, 2010; Ianora et al., 2012). These molecules could include numerous secondary metabolites, i.e., low-molecular weight organic compounds not directly involved in the make up of basic life mechanisms (Demain and Fang, 2000), belonging to different chemical groups, such as organic acids, phenolic substances, alkaloids, nitrogen-containing compounds, and PolyUnsaturated Aldehydes (PUAs) (Ianora et al., 2011). Recently, several researchers have been paying increasing interest on PUAs due to their deleterious effect on fecundity and/or hatching success of copepods (Ianora et al., 2003, 2012; Kâ et al., 2014; Lavrentyev et al., 2015; Miralto et al., 1999; Wichard et al., 2007) that is the basis of what Ban et al (1997) declared as the "paradox of diatom–copepod interactions". In addition to this effect, other important ecological functions have been described for PUAs, such as competitive inhibition of algal growth (Ribalet et al., 2007), antibacterial activity (Ribalet et al., 2008), bloom termination (Ribalet et al., 2014; Vidoudez et al., 2011) and intrapopulation signaling (Vardi et al., 2006). These biological compounds are volatile aldehydes ensuing from oxidative degradation of free PUFAs and their synthesis is widespread in plants (Delory et al., 2016), macrophytes (Alsufyani et al., 2014), cyanobacteria (Watson, 2003) and microalgae (d'Ippolito et al., 2009; Ianora et al., 2012). Diatoms are known to contribute about 40% of the marine primary production which in turn constitutes half of entire organic material produced in the planet (Rousseaux and Gregg 2014), and in particular represent the main producer groups of PUAs (Leflaive and Ten-Hage, 2009). Wichard et al., 2005 highlighted the PUA-release by 36% of the 51 diatom species examined under experimental conditions, with concentrations ranged from 0.01 to 9.8 fmol per cell. In addition to strain and species specificity (Wichard et al., 2005), it has been suggested as the PUAs production and composition

depend also by environmental factors, such as growth stage, nutrients etc. (Pohnert 2002; Ribalet et al. 2007). Regarding the metabolic pathways of PUAs, chloroplasts and plasmatic membranes represent the main sources of PUFAs, which are PUAs precursors, by lipase-mediated hydrolysis of glycolipids and phospholipids that occurs within seconds after the disrupt of the cell (Pohnert, 2000). D'Ippolito et al. (2004), describing the origin of PUAs produced by *Skeletonema costatum* with labeled precursors, showed that the metabolism of glycolipids lead to the production of hexadecatrienoic acid (C16:3), hexadecatetraenoic acid (C16:4) and eicosapentaenoic acid (C20:5) that represent the precursor, respectively, of octadienal (C8:2), octatrienal (C8:3) and heptadienal (C7:2), while phospholipids are involved only in the production of heptadienal. However, the same pool of PUFAs can constitute the precursor molecules of different PUAs, for example, in *Thalassiosira rotula*, the eicosapentaenoic acid is responsable of the decatrienal (C10:3) instead of heptadienal production (Barofsky and Pohnert 2007). Despite the clear correlation between the PUFA and PUA composition, the variety of aldehydes that a species is capable to produce depends from its pool of lipoxygenases (LOXs) (Cutignano et al., 2011). These molecules are iron nonheme enzymes able to catalyse the addition of molecular oxygen to the carbon chain of PUFAs giving the intermediate, namely fatty acid hydroperoxides (FAHs) (d'Ippolito et al., 2009). LOXs showed a species-dependent specificity and provide the direction of further reactions that consist of the FAHs trasformation, by hydroperoxide lyase (HPLS) activity, into several breakdown derivatives, i.e. PUAs (d'Ippolito et al., 2009). However, although the pathways and the effect of these oxylipins have been deeply investigated both in animals (Ianora and Miralto, 2010) and photosynthetic organisms (Ribalet et al., 2007), almost the totality of diatoms investigated belong to the planktonic domain. As a result, the real capacity of benthic organisms to produce PUAs and the effect that these compounds may have on the same benthic ecosystems remains largely unknown.

In the present study, the possible production of PUFAs and PUAs in three of the most common benthic diatoms (*Tabularia affinis*, *Proschkinia complanatoides* and *Navicula* sp.) of the Conero Riviera (northern Adriatic Sea) was investigated.

2.2 Materials and methods

Cultivation and sampling

The diatoms strains, *Tabularia affinis* (strain TAAPS0313), *Proschkinia complanatoides* (strain PCAPS0313) and *Navicula* sp. (strain NAPS0313), were obtained from microphytobenthos samples collected before summer period in the Passetto station (Conero Riviera, northern Adriatic Sea).

Single cells were isolated by the capillary pipette method (Hoshaw and Rosowski, 1973) using an inverted microscope, and monoclonal cultures were set up using multi-well plates and maintained at the following condition 20°C; 16:8 L:D photo period; irradiance of 100–110 mol $m^{-1}s^{-1}$; f/2 salt concentration (Guillard, 1978). The experiment lasted 44 days and a sample of 200 ml was taken at the days 3, 7, 9, 14, 21, 28, 35, 37, 44 from each flask for PUFAs and PUAs analysis.

PUFAs analysis

The determination and quantification of PUFAs were carried out following the method of Samorì et al. (2012). Gas chromatography–mass spectrometry (GC-MS) analyses were performed by using a 6850 Agilent HP gas chromatograph connected to a 5975 Agilent HP quadrupole mass spectrometer. The injection port temperature was 280°C. Analytes were separated by a HP-5 fused-silica capillary column (stationary phase poly[5% diphenyl/95% dimethyl]siloxane, 30 m, 0.25 mm i.d., 0.25 mm film thickness), with helium as carrier gas (at constant pressure, 33 cm s-1 linear velocity at 200°C). Mass spectra were recorded under electron ionization (70 eV) at a frequency of 1 scan s^{-1} within the 12-600 m/z range. The temperature of the column was increased from 50°C up to 180°C at 50°C min^{-1}, then from 180°C up to 300°C at 5°C min^{-1}. Tridecanoic acid and methyl nonadecanoate were utilized as internal standards for quantification of free and bounded fatty acids converted into fatty acid methyl esters (FAMEs). The relative response factors used for the quantitation were obtained by injecting solutions of known amounts of methyl tridecanoate, methyl nonadecanoate and commercial FAMEs mixture.

PUAs analysis

The quantification of PUA concentration was carried out following the method of Wichard et al. (2005). After removing of supernatant, 1 ml of derivatisation reagent (PFBHA HCl 25 mM in Tris/HCl 100 mM, pH 7) and 25 µl of internal standard (0.968 mM benzaldehyde in hexane) were added to the pellet. The sample was cooled to 4°C, an ultra-sound bath was performed for 1 min and then incubated at room temperature for 30 min. For the extraction 0.5 ml methanol and 1 ml of hexane were added to the sample that was vortexed for 1 minute and acidified with 20 µl of sulphuric acid. After that, the hexane upper layer was removed, dried over sodium sulphate, evaporated under a stream of gaseous nitrogen and taken up in 50 µl hexane. Concentrations of PUAs were then measured by GC-MS (same equipment described in PUFAs analysis) and identification of compounds was done by comparison of their retention times and of mass spectra with commercial standards (Sigma–Aldrich) when possible, or by comparison of their mass spectra with Nist libraries. Each analysis was repeated in duplicate.

Reagents

Propionaldehyde, 2,4-heptadienal, Octanal, 2-octenal, 2,4-octadienal, 6-nonenal, 2,6-nonadienal, 4-decenal, 2,4-decadienal, Undecanal, 8-undecenal, 2,4-undecadienal, benzaldeide, Tridecanoic acid, methyl nonadecanoate, Tris-HCl, O-(2,3,4,5,6-Pentafluorobenzyl) hydroxylamine hydrochloride (PFBHA), chloroform, methanol, BF_3-methanol, hexane, sulphuric acid, dimethylcarbonate, sodium sulphate were purchased from Sigma (Milan, Italy).

2.3 Results

The growth curves of *Tabularia affinis, Proschkinia complanatoides* and *Navicula* sp. used in this study are illustrated in Figure 1. The curves were characterized by an exponential growth phase lasting 28 d for *T. affinis* and *P. complanatoides* and 21 days for *Navicula* sp. Later, a short stationary phase (till day 35 for and *Navicula* sp. and *T. affinis*, till day 37 for *P. complanatoides*) and a declining phase (from day 35 to 44 for *T. affinis* and *Navicula* sp. and from day 37 to 44 for *P. complanatoides*) were observed. The highest abundances reached were of 61 x 10^3 ± 5.6 x 10^3, 63 x 10^3 ± 0.8 x 10^3 and 57 x 10^3 ± 7.9 x 10^3 cells/ml respectively for *P. complanatoides, Navicula* sp. and *T. affinis.*

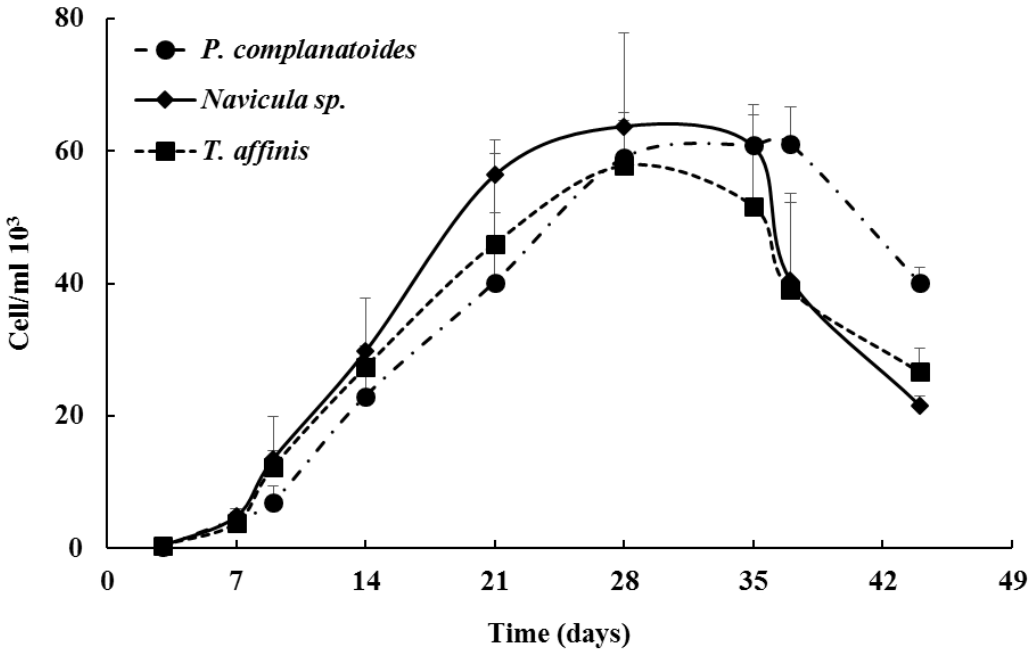

Figure 1. Cell abundances of *Tabularia affinis, Proschkinia complanatoides* and *Navicula* sp. along the growth phases. Data are means of independent replicates (n = 2) and the error bars represent standard deviations.

PUFAs analysis

The quantification of all fatty acids (saturated and unsaturated) performed on the three diatoms revealed that appreciable amounts were detected only from the day 14 in each species tested. The highest values were observed in *Tabularia affinis* (1.17 ± 0.30 ng/cell) and *Navicula* sp. (0.92 ± 0.05 ng/cell) during the decay growth phase. In the same way, the highest fatty acids concentration in *Proschkinia complanatoides* was reached at the day 44 but it never exceed the value of 0.12 ± 0.01 ng/cell in this species. Generally, the contribution of unsaturated fatty acids resulted higher respect to the one of the saturated chains along the growth curve for all the species tested (Fig. 2). In particular, these percentages ranged for 59.6 to 69.5% for *T. affinis* and *P. complanatoides* while for *Navicula* sp. it is appears more wide (55.2-71.9 %).

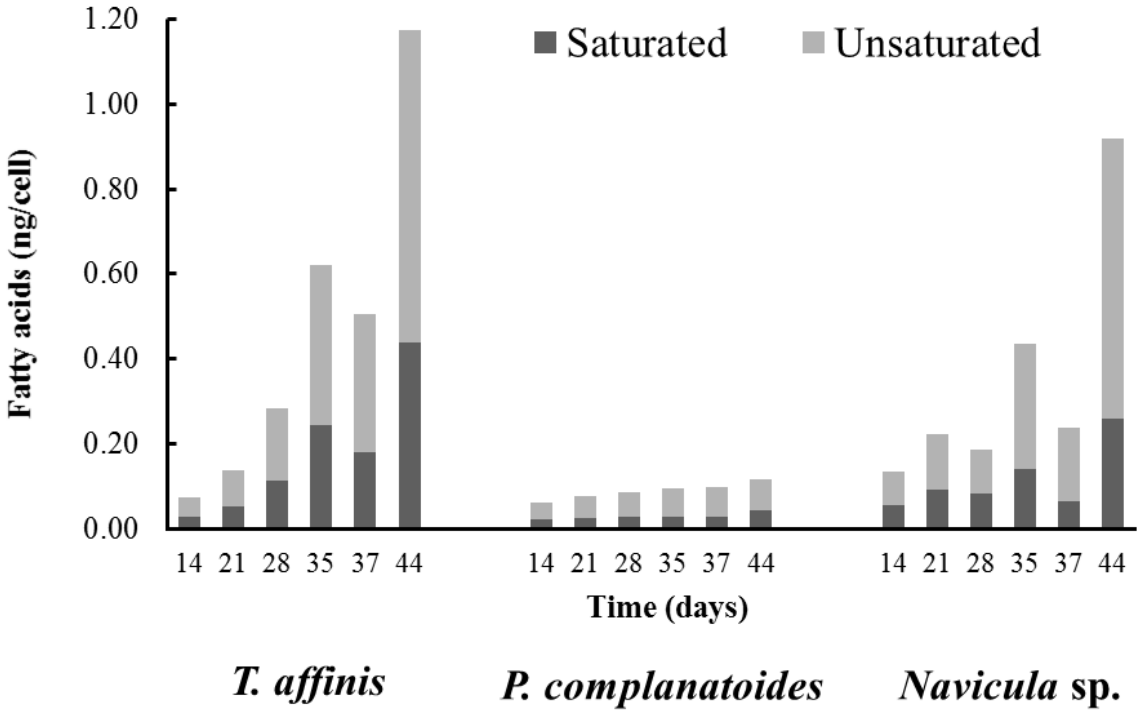

Figure 2. Fatty acids production *Tabularia affinis, Proschkinia complanatoides* and *Navicula* sp. at different days (14, 21, 28, 35, 37, 44). Data are means of independent replicates (n = 2) and the error bars represent standard deviations.

The PUFAs composition clearly highlighted that C16 and C20 fatty acids represented the prominent chains in all the samples (Fig. 3). In *P. complanatoides* culture, the combination of Hexadecadienoic acid (C16:2) plus Hexadecatrienoic acid (C16:3) and eicosapentaenoic acid (C20:5) (namely EPA) were consistently detected and their maximum values were reached at day 44 with 37.77 ± 4.03 and 19.3 ± 2.23 pg/cell respectively. Moreover high concentrations of Palmitoleic (C16:1) were measured from day 14 to day 28, then it was not observed (Fig. 3a). C18 unsaturated fatty acids (C18:1, C18:2 and C18:3) concentrations were also observed along the growth curve, but their values resulted lower than the ones of C16 and C20. A comparable PUFAs composition was observed between *Navicula* sp. and *T. affinis* with Palmitoleic acid as the main compound in both cultures. The highest concentrations of this latter compound were observed at day 44 with 502.9 ± 178.4 pg/cell for *T. affinis* and 563.7 ± 35.3 ng/cell for *Navicula* sp., while trace amounts of C18 fatty acids (C18:1 and C18:3) were revealed only in *T. affinis* (Fig. 3b,c). Regarding the saturated fatty acids, Palmitic acid (C16:0) constitute the main molecule produced by *P. complanatoides* and *Navicula* sp. while the Myristic acid (C14:0) the one by *T. affinis* (data not shown).

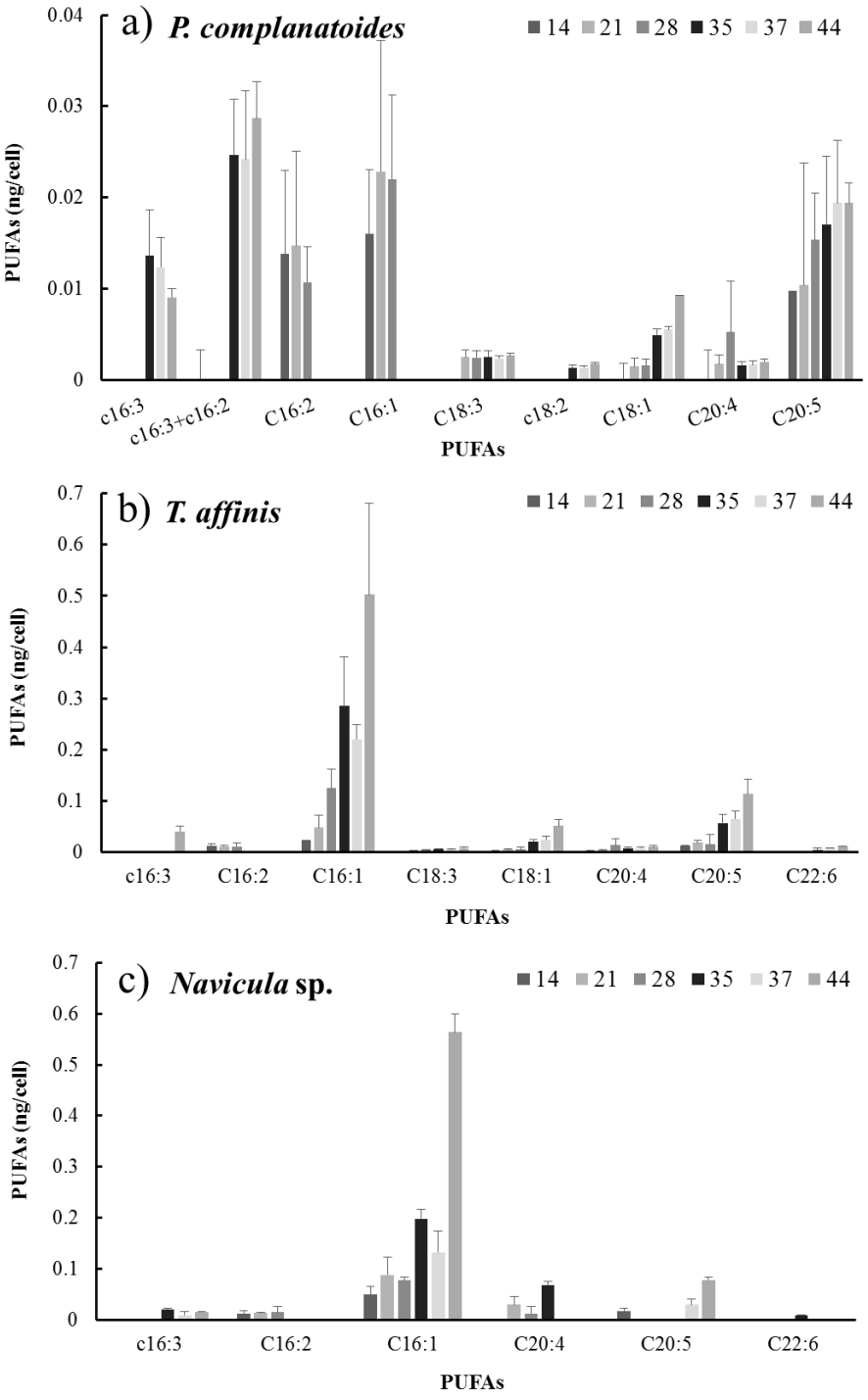

Figure 3. Polyunsaturated fatty acids production of (a) *Proschkinia complanatoides*, (b) *Tabularia affinis* and (c) *Navicula* sp. at different days (14, 21, 28, 35, 37, 44). Data are means of independent replicates (n = 2) and the error bars represent standard deviations.

PUAs analysis

The results of aldheydes quantification, that includes saturated and unsaturated compounds, are shown in Fig. 4. Some aldheydes were not identified as their fragmentation pattern in GC-MS resulted very complex and not comaprable with the ones obtained with the commercial standards or present in the Nist libraries. No aldheydes were detected until day 28 in each diatoms, then a marked increase of these compounds was observed in all three cultures tested. Their maximum peaks were reported at the day 28 for *Proschkinia complanatoides* (0.61 ± 0.43 pg/cell corrisponding to 5.78 ± 4.07 pmol/cell), 44 for *Tabularia*. *affinis* (0.67 ± 0.15 pg/cell; 6.27 ± 1.42 pmol/cell) and 44 for *Navicula* sp. (20.44 ± 4.02 pg/cell; 192.63 ± 37.94 mol/cell) (Fig. 4). Morevorer, the percentage of the polyunsaturated aldehydes (PUAs) respect to the total aldheydes showed up higher values in *Navicula* sp. (20.4 – 95.5 %) respect to *P. complanatoides* (64.2 – 67.5 %) and *T. affinis* (61.7 – 74.1 %) cultures.

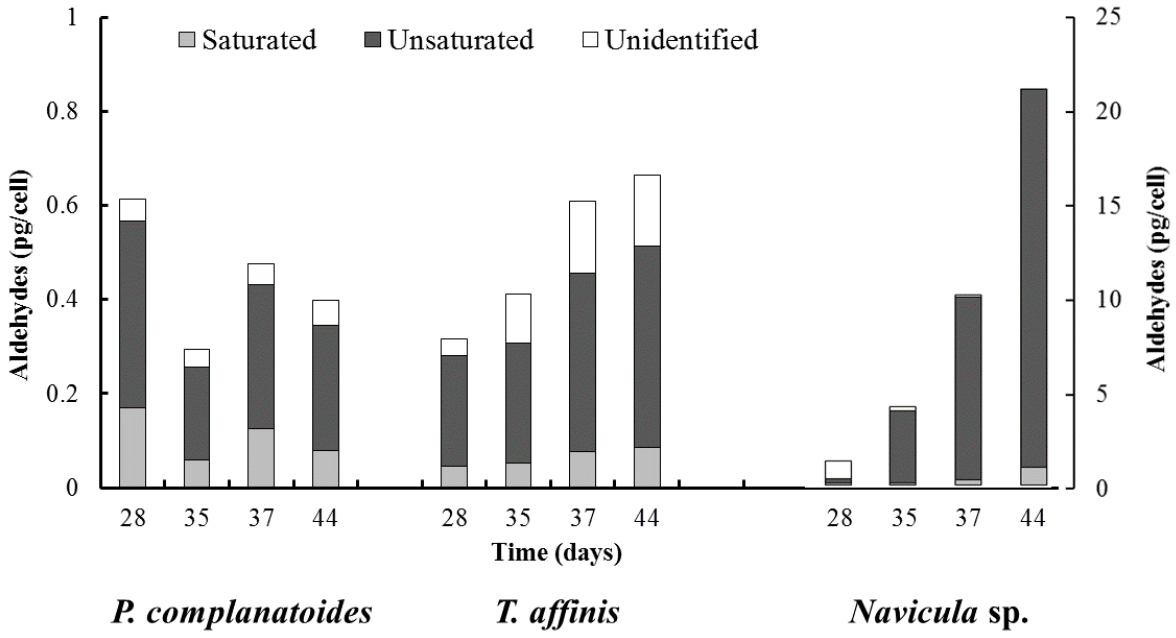

Figure 4. Aldehydes production of *Proschkinia complanatoides*, *Tabularia affinis* and *Navicula* sp. at different days (28, 35, 37, 44). Data are means of independent replicates (n = 2) and the error bars represent standard deviations.

The analysis of PUAs composition revealed a production mainly composed by medium-chained compounds for *Navicula* sp (Fig. 5c). Although several molecules such as of esadienal (C6:2), decatretraenal (C10:4), undecatetraenal C11:4 and undecapentaenal C11:5 were detected, octenal (C8:1) represented the most important molecule (93.8 %) corrisponding to 18.40 ± 3.34 pg/cell (173.38 ± 31.50 pmol/cell). On the contrary, the PUAs composition of the other two species pointed out a certain variability, but resulted similar among the two diatoms: esadienal (C6:2) contributed up to 50.56 % and 39.04 %, for *T. affinis* and for *P. complanatoides*, respectively (Fig. 5a,b). Decatretraenal (C10:4), that represented the second important PUA in these diatoms, reached the maximum values of 0.09 ± 0.01 pg/cell (0.86 ± 0.12 pmol/cell) for *T. affinis* and 0.13 ± 0.12 pg/cell (1.25 ± 1.10 pmol/cell) for *P. complanatoides* at day 44 and 28, respectively.

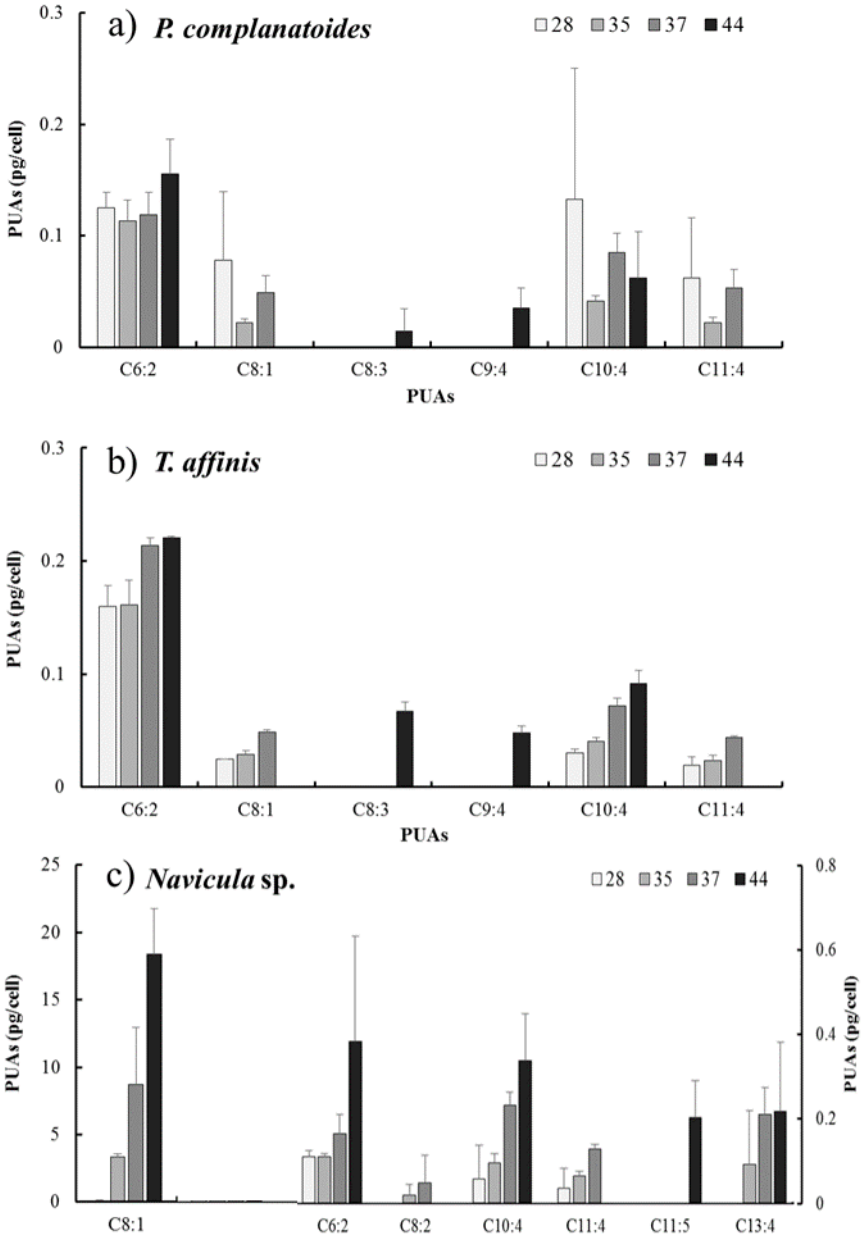

Figure 5. Polyunsaturated aldehydes production (a) *Proschkinia complanatoides*, (b) *Tabularia affinis* and (c) *Navicula* sp at different days (28, 35, 37, 44). Data are means of independent replicates (n = 2) and the error bars represent standard deviations.

2.4 Discussion

This study pointed out for the first time the production of polyunsaturated aldheydes (PUAs) by three common benthic diatoms of the northern Adriatic sea (Accoroni et al., 2016). Moreover, the quantification and characterisation of the fatty acid profiles of the same species were performed along the growing period.

In our study, the intracellular PUAs concentrations ranged from 0.29 and 20.44 pg/cell. Such values are from 2 to 4 orders of magnitude higher respect to those reported in several laboratory studies (Dittami et al., 2010; Gerecht et al. 2011), which, however, are referred to a natural release. In other words, our values reflect to the PUAs potential release of the three benthic diatoms. A similar difference between intracellular and released PUAs was also observed in field by a PUAs surface mapping performed in the Adriatic Sea, where the dissolved concentrations never reached those liberated from broken cells (Vidoudez et al., 2011).

Considering the PUAs profile of species tested, no traces of C7-chains were detected in each of three diatoms along the entire growth phase, though this compound represents one of the most common PUAs released by planktonic diatoms (Wichard et al., 2005). Similarly, most of the studies were focused on the effects of two other PUAs (octadienal and decadienal), often reported as main compounds in other algal species (e.g. *Skeletonema costatum*, D'Ippolito et al., 2004), while in the present study these molecules were not observed, except for a low amount of octadienal in *Navicula* sp. Our result highlight that there are several PUAs that could be produced and released by diatoms, particularly some with four unsaturations such as decatetraenal and undecatetraenal.

The fatty acids analysis performed on the three species revealed a molecules composition mainly constituted by myristic and palmitic acid (C14:0 and C16:0) as saturated acid, and C16-chains and eicosapentaenoic acid (C20:5, EPA) as unsaturated acids, while fewer stearic (C18:0) and other C18 fatty acids were detected. These results appear consinstent with the fatty acid profile observed in numerous studies conducted on diatom species or microalgae in general (Jüttner, 2001; Medina et al., 1998; Wichard et al., 2007). Dunstan et al. (1994) described EPA as prominent PUFA in 14 diatom

species and high amounts of palmitoleic (C16:1) acids were measured in 28 microalgae by other authors (Viso and Marty, 1993). On the contrary, although hexadecatetraenoic (16:4) acid was largerly and mainly identified in diatoms, no traces of this compound were observed in each of the diatoms tested. Starting from the exponential phase, the fatty acids are always present along the growth curve and their concentrations did not show significant variation among the diverse culture stages. This result is in accourdance with that highlighted by D'Ippolito et al. (2009) in *Pseudo-nitzschia delicatissima* cultures, but in contrast with other studies that descibed a significant increase of EPA during the stationary phase (Stonik and Stonik, 2015). Despite this presence of PUFAs in each growth phases (except for the lag one) and their decisive function in the PUAs metabolic pathway as precursors, the aldehydes production clearly occurred only during the stationary and decay growth phases in each of three diatoms tested. This discrepancy could be due to the inactivation state of enzymes essential for the catalyst of the biochemical trasformations. The crucial role of the enzyme activity for PUA chemistry was previously suggested by other authors (Ribalet et al., 2007) and would explain the contrast between the comparable PUFAs amounts of *Navicula* sp. and *T. affinis* and the great difference of the respective amounts of PUAs produced. Although the use of stable isotope labeled PUFAs was not performed in the present study, it is possible to presume some relationships between PUFAs and PUAs produced. In particular, the production of C8 and C6-chains from C16 acids and C10-chains from EPA appears consistent with the results showed by studies permorfed with radioactive precursors (D'Ippolito et al., 2006).

The outcomes of this study acquire greater significance if we consider the importance of the diatoms in the coastal benthic ecosystems especially in shallow coastal areas where contribute considerably to the total primary production (Rousseaux and Gregg, 2014). For example Accoroni et al. (2016), investigating the composition and temporal variation of the microphytobenthos communities of the Conero Riviera (northern Adriatic Sea), described the diatoms as the main group, in terms of abundances and biomass, for most of the annual cycle except the summer. Besides to represent a chemical defense towards the grazing by copepods (Kâ et al., 2014), sea urchins (Romano et al.,

2010) and other microzooplancton organisms, the PUAs produced by the benthic diatoms could affect also the dynamic population of co-occurring microalgae, including toxic bloom-forming species. However, further studies are needed to clarify the role of these secondary metabolites in the structure of the microphytobentic communities.

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CHAPTER 3. Inhibitory effect of polyunsaturated aldehydes (PUAs) on the growth of the toxic benthic dinoflagellate *Ostreopsis* cf. *ovata*

(Pichierri, S., Pezzolesi, L., Vanucci, S., Totti, C., Pistocchi, C., 2016. Inhibitory effect of polyunsaturated aldehydes (PUAs) on the growth of the toxic benthic dinoflagellate *Ostreopsis* cf. *ovata*. Aquat. Toxicol. 179, 125–133)

3.1 Introduction

The production of secondary metabolites has been reported for several classes of algae (Alsufyani et al., 2014; Ianora et al., 2012; Leflaive and Ten-Hage, 2007), vascular plants (Karuppusamy, 2009), fungi (Wang et al., 2015), invertebrates (such as sponges, hydroids and mollusks) (Bell, 2008; Macintyre et al., 2014) and fish (Kyoung Kang et al., 2015). Their multiple functions and bioactive role in chemical defense (antigrazers, antibacterial and allelopathy) have been reflected by an increasing interest of researchers towards these molecules (Agostini-Costa et al., 2012; Ianora et al., 2011; Ianora et al., 2012). Although these compounds are not directly involved in the primary metabolism of organisms, their production and release can play a crucial role in the species succession, affecting the ecology and evolution of communities of aquatic ecosystems (Takano et al., 2003; Vardi et al., 2002).

Regarding the algae, they were found to produce several secondary metabolites, such as phenolic compounds, alkaloids, peptides, oxoacids, polyketides (among which there are toxins synthesized by a number of species, Agostini-Costa et al., 2012; Pistocchi et al., 2012; Snyder et al., 2003), polyunsaturated fatty acid (PUFAs) (Grima et al., 1995; Patil et al., 2007; Tonon et al., 2002) and polyunsaturated aldehydes (PUAs) (Ianora et al 2012). PUAs derive from enzymatic degradation of PUFAs and, despite the fact that diatoms are considered as the principal PUA-producers, the synthesis of these compounds is widespread in several freshwater and marine classes of phytoplankton, such

as cyanobacteria, cryptophytes, prymnesiophytes, chrysophytes and synurophytes (Ianora et al., 2012). During the last decades, the specific production and the amount of PUAs released per cell by planktonic diatoms has been widely investigated in culture conditions and in the field (Gallina et al., 2014; Ribalet et al., 2007b, 2009, 2014; Wichard et al., 2005). Wichard et al. (2005) observed that 36% of the 51 investigated diatom species released aldehydes showing a strain-specific production with a wide range of concentrations (0.01 to 9.8 fmol per cell).

As observed also for macroalgae (e.g., *Ulva* spp., Alsufyani et al., 2014), the release of PUAs by microalgae (e.g., diatoms, haptophytes, Hansen and Eilertsen 2007; Wichard et al., 2005) in surrounding waters occurs only after mechanical distruption of cells, because only in that moment lipase enzymes bind its substrate leading to the immediate activation of the degradation of fatty acids to PUAs and other compounds altogether termed oxylipins (Pohnert, 2000).

The fact that the release of these molecules is wound-activated was confirmed in field from Ribalet et al. (2014), who showed a positive correlation between cell lysis and concentration of dissolved PUAs in the northern Adriatic Sea. The same Authors observed the highest concentrations of PUAs (2.53 nM) at the end of a diatom bloom and reported a very high spatial and temporal level's variability determined by different physiological and environmental variables such as physico-chemical parameters, growth phase (Ribalet et al., 2007b) and self-regulatory mechanisms (Vardi et al., 2006).

The temperature seems to be a crucial factor for the stability and persistence of PUAs once they are released in the surrounding environment (Bartual & Ortega, 2013). Considering the three aldehydes widely used as model compounds in allelopathy and toxicity assays (i.e., 2*E*,4*E*-decadienal, 2*E*,4*E*-octadienal and 2*E*,4*E*-heptadienal), a negative correlation between temperature and persistence of these molecules has been reported, and the 2*E*,4*E*-decadienal results perfectly stable at low temperature (10 °C) also after 200 h (Bartual & Ortega, 2013).

Considering the effects of PUAs, several deleterious consequences have been so far described for both freshwater and marine organisms, such as benthic and planktonic invertebrates (Carotenuto et

al., 2006; Hansen et al., 2004; Ianora and Miralto 2010; Juttner et al., 2005), bacterial community (Amin et al., 2012; Paul et al., 2012), and photosynthetic organisms (Casotti et al., 2005; Ribalet et al., 2007a).

In particular, the teratogenic effect of PUAs on zooplankton grazers highlights the diatom–copepod paradox that refers to the less hatching success when copepods feed on diatoms (Ianora et al., 2003). Ribalet et al. (2007a) have investigated the effects of three diverse PUAs on six species of microalgae of different taxonomic groups and shown that exposure to these compounds caused a clear reduction in the growth rate, changes in flow cytometric measures, cell granulosity and membrane permeability even at low concentrations.

However, no information is available on the effect of PUAs on harmful microalgae able to develop massive and widespread blooms and to produce toxins as are the species of the *Ostreopsis* genus (Ribalet et al., 2007a).

Ostreopsis cf. *ovata* is a toxic benthic dinoflagellate able to colonize numerous susbstrata (rocks, macrophytes, seagrasses, invertebrates and soft sediments) and to produce bloom events in worldwide coastal areas (Rhodes, 2011). During the last ten years, these phenomena have been observed along the Mediterranean coasts, including the northern Adriatic Sea, causing mass mortalities of benthic marine organisms, human intoxications and economic repercussions (Accoroni & Totti, 2016).

So far, the relationships of *Ostreopsis* bloom with several environmental parameters have been deeply investigated (Aligizaki and Nikolaidis, 2006; Cohu et al., 2013; Mabrouk et al., 2011; Mangialajo et al., 2011; Pistocchi et al., 2011; Shears and Ross, 2009; Selina et al., 2014; Totti et al., 2010; Vila et al., 2001) showing that low hydrodynamic conditions, a water temperature threshold of 25 °C, a N:P ratio around Redfield value seem to promote the occurrence of blooms (Accoroni et al., 2015a).

On the contrary, the interactions with other algal species are poorly known. Accoroni et al. (2015b) investigated the interactions between three macrophytes (*Dictyota dichotoma, Rhodymenia pseudopalmata* and *Ulva rigida*) and *O.* cf. *ovata*, highlighting that all these seaweed species exerted negative effects toward the *O.* cf. *ovata* growth, probably due to the production of allelopathic

compounds. Few studies considered the effects of *O*. cf. *ovata* culture on the growth of other dinoflagellates (*Prorocentrum minimum*, *P. lima* and *Coolia monotis*) revealing low allelopathic effects (Monti and Cecchin 2012; Pezzolesi et al., 2011). On the contrary, a field study on natural microphytobenthic community, showed that blooms of *O*. cf. *ovata* seem to not exert any negative effect on benthic diatom community (Accoroni et al., 2016).

The aim of this work was to increase our knowledge on allelopathic compounds with possible inhibitory effects on an organism whose proliferations cause negative impacts on the ecosystem; we thus investigated the effect of the most commonly studied PUAs (2*E*,4*E*-decadienal, 2*E*,4*E*-octadienal and 2*E*,4*E*-heptadienal) on growth, cell morphology and cytological features of *O.* cf. *ovata* in culture.

3.2 Materials and methods

Cultures of Ostreopsis cf. ovata

The strain of *Ostreopsis* cf. *ovata* used in this study was OOAB0801 isolated by the capillary pipette method (Hoshaw and Rosowski, 1973), using an inverted microscope from water samples collected nearby Bari (Puglia region, Italy) in 2001.

Monoclonal cultures were set up using multi-well plates, then trasferred in flasks and cultured at 20 °C under a 16:8 L:D photoperiod and an irradiance of 100-110 µmol m^{-1} s^{-1} in five-fold diluted f/2 salts concentration (Guillard, 1978).

Experimental design

The three PUAs (2*E*,4*E*-decadienal, 2*E*,4*E*-octadienal and 2*E*,4*E*-heptadienal) were purchased from Sigma-Aldrich (Milano, Italy) and diluted with absolute methanol at room temperature in order to obtain the working solutions.

The experiment was conducted in two biologically independent replicates, inoculating five concentrations (3, 6, 9, 18 and 36 µmol L^{-1}) of each of the three PUAs in 50 mL of *O*. cf. *ovata* culture (initial cell density 240 cells mL^{-1}) at exponentially growth phase in 100 mL flasks. Methanol was also added to control flasks in concentrations (5 µl MeOH/50 mL) not exceeding the threshold value affecting growth (7 µL 100% methanol mL^{-1} of culture, Ribalet et al., 2007a) as for experimental flasks. For each sample, two subsamples (5 mL) were collected after 48 and 72 h, after mixing the flask by gently shaking. The first one was preserved by adding 0.8% neutralized formaldehyde and used for countings, morphometric and nuclear observations (microscopy), while the second one (unfixed) was immediately used for lipid content analysis (microscopy) and modulated fluorescence measurements (PAM).

Light microscopy analyses

Fixed subamples were homogenized and 2.5 mL were settled in an Utermöhl chamber. Countings and measurements were carried out at the inverted light microscope (Zeiss Axiovert 135) equipped with phase contrast and epifluorescence, at 200x magnification, by differentiating aberrant from normal morphotypes. The cell dorsoventral diameter (DV) and the transdiameter (W) were measured through a micrometric ocular.

In order to observe the integrity of nucleus, after counting, the green-fluorescing SYBR Green I (Molecular Probes; dilution 1:2000) was added in the Utermöhl chamber; sample was left in the dark at room temperature for 15 minutes and then observed at the inverted microscope in epifluorescence light using the barrier filter LP 520 (blue excitation).

The analysis of the lipid content was performed on 1 mL of unfixed subsample settled in an Utermöhl chamber, after staining with Nile Red (dilution 1:100 of a stock solution - 1 mg Nile Red/mL acetone), under the inverted microscope with a filter block (excitation filter BP 450–490, dichroic beam splitting mirror FT 510, barrier filter LP 520).

Both observations in epifluorescence allowed to detect also the red autofluorescence of the chlorophyll.

Modulated fluorescence measurements

Efficiency of photosystem II of *O.* cf. *ovata*, exposed to different concentrations of the three aldehydes, was evaluated with a pulse-amplitude modulated (PAM) fluorometer. The model used consisted in: 101-PAM (H. Walz, Effeltrich, Germany) connected to a PDA-100 data acquisition system, high power LED Lamp Control unit HPL-C and LED-Array-Cone HPL-L470 to supply actinic light and saturated pulses, US-L655 and 102-FR to provide measuring light and far red light, respectively.

Samples (3 mL) were analyzed in cuvettes (10x10 mm) mounted on an optical unit ED-101US/M. Measurement of the photosynthetic efficiency was derived from the maximum quantum yield of PSII (Φ_{PSII}) (Bolhar-Nordenkampf and Oquist, 1993) and effective quantum yield of PSII (Φ'_{PSII}) (Genty et al., 1989), calculated as:

$$\Phi_{PSII} = \frac{F_m - F_0}{F_m} = \frac{F_V}{F_m}$$

$$\Phi'_{PSII} = \frac{F'_m - F}{F'_m} = \frac{\Delta F}{F'_m}$$

where all parameters were given by induction curve measurements. The minimal fluorescence (F_0) was measured on 20 min dark-adapted cultures, by using modulated light of low intensity (2 µmol m^{-2} s^{-1}). Then, a short saturating pulse of 3000 µmol m^{-2} s^{-1} for 0.8 s induced the maximal fluorescence yield (F_m). Changes of the maximal fluorescence yield (F'_m) were induced on light adapted cultures by saturating flashes given periodically every 60 s. The steady-state value of fluorescence immediately prior to the flash is termed F.

Data analysis

The acute toxicity of PUAs was reported as half maximal effective concentration (EC_{50}), which is the concentration of PUA inducing a reduction of 50% in *O*. cf. *ovata* growth respect to the control, after 48 or 72 h exposure to 2*E*,4*E*-decadienal, 2*E*,4*E*-octadienal and 2*E*,4*E*-heptadienal.

The EC_{50} of each aldehyde was estimated by fitting the experimental concentration-response curves to a logistic model:

$$y = bot + \frac{(top - bot)}{(1 + (x/EC_{50})^c)}$$

Where: y = endpoint value; x = aldehyde concentration; *bot* = expected endpoint value when the concentration of the aldehyde is infinite (bottom asymptote); *top* = expected endpoint value when the concentration of the aldehyde is zero (top asymptote); c = slope parameter.

The parameters of the equation, including the EC_{50}, were estimated using the non-linear regression procedures implemented in Statistica (Statsoft, Tulsa, OK, USA). The same equation was used to calculate the value of statistical no effect concentration (SNEC) and the "slope" (*sensu* Ribalet et al. 2007a) that represents the responsiveness of *O*. cf. *ovata* to increasing PUA concentrations.

The differences in the cell dimensions, EC_{50} values and in the photosynthetic efficiency were evaluated by analysis of variance (ANOVA). Whenever a significant difference for the main effect was observed ($P<0.05$), a Tukey's pairwise comparison test was also performed.

3.3 Results

Effect of PUAs on Ostreopsis *cf.* ovata *growth*

Figure 1 shows the percent growth, normalized to the control (untreated), of *O.* cf. *ovata* cells after 48 h and 72 h exposition to different concentrations of three different PUAs.

The results show a marked decrease of *O.* cf. *ovata* cell yields when grown in the presence of all PUAs; in particular, 2*E*,4*E*-decadenial and 2*E*,4*E*-octadenial caused a higher inhibition than 2*E*,4*E*-heptadienal. The latter, after 48 h, determined 33 ± 9% growth inhibition at the highest tested concentrations, contrarily to the cultures exposed to 2*E*,4*E*-decadenial and 2*E*,4*E*-octadenial where inhibition reached maximum values of 94 ± 2 and 77 ± 1%, respectively. In addition, a concentration-dependent decrease of the culture growth was observed for each tested PUAs, being more marked for long-chained molecules. After 72 h of exposition, the growth decrease (respect to the control), at the maximum concentration tested, was 92.4 ± 4%, 81.5 ± 3% and 48.6 ± 3% for 2*E*,4*E*-decadienal, 2*E*,4*E*-octadienal and 2*E*,4*E*-heptadienal, respectively. The same effects were confirmed by the EC_{50}, SNEC and "slope" values obtained from the growth curve. The EC_{50} values, calculated at 48 h, resulted significantly higher for 2*E*,4*E*-octadienal (17.9 ± 2.6 µmol L^{-1}) than for 2*E*,4E-decadienal (6.6 ± 1.5, ANOVA, $p < 0.05$); whereas the EC_{50} value for 2*E*,4*E*-heptadienal, due to the lower toxic effect, was obtained only after 72 h, being 18.4 ± 0.7 µmol L^{-1}. The SNEC values were 3.3, 12.2, and 16.7 µmol L^{-1} for 2*E*,4*E*-decadienal, 2*E*,4*E*-octadienal and 2*E*,4*E*-heptadienal, respectively; the responsiveness of *O.* cf. *ovata* to the increase of PUAs concentrations (i.e., the 'slope') was quite similar for the 2*E*,4*E*-decadienal and 2*E*,4*E*-octadienal (1.7 and 4.3 respectively), while a higher value was determined for 2*E*,4*E*-heptadienal (32.8) (Table 1).

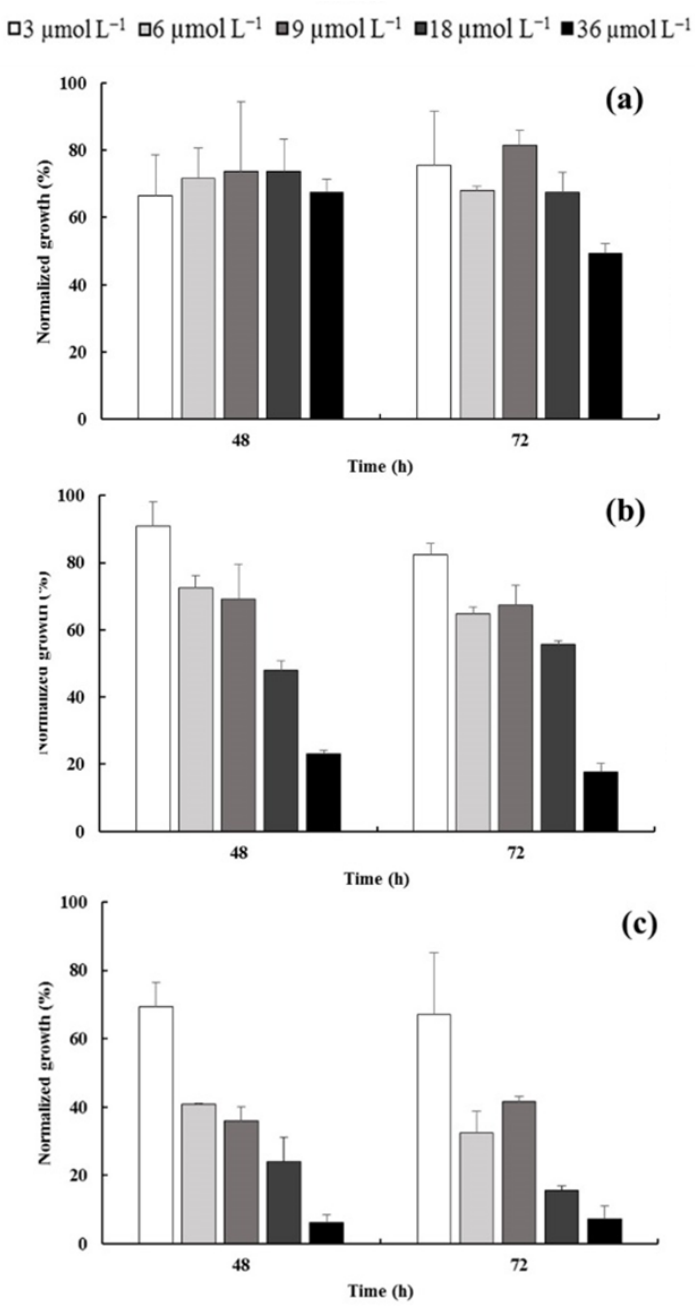

Figure 1. Effect of different PUA concentrations (3, 6, 9, 18, 36 µmol L^{-1}) on *Ostreopsis* cf. *ovata* growth (in percentage respect to the controls) after 48 and 72 h: (a) 2*E*,4*E*-heptadienal; (b) 2*E*,4*E*-octadienal; (c) 2*E*,4*E*-decadienal. Data are means of independent replicates (n = 2) and the error bars represent standard deviations.

Table 1. EC_{50} values (µmol L^{-1}) for 2*E*,4*E*-heptadienal (72h), 2*E*,4*E*-octadienal (48h) and 2*E*,4*E*-decadienal (48h) and statistical no effect concentration (SNEC, µmol L^{-1}) and "slope" of *Ostreopsis* cf. *ovata* growth curve exposed to the three PUAs.

	2*E*,4*E*-heptadienal	**2*E*,4*E*-octadienal**	**2*E*,4*E*-decadienal**
EC_{50} (µmol L^{-1})	18.4 ± 0.7	17.9 ± 2.6	6.6 ± 1.5
SNEC (µmol L^{-1})	16.7	12.2	3.3
Slope	32.8	4.3	1.7

Microscopy analysis

The morphological analysis of *O.* cf. *ovata* cells revealed the presence of aberrant forms only in the cultures exposed to the highest concentrations of 2*E*,4*E*-decadienal (i.e., 9, 18 and 36 µmol L^{-1}). In particular, these forms appeared motionless and showed a significant decrease in dimensions (31 ± 5 µm (DV) and 24 ± 6 µm (W) respect to 43 ± 5 µm (DV) and 34 ± 5 µm (W) of the control, ANOVA, $p < 0.05$), a contraction of cytoplasm and the formation of abnormal vesicle-like structures (Fig. 2c). No abnormal forms were observed in the cells exposed to any concentrations of 2*E*,4*E*-octadienal and 2*E*,4*E*-heptadienal. Taking into account the percentage of the abnormal forms respect to the total cells counted, the highest values (79 ± 2%) were observed after 48 h at 36 µmol L^{-1} of 2*E*,4*E*-decadienal. After 72 h, the percentages decreased in each of three concentrations tested and some dead cells were observed (Fig. 2a).

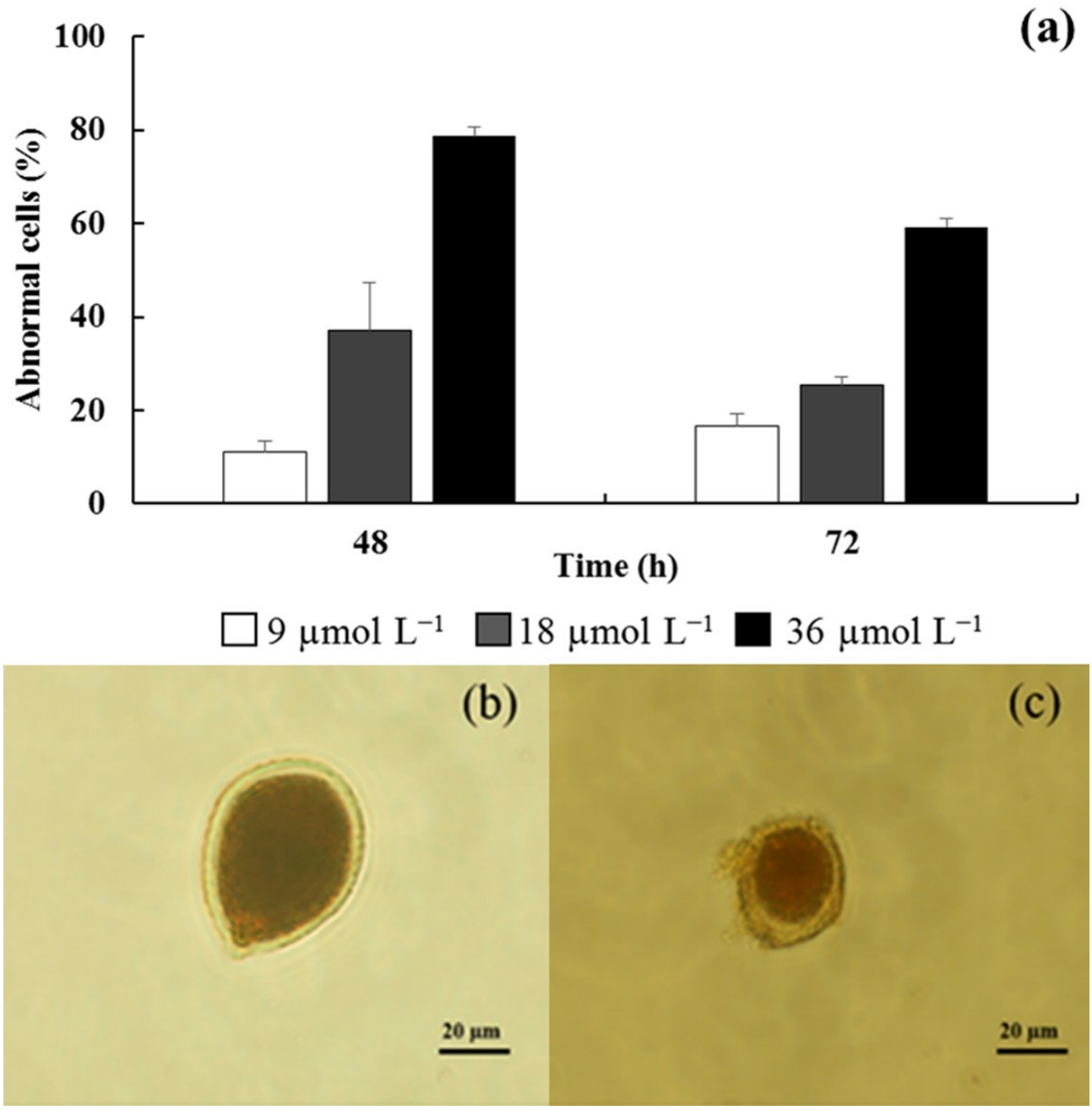

Figure 2. Morphological analysis of *Ostreopsis* cf. *ovata* under light microscopy. (a) Percentage of abnormal forms in cells exposed to 9, 18, 36 µmol L^{-1} of 2*E*,4*E*-decadienal after 48 and 72 h. (b) Light micrograph of a normal shaped cell. (c) Light micrograph of an abnormal shaped cell. Data are means of independent replicates (n = 2) and the error bars represent standard deviations.

Morphological observations under epifluorescence allowed to determine cell integrity and to analyze some cytological features. Untreated cells exhibited the majority of the DNA circumscribed in the nucleus, which was located in the dorsal part of the cell, very small green fluorescent drops representing chloroplast DNA and several yellow fluorescent lipid droplets in the peripheral cytoplasm (Fig. 3).

In cells exposed to PUAs, several modifications were observed, already after 48 h. The highest concentrations of 2*E*,4*E*-decadienal caused a clear DNA damage resulting in the chromatin dispersion to the outlying cytoplasm and in the lack of red autofluorescence of the chlorophyll. Lipid bodies, which in the control had a diameter of 100-300 nm, appeared larger until almost occupying the whole cell.

Although less intense, deleterious effects were visible also with the other two aldehydes tested: in *O.* cf. *ovata* exposed to 2*E*,4*E*-octadienal or 2*E*,4*E*-heptadienal, the nucleus appeared larger with an irregular shape and lipid droplets became more numerous and larger.

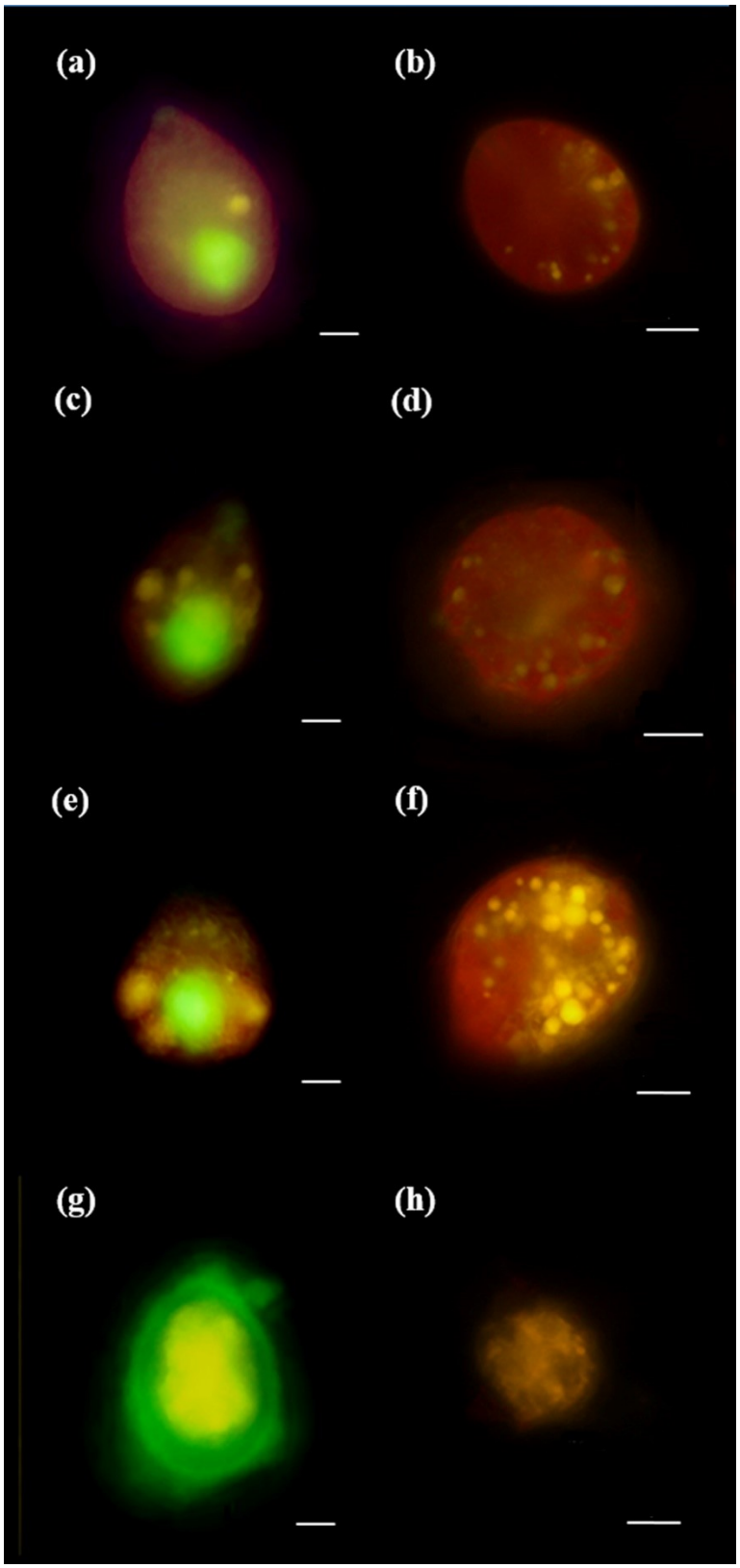

Figure 3. Observations of *Ostreopsis* cf. *ovata* under epifluorescence microscopy: (a,b) untreated cells; (c-h) cells exposed for 72 h to 36 µmol L^{-1} of (c,d) 2*E*,4*E* heptadienal, (e,f) 2*E*,4*E*-octadienal and (g,h) 2*E*,4*E*-decadienal. The left panels show cells stained with SYBR Green I (green fluorescence) while the right ones show cells stained with Nile Red (yellow fluorescence). Scale bar: 10 µm.

Modulated fluorescence measurements

The maximum (Φ_{PSII}) and effective (Φ'_{PSII}) quantum yields of photosystem II were evaluated for the control and *Ostreopsis* cf. *ovata* cultures exposed to the different concentrations (0-36 µmol L^{-1}) of each aldehydes, after dark adaptation or light exposure, respectively. After 72 h, Φ_{PSII} and Φ'_{PSII} of the controls were 0.23 and 0.19, respectively, while the values measured in presence of the three PUAs were significantly lower at the highest tested concentrations (18 and 36 µmol L^{-1}) respect to the controls (Fig. 4) (ANOVA, $p < 0.05$). The decrease in photosynthetic efficiency was concentration-dependent for each aldehyde, determining values of 0.096-0.152 and 0.081-0.131 for Φ_{PSII} and Φ'_{PSII}, respectively. The maximum and effective quantum yields showed different pattern upon exposure to the different compounds: cultures exposed to 2*E*,4*E*-decadienal reported the lowest effective quantum yields, particularly at the highest concentrations, while maximum quantum yields decreased more gradually in the presence of the intermediate concentrations of this compound respect to what observed with the other two PUAs (Fig. 4).

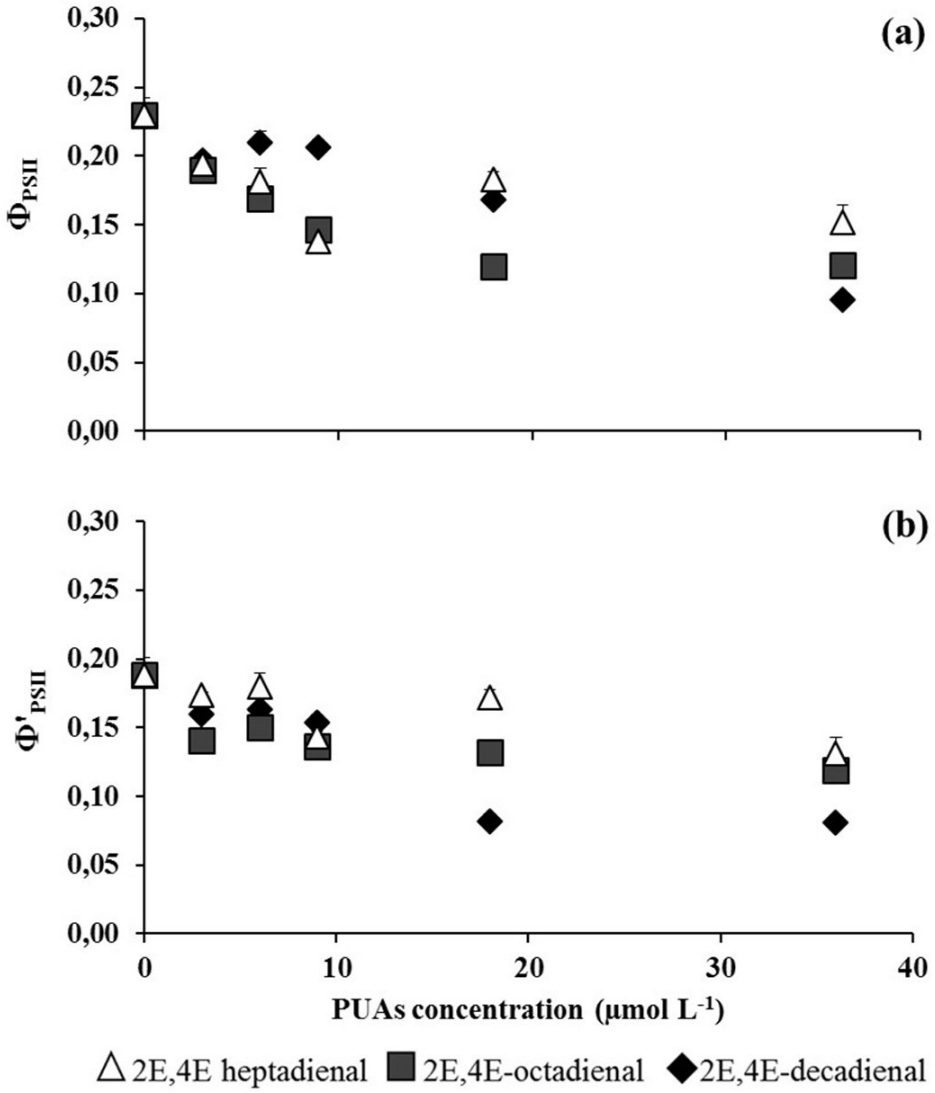

Figure 4. Effect of the three PUAs (2*E*,4*E* heptadienal, 2*E*,4*E*-octadienal, 2*E*,4*E*-decadienal) at different concentrations (3–36 µmol L^{-1}) on (a) maximum and (b) effective quantum yields of *Ostreopsis* cf. *ovata* photosystem II after 72 h respect to the control (concentration = 0). Data are means of independent replicates (n = 2) and the error bars represent standard deviations.

Photosynthetic efficiency measured in the presence of 2*E*,4*E*-decadienal at the two highest concentrations (36 and 18 µmol L^{-1}, Fig. 5) resulted significantly lower (about 60%, ANOVA, $p < 0.05$) already after 48 h respect to the control; the decrease was even more marked (43-70%) after 72 h for all the cultures exposed to the highest concentrations of aldehydes (18 and 36 µmol L^{-1}), while photosynthetic efficiency maintained a constant value (90-95%) for cells exposed to 18 µmol L^{-1} of 2*E*,4*E*-heptadienal. Generally, in cultures exposed to 2*E*,4*E*-decadienal, the PSII efficiency showed

significantly lower values (0.096 for ΦPSII at 36 µmol L^{-1}) respect to the control (0.23 for ΦPSII) (ANOVA, $p < 0.05$).

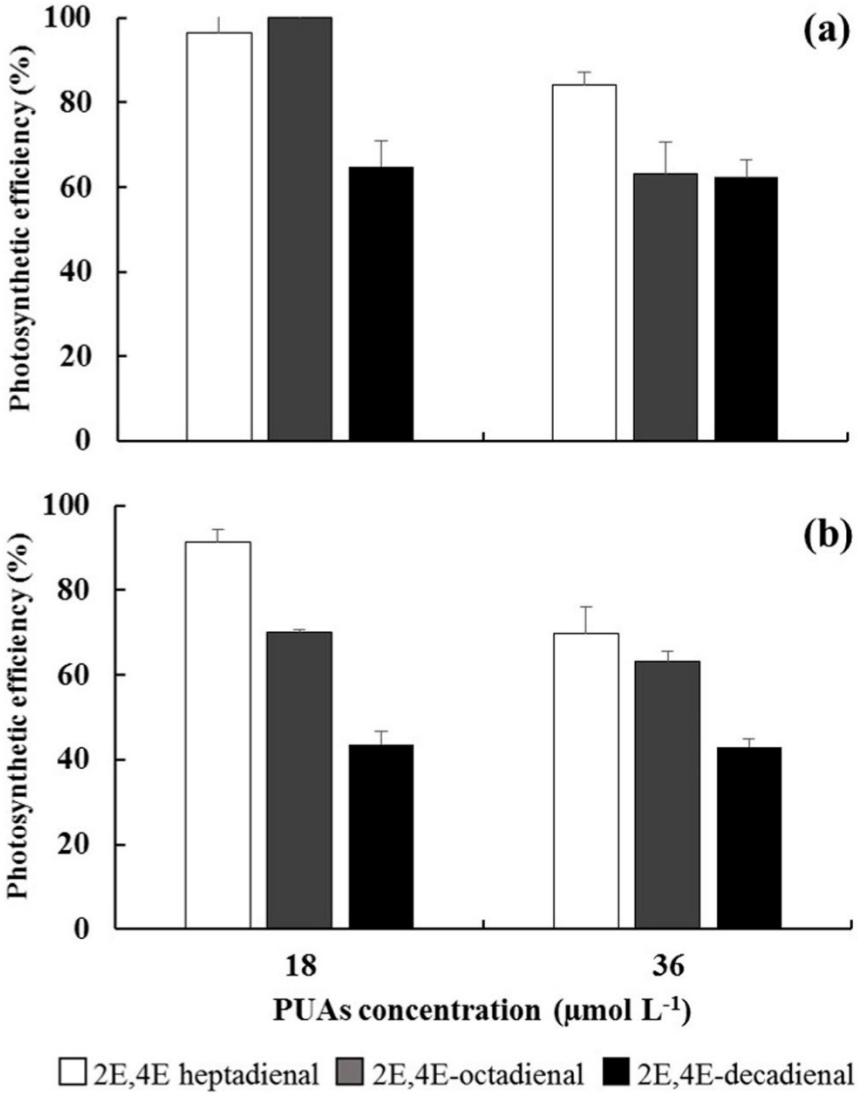

Figure 5. Photosynthetic efficiency, normalized respect to the controls, of *Ostreopsis* cf. *ovata* cells exposed to 36 and 18 µmol L^{-1} of 2*E*,4*E*-decadienal, 2*E*,4*E*-octadienal, 2*E*,4*E* heptadienal after (a) 48 h and (b) 72 h. Data are means of independent replicates (n = 2) and the error bars represent standard deviations.

3.4 Discussion

The exposure of *Ostreopsis* cf. *ovata* to three polyunsaturated aldehydes induced a marked decrease of growth even at low doses, and several modifications of important cytological features.

The growth inhibition varied considering the type and concentration of aldehyde molecules. Although a concentration-dependent decrease of growth was observed for each of the three PUAs tested, the effect was stronger with longer-chained molecules (i.e. 2*E*,4*E*-decadienal) than with shorter-chained ones. In fact, intermediate concentrations (9 and 18 μmol L^{-1}) of 2*E*,4*E*-decadienal caused a higher inhibition respect to the ones reported for the other shorter-chain molecules, even at the highest concentrations.

Our results agree with those obtained both in bioassays, that showed a positive relationship between length and biological activity of molecule (Adolph et al., 2003), and in toxicological studies performed on phytoplankton and invertebrate species (Hansen et al., 2004; Ribalet et al., 2007a).

In this study, the values of EC_{50}, SNEC and "slope" confirmed the strongest negative effect of longer chains, although their values were higher than those reported by Ribalet et al. (2007a) for other phytoplankton species exposed to the same molecules, highlighting that *Ostreopsis* seems to be less affected by these molecules than other microalgae. Taking into account only the statistical parameters referred to 2*E*,4E-decadienal, the EC_{50} and SNEC values for *O.* cf. *ovata* were about twice respect to those measured by Ribalet et al. (2007a) for *Skeletonema marinoi*, which in that study represented the species less affected by the exposure to the PUAs. This difference could be partially explained by cytological and/or metabolic features of *O.* cf. *ovata*; in fact, the uptake of PUAs appears to depend on several factors, such as cell size, cell wall properties and total lipid content. In particular, it was reported that species with a mineralized or highly structured cell wall, a low surface-volume ratio and lipid content can limit the ability of these lipophilic compounds to diffuse across lipid membranes (Hutchinson et al., 1980; Ribalet et al., 2007a). The presence of a rigid outer cell covering and the high cell volume could explain the apparent lower sensitivity of *O.* cf. *ovata* to the aldehydes respect to other phytoplankton species. In addition, it is important to point out that a phase-dependent

susceptibility to PUAs has been previously reported (Leflaive & Ten-Hage, 2009; Ribalet et al., 2007b), as algal cells in the stationary phase resulted more sensitive than young cells. *O*. cf. *ovata* cells might thus be more affected by the presence of PUAs, expecially at a late stage of the long lasting blooms which are tipical of this dinoflagellate in temperate areas (Mangialajo et al., 2011).

Morphological analysis conducted by light microscopy highlighted several cytological anomalies only in the cells exposed to the highest concentrations of 2*E*,4*E*-decadienal. In fact, a decrease of cell size and motility reduction were not observed in algal cells exposed to the other two molecules tested. On the contrary, observations of cells stained with specific fluorescent dyes highlighted a gradual DNA degradation and the increase of lipid droplets not only in presence of 2*E*,4*E*-decadienal but with all tested PUAs. The ability of these compounds to cause DNA fragmentation and variations in the cellular glutathione level was described both in marine animals, such as copepods and sea urchin embryos (Varella et al., 2014), and microalgae species (Ribalet et al., 2007a). The genotoxicity of these molecules is related to their ability to bind to nucleic acids and proteins and to create numerous covalent adducts (Carvalho et al., 2000). Moreover, an irreversible blockage of cells in G1-phase of the cell cycle has been observed by Casotti et al. (2005) in *Thalassiosira weissflogii* exposed to 2*E*,4*E*-decadienal, preventing the DNA transcription necessary for the synthesis of structural components.

The significant expansion of cytoplasmic lipid bodies that we observed in cells exposed to PUA molecules agrees with what described by Honsell et al. (2013) during the stationary growth phase of *O*. cf. *ovata*, suggesting that an increase of lipid content could be interpreted as a response to environmental stress (such as those caused by heavy increase/decrease of temperature and nutrients), as reported for other microalgae and also vascular plants (Chen et al., 1999; Haywood, 1974; Murphy, 2001; Wang et al., 2009).

Modulated fluorescence measurements in the controls pointed out the typical effective and maximum quantum yields values reported during *O*. cf. *ovata* exponential growth phase (Pezzolesi et al., 2016), while a significant reduction of the photosynthetic activity was observed in cultures exposed to all

three molecules tested. Although the cells were exposed to each PUA for only 72 h, it was possible to observe a progressive decrease of photosynthetic efficiency respect to the control, confirming a leak of the photopigments and the consequent decline of chlorophyll autofluorescence. These results suggest that in absence of DNA repair mechanisms, these cytological anomalies could lead to the inhibition of *O.* cf. *ovata* cell proliferation, even if low doses of PUAs are present, raising concern on the potential effects of these compounds in the marine environment.

The PUAs concentrations used in our experiments are similar to those used in other studies (Ka et al., 2014; Hansen et al., 2007; Ribalet et al. 2007a, 2008; Romano et al., 2010) and were chosen in order to mimic the conditions likely occurring in seawater during and after a diatom bloom. The concentrations used in our experiments were 1-2 orders of magnitude higher than those measured in field (see also Lavrentyev et al. 2015). PUAs measurements in seawater samples collected in the Passetto station (Conero Riviera), a coastal area where *Ostreopsis* blooms are recurrent (Accoroni and Totti, 2016), revealed concentrations ranging from 0.14 to 0.21 μmol l^{-1} in the period before *Ostreopsis* bloom (Pezzolesi, unpublished data). Following Ribalet et al. (2007a), we considered that the real amount of PUAs in seawater is a function of the distance between the producer and target species, and, when cell lysis takes place as occurs after massive diatom bloom, the local PUAs concentrations in the immediate surroundings of each single producer cell can reach up to 1.25 μM. Moreover, we considered the values used in our experiments quite realistic considering that in field conditions: (i) rapid settling of planktonic PUA-producing diatoms at the end of massive blooms could potentially result in their accumulation at the sediment-water interface where *Ostreopsis* live, (ii) *Ostreopsis* cells are strictly associated to a rich benthic diatom populations (Accoroni et al., 2016) and (iii) the mixotrophic behaviour suggested for *Ostreopsis* species could get them exposed to intracellular PUA by feeding on diatoms (Barone 2007; Burkholder et al. 2008).

The PUAs values measured in seawater of the Passetto station are much higher respect to the concentrations reported by other authors for the northern Adriatic Sea (Ribalet et al., 2014; Vidoudez et al., 2011) and in the Atlantic Ocean (Lavrentyev et al., 2015). We explain this result considering

that this site is a sheltered and shallow coastal area colonized by a well structured phytobenthic (micro and macrophytobenthos) community and characterized by high primary production and biomass. Such high PUAs concentrations are probably affected by the contribution of some components that are absent in offshore area, i.e. benthic microalgae and macroalgae (e.g., *Ulva* spp., Akakabe et al., 2000; Alsufyani et al., 2014).

With this in mind, further studies are needed to identify other benthic organisms able to produce aldehydes so as determine the effective capacity of PUAs production by the benthic communities. Moreover, additional field samplings are in progress in order to have a more spatial-temporal detailed distribution of PUAs (Pezzolesi, unpublished data) and to understand a possible role of these molecules in affecting the bloom dynamics of *O*. cf. *ovata* and/or other marine benthic organisms.

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CHAPTER 4. Effect of several diatoms PUAs-producers on *Ostreopsis* cf. *ovata* growth

4.1 Introduction

Harmful algal blooms (HABs) constitute a serious environmental problem for aquatic ecosystems, economic activities and public health due to the negative effects induced on both humans and marine organisms (e.g., invertebrates, fish, marine mammals, Bownik 2016; Shears and Ross 2009). The occurrence of these phenomena has become increasingly common in recent decades affecting numerous coastal areas of tropical and temperate regions of world (Glibert et al., 2005; Rhodes, 2011). Variation of physicochemical environmental factors such as temperature, light, and nutrients are traditionally used to explain the HABs dynamics (Smetacek and Cloern et al., 2008). However, the recent development of the chemical ecology highlighted a higher complexity of the microbial web than that observed in previous models, underlining the necessity to consider the biochemical interactions between microalgal species for better understanding the bloom dynamics (Ianora et al., 2012). Several studies showed that secondary metabolites (low-molecular weight organic not directly involved in the make up of basic life mechanisms produced by numerous organims) considerably affect the growth dynamics of harmful algal blooms (Fistarol et al., 2003; Yamasaki et al., 2010; Uchida et al., 1999) and the algal succession (Fistarol et al., 2004), and hatch success of zooplankton population (Ianora et al., 2003, 2004). For example, Uchida et al. (1999) hypothesized that the dominance of the red tide dinoflagellate *Heterocapsa circularisquama* on the phytoplankton community could be explained by its ability to produce secondary metabolites with inhibitory effect on the growth of the co-occurring flagellates. Moreover, Prince and Myers (2008) reported that extracellular extracts produced during the bloom of the toxic dinoflagellate *Karenia brevis* strongly inhibited the growth of competitor species, causing changes in the composition of the plankton community.

Ostreopsis cf. *ovata* is a harmful benthic dinoflagellate causing massive blooms in several tropical and temperate regions and is widespread in the Mediterranean Sea (Accoroni and Totti, 2016; Mangialajo et al., 2011; Rhodes, 2011). It is able to colonize both biotic (macrophytes, seagrasses and invertebrates) (Accoroni et al., 2015b; Turki, 2005; Vila et al., 2001) and abiotic substrata (soft sediments and rocks) (Totti et al., 2010) and, in concomitance with *Ostreopsis* blooms, mass mortalities of benthic marine organisms and human intossications have been observed, due to the production of palytoxin-like compounds (Brissard et al., 2015; Scalco et al., 2012; Uchida et al., 2013). Studies on *Ostreopsis* bloom dynamics mainly focused on the effects of the main environmental factors such as temperature, nutrient concentrations, hydrodinamics (Accoroni et al., 2015b; Mangialajo et al., 2011; Pezzolesi et al., 2012, 2016; Pistocchi et al., 2011; Vanucci et al., 2012). Recently, also the relationships of this dinoflagellate with macroalgae and microphytobenthos have been addressed (Accoroni et al., 2015b, 2016). Authors highlighted that (i) macroalgal species commonly occurring in the benthic flora of blooming area may negatively affect *Ostreopsis* growth and (ii) the diversity of microphytobenthos was significantly lower during the *O*. cf. *ovata* bloom respect to the rest of the year, suggesting a possible negative interactions between diatoms and this benthic dinoflagellate.

Diatoms are the main producers of polyunsaturated aldehydes (PUAs) (Ianora and Miralto, 2010; Ianora et al., 2011; Wichard et al., 2005). Although the main effect of PUAs is reported to be the inhibition of copepod fecundity and the growth of its larvae (Carotenuto et al., 2006; Ianora et al., 2003; Ianora and Miralto 2010), in the last decade several studies documented a negative impact also on phytoplanktonic species (Ribalet et al., 2007). Recently, Pichierri et al (2016) highlighted a clear decrease of growth, DNA degradation and stress signals in the toxic benthic dinoflagellate *Ostreopsis* cf. *ovata* cells exposed to three commercial molecules of PUAs (2E,4E-decadienal, 2E,4E-octadienal and 2E,4E-heptadienal) commonly produced by diatoms, and we demonstrated (see Chapter 2) that some benthic diatoms could release PUAs compounds. Despite the importance of diatoms in marine ecosystems, in terms of abundance and primary productivity (Uitz et al., 2010), and the negative

impact of their secondary metabolites on several organisms (Barreiro et al., 2011; Hansen and Eilersten 2007; Ianora et al., 2012), no information is available on the direct effect of the PUAs producer diatoms on harmful benthic dinoflagellates such as *Ostreopsis* cf. *ovata*.

The aim of this study was to investigate the direct effect of some diatom PUAs-producers (both planktonic and benthic) on the *O.* cf. *ovata* growth and cytosolic features.

4.2 Materials and methods

Experimental design

The diatoms utilized for growth assays were two planktonic (*Thalassiosira* sp. and *Skeletonema marinoi*) and three benthic (*Tabularia affinis*, *Proschkinia complanatoides* and *Navicula* sp.) species. They were selected for the following reasons (i) ability to produce PUAs (Dittami et al., 2011; Gallina et al., 2014; Chapter 2), (ii) recurring presence and/or extensive bloom forming in the northern Adriatic Sea by planktonic species (Bernardi-Aubry et al., 2004; Totti et al., 2000, 2005), (iii) co-occurrence in the same microenvironment of *O.* cf *ovata* by benthic species (Accoroni et al., 2016).

The experiments performed with the two planktonic diatoms constituted a preliminary assay and only their effect on *Ostreopsis* growth was considered, while in the case of three benthic diatoms also the cellular morphological and DNA changes, of *Ostreopsis* were evaluated.

Cultures setting up

Single cells were isolated by the capillary pipette method (Hoshaw & Rosowski, 1973) using an inverted microscope, and monoclonal cultures were set up using multi-well plates and maintained in the following conditions: 21°C, 12:12 h L:D cycle, light intensity 80-90 µmol m^{-2} s^{-1}, in the f/2 growth medium for the diatom species and in modified f/4 medium prepared by adding macronutrients and selenium without silica to filtered and autoclaved natural seawater (salinity 35) for *O.* cf *ovata* (Guillard and Ryther, 1962).

Three strains of *Ostreopsis* cf. *ovata* (OOAPN0808B2, OOAPS0914B4, OOAN0816) were isolated from samples collected during summer blooms in the Passetto station (Conero Riviera, northern Adriatic Sea) in 2008, 2014 and 2016, respectively. The benthic diatom strains *Tabularia affinis* (TAAPS0313Y4), *Proschkinia complanatoides* (PCAPS0313N1), *Navicula* sp. (NAPS0313N2), were obtained from microphytobenthos samples collected in 2013 while the planktonic diatom *Thalassiosira* sp. (THAN0801) from seawater samples collected in the Passetto station in 2001; *Skeletonema marinoi* was purchased by NCMA (National Center for Marine Algae and Microbiota (CCMP2497).

Ostreopsis *cf.* ovata *growth in the diatom filtrates*

The experiments were performed at the experimental conditions indicated above, culturing *O*. cf. *ovata* in 250 ml Erlenmeyer flasks containing cell-free medium where the diatom species were previously grown. Culture media were filtered (pore size 0.22 μm) and used to prepare the medium for the experiment: the pH was adjusted to 8 and nutrients were added to obtain the same medium used for the control. The filtrates of diatom cultures were collected at the stationary phase for all tested species with an additional *Proschkinia complanatoides* filtrate collected at decay phase. Counting of algal cells was carried out using an Utermöhl chamber at the inverted light microscope (Zeiss Axiovert 135) equipped with phase contrast and epifluorescence at 200x magnification.

Preliminary experiments with planktonic species were performed by growing *O*. cf. *ovata* (inoculum density of $3x10^2$ cells ml^{-1}) for 18 days in the undiluted filtrates of *Skeletonema marinoi* ($1.96x10^6$ cells ml^{-1}) and *Thalassiosira* sp. ($9.1x10^3$ cells ml^{-1}) collected at the stationary growth phase.

The following experiments took into consideration the natural ratio between diatoms and *O*. cf. *ovata* cells (ratio 100:1), thus each diatom filtrate was diluted in order to maintain this ratio.

Each test lasted 30 days and every 2 days a subsample (3 ml) was taken from each flask, fixed with 0.8% hexametylentetrammine neutralized formaldehyde and stored at 4 °C in the darkness until the analyses. Moreover, the aberrant forms were distinguished from normal morphotypes.

Morphological observations and DNA integrity analysis

The morphological observations and integrity DNA analysis were performed only for *O.* cf. *ovata* cells grown in *Proschkinia complanatoides* and *Navicula* sp. filtrates using an inverted light microscope (Zeiss Axiovert 135) equipped with phase contrast and epifluorescence at 200x magnification. The aberrant morphotypes were separately counted.

For the DNA analysis, the green-fluorescing SYBR Green I (Molecular Probes) was added in the Utermöhl chamber; sample was incubated in the dark at room temperature for 15 minutes and then observed under an inverted light microscope in epifluorescence light using the barrier filter LP 520 (blue excitation). Moreover, observations in epifluorescence allowed to detect also the red autofluorescence of the chlorophyll.

Nutrient analysis

Nitrate and phosphate were analysed spectrophotometrically (UV/VIS/NIR, JASCO V-650, Tokyo, Japan) according to Strickland and Parsons (1972).

Statistical analysis

Differences in cell densities between the control and the cells of *Ostreopsis* cf. *ovata* exposed to each diatom filtrates were assessed through a one-way analysis of variance (ANOVA). When significant differences for the main effect were observed ($p < 0.05$), a Tukey's pairwise comparison test was also performed. Statistical analyses were performed using Statistica (Statsoft) software. The average lethal times (LT_{50}) represents the time at which 50% of the *Ostreopsis* cf. *ovata* cells were dead.

4.3 Results

Effect of the Skeletonema marinoi *and* Thalassiosira *sp. filtrates on* Ostreopsis *cf.* ovata *growth*

Inibition of *O.* cf. *ovata* growth in the undiluted extracellular medium of *Skeletonema marinoi* and *Thalassiosira* sp. were observed. In fact, as reported in Table 1, the final cell yields of the cultures grown in these filtrates was 56 and 78% of those measured in the controls, respectively for *S. marinoi* and *Thalassiosira* sp. With both filtrates decreasing numbers of *O.* cf. *ovata* cells were observed starting from day 2-3 of inoculum (data not shown). A number of aberrant forms were also observed in the inhibited cultures although their quantification was not performed.

Table 1 - *Ostreopsis* cf. *ovata* grown for 18 days in standard medium (control) or in filtrates obtained from cultures of the diatoms *Skeletonema marinoi* and *Thalassiosira* sp. collected at the stationary growth phase.

***Ostreopsis* cf. *ovata* treatment**	**Final cell yield (day 18)**	**% inhibition**
Control	5681 ± 48	
Skeletonema marinoi filtrate, day 17	3222 ± 259	57
Thalassiosira sp. filtrate, day 17	1666 ± 89	78

Effect of the Tabularia affinis *filtrate on* Ostreopsis *cf.* ovata *growth*

The Figure 1 shows a gradual decrease of *O.* cf. *ovata* growth respect to the control when exposed to *Tabularia affinis* filtrate. At the end of the experiment (day 30), the cell abundances of *O.* cf. *ovata* were significantly lower in the *Tabularia* filtrate than in the control ($p < 0.001$). The maximum yield was reached at the day 28 (5474 ± 172 cells ml^{-1}) in the control, while in the *Tabularia* filtrate it was reached at the day 20 (2003 ± 80 cells ml^{-1}). Moreover, only slight differences between the cells exposed to the *T. affinis* filtrate and the control were observed until day 8. On the contrary, the main part of *O.* cf. *ovata* growth inhibition started in the second part of the experiment (days 10-20) and

has become even more evident in the last part (days 22-30). The average lethal times (LT_{50}) was of 10 days.

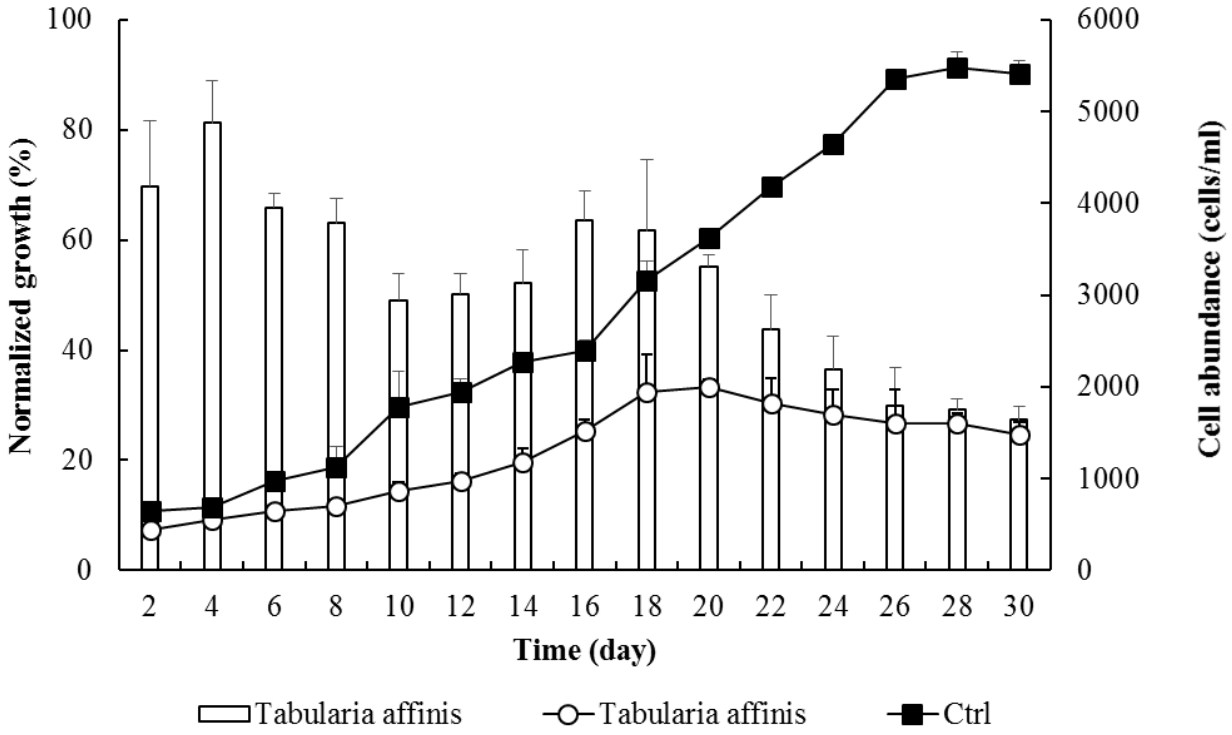

Figure 1. Effect of *Tabularia affinis* filtrate on *Ostreopsis* cf. *ovata* growth: bars indicate the growth in percentage respect to the controls (1st left y-axis), white circles indicate *Ostreopsis* cf. *ovata* abundance grown in *T. affinis* filtrate expressed in cells/ml (2nd left y-axis), black squares indicate the abundance of *Ostreopsis* cf. *ovata* control expressed in cells/ml (2nd left y-axis) Data are means of independent replicates (n = 3) and the error bars represent standard deviations.

Effect of the Proschkinia complanatoides *filtrate on* Ostreopsis *cf.* ovata *growth*

The Figure 2 shows a clear negative effect of the *Proschkinia complanatoides* filtrate on *O.* cf *ovata* growth. The maximum cell yield was reached at the day 26 in the control (8337 ± 498 cells ml^{-1}), while in the culture medium filtrate it was observed at the day 12 with 1570 ± 236 cells ml^{-1}. Only a moderate growth inhibition was observed until day 4; on the contrary, a clear effect is evident in the second part (days 8-20) and in the last part (days 22-30) of the experiment, where the cell abundances of the diatom filtrate were significantly lower than the control ($p < 0.001$ and $p < 0.005$, respectively). The average lethal times (LT_{50}) was of 8 days.

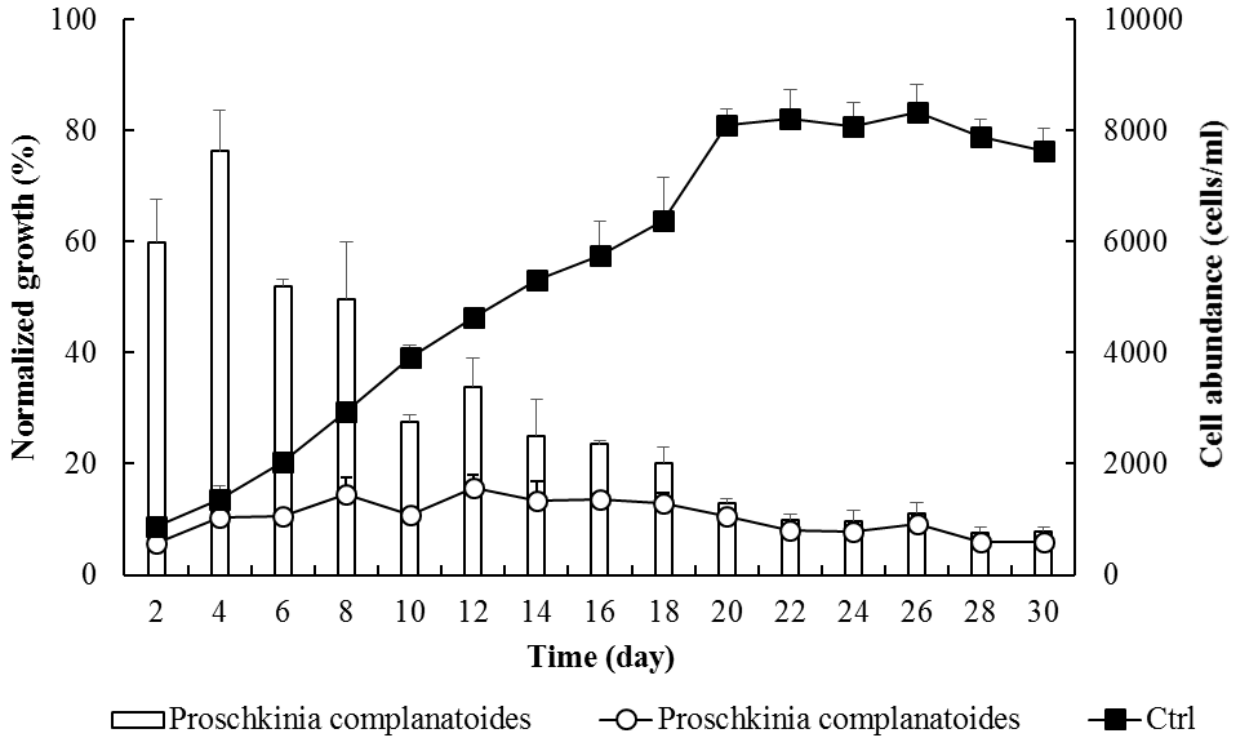

Figure 2. Effect of *Proschkinia complanatoides* filtrate on *Ostreopsis* cf. *ovata* growth. Bars indicate the growth in percentage respect to the controls(1st left y-axis), white circles indicate *Ostreopsis* cf. *ovata* abundance grown in *Proschkinia complanatoides* filtrate expressed in cells/ml (2nd left y-axis), black squares indicate the abundance of *Ostreopsis* cf. *ovata* control expressed in cells/ml (2nd left y-axis). Data are means of independent replicates (n = 3) and the error bars represent standard deviations.

Effect of Navicula *sp. filtrates (stationary and decline phase) on* Ostreopsis *cf.* ovata *growth*

A strongly decrease of *Ostreopsis* cf *ovata* growth was observed when it was exposed to the *Navicula* sp. filtrates collected in the decline phase respect to the control (Fig. 3). In this case, the maximum cell yield was reached at the day 16 with 2357 ± 405 cells ml^{-1} and the average lethal times (LT_{50}) was of 8 days. On the contrary, no inhibition of *O*. cf. *ovata* growth was observed when the cells were cultured in the stationary phase of the same diatom filtrate with growth percentage respect to the control ranged from 80 to 99%, except for first 6 days.

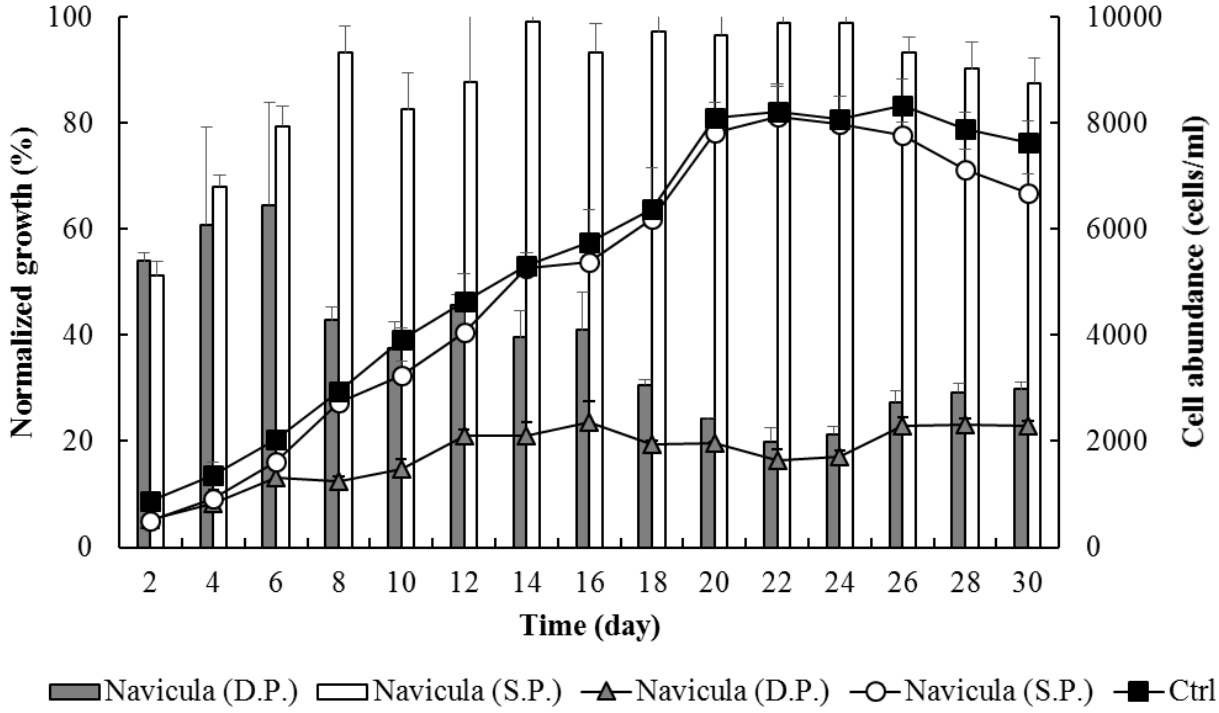

Figure 3. Effect of *Navicula* sp. filtrate on *Ostreopsis* cf. *ovata* growth. White and grey bars indicate the *Ostreopsis* cf. *ovata* growth,in percentage respect to the controls, grown in *Navicula* sp. filtrates collected rispectively at Stationary Phase and Decay Phase (1st left y-axis), white circles indicate *Ostreopsis* cf. *ovata* abundance grown in *Navicula* sp. filtrate collected at the Stationary Phase expressed in cells/ml (2nd left y-axis), grey triangles indicate *Ostreopsis* cf. *ovata* abundance grown in *Navicula* sp. filtrate collected at the Decay Phase expressed in cells/ml (2nd left y-axis) and black squares indicate the abundance of *Ostreopsis* cf. *ovata* control expressed in cells/ml (2nd left y-axis). Data are means of independent replicates (n = 3) and the error bars represent standard deviations. D.P. = decline phase, S.P. = stationary phase

Microscopy observations and analysis

The morphological analysis performed on *Ostreopsis* cf. *ovata* cells exposed to the *Proschkinia complanatoides* and *Navicula* sp. filtrates (day 35) highlighted the presence of some aberrant forms (Fig. 4c). These morphotypes were characterized by lack of motility, a formation of abnormal vesicle-like structures and a contraction of cytoplasm. The abnormal forms appeared since the exponential growth phase (day 8 and 10 for *P. complanatoides* and *Navicula* sp. filtrates respectively) until the end of the experiment (day 30). The abnormal forms ranged from 6 to 83.8% of the total cells, with the maximum observed at day 10. No differences in the trend of abnormal forms were observed between the two algal filtrates (Fig. 4a).

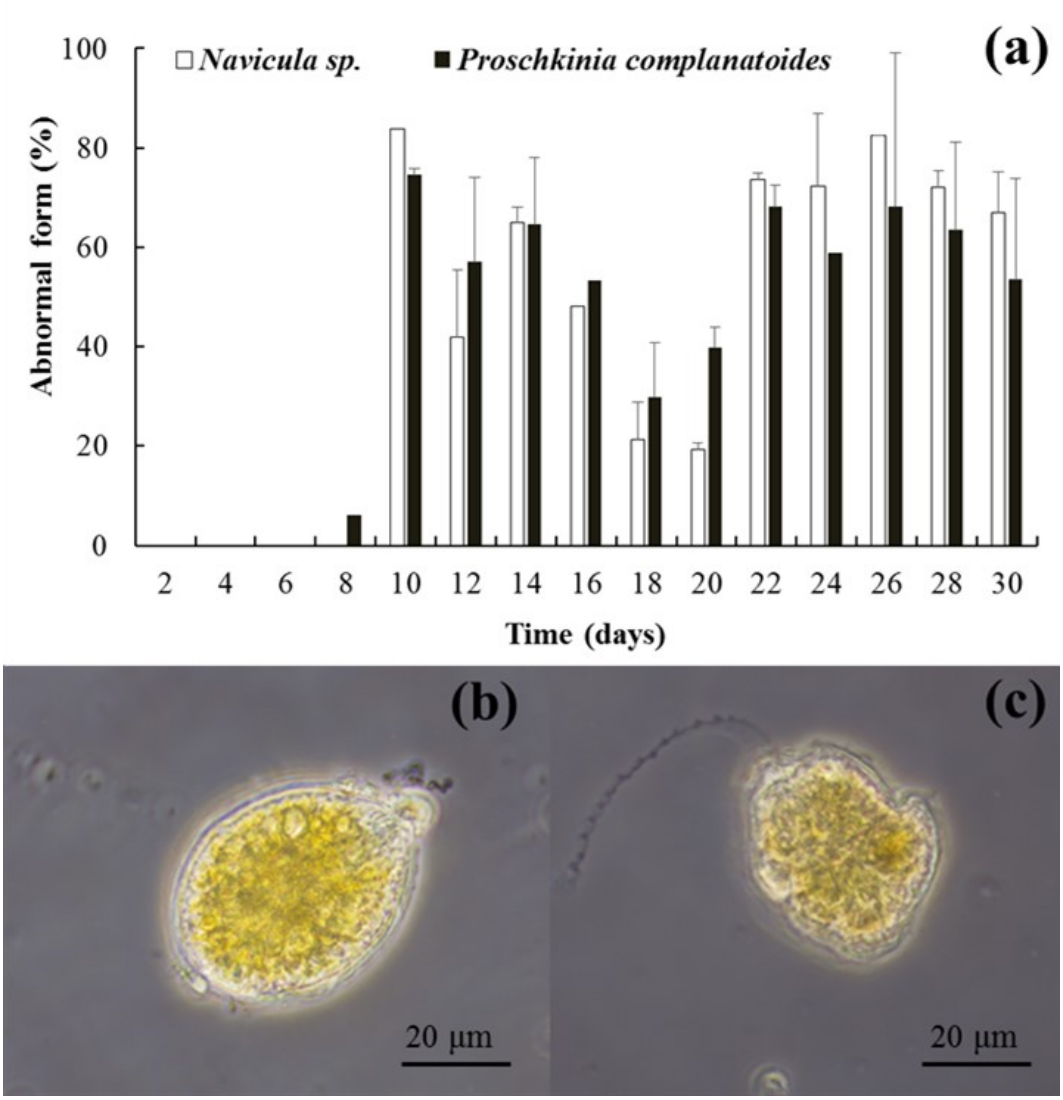

Figure 4. Morphological analysis of *Ostreopsis* cf. *ovata* under light microscopy. (a) Percentage of abnormal forms in cells exposed to *Proschkinia complanatoides* and *Navicula* sp. filtrates (b) Light micrograph of a normal shaped cell. (c) Light micrograph of an abnormal shaped cell. Data are means of independent replicates (n = 2) and the error bars represent standard deviations.

The DNA analysis performed on cells stained with SYBR Green I showed a clear genotoxic damage on cells exposed to the *Proschkinia complanatoides* and *Navicula* sp. filtrates. The control cells exhibited the majority of the DNA circumscribed in the nucleus (located in the dorsal part of the cell) during all the experiment, while in cells exposed to the two filtrates the shape, both size and position of the nucleus were affected. In particular, the chromatin damage caused the chromatin dispersion to the outlying cytoplasm and a gradual enlargement of the nucleus from a diameter of 10 nm (day 0) to about 15 nm in the exponential phase (day 12) and 18 nm in the stationary phase (day 20) in the cells

exposed to the *P. complanatoides* and *Navicula* sp. filtrates (Fig. 5). Moreover, lack of red autofluorescence of the chlorophyll respect to the control was observed in both cells.

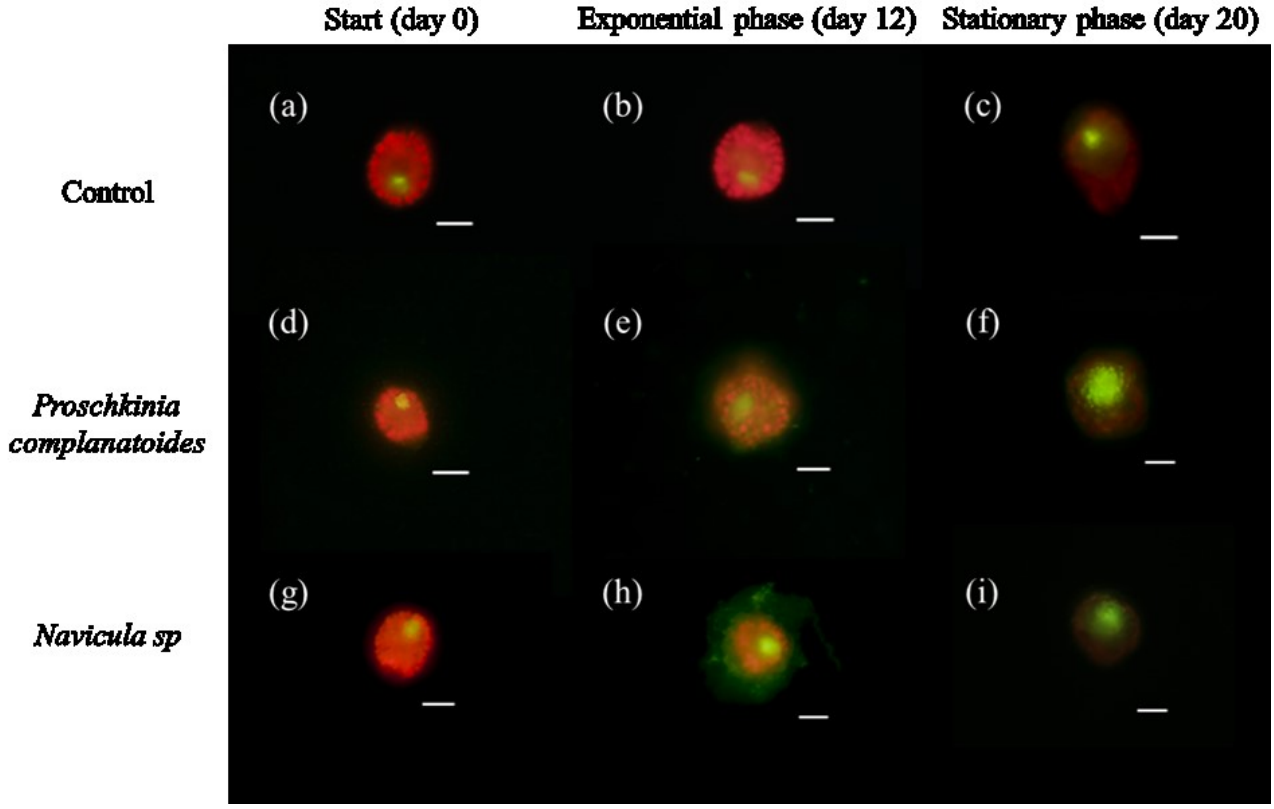

Figure 5. Observations of *Ostreopsis* cf. *ovata* cells stained with SYBR Green I (green fluorescence) under epifluorescence microscopy: (a-c) untreated cells; (d-f) cells exposed to *Proschkinia complanatoides* filtrates; (g-i) cells exposed to *Navicula* sp. filtrates. Scale bar: 20 µm.

4.4 Discussion

Several studies have been carried out in order to clarify which conditions influence *O.* cf. *ovata* bloom dynamics in different world areas, addressing mainly on abiotic factors such as hydrodynamics, water temperature and nutrients (Accoroni et al., 2015a; Mabrouk et al., 2011; Mangialajo et al., 2011; Pezzolesi et al., 2012, 2016; Pistocchi et al., 2011; Selina et al., 2014; Shears and Ross, 2009; Totti et al., 2010; Vanucci et al., 2012; Vila et al., 2001). Recently, some authors started to investigate also the role of biotic factors, highlighting important interactions between *O*. cf. *ovata* and other organisms. For example, *Ostreopsis* populations seem to affect both bacterial and benthic diatom communities (Accoroni et al. 2016; Vanucci et al. 2016). Moreover, a certain allelopathic activity has been highlighted by *O*. cf. *ovata* towards the growth of many co-occuring benthic dinoflagellates (García-Portela et al. 2016; Monti and Cecchin 2012), as already documented for other toxic dinoflagellates which affect co-occurring microalgae under unfavorable environmental conditions (Fistarol et al., 2003, 2004; Granéli and Hansen, 2006; Granéli and Johansson, 2003; Prince et al., 2008).

However, allelopathic activity has been observed towards *Ostreopsis* too. In studies carried out on natural populations of *O*. cf. *ovata*, significantly higher abundances were reported on pebbles than on macroalgae (Accoroni et al., 2011; Totti et al., 2010), suggesting that living substrata allow lower concentration of epibionts than any other substrate. Indeed, a study conducted to assess possible allelopathic interactions between *O*. cf. *ovata* and macroalgae showed that several seaweed species could exert negative effects toward this benthic dinoflagellate.

In the same way, an effect of diatoms towards *Ostreopsis* growth could be hypothesized. In this regards, diatoms are able to produce and release different types of PUAs compounds (D'Ippolito et al., 2004, 2006; Wichard et al., 2005; Chapter 2), known to exert negative effects on the growth, cytological features and cell morphology of *O*. cf. *ovata* (Pichierri et al., 2016).

This study highlighted for the first time a deleterious impact on growth, cell morphology and cytosolic features of *Ostreopsis* cf. *ovata* cells when exposed to filtrates of several diatom species, both

planktonic and benthic. Although unfortunately, the analysis of PUAs concentrations in filtrates was not carried out, we hypothesize that these negative effects could be due to the production of PUAs molecules by these diatoms, as they were proved to produce large amounts of such compounds (see Chapter 2).

In our first experiments, we performed a preliminary test using two planktonic species, i.e. *Skeletonema marinoi* and *Thalassiosira* sp., that are responsibles for recurrent and extensive blooms in several temperate areas, as well in the northern Adriatic Sea (Bernardi-Aubry et al., 2004; Fonda Umani et al., 1992). Given that the coastal areas generally affected by *Ostreopsis* blooms are characterized by shallow waters (Totti et al., 2010), we consider the exposition of microphytobenthic communities to planktonic secondary metabolites a quite realistic scenario. The highest inhibition percentage reported in the *Thalassiosira* filtrate (78%) seems to indicate a stronger inhibitory effect of this diatom respect to *S. marinoi.* These results agree with data reported in literature on the PUAs production by these species, showing PUA concentrations 20 times higher in *Thalassiosira* than in *S. marinoi* (up to 40.2 and 0.1-2.1 fmol/cell respectively, Dittami et al., 2010; Gerecht et al. 2011; Vidoudez and Pohnert et al., 2008).

As regard to the benthic diatoms, we tested the effect of three species that live in close contact with *O.* cf *ovata*, i.e. *Proschkinia complanatoides, Tabularia affinis* and *Navicula* sp. A clear allelopathic effect towards *O.* cf *ovata* growth was pointed out also for all these species and the strongest impact was observed in the exposure to *P. complanatoides* filtrate. In this case, although our results seem to be in contrast with what observed in previous experiments (see Chapter 2), i.e., that *Navicula* sp. produces higher concentrations of PUAs than *P. complanatoides* and *T. affinis* (34.9, 3.75 and 2.21 fmol/cell respectively), this could be explained taking in consideration the type molecules released into the medium from different diatom species instead of the total amount. In this regard, a strong positive relationship has been highlighted between the length of PUAs molecules and their cytotoxic effect, in both bioassays performed using synthesized molecules and toxicological studies conducted on phytoplankton and invertebrate species (Carvalho et al., 2000; Ribalet et al., 2007; Wichard et al.,

2005). Pichierri et al. (2016) showed more deleterious effects in *O*. cf. *ovata* cells exposed to 6 µmol l^{-1} of 2E,4E-decadienal respect to 36 µmol l^{-1} of 2E,4E-heptadienal. The lower inhibition exerted by *Navicula* sp. filtrates respect to those of *P. complanatoides* could be explained considering that the latter species produces mainly C8 chains and the former mainly C10 and C11 chains (see Chapter 2), in addition to variability production due to the different culture conditions (Dittami et al., 2010).

The growth phase of diatom culture represents a crucial factor for the *O*. cf. *ovata* inhibition, because a stronger effect was observed with increasing length of the culture period in benthic diatom experiments. These results comfirm the wound activated process that has been described in all PUAs-producers, i.e. the release of these compounds only after cellular distruction (Adolph et al., 2003; Pohnert 2000).

The morphological and DNA analysis brought to light some important cytosolic anomalies, such as a gradual enlargement of nucleus and the presence of some abnormal forms characterized by a contraction of cytoplasm and vesicle-like structures in the peripheral part. These abnormal forms represent a clear state of cellular stress and they were found in all *O*. cf. *ovata* cultures grown in filtrates for which growth inhibition was observed. Changes of nucleus size resulted from chromatin fragmentation, in addition to a clear dispersion of DNA to the outlying cytoplasm observed in the cells exposed to *P. complanatoides* and *Navicula* sp. filtrates. All these cytotoxic effects, even though more intense, were previously highlighted in *O*. cf. *ovata* cells exposed to high concentrations (3 – 36 µmol l^{-1}) of commercial PUAs (Pichierri et al., 2016). In the absence of DNA repair mechanisms, they are considered as a previous step to the cell death as they cause an irreversible blockage in G1-phase of the cell cycle and preclude the synthesis of structural components (Carvalho et al., 2000; Casotti et al., 2005). Although the DNA analysis performed in this study does not allow us to determine when the bond aldehydes-nucleic acids occurred, we can hypothesize a formation of the first covalent DNA adducts immediately after the *O*. cf. *ovata* inoculum in the diatom filtrates, due to the scarce persistence of PUAs at temperatures of 20°C (Barutal and Ortega, 2013). Therefore, the cytotoxicity observed in the second part of experiment could represent the result of irreversible

biological reactions occurred in the first days of exposition. Despite of the intrinsic limitations of using cell-free media for investigating allelopathic relationships, our results suggest that similar interactions might occur also in field conditions. Considering the composition and the temporal variation of microphytobenthos in the northern Adriatic Sea, where diatoms represented the most conspicous microalgal fraction for most of the annual cycle, with a density ratio between diatoms and dinoflagellates slightly higher than 100:1 (Accoroni et al., 2016), the concentrations tested in our experiments were quite realistic. Moreover, the naviculoid diatoms, that include *P. complanatoides* and *Navicula* sp., represent the most abundant fraction of the entire diatom group while *Tabularia* constitutes the main taxa of the erect growth forms. Taking into account the negative relationship of PUAs with high temperature (Bartual and Ortega 2013), it is reasonable to suppose a clear and gradual decrease of PUAs concentrations in field during the summer, promoting conditions suitable for the development of the *Ostreopsis* bloom. In this sense, preliminary PUAs measurements performed during summer in the Conero Riviera, revealed low values of these compounds (0.14 to 0.21 µmol l^{-1}) (Pezzolesi, unpublished data), in agreement with that hypothesis.

This study highlighted that secondary metabolites with allelopathic effects should be taken into account besides the abiotic factors to better understand the HAB dynamics. However, further studies are required to assess the trend of PUAs concentrations in the areas affected by *Ostreopsis* proliferation.

4.5 References

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