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Ecology of the toxic dinoflagellate *Ostreopsis* cf. *ovata* along the coasts of the Campania region (Tyrrhenian Sea, Mediterranean Sea)

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**“Open your mouth wide
The universal sigh
And while the ocean blooms
It's what keeps me alive
So why does it still hurt?
Don't blow your mind with why**

**I'm moving out of orbit
Turning in somersaults
I dive into those eyes
Jellyfish swim by”**

“Bloom”

Radiohead

The King of Limbs (2011)

Chapter 1

General introduction

1.1 Harmful algal blooms

Microalgae are unicellular photosynthetic organisms capable to produce organic carbon from CO_2 using light energy from the Sun. Their ability to fix the atmospheric carbon makes them primary producers in the trophic web. Ecologically speaking these organisms can be separated into two groups: phytoplankton and microphytobenthos, which inhabit the lit upper water column and sea bottom, or other substrates, respectively. While being the main primary producer in the plankton, microalgae share the role of producers in the benthic realm with macroalgae and aquatic phanerogams. The presence or the massive proliferation of microalgae in coastal waters may cause at times harmful algal blooms (HABs) which negatively impact the environment, human health and economic activities (Zingone and Wyatt, 2005). More than defining a natural group of microorganisms, the term HAB is a societal concept applied to any negative effect exerted by microalgae (Hallegraeff, 2014). In the last two decades, the incidence of HAB events has been increasing in terms of frequency, intensity and geographic distribution throughout the world's seas (Hallegraeff, 1993). Human-induced processes such as climate change (Hallegraeff, 2010), coastal eutrophication (Anderson, 2009) and dispersal (via ballast waters or shellfish translocation) (Hallegraeff, 1998) have been called into question as potential agents of such increase. However, more frequent interactions with the sea and more investigations are also likely to cause an increase of records for these phenomena (Zingone and Enevoldsen, 2000; Zingone and Wyatt, 2005).

HAB species may differ in the mechanism by which cause negative impacts: while some species may cause deleterious effects by reaching high biomasses (water discoloration, oxygen depletion of water column, gills clogging and abrasion in fishes) others are also toxin producers (Black, 2000). Among the 5000 marine phytoplankton species described (Hallegraeff, 2003), about 200 are considered noxious species of which 115 (Moestrup et al., 2009) are currently known to be toxic, distributed among several taxonomic groups (diatoms, haptophytes, dinoflagellates, raphidophytes, dictyochophyceans, cyanobacteria). As the research efforts increase, this list is steadily increasing with the addition of new discovered toxic species, new toxic mechanisms in already known species and also with the intensification of the interactions with man and coastal environment (Zingone & Wyatt, 2005). The diversity of HABs causative organisms results in a wide variety of toxins and relative mechanisms of actions (Zingone & Enevoldsen, 2000), which in turn display a wide array of specific symptoms. At least six human syndromes associated to the consumption of seafood by algal toxins have been described so far: Amnesic Shellfish Poisoning (ASP), Azaspiracid Poisoning (AZP), Ciguatera Fish Poisoning (CFP), Diarrhetic Shellfish Poisoning (DSP), Neurotoxic Shellfish Poisoning (NSP) and Paralytic Shellfish Poisoning (PSP).

1.2 Benthic harmful algal blooms

The first and well known problem caused by harmful benthic microalgae is ciguatera fish poisoning (CFP), a human syndrome which is frequently reported in tropical and subtropical waters (Skinner et al., 2011). This syndrome causes gastrointestinal (nausea, vomiting and diarrhea) and neurological symptoms (headaches, muscle aches, paresthesia, numbness, ataxia, vertigo and hallucinations) (Swift and Swift, 1993). The toxic benthic dinoflagellate *Gambierdiscus toxicus* has been identified as the causative agent of this illness. This organism produces ciguatoxins and maitotoxins which bioaccumulate in reef fishes. Despite no confirmed case of ciguatera fish poisoning has ever been documented in the Mediterranean Sea (Ferrante et al., 2013), suspected cases have been reported in Israel coasts (Herzberg, 1973; Spanier et al., 1989). However, the occurrence of *Gambierdiscus* spp. in Crete since 2003 may imply the onset of the disease also in this area (Aligizaki and Nikolaidis, 2008).

A number of other benthic dinoflagellates are associated with *Gambierdiscus* in tropical benthic waters. Among these are those belonging to the genus *Ostreopsis*, since 1995 (Usami et al., 1995) known to produce a different kind of toxins, of the family of the palytoxins. Initially all the problems caused by benthic microalgae were lumped under the CFP issue, but subsequently *Ostreopsis* has been associated with another syndrome, clupeotoxism. In addition, the widespread occurrence of *Ostreopsis* in temperate areas (Rhodes, 2010), where *Gambierdiscus* is rarely found, has required the two genera and the problems they cause be handled as separated.

In the Mediterranean Sea, threats emerging from organisms of the genus *Ostreopsis* are relatively new. Firstly spotted in Villefranche-sur-mer (Mediterranean Sea) in 1972 (Taylor, 1979), *Ostreopsis* outbreaks have increased their frequency and intensity in the Mediterranean Sea (Vila et al., 2001; Penna et al., 2010; Totti et al., 2010; Mangialajo et al., 2011). During *Ostreopsis* blooms, severe faunal damage and respiratory problems in humans have been reported (Sansoni et al., 2003; Ciminiello et al., 2006; Shears and Ross, 2009; Totti et al., 2010). Other benthic dinoflagellates capable to produce harmful events belong to the genus *Prorocentrum* and *Amphidinium*, while other benthic algal groups (diatoms of the genus *Nitzschia* and *Halamphora*, and freshwater cyanobacteria) have been shown to cause negative effects to the public health and to the environment (Moestrup et al., 2009; Quiblier et al., 2013). Based on the ecological region where these species preferentially live and on the negative effects on human health and the environment they cause, their proliferations are collectively referred as benthic harmful algal blooms (BHABs).

1.3 The genus *Ostreopsis*

Organisms of the genus *Ostreopsis* Schmidt (fig. 1.1) are unicellular algae growing epiphytically on benthic macroalgae but also found on sand, rocks, shells of benthic animals and in surface waters (Fukuyo, 1981; Ballantine et al., 1988; Faust et al., 1996; Totti et al., 2010). The genus is part of Dinophyceae (dinoflagellates), a widespread class including both autotrophic and heterotrophic unicellular species, abundant mainly in the plankton, characterized by size generally ranging mostly from 10 to 300 µm. Six of the nine species of *Ostreopsis* genus have been shown to be toxic (Rhodes, 2010) being capable to produce structural analogues of the palytoxin, one of the most powerful known neurotoxins (Wiles et al., 1974), which can contaminate a wide range of edible marine organisms, including crustaceans, molluscs, echinoderms and fishes (Aligizaki et al., 2011). Dermatological syndromes and development of toxic aerosols have also been reported during *Ostreopsis* blooms (Gallitelli et al., 2005; Ciminiello et al., 2014). Proliferations of these organisms may also be visible with the naked eye as a rusty brown mucilaginous film covering benthic substrates (Faust et al., 1996), which can be easily resuspended in the water column by mechanical actions of waves, giving rise to flakes and at times sea-surface foams (Totti et al., 2010).

1.3.1 Taxonomy

The type species of the class Ostreopsidaceae (order Gonyaucales) and genus *Ostreopsis* (tab. 1.1), *O. siamensis* Schmidt was described at the beginning of the last century (1901) in the area formerly known as Gulf of Siam (now Gulf of Thailand). Late in the century Fukuyo (1981) redescribed *Ostreopsis siamensis* and described two new species from French Polynesia waters and Ryukyu Islands, *O. lenticularis* Fukuyo and *O. ovata* Fukuyo. In the following years, new species were described from Florida Keys, *O. heptagona* Norris, Bomber and Balech (Norris et al., 1985), *O. mascarenensis* Quod (Quod, 1994) from southwest Indian Ocean and *O. labens* Faust & Morton from Belize waters. In 1999, three species were added to the genus with the description of *O. belizeanus* M.A. Faust, *O. caribbeanus* M.A. Faust, and *O. marinus* M.A. Faust from Caribbean Sea and southwest Indian Ocean samples (Faust, 1999). Despite the intense work of taxonomists, the number of species of the genus *Ostreopsis* at present time is still unknown. In fact, none of the visual taxonomic characters proposed until now (cell shape, size, plate tabulation and thecal pore size) have been proved to allow for a reliable distinction among the species, while a wide genetic diversity has been discovered in some taxa (Sato et al., 2011).

Generally, species of the genus are anterior-posteriorly compressed, which gives them almond-like to egg-like shapes (Berdalet et al., 2012). Cell size has been formerly used to define new *Ostreopsis* species: Fukuyo (1981) distinguished *O. ovata* cells by having smaller size compared to *O. lenticularis* and *O. siamensis.* Larger cell size was attributed to *O. heptagona* Norris, Bomber, and Balech compared to the previously described species, while in an extensive work of Faust et al., 1996, *O. siamensis* cells were found to be bigger than *O. lenticularis*. Recent works considered cell shape as a taxonomic character, by using the measure of two cell axes and their ratios, the dorso-ventral/antero-posterior (DV/AV) and dorso-ventral/transdiameter (DV/W) (Penna et al., 2005; Aligizaki and Nikolaidis, 2006; Selina and Orlova, 2010; Parsons et al., 2012). However, several studies showed that there is a substantial overlap of cell size in many *Ostreopsis* species (Totti et al., 2010; Sato et al., 2011; Accoroni et al., 2012a). For instance, Mediterranean and Atlantic strains of *Ostreopsis ovata* and *Ostreopsis siamensis* showed comparable cell size (Accoroni et al., 2012b; David et al., 2012), despite the latter was found smaller in many studies (Tognetto et al., 1995; Penna et al., 2005; Aligizaki and Nikolaidis, 2006; Honsell et al., 2011). A source of misidentification can also be attributable to the high variability in the morphology of cells collected from field material (Aligizaki and Nikolaidis, 2006; Accoroni et al., 2012b), which in some cases may derive from coexisting species, and also from morphological variability in cultured material (Pin et al., 2001; Penna et al., 2010; Pagliara and Caroppo, 2012; Scalco et al., 2012; Accoroni et al., 2014).

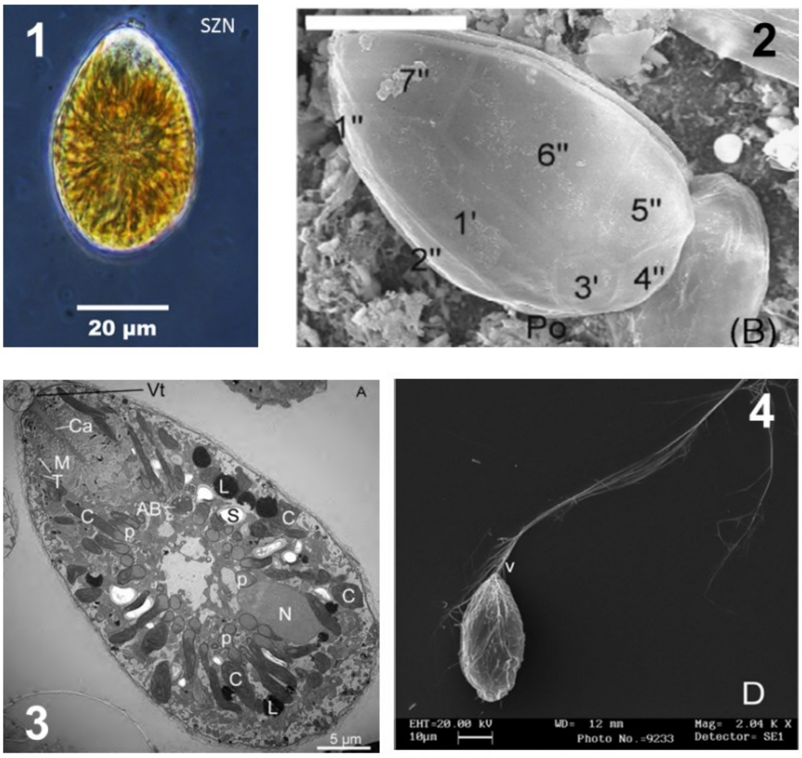


Figure 1.1. 1) *Ostreopsis* cf. *ovata* from Gulf of Naples in light microscopy.
2) Epithecal view of *Ostreopsis ovata* in light microscopy. Scale bar, 20 μm (from Aligizaki and Nikolaidis, 2006).
3) Longitudinal section of *Ostreopsis* cf. *ovata* cells from field sample, observed by transmission electron microscopy. AB, accumulation body; C, chloroplast; Ca, mucus canal; G, Golgi apparatus; L, lipids; M, mucocysts; N, nucleus; p, pyrenoid; S, starch; T, trichocysts; Vt, ventral tube (from Escalera et al., 2014).
4) *Ostreopsis* cf. *ovata* cell with a long filament departing from its ventral side (v) observed by scanning electron microscopy. Scale bar, 10 μm (from Honsell et al., 2013).

Plate tabulation, one of the most used morphological characters in thecate dinoflagellate identification, was used to distinguish *O. heptagona* from all the other *Ostreopsis* species, which all share the same plate formula (Penna et al., 2005). However, the autoapomorphic character in *O. heptagona* (contact between the 4' and 4'' plates) was also observed in some cells of *O.* cf. *ovata* belonging to an Indonesian strain (Penna et al., 2010).

The size of pores on the thecal plates has also been proposed as a distinctive taxonomic character: two pore classes were described in *O. lenticularis* (Fukuyo, 1981) while only one class was reported for *O. ovata* (Penna et al., 2005; David et al., 2012). Two different pore size classes were observed in *O. siamensis* from Atlantic coasts of Iberian Peninsula and in the Mediterranean Sea, while the original description of Fukuyo (1981) reported just one kind of pores for this species. All the studies enlisted above highlight the current taxonomic uncertainty using morphologic characters for defining *Ostreopsis* species. Most of the efforts are now focused on the use of molecular markers for species resolution. While the taxonomy of the genus is under revision, at present time the use of the suffix cf. (confer, confront) for *Ostreopsis ovata* and *O. siamensis* is needed (Penna et al., 2010; Sato et al., 2011).

Table 1.1. *Ostreopsis* species, type locality and selected references. Type species is shown in bold (from Berdalet et al., 2012).

Species	Type locality	References
***Ostreopsis siamensis* Schmidt,1901**	Trat Province, Gulf of Thailand	Schmidt (1901); Fukuyo (1981); Penna et al. (2005; 2010); Sato et al. (2011)
Ostreopsis lenticularis Fukuyo,1980	Gambier Islands and Society Islands, French Polynesia, South Pacific Ocean	(Fukuyo, 1981; Norris et al., 1985; Larsen and L., 2004)
Ostreopsis ovata Fukuyo, 1980	French Polynesia, New Caledonia and Ryukyu Islands, South and West Pacific Ocean	Fukuyo (1981); Penna et al. (2005; 2010); Sato et al. (2011)

Species	Type locality	References
Ostreopsis heptagona, Norris, Bomber et Balech, 1985	Florida Keys, Gulf of Mexico	Norris et al. (1985)
Ostreopsis mascaranensis Quod, 1994	Reunion Island, West Indian Ocean	Quod (1994)
Ostreopsis labens Faust et Morton, 1995	Belize, Central America, Caribbean Sea	Faust and Morton (1995)
Ostreopsis marinus Faust, 1999	Belize, Central America, Caribbean Sea	Faust (1999)
Ostreopsis belizeanus Faust, 1999	Belize, Central America, Caribbean Sea	Faust (1999)
Ostreopsis caribbeanus Faust,1999	La Parguera, Puerto Rico, Caribbean Sea	Faust (1999)

1.3.2 Phylogeny

The use of genetic molecular markers in the taxonomy of *Ostreopsis* species is relatively recent and seminal studies have started only during the last decade with the first phylogenetic assessment on Malaysian strains (Pin et al., 2001), who genetically defined "*O. ovata"* and "*O. lenticularis".* These analyses, which were totally missing in the first description of the nine species, have provided the first sound taxonomic assessment of the genetic diversity in some species of the genus. Relevant phylogenetic analyses were performed with ribosomal DNA regions (rDNA) of the small (5.8S rDNA) and the large subunit (LSU) and with the internal transcribed spacers (ITS) (Battocchi, 2009; Penna et al., 2010; Laza-Martinez et al., 2011; Sato et al., 2011; David et al., 2012; Nascimento et al., 2012). Most of the recent studies mainly focus on *O.* cf. *ovata*, which appears to be the most widespread taxon in the genus, and is the one that dominates across the Mediterranean Sea.

These studies have highlighted profound differences at phylogeographic level: while genetic surveys performed on *Ostreopsis* cf. *ovata* using ITS and 5.8S rDNA regions have identified a rather uniform population in the Mediterranean and Atlantic Sea

(Penna et al., 2010), a “surprisingly high” diversity was found even at very small spatial scale on isolates collected in Japanese waters (fig. 1.4, Sato et al., 2011), harbouring a high number of distinct clades. This study also suggested a recent radiation of *O.* cf. *ovata* from Indo/Pacific to the Mediterranean/Atlantic region. A man-mediated transport is likely involved in this process: the vector this time would not be ballast waters (commonly responsible of dinoflagellates cysts translocations (Hallegraeff, 1998) but rather aquacultured shellfish imported from Pacific to Atlantic/Mediterranean areas, though this hypothesis needs further studies to be confirmed (Zingone, 2014).

Several nuclear rDNA sequences from *Ostreopsis* isolates obtained worldwide are now available. A recent phylogeographic survey on ITS - 5.8S and LSU gene sequences has confirmed the occurrence of at least five clades of *Ostreopsis* species (fig. 1.3): three *Ostreopsis* cf. *ovata* clades (the Atlantic/Mediterranean/ Pacific, the Atlantic/Indian/Pacific and the Pacific clade), an Atlantic/Mediterranean *Ostreopsis* cf. *siamensis* clade, two undefined *Ostreopsis* species clade, and two separate clades for *Ostreopsis* cf. *labens* and *Ostreopsis* cf. *lenticularis* in the Pacific Ocean (Penna et al., 2014). In this study, the presence of three geographically defined subgroups within the Atlantic/Mediterranean/Pacific clade of *O.* cf. *ovata* has also been suggested. The high genetic diversity of *Ostreopsis* cf. *ovata* has recently been confirmed also in other Pacific areas (Kang et al., 2013; Tawong et al., 2014). Given the bulk of studies supporting the high diversity of *Ostreopsis* cf. *ovata* at intraspecific level, it has been suggested that this species may be a species complex (Penna et al., 2010). The huge genetic diversity observed in *Ostreopsis* cf. *ovata* is compatible with the definition of several new species, if compared with other dinoflagellates (Litaker et al., 2007).

Nowadays other molecular markers are under investigation to describe the genetic diversity of *Ostreopsis* species: while mitochondrial genes (COI and cob) have been found very similar among different species and thus unable to resolve the interspecific genetic diversity (Penna et al., 2014), a DNA fingerprinting technique is now providing emerging evidences about the presence of two distinct populations in the Eastern and Western basins (Italiano et al., 2014), in agreement with physiological (Guerrini et al., 2010) and ecological data (Totti et al., 2010; Mangialajo et al., 2011). More recently, in addition to phylogenetic studies, molecular techniques (such as qPCR and qrtPCR) have also been used for monitoring purposes (Battocchi et al., 2010; Perini et al., 2011; Casabianca et al., 2013).

1.3.3 Biogeography

The genus *Ostreopsis* has a worldwide distribution (fig 1.2), and was initially found in tropical and subtropical areas; in recent years, an increasing number of reports have been documented for *O.* cf. *ovata* and *O.* cf. *siamensis* in warm and cold temperate areas such as New Zealand, the Mediterranean Sea and the Japanese coasts (Vila et al., 2001, Shears & Ross, 2009, Sato et al., 2011) expanding the distribution range to the 45° Northern (Monti et al., 2007) and the 45° Southern parallel (Rhodes et al., 2010). Six of the nine species of the genus can be considered to have a pantropical distribution: *O.* cf. *heptagona*, *O.* cf. *labens*, *O.* cf. *marinus*, *O.* cf. *caribbeanus*, *O.* cf. *belizeanus* and *O.* cf. *mascarenensis* while *O.* cf. *ovata*, *O.* cf. *siamensis* and *O.* cf. *lenticularis* occur from tropical to temperate waters (Rhodes, 2010).

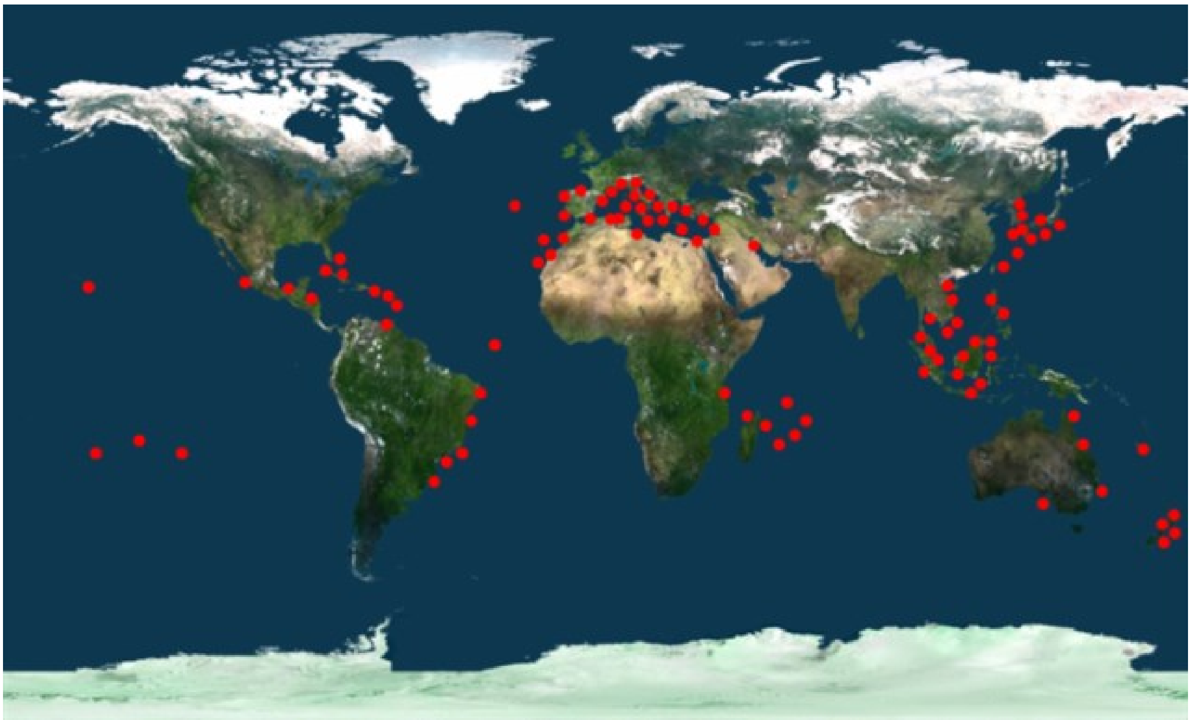


Figure 1.2. *Ostreopsis* genus distribution (based on Rhodes, 2010 and updated to March 2015).

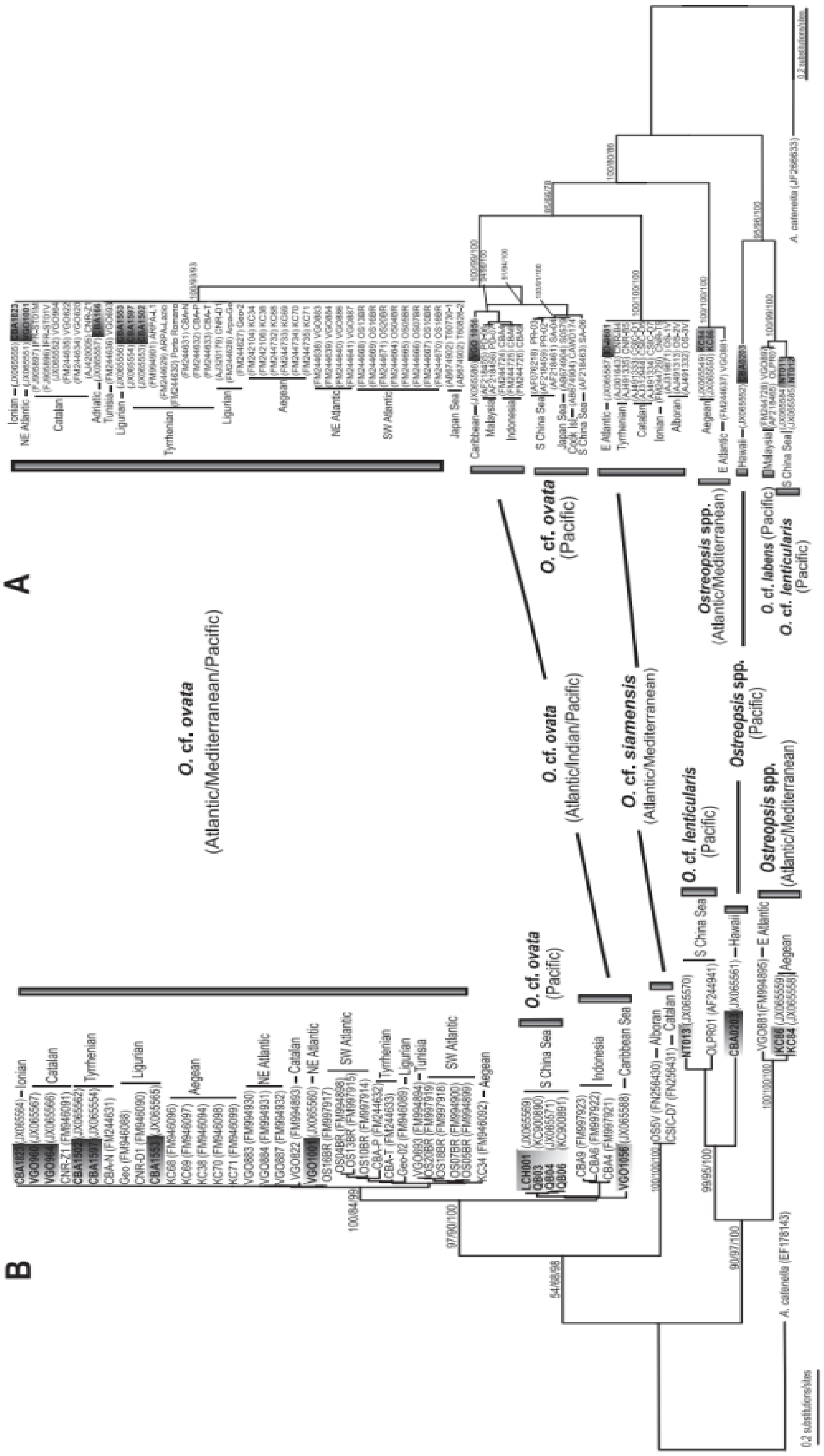


Figure 1.3. Phylogenetic tree of the genus *Ostreopsis* inferred from ITS-5.8S (A) and LSU (B) ribosomal gene sequences. The trees are rooted with *Alexandrium catenella* as outgroups (from Penna et al., 2014).

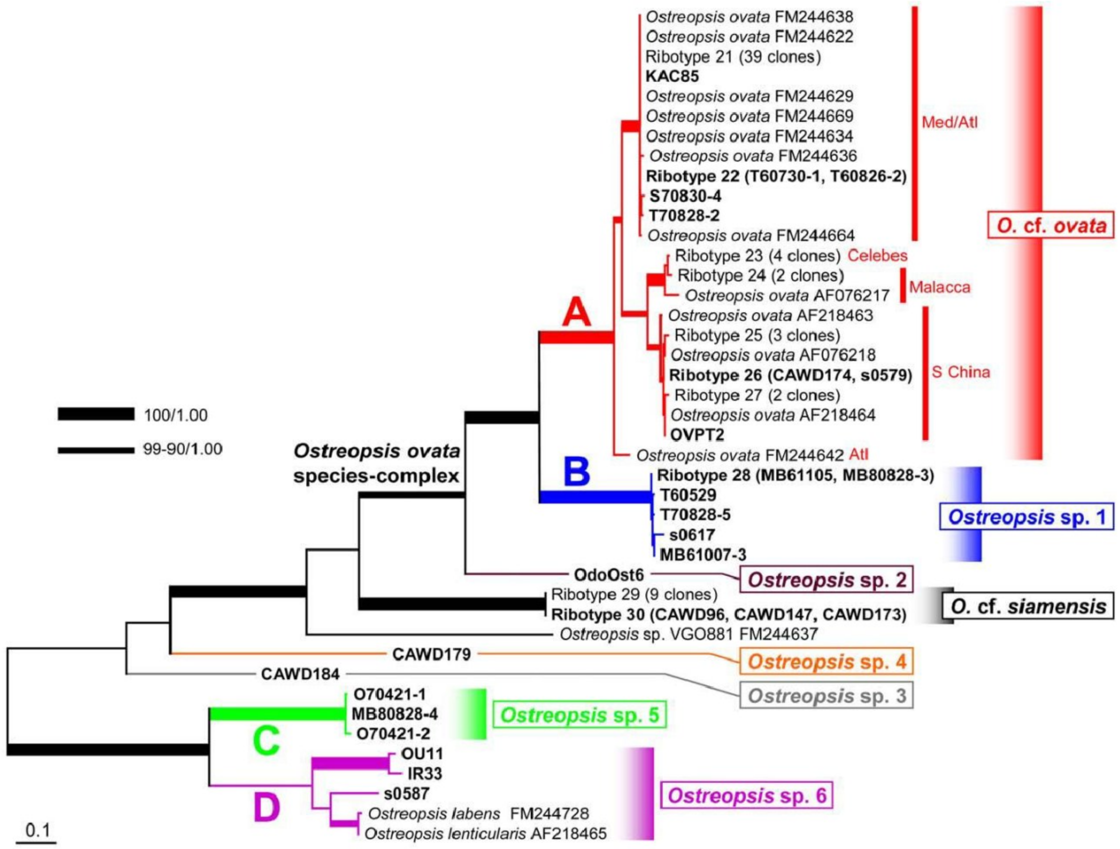


Figure 1.4. Maximum likelihood phylogeny of *Ostreopsis* inferred from ITS sequences. Nodes with strong supports are shown as thick lines. Geographic origins of *O.* cf. *ovata* clone are indicated (from Sato et al., 2011).

Ostreopsis cf. *ovata* is the most widely distributed species of the genus and it is present in both the hemispheres. Its occurrence in the Mediterranean Sea (fig. 1.5) dates back to 1972, when it was first spotted in the harbour of Villefranche-sur-mer (Taylor, 1979) but misidentified as *Ostreopsis siamensis*, the only species known at that time (Taylor, pers. comm. in Zingone et al., 2006; Zingone et al., 2010). *Ostreopsis* cf. *ovata* was later reported in Italy in the Tyrrhenian Sea in 1989 (Zingone, pers. comm. in Tognetto et al., 1995). In recent years, the presence of this species in the Mediterranean Sea has been confirmed by several studies (Turki, 2005; Aligizaki and Nikolaidis, 2006; Ciminiello et al., 2006; Riobó et al., 2006; Zingone et al., 2006; Monti et al., 2007; Vila et al., 2008; Bushati et al., 2010; Guerrini et al., 2010; Totti et al., 2010; Mangialajo et al., 2011; Illoul et al., 2012; Pfannkuchen et al., 2012; Abboud-Abi Saab et al., 2013) and by several monitoring surveys conducted at national level in Mediterranean countries (ISPRA, 2012; Lemée et al., 2012). *Ostreopsis* cf. *ovata* is also present in the Atlantic Sea (Silva et al., 2010; Nascimento et al., 2012; Penna et al., 2014). The other species occurring in the Mediterranean area is *O.* cf. *siamensis*. This species is mostly found on Northern African coasts (Abboud-Abi Saab and El-Bakht, 1997; Turki et al., 2006; Ennaffah et al., 2011; Mabrouk et al., 2012a) and on the Iberian coasts of the Atlantic Ocean (Laza-Martinez et al., 2011; David et al., 2012) but its presence is documented also in Siciliy (Penna et al., 2005; Gangemi et al., 2006; Amorim et al., 2011), Greece (Aligizaki & Nikolaidis, 2006) and Iberian Mediterranean coasts (Vila et al., 2001; Penna et al., 2005). Furthermore, in the Mediterranean Sea, recent phylogenetic analyses are showing interspecific diversity and new clades based on material from Canary Islands, Greece and Crete (Penna et al., 2012) and Cyprus (Giussani et al., 2014b) are under investigation to clarify if they could rank as new species.

The increasing spatial distribution of *Ostreopsis* in temperate and tropical waters does not appear to be driven by climate, as toxic blooms of the species are reported in a wide latitudinal range. The dispersion capabilities of the genus combined with the continuous efforts in the research will likely enlarge the geographic range of the genus in future years (Parsons et al., 2012).

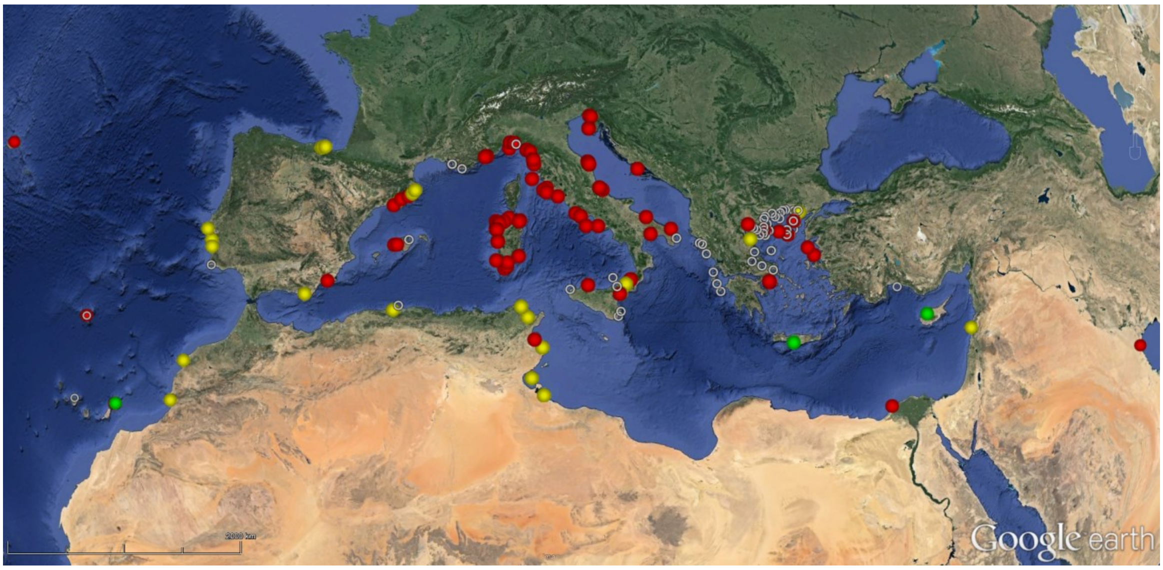


Figure 1.5. Distribution of *Ostreopsis* species in the Mediterranean Sea and Western Atlantic Sea: *O.* cf. *ovata* (red dots), *O.* cf. *siamensis* (yellow dots), *Ostreopsis* undetermined species (empty circles). A new species from Crete, Cyprus and Canary islands currently under description is also shown (green dots). See Appendix B of this thesis for location references on this map.

1.3.4 Toxicity

Species of the genus *Ostreopsis* are capable to produce substances of the palytoxin group (PLTX) (Usami et al., 1995). Palytoxin (fig. 1.6) was firstly isolated from the tropical coral *Palythoa* spp. (Moore and Scheuer, 1971) and is one of the most potent non-proteinaceous toxins ever found in nature (Deeds and Schwartz, 2010). PLTX has a molecular formula of $C_{129}H_{221}N_3O_{54}$ and a molecular weight of 2680 Da (Moore and Bartolini, 1981). The complex structure of this molecule consists of lipophilic and hydrophilic areas. The mechanism of action is known: this molecule binds selectively to Na^+/K^+-ATPase converting the pump into an ion channel (Artigas and Gadsby, 2003), causing the impairment of cellular osmoregulation. Secondary pharmacological actions of this molecule include nausea, vomiting, hypersalivation, diarrhoea, numbness, abdominal cramps, muscular spasms and respiratory distress (Yasumoto et al., 1986; Alcala et al., 1988; Kodama et al., 1989).

Six of the nine species of *Ostreopsis* genus have shown to be toxic (Rhodes, 2010) and some of them capable to produce structural analogues of the palytoxin, known as PLTX-like molecules (Ciminiello et al., 2008). *Ostreopsis* species were considered to produce palytoxin itself or a similar chemical compound of the same molecular weight, which was provisionally called putative palytoxin (Onuma et al., 1999). A recent study showed that the molecule produced is instead a structural isomer of palytoxin (García-Altares et al., 2014) and thus the name of isobaric palytoxin was proposed. *Ostreopsis* spp. have been found to produce a large number of palytoxin isomers: the first molecular analogue discovered was ostreocin-D, isolated from *O.* cf. *siamensis* (Usami et al., 1995; Ukena et al., 2002). Mascarenotoxins (McTX) is another class of palytoxin-like molecules: initially isolated from *O.* cf. *mascarenensis* (Lenoir et al., 2004), different isomers were later identified also in *O.* cf. *ovata (Rossi et al., 2010)*.

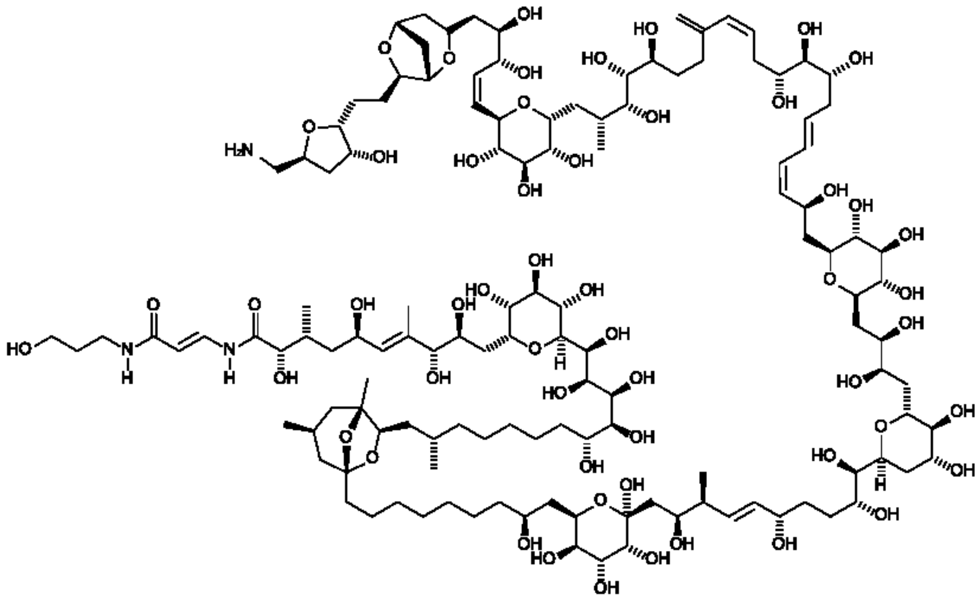


Figure 1.6. Molecular structure of palytoxin.

The largest group of palytoxin analogues produced by *Ostreopsis* species are the ovatoxins (OVTX). In recent times the number of chemical studies on ovatoxins has continuously increased, leading to the identification of several compounds belonging to this group, from ovatoxin-a to ovatoxin-h (Ciminiello et al., 2008; Rossi et al., 2010; Ciminiello et al., 2012; García-Altares et al., 2014; Brissard et al., 2015) (tab. 1.2). These molecules are also the most abundant toxins produced in *O.* cf. *ovata* cultures (Ciminiello et al., 2012), with ovatoxin-a being the major toxin produced. Despite the small structural divergences among these molecules, their toxicological profiles could be very different. Ostreocin-d, a very similar compound of palytoxin, showed mouse lethality as palytoxin, but a significantly reduced cytotoxicity and hemolytic potency (Ukena et al., 2002). Yet, toxicity effects of most of the pure single compounds are still unknown.

Other toxic compounds, referred as non-PLTX analogues, were found in *O.* cf. *lenticularis*, ostreotoxin-1 and ostreotoxin-3 (Mercado et al., 1994) from *O.* cf. *lenticularis*. More recently ostreol A, another cytotoxic non-palytoxin derivative, was identified in a strain of *O.* cf. *ovata* from Pacific area (Hwang et al., 2013). Difference in toxin production at intraspecific level may also exist: *O.* cf. *siamensis* in Mediterranean and Atlantic waters showed to be non-toxic (Paz et al., 2011; Ciminiello et al., 2013) while Pacific O. cf. *siamensis* populations caused negative effects on the health of the sea urchin *Evechinus chloroticus* in New Zealand (Shears and Ross, 2009). The on-going taxonomic revision of the genus will likely shed a light on variations on toxin produced by *Ostreopsis* at interspecific and intraspecific level.

Effects on human health

Human poisoning due to the consumption of seafood contaminated by palytoxin has been demonstrated in crabs (Alcala et al., 1988) and fishes (Fukui et al., 1987; Kodama et al., 1989; Onuma et al., 1999; Taniyama et al., 2003) from tropical areas, causing severe intoxications, in some case lethal. Clupeotoxism is the name of the syndrome associated with the consumption of fishes contaminated by palytoxin. *Ostreopsis* toxins have been hypothesized as source of seafood contamination for this syndrome (Randall, 2005) but their relationship remains elusive since case reports often suffer from the absence of chemical analytic confirmation of the toxins in the leftover food or in clinical samples (Tubaro et al., 2011).

Table 1.2. Summary of the toxic molecules known in *Ostreopsis* spp.

Chemical group	Chemical compound	Species	Bibliographic references
PLTX analogues	Isobaric palytoxin	*O.* cf. *ovata*	Onuma et al., 1999; Garcìa-Altares et al., 2014
	Ovatoxin-a	*O.* cf. *ovata*	Ciminiello et al. 2008
	Ovatoxin-b	*O.* cf. *ovata*	Rossi et al., 2010
	Ovatoxin-c	*O.* cf. *ovata*	Rossi et al., 2010
	Ovatoxin-d/e	*O.* cf. *ovata*	Rossi et al., 2010
	Ovatoxin-f	*O.* cf. *ovata*	Ciminiello et al., 2012b
	Ovatoxin-g	*O.* cf. *ovata*	Garcìa-Altares et al., 2014
	Ovatoxin-h	*O.* cf. *ovata*	Brissard et al., 2015
	Mascarenotoxin-a	*O.* cf. *mascarenensis* *O.* cf. *ovata*	Lenoir et al., 2004 Rossi et al., 2010
	Mascarenotoxin-b	*O.* cf. *mascarenensis*	Lenoir et al., 2004
	Mascarenotoxin-c	*O.* cf. *ovata*	Rossi et al., 2010
	Ostreocin-d	*O.* cf. *siamensis*	Usami et al., 1995; Ukena et al., 2001
Non-PLTX analogues	Ostreotoxin-1	*O.* cf. *lenticularis*	Mercado et al., 1994
	Ostreotoxin-3	*O.* cf. *lenticularis*	Mercado et al., 1994
	Ostreol A	*O.* cf. *ovata*	Hwang et al., 2013

While the mortality for clupeotoxism is quite high in tropical areas, at present day no episode related to this illness has ever been reported in temperate areas. Oral ingestion of palytoxin-contaminated seafood includes general malaise, gastrointestinal symptoms, myalgia, muscle cramps, cardiac dysfunctions, respiratory problems and cyanosis and occasional metallic and/or bitter taste of seafood (Tubaro et al., 2011). In temperate areas, molecules of the group of the palytoxin have been shown mainly in sea urchins (Ciminiello et al., 2006; Ciminiello et al., 2008; EFSA, 2009; Ciminiello et al., 2010; Rossi et al., 2010) and in shellfish (Briggs et al., 1998; Pearce et al., 2001; Aligizaki et al., 2008; EFSA, 2009), in coincidence with and after the occurrence of *Ostreopsis* blooms (Aligizaki et al., 2008). The emerging risk of exposure to seafood contaminated by palytoxins has led recently the European Food Safety Authority to propose a regulatory level of 30 µg of palytoxin and ostreocin-d per kg of body tissue in shellfish resources (EFSA, 2009).

Ingestion of contaminated seafood is not the only exposure way to molecules of PLTX-group: in 2005 and in 2006, intense blooms of *Ostreopsis* cf. *ovata* along the northern Tyrrhenian coasts (Brescianini et al., 2006; Durando et al., 2007) caused the intoxications of more than 200 hundred people, possibly by the dispersion of *Ostreopsis* cells and/or toxins in the marine aerosol, (Gallitelli et al., 2005; Ciminiello et al., 2006; Kermarec et al., 2008; Tichadou et al., 2010; Honsell et al., 2011). The symptoms reported were fever, sore throat, cough, dyspnea, headache, nausea, rhinorrhea, conjunctivitis and skin irritations (Durando et al., 2007). The occurrence of *Ostreopsis* cf. *ovata* cells and palytoxin-like molecules in the aerosol during the seasonal blooms of *O*. cf, *ovata* has been recently confirmed (Casabianca et al., 2013; Ciminiello et al., 2014). Intoxication by contaminated aerosol has been reported in French (Tichadou et al., 2010) and Spanish coasts (Riobó et al., 2010) and in several other Italian littorals (Gallitelli et al., 2005; Martinelli et al., 2009; ISPRA, 2012). On the other hand, case reports of PLTX exposure by contact (cutaneous and ocular) are limited probably due to the transient nature of the symptoms (24-48 h), which may be overlooked and underreported. Dermatitis and ocular mucous irritations have been reported after exposition to marine aerosol during *O.* cf. *ovata* proliferations (Gallitelli et al., 2005; Durando et al., 2007; García et al., 2008; Kermarec et al., 2008; Tichadou et al., 2010). A case report of dermatitis associated with the direct contact of contaminated material, involved the handling of fishing nets by fishermen in Southern Italy (Tubaro et al., 2011) where high *Ostreopsis* cells concentration were subsequently found. Other cases of PLTX exposure by contact are reported instead during aquaria maintenance where contaminated coral were present (Hoffmann et al., 2008).

Effects on the environment

An unpleasant aspect of the water such as a brownish mat covering the benthos, suspended mucous flakes in the water column and superficial foams have been first reported during *Ostreopsis* blooms in Tuscany, in the vicinity of partially submerged artificial structures to protect the coastline (Sansoni et al., 2003). These phenomena, along with the above-mentioned cases of intoxication may be detrimental to tourism and recreational activities. Another important aspect of the detrimental impact of the blooms is related to the negative effects occurring at ecosystem level. During some blooms events, health impairments in benthic invertebrates were found, initially attributed to hypoxia and/or anoxia (Simoni et al., 2003). In particular, mussels (*Mytilus galloprovincialis*) showed byssus loosening and many empty shells on the benthos, increasing the water turbidity. Reduction in the size of the population of other invertebrates such as *Patella* spp., *Monodonta turbinata*, *Actinia equina* were also found (Funari et al., 2014). Mass mortalities in sea urchins were reported in New Zealand (Shears and Ross, 2009) and in Italy (Simoni et al., 2003; Accoroni et al., 2011). During the blooms, spines folding or their loss in sea urchins and anomalous arrangement or arms loss in sea stars were also observed (Sansoni et al., 2003). Also some edible species such as the annular sea bream *Diplodus annularis*, the common cuttlefish *Sepia officinalis* and the common octopus *Octopus vulgaris* showed signs of suffering and death. Negative effects of the blooms were also reported in the phytobenthos: in the Adriatic Sea, reduction in macroalgae covering and the whitening of the thalli were found (Accoroni et al., 2011).

The environmental issues related to *Ostreopsis* blooms showed variable intensity and at present time they are hardly predictable. Several marine organisms can accumulate high concentrations of *Ostreopsis* toxins without showing any apparent pathologic symptoms (Aligizaki et al., 2011). However, closer analysis have shown that apparently healthy mussels collected in areas affected by blooms, like the Gulf of Naples, may have histopathological alterations (Carella et al., 2015). Pathological studies have been performed *in vitro* on several marine organisms of interest to bridge the existing gap of knowledge at ecotoxicologic level. Experiments on different developmental phase of the sea urchin *Paracentrotus lividus* showed inhibition of embryos development (Pagliara and Caroppo, 2012) and several deleterious effects (reduction of the spawning and of fertilization, stimulation of the stress response) in the adult phase, possibly leading to negative effects in the recruitment of the population (Migliaccio et al., in preparation). Hystopathological studies on the mussel *Mytilus galloprovincialis* showed instead impairment of metabolic functions such as the activation of inflammatory response (Carella et al., 2015) and decrease of the immunologic activity and tissue degeneration (Gorbi et al., 2013).

At ecosystem level, the intensity of the toxic effects of *Ostreopsis* blooms may be modulated by environmental variables (temperature, dissolved oxygen), by the exposure routes (ingestion of cells, contact with gills) (Faimali et al., 2012; Gorbi et al., 2013) and blooming phase (Simonini et al., 2011). Also physiological features (i.e. trychocysts and mucus production) may modulate the toxicity: *Ostreopsis* cells are capable to produce a mucous web (fig. 1.7), which entangle nematods and crustaceans (Barone and Prisinzano, 2006), sometimes even bigger (Barone, 2007). In an experimental study, the presence of this mucous trap showed to exert an increased mortality in invertebrates and cnidarian larvae, compared with the toxins themselves, suggesting an active role of the mucus in the toxic delivery (Giussani et al., 2014a).

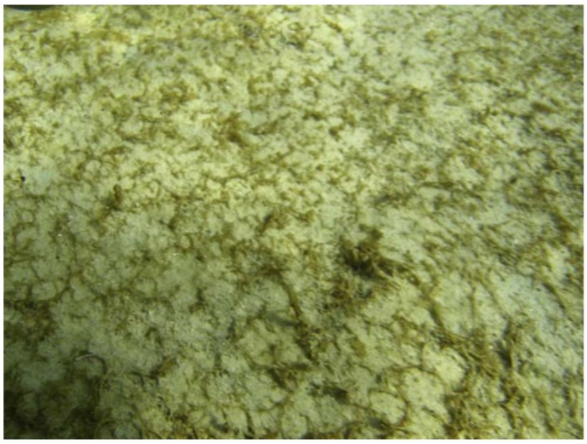


Figure 1.7. *Ostreopsis* cf. *ovata* forming a brownish spotted mat on rocks in Conero Riviera, North Adriatic Sea (from Totti et al., 2010).

1.3.5 Ecology

A number of studies have been conducted on the potential role of environmental variables on the dynamic of *Ostreopsis* blooms. We present here some general highlights on the most studied environmental factors, i.e. temperature, salinity, depth, nutrients, light, wave energy and substrate, with an overview of their influence on the distribution, survival, growth and bloom formation of *Ostreopsis* species. In many cases, contrasting results are found for the same taxon from different areas, probably related to wide genetic variability and cryptic speciation in the *Ostreopsis* genus, which was unknown until recently.

Temperature

Several studies have proposed temperature as an important driver variable in *Ostreopsis* ecophysiology: high sea water temperature has been indicated among the major causes of *Ostreopsis* blooms in the Mediterranean Sea (Tognetto et al., 1995; Aligizaki and Nikolaidis, 2006; Mangialajo et al., 2008) and proposed as a main factor responsible for the geographical expansion of the genus and the recent occurrence of *O.* cf. *ovata* in temperate areas (Granéli et al., 2010; Hallegraeff, 2010). On the other hand, a number of field and experimental studies have demonstrated that in the genus *Ostreopsis* there exists a quite wide range of interspecific and intraspecific diversity in the response to temperature (Yamaguchi et al., 2012a; Yamaguchi et al., 2012b)

Ostreopsis cf. *siamensis* from the Pacific area is adapted to temperature ranging from 7 to 25°C (Morton et al., 1992; Rhodes et al., 2000; Selina and Orlova, 2010), with the production of resting stages occurring at 10 °C (Pearce et al., 2001)). At the opposite, *Ostreopsis* cf. *siamensis* from the Mediterranean Sea, which is genetically very distinct from that from the Pacific area, probably tolerates higher temperatures, since it occurs in North Western waters from March to September (Vila et al., 2001) when water temperatures may reach values as high as 28 °C (Mangialajo et al., 2011).

Ostreopsis cf. *lenticularis* is negatively correlated with temperature in the Caribbean Sea (Carlson and Tindall, 1985) but experimental studies found that high seawater temperature (31 °C) was able to stimulate the highest growth rate and toxicity (Tosteson et al., 1989; Ashton et al., 2003).

In agreement with the high wide genetic variability revealed for *O.* cf. *siamensis*, contrasting results were shown also for *O.* cf. *ovata*: the extreme cases would be the negative correlation with temperature found in a field study in the Hawaii (Parson and Preskitt, 2007) and the finding of blooms at 28 - 29 °C along the Latium coasts in the Mediterranean Sea (Tognetto et al., 1995). However, these edge cases have been smoothened by several findings coming from experimental studies and field observations. Experimental evidences show that *O.* cf. *ovata* in the Mediterranean Sea is capable to grow from 16 to 30 °C (Graneli et al., 2010), with optimal values of growth at 26 °C (Scalco et al., 2012). Field observations confirm this trend, with bloom concentrations attained over a wide range of temperature values, i.e. at low temperature (>22 °C) in the Adriatic Sea (Monti et al., 2007; Totti et al., 2010; Accoroni et al., 2011), at intermediate values (24 - 26 °C) in Tyrrhenian and South Adriatic waters (Ungaro, 2005; Ciminiello et al., 2006; Gangemi et al., 2006; Ingarao and Pagliani, 2009) and at high seawater temperature (<28 °C) in the above mentioned case of the Latium coasts (Tognetto et al., 1995).

These conflicting results may likely reflect the occurrence of cryptic species and/or geographical differences (Parsons et al., 2012). Further studies and observations are needed to clarify this aspect of the ecophysiology of *O.* cf. *ovata*. Nevertheless, the wide tolerance to different temperatures may have contributed to the global expansion of this species and elicited the colonization of new areas, as emerging evidences of a recent introduction of *O.* cf. *ovata* in the Mediterranean and Atlantic Seas are demonstrating (see paragraph 1.3.2).

Salinity

While *Ostreopsis* species in the Mediterranean Sea occur at relatively high values of salinity (>37) (Berdalet et al., 2012) and their presence has never been detected in correspondence of rivers (Pistocchi et al., 2011b), *O.* cf. *heptagona* and *O.* cf. *siamensis* have been observed at lower salinity values (28 - 34) (Morton et al., 1992; Rhodes et al., 2000). Several studies showed conflicting results in salinity preferences of *Ostreopsis* cf. *ovata*: though a negative correlation has been found in Hawaiian coasts (Parsons and Preskitt, 2007), experiments performed on Mediterranean strains demonstrate a strong tolerance to salinity (26 - 40) (Pezzolesi et al., 2012) while strains from South China Sea and Thailand Sea showed semioptimal conditions at low (24 - 30) and intermediate (28.7 - 35) values, respectively (Tawong et al., 2015). Also in this case, the wide genetic variability and cryptic speciation observed in the *Ostreopsis* genus may be responsible of the differences at basin level.

Nutrients

The relationships between nutrient enrichment and HAB is still under debate: while a number of studies highlight a connection of HABs occurrence with anthropogenic nutrient loading (Glibert et al., 2014), others present a more complex view of the phenomena, where nutrients could affect only high biomass blooms (Anderson et al., 2002; Zingone and Wyatt, 2005; Smayda, 2008), suggesting that other variables such hydrodynamism and other location-specific factors may promote the bloom formation (Davidson et al., 2014). The validity of the relationship between nutrient enrichment and HAB remains elusive also for benthic dinoflagellates (Grzebyk et al., 1994) and in particular for *Ostreopsis* (Pistocchi et al., 2011). *Ostreopsis* species are present worldwide in eutrophic (Heisler et al., 2008; Hallegraeff, 2010) and oligotrophic areas (Tindall and Morton, 1998). While some work performed in the field showed no correlation between *Ostreopsis* species and nutrient availability (Delgado et al., 2006; Shears and Ross, 2009), a study in Hawaiian waters found positive correlations (Parsons and Preskitt, 2007), although these differences may be related to genetic diversity of the species. Such relationship is not yet clearly demonstrated also in the Mediterranean Sea (Vila et al., 2001), but opposite correlations were found with silicates (-) (Cohu et al., 2011) and phosphates (+) (Asnaghi et al., 2012). The mutual interplay of physiological characteristics (Pezzolesi et al., 2014), environmental drivers (Burkholder et al., 2008; Parsons et al., 2012) and behavioural traits (Barone and Prisinzano, 2006; Burkholder et al., 2008; Escalera et al., 2014) may also be responsible of the complexity of the trophic capabilities observed in the genus.

Light

While experimental studies have shown the preference of *Ostreopsis* for low light regimes (Scalco et al., 2012), several studies found that *Ostreopsis* cells preferentially occur in shallow waters (Totti et al., 2010; Cohu and Lemee, 2012), showing a negative correlation with depth (Richlen and Lobel, 2011). This apparent contrast may be explained with the capabilities of these organisms to modulate light intensity by behavioural traits such as macroalgal shading (Ballantine et al., 1988) or mucus production (Heil et al., 1993) and daily cycle-based photoacclimative mechanisms (Fraga et al., 2012). Other complex relationships with photoperiod may also be involved (Berdalet et al., 2012).

Wave energy

A general consensus about the effect of wave action on *Ostreopsis* concentrations has yet to be reached. Several studies have shown that stable conditions are more favourable for *Ostreopsis* species (Carlson and Tindall, 1985; Taylor et al., 1985; Tindall and Morton, 1998; Chang et al., 2000; Barone, 2007; Shears and Ross, 2009; Totti et al., 2010; Richlen and Lobel, 2011; Mabrouk et al., 2012b) while other studies pointed out a preference of *Ostreopsis* for high energy waters (Vila et al., 2001; Parsons and Preskitt, 2007; Kibler et al., 2008; Richlen and Lobel, 2011). A complex of environmental (water motion, substrate, light exposure) (Grzebyk et al., 1994) and genetic factors (Parson et al., 2012) have been suggested as causes modulating the observed differences in hydrodynamic preferences. Temporal variability in the planktonic concentration of *Ostreopsis* may also be linked to the resuspension of cell in the water column during stormy events, which may be re-established after some days of more stable conditions (Accoroni et al., 2012).

Substrate preferences

O. cf. *ovata* is capable to grow on abiotic substrata (rocks, invertebrate shells, water column, plastic debris) (Bottalico et al., 2002; Masò et al., 2003; Sansoni et al., 2003; Simoni et al., 2003; Totti et al., 2010) as well as biotic substrata (coral rubble, macroalgae, marine phanerogams) (Fukuyo, 1981; Norris et al., 1985; Turki, 2005; Aligizaki and Nikolaidis, 2006; Turki et al., 2006; Battocchi et al., 2010; Totti et al., 2010; Mabrouk et al., 2012b). *Ostreopsis* cells can adhere to the substrate by the formation of filaments (trichocysts) (Honsell et al., 2013) and mucillagenous substances, which may cover the benthos, appearing as a brownish mat, sometimes spotted (Totti et al., 2010). *Ostreopsis* cells may be embedded in the mucous matrix or either unlinked to the mat, and thus able to disperse in the plankton (Faust and Morton, 1995; Tognetto et al., 1995; Chang et al., 2000; Parsons et al., 2012; Zingone, 2014). The presence of mucilage may offer many adaptive benefits to individual algae, such as increased settling rates, aid in the sequestration of vital nutrients, reduction of the palatability of algae and defence against grazing (Reynolds, 2007).

Several evidences show that *Ostreopsis* cells display algal host preferences, with specie-specific differences: red and brown algae are preferentially epiphytized by *Ostreopsis* cf. *ovata* (Monti et al., 2007; Parson & Preskitt, 2007) compared to green algae and seagrasses (Aligizaki & Nikolaidis, 2006), while *O.* cf. *siamensis* was found on seagrasses in Tasmania waters (Pearce et al., 2001) and in Tunisia (Mabrouk et al., 2012a). Other factors related to the nature of the macrophytic substrates (surface area, spatial structure, presence of allelopathic compounds) may explain the host preference of the species (Lobel et al., 1988; Bomber et al., 1989; Grzebyk et al., 1994).

Framework and aims of the study

The Veterinary sector of the Assessorato alla Salute of Campania Region convened in 2007 a board of experts, as indicated in the Guidelines on *Ostreopsis ovata* of the Italian Ministry of Public Health, and funded a Plan for the monitoring and the management of the risk associated to *Ostreopsis* blooms. This program is still active and it is carried out by five institutions: Agenzia Regionale per la Protezione dell'Ambiente (ARPA Campania), Osservatorio Regionale per la Salute Alimentare (ORSA), Stazione Zoologica Anton Dohrn (SZN), Instituto Zooprofilattico Sperimentale del Mezzogiorno (IZSM) and Dipartimento of Chemistry of Natural Substances of the University Federico II.

This thesis is based on the analysis of a multiannual dataset gathered at different coastal sites along the Gulf of Naples and a sampling campaign on the Gulf of Salerno, in the framework of the above-mentioned regional environmental monitoring plan. Three main sites were investigated: 1) the area of Posillipo, in the Gulf of Naples, where the most intense blooms of *O.* cf. *ovata* for the Campania Region are regularly recorded; 2) the Ischia Island coasts, which are subjected to natural acidification due to the presence of volcanic vents; 3) the sandy area of the south eastern area of Gulf of Salerno littoral, where structures for the defence from coastal erosion will be built, and where *O.* cf. *ovata* has not been found in previous researches.

The present work intends to answer to the following questions:

- how much is variable the temporal distribution (inter-annual, seasonal and intra-seasonal) of *Ostreopsis* cf. *ovata* in Posillipo area?
- how much the environmental variables affect the intensity of the blooms?
- do changes in coastal oceanography caused by anthropogenic pressures (rise in temperature, eutrophication, ocean acidification) will play a role in *Ostreopsis* distribution?
- do artificial breakwaters for the defence to coastal erosion will provide an optimal substrate compared to the sandy bottoms of the Gulf of Salerno?
- can an ecologic assessment of the toxic blooms of *Ostreopsis* offer a better understanding and offer tools for the prediction of the effects on the environment, the benthic fauna and the human health?
- does the regional monitoring plan of *Ostreopsis* blooms along Campania coasts represent a valid tool for the defence of public health?

Chapter 2

Temporal distribution of *Ostreopsis* cf. *ovata* in the Gulf of Naples

ABSTRACT

The expansion and the intensification of *Ostreopsis* blooms are posing new threats to human and environmental health in temperate waters, and particularly in the Mediterranean Sea. Nonetheless, the reasons for this increase and predictions of future trends are still out of our reach. The phenology and ecology of *Ostreopsis* cf. *ovata* were investigated in the Posillipo coasts of the Gulf of Naples (Tyrrhenian Sea, Mediterranean Sea) to assess distribution patterns and evaluate factors affecting the development of the blooms. Sampling was carried out weekly from 2007 to date during summer/early autumn at two stations of the Posillipo coasts, Gaiola and Rocce Verdi. *O.* cf. *ovata* distribution showed a decreasing trend over the years and a marked seasonality, with a bimodal distribution: a first, more intense bloom in July ($< 3.8 \cdot 10^5$ cells g^{-1} fw) was followed by a second, minor and less constant bloom in late September ($< 2.4 \cdot 10^5$ cells g^{-1} fw). Temperature, salinity and nutrient availability did not appear to represent primary drivers for bloom timing and intensity, while low hydrodynamic regimes likely affects the intensity of *O.* cf. *ovata* blooms.

2.1 Introduction

Blooms of the toxic dinoflagellate *Ostreopsis* genus are a recurrent phenomenon occurring in tropical and temperate regions worldwide (Rhodes, 2010). In particular, the most distributed species in the Mediterranean Sea, *Ostreopsis* cf. *ovata*, is causing blooms with increasing intensity, frequency and distribution (Vila et al., 2001; Aligizaki and Nikolaidis, 2006; Mangialajo et al., 2011). These blooms have been associated with respiratory problems and skin irritations in humans (Gallitelli et al., 2005; Tubaro et al., 2011), causing major intoxication events in Italian coasts in 2005 and 2006 (Ciminiello et al., 2006; Mangialajo et al., 2008). The negative impacts of these toxic blooms extend also at ecosystem level (Ramos and Vasconcelos, 2010): suffering of macroalgae (Vale and Ares, 2007) and mortality in benthic fauna (sea urchins, mussels and fishes) have been reported on the coasts of New Zealand (Shears and Ross, 2009) and in the Mediterranean Sea (Sansoni et al., 2003; Simoni et al., 2003; Ciminiello et al., 2006; Totti et al., 2010). Chemical analyses conducted on *Ostreopsis* cultured biomass have shown that the causative agent of these toxic events are molecules related to palytoxin (Rossi et al., 2010; Ciminiello et al., 2012; García-Altares et al., 2014), one of the most potent non proteic substance ever found in nature (Deeds and Schwartz, 2010). Ecological surveys conducted in the Mediterranean Sea have found different seasonality of the blooms in two basins: early summer in the NW Mediterranean Sea and in late summer/autumn in the Adriatic Sea (Totti et al., 2010; Mangialajo et al., 2011; Pistocchi et al., 2011a). In the last years, the increased awareness of the risks associated to these blooms led to establish specific monitoring programmes for *Ostreopsis* bloom detection in different countries (Lemée et al., 2012; Vila et al., 2012). Since the first detection in Tyrrhenian coasts (Tognetto et al., 1995), this species has been found in almost all the Italian coastlines (Funari et al., 2014). The occurrence of *Ostreopsis* cf. *ovata* along the coasts of the Campania region (Tyrrhenian Sea), in particular, is reported back to 2004, when it was firstly identified during a macroalgae sampling along the Posillipo area of the Gulf of Naples (Zingone et al., 2005; Zingone et al., 2006). A targeted survey conducted in 2007 in the framework of *Ostreopsis* monitoring in the same areas led to the identification of two “hot spots” of proliferation of this species on the rocky coasts of Sorrento and Posillipo (ARPAC, 2008). Here we provide an ecological and phenological assessment of the bloom of *Ostreopsis* cf. *ovata* in Posillipo coast (Gulf of Naples), one of the most impacted areas of the Southern Mediterranean Sea. We will also define the most favourable conditions for *Ostreopsis* blooms, in relation to abiotic and biotic factors.

2.2 Specific aims of the chapter

The objectives of the study of the development of *Ostreopsis* cf. *ovata* blooms is the analysis of inter-annual, seasonal and intra-seasonal variability and the influence of the environmental variables on the intensity of the blooms.

This study aims to verify:

1. the seasonality of the blooms of *O.* cf. *ovata* in relation to environmental parameters;

2. their tendency to diminish or increase in intensity and duration over the years;

3. the effect of environmental variables on the intensity of the blooms (temperature, nutrients, light exposure, hydrodynamic regime);

4. the presence of microalgae in surface waters, potentially able to disperse with marine aerosol;

2.3 Study areas: Posillipo coast

In the framework of the “Regional monitoring program for *Ostreopsis* blooms along the Campania coasts”, the dynamic of *Ostreopsis* blooms were followed at two fixed stations in the Naples area, Gaiola and Rocce Verdi (fig. 2.1, tab. 2.1), both located along the Posillipo coastline, in the northwestern part of the Gulf of Naples. This is the only stretch of coastline in 50 km that has been interested by very low anthropogenic activity over centuries, preserving its original environment (Simeone et al., 2012). Moreover, in 2007 a regional survey for *Ostreopsis* occurrence at these two stations has shown the presence of the most intense blooms of *Ostreopsis* along the Campania coast. Gaiola station is located in the Marine Protected Area (MPA) “Gaiola Underwater Park”. This MPA is the smallest of Italy, covering 41.6 hectares and it is a naturalistic and archaeological site of interest. In this station there is a considerable concentration of submerged or semi-submerged archaeological structures of the Roman times (Simeone and Masucci, 2009). Gaiola sampling station is within the “Integral Reserve Area” (A Zone) of the MPA, where maritime traffic and bathing are forbidden. Rocce Verdi station is located at 0.5 nm east from Gaiola station, far off the MPA. Both stations are characterized by rocky bottom. An additional sampling site was added in 2011, Cala San Basilio (fig. 2.1, tab. 2.1), characterized by shallow waters, low hydrodynamism and high density of bathers, especially during July-August. This site is very close to Gaiola station but right outside the boundaries of the MPA “Gaiola Underwater Park”.

Table 2.1. Sampling sites and coordinates (WGS 84) of stations along Posillipo coasts.

Station	Latitude (N)	Longitude (E)
Gaiola	40° 47’ 31’’	14° 11’ 17’’
Rocce Verdi	40° 47’ 50’’	14° 12’ 04’’
Cala San Basilio	40° 47’ 34’’	14° 11’ 16’’

Figure 2.1. Sampling sites along Posillipo coasts.

2.4 Material and methods

2.4.1 Sampling methods

Sampling was carried out over eight years, from 2007 to 2014, from middle of June until the end of the bloom at two stations of the Gulf of Naples, Gaiola and Rocce Verdi, with the support of the SZN R/V “Federico Raffaele” or with the R/V “Vettoria” and in the 2012 with the logistic support of the C.S.I. Gaiola Onlus R/V “Aquavision”. In case of unavailability of the boats, the sampling was performed only at Gaiola station. The sampling was suspended at Gaiola station in July-August 2010 and at Rocce Verdi station at the beginning of 2014 sampling campaign for technical issues. Macroalgal samples of red and/or brown algae (*Asparagopsis taxiformis*, *Jania rubens, Corallina mediterranea* within the red algae and *Halopteris scoparia*, *Dictyota dichotoma,* within the brown algae) were collected in triplicates by SCUBA divers at a depth comprised from 1.7 m to 7.4 m, within 5 m of distance. The green alga *Flabellia petiolata* was sampled in case of unavailability of other macroalgal substrate. Macroalgal samples were collected following two methods: the method in the Guidelines of the Italian Ministry of Health (Di Girolamo et al., 2007) (referred here as “official method”) and the Falcon method (ISPRA, 2012). Superficial water was collected using 250 ml plastic bottles. At Gaiola and Rocce Verdi, macroalgal samples were collected from the same thallus following both methods. At Cala San Basilio station, samples were collected instead only with the Falcon method.

Official method

The method in the Guidelines of the Italian Ministry of Health "Gestione del rischio associato alle fioriture di *Ostreopsis ovata* nelle coste italiane" (Di Girolamo et al., 2007) consists of the following operations:

- covering a macrophyte with a plastic bag, cutting the thallus at the base and rapidly closing the bag, collecting the macroalgae together with the adjacent water;

- scratching the surface, collecting several adjacent tufts and the adjacent water in the plastic bag, in case the macrophytes are present in very small clusters.

After the collection, on board of the R/V, macroalgal samples were immediately transferred to 2 litres plastic cylinders and kept in the dark and at room temperature until the arrival in the laboratory. The collected seawater exceeding 2 litres limit was left in the plastic bag; it was kept in the same condition as the macroalgal sample and then merged to the corresponding macroalgal/water sample. In the laboratory, samples in the cylinders were vigorously shaken (ca. 40 times) to allow the detachment of epiphytic cells. The collected water was then transferred to a beaker. Additional filtered sea water (0.22 µm, 36‰ salinity) was added to the cylinder to rinse the macroalgal sample. Macroalgal samples were weighted using a precision balance using pre-weighted aluminium foils and fresh weight was determined (fw). Seawater volume was measured with a graduated cylinder and then filtered using a metallic sieve with a 98 µm mesh size). A subsample (250 ml) of this fraction was then fixed with a 19% aqueous formaldehyde solution at a final concentration of 0.7% for following counting. *Ostreopsis* cf. *ovata*, *Prorocentrum lima* and *Coolia monotis* cells obtained from macroalgal and water surface samples were counted using an inverted microscope (Zeiss Axiovert 200). At this purpose, aliquots of 3 or 10 ml (in the case of subsamples from macroalgae) or 50 or 100 ml (in the case of surface water samples) were placed in settling chambers according to the Utermöhl sedimentation method (Hasle, 1978) and observed after a minimum time of 16 and 48 hours, respectively. Counting was performed from 2 transects to the whole chamber, depending on sample cell density, from 100 to 400x magnification. Where possible, at least 100 *Ostreopsis* cf. *ovata* cells were counted, but the number of cells observed in the poor samples was much lower. Average and standard deviation of the three replicates was calculated and abundance values were expressed as number of cell per g of fresh weight macroalgae (fw). When benthic abundances exceeded 10000 cells g^{-1} fw, a counting was performed also on the corresponding water samples and planktonic concentration values were calculated (cell · l^{-1}).

Falcon method

The Falcon method, as proposed and specified in the Operative Protocols of ISPRA (ISPRA, 2012), consists of the following operations:

- cutting the thallus at the base and rapidly putting it into a 50 ml Falcon tube, taking care to do not shake the thallus during the collection. Close it promptly underwater. The thallus should be at least of 3 g fw and easily fitting in the 50 ml tube;

- filling the tube with seawater surrounding the sample or with filtered seawater;

- store the labelled tubes in dark conditions and field temperature until the treatment in laboratory. Fix the samples (Lugol, formaldehyde…) if will not be treated in the same day.

Macroalgal samples were fixed at a final concentration of 0.7% neutralized formaldehyde and stored in the dark until the analyses. Falcon tubes were vigorously shaken at least 20 times to allow the detachment of benthic cells. Macroalgal thalli were rinsed with fresh water, dry-blotted with paper on a pre-weighted aluminium foil, and then weighed to determine fresh weight (g fw). The volume of the water sample containing detached cells inside the Falcon tube was measured (ml). Cell counting was performed only on *Ostreopsis* cells, using a Zeiss Axioskop 2 plus, at a magnification of 125x, settling 1 ml of the water sample in Sedgewick-Rafter slides. Abundances of *Ostreopsis* cells were finally expressed as cells g^{-1} fw. For an extensive insight about the comparison between abundance values obtained with the two methods, see Appendix I of this thesis.

2.4.2 Environmental variables

Temperature and salinity

Water temperature, salinity and density values were measured with two CTD: from 2007 to 2011 data were acquired with a SBE 19plus V2 SeaCAT Profiler CTD; from 2012 to 2014 an YSI CastAway™ CTD was used instead.

Light

Light intensity data were acquired at Gaiola station with HOBO Data Loggers (Onset®). Data loggers were deployed at a depth comprised from 2.5 to 3.5 m, in 2011 to 2014 sampling campaigns, with a sampling frequency of 15’ (2011) and 10’ (2012 - 2014). Daily integrated light intensity values were obtained converting loggers values (in lux) in μmol photons $\cdot$ $m^{-2} \cdot s^{-1}$ using a conversion factor (Thimijan and Heins, 1983). Biofilms and organisms found on the devices were removed by SCUBA divers during the samplings. Logistic issues in the sampling and manual removal of data loggers by sea-goers did not allow the collection of continuous data in 2011 and 2014.

Hydrodynamic regimes

A total number of 78 samples were collected in triplicates with the official and Falcon method at Gaiola and Cala San Basilio stations to evaluate hydrologic preferences of *Ostreopsis*. Abundances values obtained with the official method in 2012 and 2013 at Gaiola station have been converted with a conversion factor to allow a comparison with the abundances at Cala San Basilio station.

Nutrients

Water samples for nutrient analysis (nitrates, nitrites, ammonia, ortophosphates and silicates) were collected and stored in polyethylene bottles at -20°C and unfreezed before the analysis at 37°C in a water bath. Analysis were performed following the method reported in “Metodologie Analitiche di Riferimento” (Cicero and Di Girolamo, 2001), using a continuous flux analyzer (Flow-Sys Systea). Detectability limits were 0.05 μmol $\cdot$ l^{-1} for ammonia and silica and 0.01 μmol $\cdot$ l^{-1} for nitrites, nitrates and orthophosphates.

2.4.3 Statistical analyses

All the analysis were performed on $\log_{10}(x+1)$-transformed data of benthic cell abundances to stabilize the variance. Normality assumption of benthic cell abundances was assessed by visual check (Q-Q plot) and evaluated with Shapiro-Wilk test. Significance level value was set for $p<0.05$. All tests were performed in XLStat.

Temporal distribution

Differences in the temporal distribution of *Ostreopsis* abundances between Gaiola and Rocce Verdi were evaluated with an unpaired sample t-test. Abundances temporal trends at each site were evaluated using a weighted regression analysis.

Principal component analysis

A principal component analysis (PCA) was performed on physical (temperature and salinity) and chemical variables (dissolved inorganic nitrogen (DIN), phosphates and silica) to identify significant relationships among the variables in the dataset. Single forms of nitrogen species were not included in the analysis to avoid redundancy.

Canonical correspondence analysis

A constrained (canonical) correspondence analysis (CCA) was used to assess relationships between benthic cell abundances and significant environmental variables, selected with *envfit* function of “vegan” package (R Development Core Team, 2001). CCA is based on unimodal species-environment relationship but is robust even when species display bimodal responses (Ramette, 2007).

2.5 Results

2.5.1 Environmental characterization of Posillipo coast

Temperature and salinity

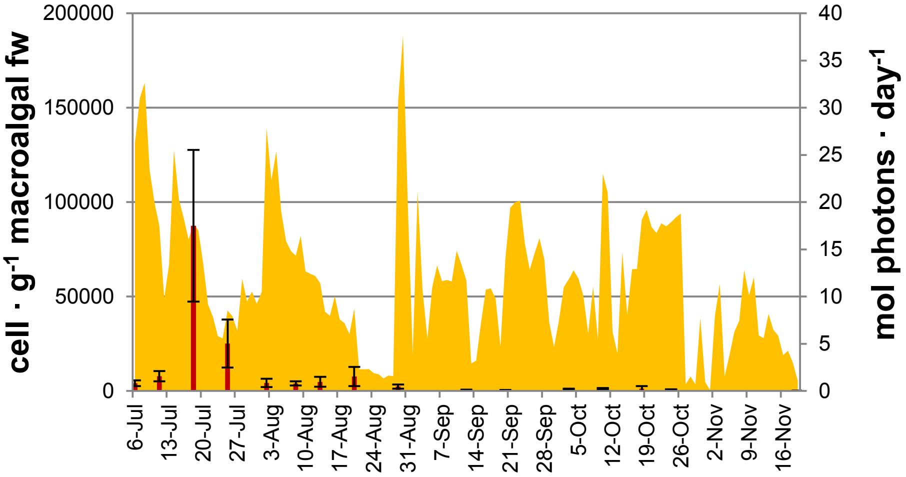
Temperature values showed similar distributions at both stations during the season, following unimodal patterns. Increasing temperatures were reported at both stations at the beginning of each sampling (May - June; ca. 22 °C), reaching maxima in August (26.35 ± 0.42 °C) and decreasing to minima in November (Rocce Verdi; 19.3 °C). At interannual scale, average values followed a decreasing trend over the years, showing the highest variability in 2013 (fig. 2.3). Lowest temperatures reported in 2007 are allegedly due to the low frequency of the sampling activity occurred at the beginning of the sampling campaign (fig. 2.3). Salinity values showed a linear increase from June (37.24 ± 0.21) to September - early October (37.82 ± 0.22) at both stations. At interannual scale, salinity showed very constant values over the years, but high seasonal variability was shown in early July and late September (fig. 2.3).

Light

Light intensity, calculated as moles of photons per day, showed very irregular patterns in 2012 and 2013 (fig. 2.2). Several spikes are present along the sampling period and a sudden light intensity increase occurred each year at the end of August (< 30 mol photons · day^{-1}). However, this peak is probably due to the reprise of the sampling, which involved a periodic removal of biofouling products accumulated on the logger during the week. Average light intensity values were similar for each year (ca. 12 mol photons · day^{-1}) and maximum values were reached at the beginning of September. Light intensity during *Ostreopsis* seasonal maxima were 12.36 and 22.10 mol photons · day^{-1} in 2012 and 2013, respectively.

Nutrients

Nutrient concentrations were similar at Gaiola and Rocce Verdi stations. The water was oligotrophic: nutrient (DIN, phosphates and silica) concentrations at both stations showed generally constant values over the years with a moderate degree of variability at seasonal scale (figg. 2.4 and 2.5). The average DIN concentration, obtained as the sum of nitrate (NO_3), nitrite (NO_2) and ammonium (NH_4), ranged from 0.32 (Gaiola, 2nd October 2014) to 8.27 µmol · l^{-1} (Rocce Verdi, 18th September 2009). DIN concentration at temporal scale showed a no significant, steady low increase (comprised from 1 to 2 µM) at seasonal and interannual scale (fig. 2.4). Phosphates concentration ranged from 0.01 to 0.32 (Rocce Verdi, 15th July 2009). Phosphates values showed constant values over the season and among the years, with values comprised between 0.05 and 0.1 µM. Silica concentrations displayed a broader range of values compared to other nutrients, ranging from 0.09 and 4.95 µM. Concentrations were rather constant over the season, with the occurrence of peaks in July and during the fall (fig. 2.5). Silica values showed a constant and significant increase at interannual scale.



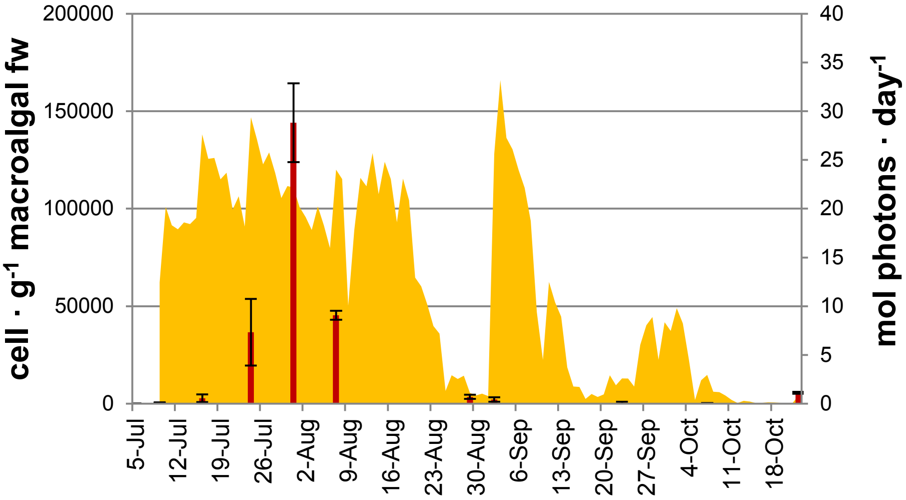


Figure 2.2. *Ostreopsis* abundances (as average of the three replicates) and daily integrated light intensity pattern in 2012 and 2013 at Gaiola station.

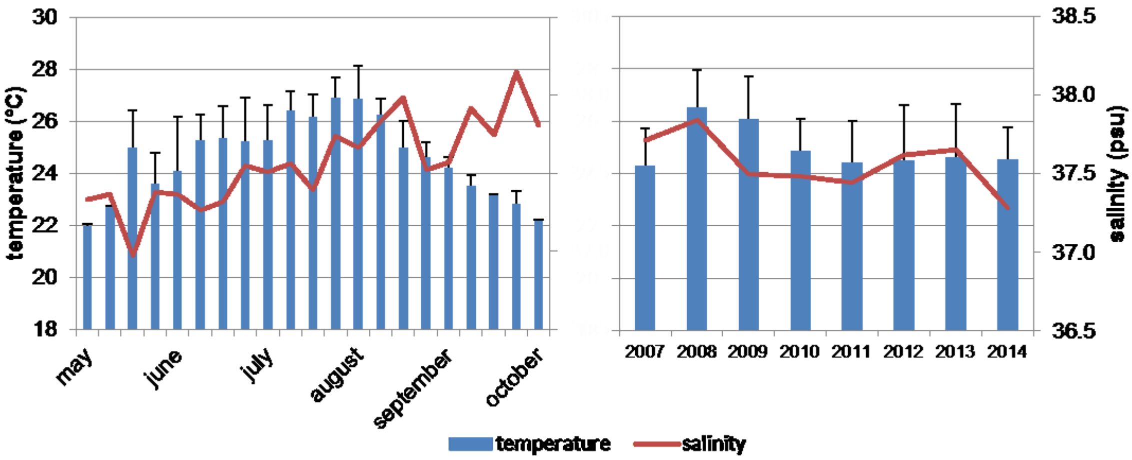


Figure 2.3. Weekly and interannual variability of temperature and salinity values at Posillipo coasts during the sampling season (from late May to late October). Each point is the average of 6 data, 3 for Gaiola and 3 for Rocce Verdi, except for 2007, when no replicates were sampled.

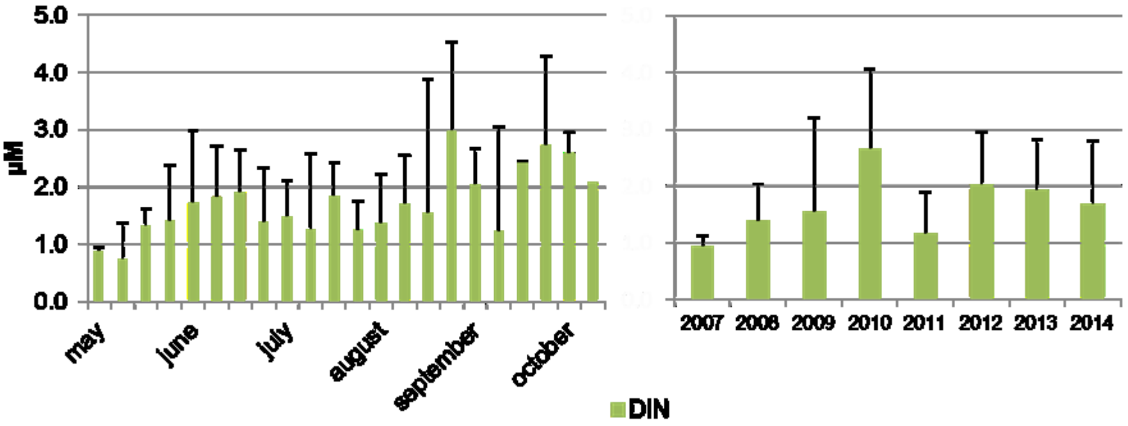


Figure 2.4. Weekly and interannual variability of dissolved inorganic nitrogen concentrations (DIN) at Posillipo coasts, obtained from averaged values of Gaiola and Rocce Verdi stations, during the sampling season (from late May to late October).

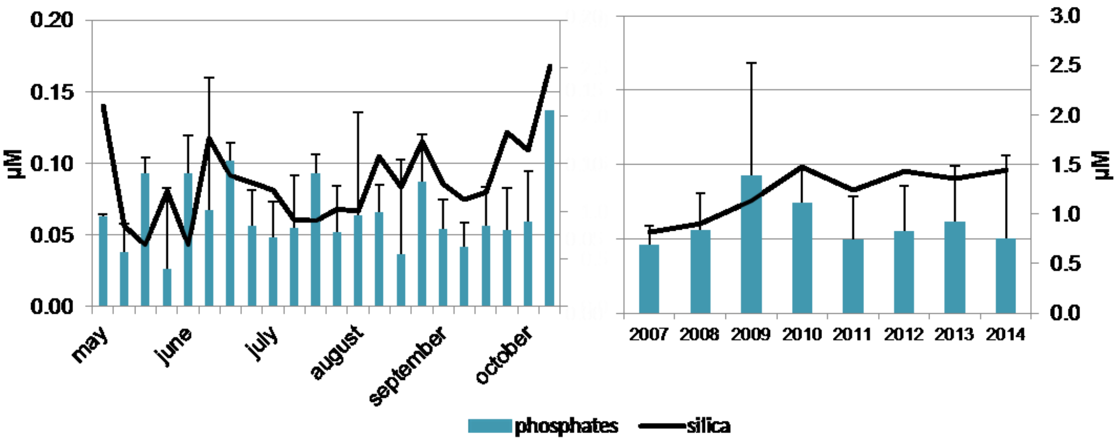


Figure 2.5. Weekly and interannual variability of phosphates and silica concentrations at Posillipo coasts (obtained from average values from Gaiola and Rocce Verdi stations) during the sampling season (from late May to late October).

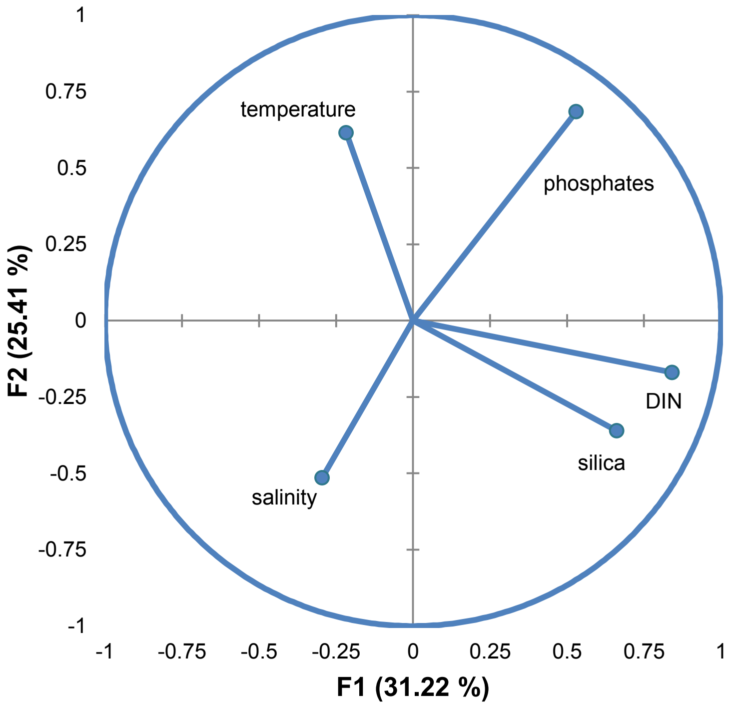


Figure 2.6. Principal component analysis (PCA) based on the selected environmental variables at Gaiola and Rocce Verdi stations.

Table 2.2. Correlation matrix of the principal component analysis (Spearman) based on the combined selected environmental variables at Gaiola and Rocce Verdi stations.

Variables	temperature	salinity	DIN	phosphates	silica
temperature	**1**	0.03	**-0.18**	**0.17**	-0.08
salinity	0.03	**1**	0.01	**-0.25**	-0.05
DIN	**-0.18**	0.01	**1**	**0.31**	**0.41**
phosphates	**0.17**	**-0.25**	**0.31**	**1**	-0.01
silica	-0.08	-0.05	**0.41**	-0.01	**1**

PCA axes 1 and 2 accounted for 56.63% of the variation of observed environmental variables (fig. 2.6). DIN and silica concentrations showed the highest factor loadings on axis 1 (0.84 and 0.66 respectively) and a significant correlation (0.41, tab. 2.2). PCA axis 2 explained most of the observed variation of temperature and phosphates concentration, and low but significant correlation (0.17, tab. 2.2).

2.5.2 Temporal distribution of benthic abundances

Ostreopsis cf. *ovata* cells occurred at both stations of Gaiola and Rocce Verdi from the end of May until the decay of the bloom in November. *Ostreopsis* cf. *ovata* peaks showed a marked seasonality over the years, with a bimodal distribution consisting of a first, more intense bloom in half July-early August, which was followed by a second, minor and less constant bloom in September. The intensity of the peaks was comparable between the two stations but higher cell concentrations were generally attained at Gaiola station. However, the temporal distribution did not significantly differ between sites over the years (unpaired two-sample t-test: p-value = 0.30). At both stations, interannual abundance pattern showed a very low non-significant decreasing trend over the years (regression analysis; Gaiola; F = 0.54, p-value = 0.46, Rocce Verdi; F = 1.28, p-value = 0.26), with highest peaks occurring during the initial years of the sampling campaign (2009, Gaiola station; $3.79 \pm 0.88 \cdot 10^5$ cells · g^{-1} fw) and minimum values in 2011 ($5.44 \pm 3.01 \cdot 10^4$ cells g^{-1} fw) (fig. 2.8). The other benthic dinoflagellates species, *Coolia monotis* and *Prorocentrum lima*, showed lower concentrations than *Ostreopsis* at both stations, reaching 1 and 2 orders of magnitude lower maximum abundances, respectively. In general, *Coolia monotis* blooms occurred at the end of June-early July, reaching maximum value on July 2010 (Gaiola; $3.26 \pm 0.81 \cdot 10^4$ cells g^{-1} fw). *Prorocentrum* spp. showed instead more constant abundances over the season, reaching maximum concentrations in

Rocce Verdi (7th July 2011, 2.15 ± 1.92 · 10^3 cells g^{-1} fw). *Ostreopsis* cf. *ovata* planktonic concentrations were scarcely abundant, rarely exceeding the limit of 10000 cells · l^{-1}, always in coincidence of or one week later the seasonal benthic bloom.

2.5.3 Relationships between benthic cell abundances and environmental variables

Temperature and salinity

No evident relationship between benthic abundances of the three species and temporal trends of temperature and salinity were observed, despite the latter resulted significant in the *envfit* analysis (goodness of fit: 0.19, p-value: 0.001, tab. 2.3). In particular, benthic abundances of *Ostreopsis* and *Prorocentrum* showed a null effect of salinity, while a moderate negative correlation was found with *Coolia*. Abundances peaks of *Ostreopsis* occurred in all the range of salinity (37 - 38) detected along Posillipo coasts. *Ostreopsis* blooms occurred at intermediate temperature values (24 - 26 °C) of the range of the area (19.6 - 28.0 °C); no peaks were found at extreme seasonal temperature values.

Light

Light intensity (as daily integrated values) showed higher values in July (2012: 15.80 ± 7.35; 2013: 21.95 ± 3.92 photons day^{-1}) which decreased in the following months (September, ca. 10 mol photons day^{-1}). Decreasing values detected between the samplings (fig. 2.2) are allegedly due to the increased biofouling on the loggers which were manually removed during the sampling. Nonetheless, high *Ostreopsis* concentrations were generally observed in a range of light intensity comprised between 6 and 28 mol photons per day (fig. 2.2)

Hydrodynamic regimes

The role of hydrodynamics was investigated comparing abundances values in Gaiola and Cala San Basilio stations (fig. 2.10). Higher *Ostreopsis* abundances values were generally found at the sheltered site (Cala San Basilio; 1.07 · 10^5 cell · g^{-1} macroalgal fw), compared to the exposed one (Gaiola; 2.91 · 10^4 g^{-1} macroalgal fw, tab. 2.5), with significant difference between the two stations ($p < 0.05$). In 11th July 2014, at Cala San Basilio station an *Ostreopsis* bloom was macroscopically evident, forming a dense mat on a macroalgal turf (fig 2.11). On this date, benthic concentrations showed the highest values of the season (1.37 ± 0.45 · 10^6 g^{-1} macroalgal fw). During the bloom *Ostreopsis* cells showed high concentrations also in the water column (5.7 · 10^4 cell l^{-1}).

Nutrients

Highest *Ostreopsis* abundance always occurred at relatively low nutrient concentrations (DIN < 1 µM; phosphates < 0.05 µM; silica < 1.5 µM), yet their values did not show any evident pattern in relation to *Ostreopsis* blooms, with only DIN and phosphates being significant but with a very low goodness of fit (tab. 2.6). Benthic abundances of *Ostreopsis*, *Prorocentrum* and *Coolia* spp. showed negative non-significant relationship for DIN and silica at the seasonal scale, but positive non-significant relationship with the same variables at the interannual scale (tab. 2.6). Canonical correspondence analysis showed that the total mean squared contingency coefficient (inertia) was 0.36, of which environmental variables explained only the 10.43% of the variance (tab. 2.4). CCA axis 1 accounted for 99.18% of the variability between environmental variables and benthic cell abundances, being represented mostly by salinity.

Table 2.3. Vectors of envfit analysis, goodness of fit (r^2) and relative p-values (based on 999 permutations). Asterisks represent the significance level (0 '*', 0.001 '**', 0.01 '*', 0.05 '.', 0.1 ' ', 1).**

	CCA1	CCA2	r^2	p-value
temperature	0.14	0.99	0.00	0.435
salinity	-0.98	0.19	0.19	0.001***
DIN	-0.81	0.59	0.03	0.002**
PO_4	0.95	-0.32	0.02	0.011*
silica	0.04	1.00	0.01	0.122

Table 2.4. Total mean squared contingency coefficient (inertia) of the canonical correspondence analysis.

	Value	%
Total	0.36	100.00
Constrained	0.04	10.43
Unconstrained	0.33	89.57

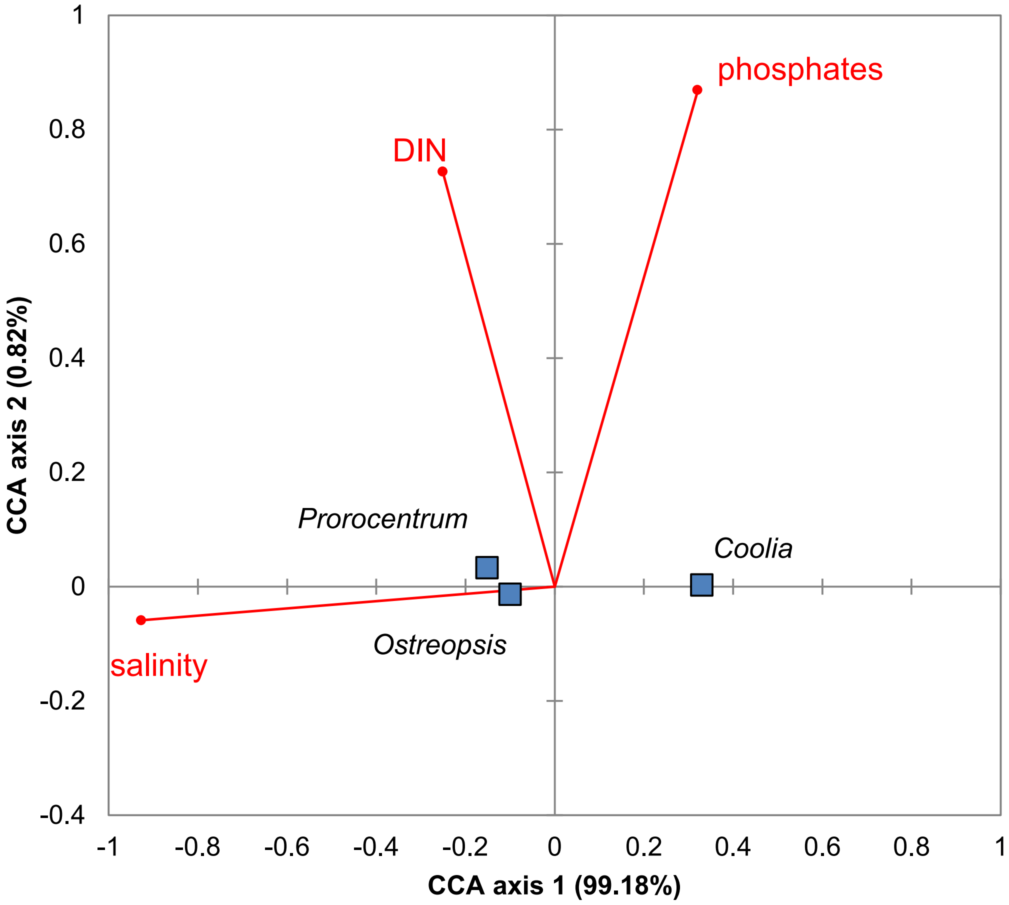


Figure 2.7. Canonical correspondence analysis of benthic species (blue squares) and the significant environmental variables (red dots).

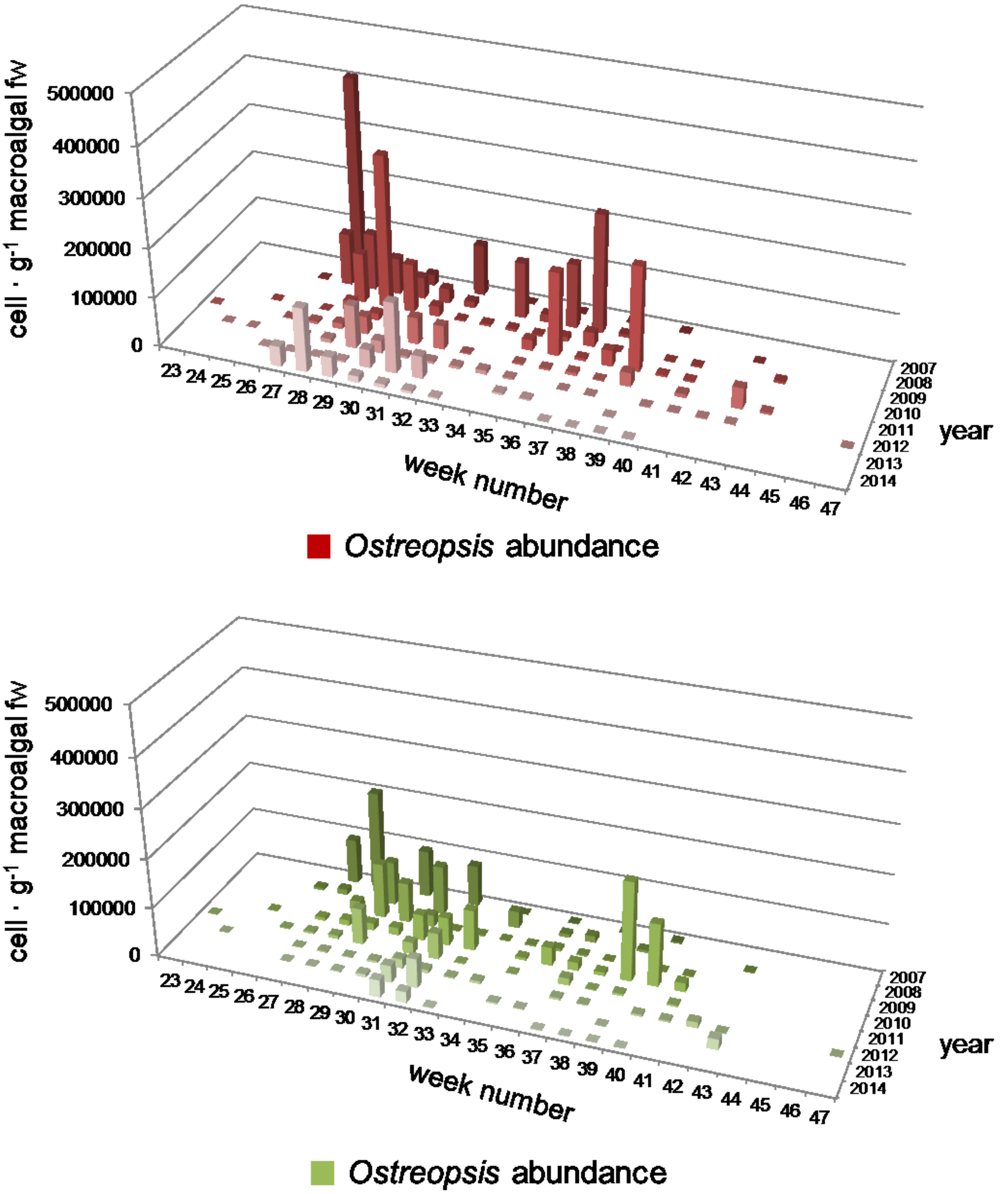


Figure 2.8. ***Ostreopsis*** **abundances (average of triplicates) at Gaiola (red bars) and Rocce Verdi (green bars) stations, at multiannual scale. Missing squares represent dates where no sampling was performed. The week number axis spans from end of May to late October**

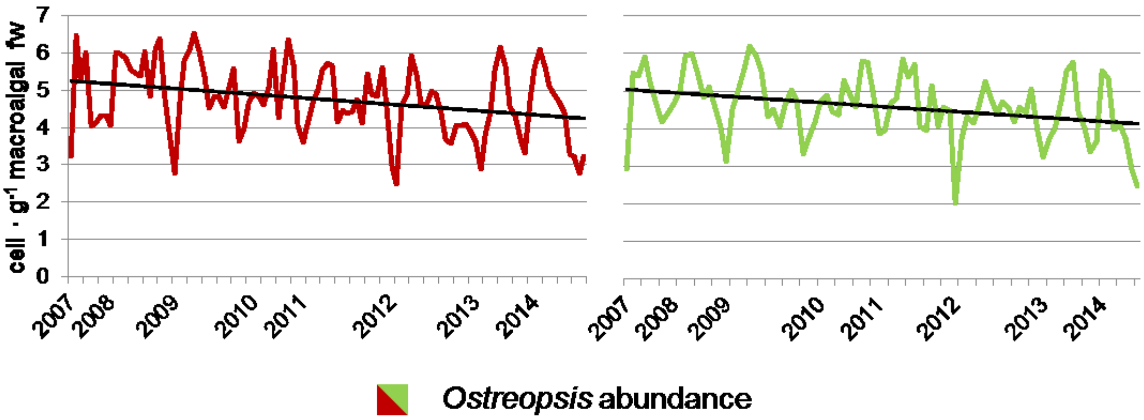


Figure 2.9. Multiannual trends of *Ostreopsis* abundances (in logarithmic values) at Gaiola (red line) and Rocce Verdi (green line) stations, calculated as average values of triplicates. Linear regression shows a decreasing trend of abundances at both stations (black line).

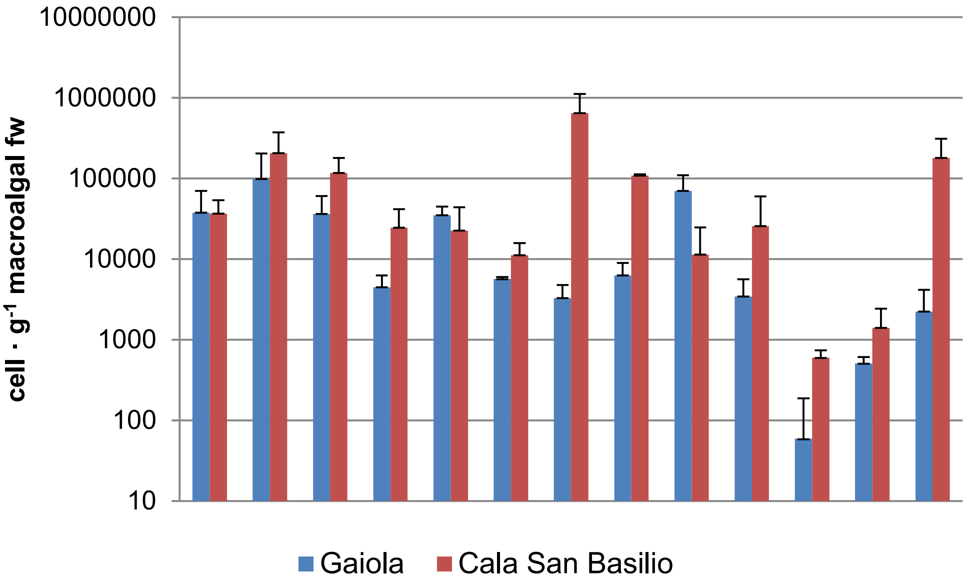


Figure 2.10. Comparison between *Ostreopsis* abundances at exposed site (Gaiola) and sheltered site (Cala San Basilio) recorded on the same dates. Abundance values are expressed as cell g^{-1} macroalgal fw.

Table 2.5. *Ostreopsis* abundances at exposed (Gaiola) and sheltered (Cala San Basilio) stations. Values (avg.) are expressed as cell g^{-1} macroalgal fw.

date	method used	exposed	sheltered
13/07/2011	Falcon/falcon	47103.64	36797.28
27/07/2011	Falcon/falcon	122436.06	205737.12
03/08/2011	Falcon/falcon	45406.32	117147.09
21/09/2011	Falcon/falcon	5583.36	24439.42
19/10/2011	Falcon/falcon	43728.88	22645.18
27/10/2011	Falcon/falcon	7064.91	11137.23
06/07/2012	Falcon/normal	4100.77	646924.80
11/07/2012	Falcon/normal	7836.13	108500.43
18/07/2012	Falcon/normal	87511.64	11362.48
02/08/2012	Falcon/normal	4301.61	25674.88
05/07/2013	Falcon/normal	73.83	596.62
09/07/2013	Falcon/normal	632.92	1400.45
16/07/2013	Falcon/normal	2799.06	179149.01

Figure 2.11. *Ostreopsis* cf. *ovata* forming a mat on a macroalgal turf dominated by *Jania* spp.

Table 2.6. Seasonal and interannual coefficients of correlation of benthic abundances and environmental variables. Values in bold are different from 0 with a significance level alpha = 0.05. Seasonal coefficients are calculated as average value of the variable on a weekly scale.

Seasonal

Variables	*Ostreopsis*	*Prorocentrum*	*Coolia*	temperature	salinity	DIN	phosphates	silica
Ostreopsis	**1**	0.30	0.24	0.33	-0.03	-0.25	0.16	-0.05
Prorocentrum	0.30	**1**	0.30	**0.49**	-0.24	-0.23	0.31	-0.13
Coolia	0.24	0.30	**1**	0.32	**-0.62**	-0.36	0.32	-0.12
temperature	0.33	**0.49**	0.32	**1**	0.07	**-0.55**	0.11	-0.39
salinity	-0.03	-0.24	**-0.62**	0.07	**1**	-0.10	**-0.58**	-0.15
DIN	-0.25	-0.23	-0.36	**-0.55**	-0.10	**1**	0.33	**0.55**
phosphates	0.16	0.31	0.32	0.11	**-0.58**	0.33	**1**	-0.01
silica	-0.05	-0.13	-0.12	-0.39	-0.15	**0.55**	-0.01	**1**

Interannual

Variables	*Ostreopsis*	*Prorocentrum*	*Coolia*	temperature	salinity	DIN	phosphates	silica
Ostreopsis	**1**	0.62	**0.78**	0.48	0.08	0.23	0.06	0.57
Prorocentrum	0.62	**1**	0.65	0.10	0.03	0.14	-0.28	0.62
Coolia	**0.78**	0.65	**1**	0.37	-0.42	0.47	0.27	0.73
temperature	0.48	0.10	0.37	**1**	0.40	0.32	0.44	0.29
salinity	0.08	0.03	-0.42	0.40	**1**	-0.49	-0.41	-0.35
DIN	0.23	0.14	0.47	0.32	-0.49	**1**	0.69	**0.80**
phosphates	0.06	-0.28	0.27	0.44	-0.41	0.69	**1**	0.24
silica	0.57	0.62	0.73	0.29	-0.35	**0.80**	0.24	**1**

2.6 Discussion

2.6.1 Temporal variability of *Ostreopsis* abundances

Ostreopsis cf. *ovata* occurrence along the Posillipo area (Gulf of Naples) is known since 2004 (Zingone et al., 2005; Zingone et al., 2006) but no report was available about the intensity and temporal variability of its blooms in this area. In this study, we showed the occurrence and the variability at temporal scale of *Ostreopsis* blooms along the Posillipo coast (Eastern Central Tyrrhenian Sea). In that area, *Ostreopsis* blooms did not attain intensities comparable to the one reported in the Adriatic Sea ($1.7 \cdot 10^6$ cells g^{-1} fw; Totti et al., 2010) or to some station of the North Western Mediterranean (Mangialajo et al., 2011). Blooms showed instead comparable intensity with other sites of the North Western Mediterranean Sea, but higher than the ones reported in Greece seas (Vila et al., 2001; Aligizaki and Nikolaidis, 2006). *Ostreopsis* cf. *ovata* at the Posillipo coast showed regular seasonal blooms from July to early August in all the years of the sampling, a pattern shown also in other Western Mediterranean sites (Mangialajo et al., 2011; Cohu and Lemee, 2012). A second bloom was occasionally found in fall during the first years of the sampling (fig. 2.8), as reported also in the Catalan coasts (Berdalet et al., 2012), though in other Eastern Mediterranean sites fall bloom occurred with regular frequency (Monti et al., 2007; Totti et al., 2010; Accoroni et al., 2011). At multiannual scale, *Ostreopsis* abundances at Gaiola and Rocce Verdi showed a decreasing trend of abundance values (fig. 2.9), and the same was shown for *Prorocentrum* and *Coolia* spp. At present, a very limited number of studies on interannual *Ostreopsis* abundance trends are available. These are restricted to planktonic concentrations (Asnaghi et al., 2012) or to very few years of sampling (Mangialajo et al., 2011), making it hard to compare the negative trend observed in the Gulf of Naples to other areas. Though this study is, to our knowledge, the longest *Ostreopsis* monitoring survey conducted in the Mediterranean Sea, the relatively short time span of our investigation do not allow to assess if the observed linear decrease of bloom intensities may represent a fraction of a more complex temporal pattern (Southward, 1995) (i.e. cyclic or chaotic trend) which typically occur at higher temporal scale (Wyatt, 2003). Nevertheless, the increased monitoring surveys of this species along the coasts of Mediterranean Sea

(Lemée et al., 2012; Vila et al., 2012) may soon allow a comparison between other basins, influenced by different environmental characteristics.

The lack of clear relationships with environmental variables does not allow interpretating the observed trend as an effect of an external forcing, at least based on the factors considered in this study. One possible explanation, although very speculative, could reside in the suspected status of *Ostreopsis* cf. *ovata* as an alien species. This hypothesis is based on the observation of a wide genetic diversity of *O.* cf. *ovata sensu lato* in the Pacific ocean (Japan Sea) compared to the Mediterranean area, and on the finding in that area of strains identical to the Mediterranean ones (Sato et al. 2011). A decreasing trend of *O.* cf. *ovata* in the Mediterranean area, if confirmed with observation from other sites, could be in line with what observed for invasive alien species, which often show a phase of explosion in the new area followed by a decrease, probably due to competition or parasitism with other species or by unknown effects (Simberloff and Gibbons, 2004).

2.6.2 Relationship between benthic abundances and environmental variables

Several studies have highlighted temperature as an important driver in *Ostreopsis* ecophysiology: high sea water temperature has been indicated as major cause of *Ostreopsis* blooms in Mediterranean Sea (Tognetto et al., 1995; Aligizaki and Nikolaidis, 2006; Mangialajo et al., 2008) and proposed as a main factor responsible for the occurrence of *O.* cf. *ovata* in the Tyrrhenian Sea (Granéli et al., 2010). On the other hand, several authors found negative (Parsons and Preskitt, 2007) or no correlation of *Ostreopsis* abundance with temperature (Vila et al., 2001; Monti et al., 2007; Selina and Orlova, 2010; Totti et al., 2010; Accoroni et al., 2011). Our findings are in agreement with the latter authors: though *Ostreopsis* cf. *ovata* in the Gulf of Naples reached high concentration (> 50000 cell g^{-1} macroalgal fw) on a wide range of temperatures, abundance peaks were always found at intermediate values in the season (ca. 24 °C), as often reported in other areas of the Western Mediterranean Sea (Ciminiello et al., 2006; Bianco et al., 2007). Experimental studies on strains from the Gulf of Naples seem to confirm this finding: cells grown in laboratory perform better at intermediate values than at extreme temperature (18 - 30 °C), showing highest growth rate at 22 - 26 °C (Zingone et al., 2010; Scalco et al., 2012). At seasonal and multiannual scale, *Ostreopsis* cf. *ovata* bloom intensities showed a moderate but non-significant correlation with temperature values. This result suggests that the development of the *Ostreopsis* blooms may need a threshold temperature value to be reached (Mangialajo et al., 2011) or may be triggered by an indirect signal of seasonal effects (influenced by temperature).

Ostreopsis cf. *ovata* in the Mediterranean Sea occurs at relatively high values of salinity (>37) (Berdalet et al., 2012) and its presence has never been detected in correspondence of rivers (Pistocchi et al., 2011a). Though a negative correlation with salinity has been found in Hawaiian coasts (Parsons and Preskitt, 2007), ecophysiology experiments performed on Mediterranean strains of *Ostreopsis* cf. *ovata* at different salinity conditions (26 - 40) demonstrate a strong tolerance of salinity and weak influence on the growth rate (Pistocchi et al., 2011a; Pezzolesi et al., 2012). In our area, *Ostreopsis* cells were observed to reach bloom concentrations on a salinity range between 37 and 38, while no relationship with salinity was found at temporal scale. *Coolia* abundance in benthic samples instead showed strong negative correlation with salinity over the season.

If the monophasic bloom of *Coolia* at the beginning of the season may simply explain this finding, the null effect of salinity displayed by *Ostreopsis* and *Prorocentrum* (fig. 2. 7) may be justified by the contrast of their phenologic characteristics (a biphasic bloom and a rather homogeneous seasonal distribution, respectively) with the steady increase of salinity increase from May to late autumn, a well know basin scale feature of the Mediterranean Sea.

Light intensity data collected in this study are not suitable to establish a reliable comparison with the dynamic of *Ostreopsis* blooms. Nonetheless, general assumptions about light regime preferences in the field could be pointed out: the bloom phase did never coincide with maximum values of daily light intensity, while high *Ostreopsis* concentrations were attained at decreasing values of light intensity. Experimental studies have already shown the preference of *Ostreopsis* for low light regimes (Scalco et al., 2012). While our field data confirm these findings for lower values of light intensity, high *Ostreopsis* abundances were also found at values exceeding the ones reported in experimental studies (Morton et al., 1992; Scalco et al., 2012). Nevertheless, these latter results could be explained by the capability of adaptation to different light regimes offered by macroalgal shading (Ballantine et al., 1988) or by mucus production (Heil et al., 1993).

Generally, *Ostreopsis* cf. *ovata* attained higher concentrations at the sheltered site of Cala San Basilio than in the exposed one. While this in contrast with data collected from another site of the Gulf of Naples (see chapter 3 of this thesis), there is no general consensus about the effect of wave action on *Ostreopsis*. Several studies have shown that stable conditions are more favourable for *Ostreopsis* species (Carlson and Tindall, 1985; Taylor et al., 1985; Tindall and Morton, 1998; Chang et al., 2000; Shears and Ross, 2009; Totti et al., 2010; Richlen and Lobel, 2011) while other studies pointed out a preference of *Ostreopsis* for high energy waters (Vila et al., 2001; Parsons and Preskitt, 2007; Kibler et al., 2008). A complex of environmental (water motion, substrate, light exposure (Grzebyk et al., 1994) and genetic factors (Parsons et al., 2012) have been suggested as causes which modulate the observed differences in hydrodynamic preferences.

Very low concentrations of *Ostreopsis* cells in the water column were observed at both stations: this result is in contrast with what is reported in other Mediterranean areas where planktonic cell concentrations were higher and correlated with benthic ones (Vila et al., 2001; Mangialajo et al., 2011). Nevertheless, the purported absence of syndromes by inhalation in beach-goers along Posillipo coasts seems to further support this finding. The delay of planktonic peaks observed at Posillipo coasts showed instead similar patterns with NW Mediterranean areas (Mangialajo et al., 2011): cell re-suspension caused by wave action (Totti et al., 2010) or by daily cycles of vertical migration (Vila et al., 2008) has been invoked to explain these differences.

The occasional occurrence of *Ostreopsis* cells during the summer bloom has been reported at 2 nm offshore Posillipo area, at the long-term sampling station of the Gulf of Naples MareChiara (Sarno, personal communication). The presence of *Ostreopsis* in the water column, far from the benthic environment has already been shown in other areas highlighting the role of hydrodynamic in the dispersion capabilities of this species in the plankton domain.

The relationships between nutrient enrichment and HAB is still under debate: while a number of studies highlight a connection of HABs occurrence with anthropogenic nutrient loading (but see Glibert et al., 2014), others did not observe such connection (Anderson et al., 2002; Zingone and Wyatt, 2005; Smayda, 2008), and the role of hydrodynamic conditions and other pressures at local scale may be capable to over-ride nutrient effects (Davidson et al., 2014). The validity of this link remains elusive also for benthic dinoflagellates (Grzebyk et al., 1994) and in particular for *Ostreopsis* (Pistocchi et al., 2011). *Ostreopsis* species are present worldwide in eutrophic (Heisler et al., 2008; Hallegraeff, 2010) and oligotrophic areas (Tindall and Morton, 1998; Zeldis et al., 2004). While some work performed on the field showed no correlation between *Ostreopsis* species and nutrient availability (Delgado et al., 2006; Shears and Ross, 2009), a study in Hawaiian waters found positive correlation (Parsons and Preskitt, 2007), although these differences may be related to genetic diversity of the species. Such link is not yet clearly demonstrated in the Mediterranean Sea (Vila et al., 2001), and opposite relationships were found with silicates (-) (Cohu et al., 2011) and phosphates (+) (Asnaghi et al., 2012; Cohu et al., 2013). While coastal zones are generally richer in nutrient than open waters, our data show that Gaiola and Rocce Verdi have a quite low nutrient content. Our dataset confirm data obtained from the Mediterranean Sea: although no significant correlation between benthic abundances and nutrient concentrations were found, DIN and phosphates showed weak correlation with the abundances of the three species analysed. Relationships derived from physiological characteristics (Pezzolesi et al., 2014), environmental drivers (Burkholder et al., 2008) and behavioural traits (Burkholder et al., 2008; Escalera et al., 2014) and the potential role of other nutrients (Cohu et al., 2013) may be responsible of the complexity of the trophic capabilities of this species.

This study represents a first characterization of temporal trends of *O.* cf. *ovata* in Eastern Tyrrhenian sector of the Mediterranean Sea: despite the complexity of the relations existing between the phenology and the ecology of this species, further studies may allow to build a model of the development of *Ostreopsis* to be used for ecologic and sanitary risk assessment in the Gulf of Naples.

2.7 Chapter summary

The monitoring survey of *Ostreopsis* cf. *ovata* along the Posillipo coasts of the Gulf of Naples from 2007 to 2014 showed:

- marked seasonality of the blooms, with one peak in July and a second, less constant peak in September;
- decreasing trend of abundance peaks over the years;
- seawater temperature and nutrient availability do not seem to play a major role in bloom development;
- environmental variability does not account for the observed interannual trends of *Ostreopsis* benthic abundances.

Acknowledgements

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Chapter 3

Spatial distribution of *Ostreopsis* cf. *ovata* along a naturally occurring carbon dioxide and pH gradient

ABSTRACT

Ocean acidification caused by increasing atmospheric CO_2 concentrations could influence benthic communities, including harmful microalgae living therein. With the aim of assessing the possible effects of pH decrease on benthic harmful algal blooms (BHABs), the distribution of *Ostreopsis* cf. *ovata* was investigated at volcanic CO_2 vents of the Ischia Island (Tyrrhenian Sea, Mediterranean Sea). Samples were collected on 13 sampling dates from the brown alga *Dictyota dichotoma*, at 6 stations located along marked pH gradients (6.8 - 8.1), of which 3 at a sheltered and 3 at an exposed site. *Ostreopsis* abundance was significantly higher in the exposed site than in the sheltered one, but did not vary significantly along the pH gradient within each site. These results indicate that *O.* cf. *ovata* is tolerant to a wide range of pH values, while it is affected, in terms of abundance, by hydrodynamic factors and/or by their effects on the benthic biota. Apparently, ocean acidification is not likely to represent a hindrance to the spreading and intensification of *Ostreopsis* blooms, while, a relevant rôle could be played by hydrographic variations in coastal environments.

3.1 Introduction

Anthropogenic gas emissions have increased CO_2 levels in the atmosphere since the industrial revolution, causing a shift from 280 to 400 ppm at present day (Dlugokencky and Tans, 2015) and leading to climate system and ocean chemistry alterations. The latter process is collectively called ocean acidification (OA) and it is expected to provoke a drop in seawater pH of approximately 0.4 units at the end of this century (Orr, 2011), with profound impacts on marine ecosystems (Guinotte and Fabry, 2008; Hoegh-Guldberg and Bruno, 2010; Doney et al., 2012) through chemico-physical and biological modifications. Multiple experiments show that ocean acidification will likely cause declines in calcification rates in marine calcifying organisms (Guinotte and Fabry, 2008; Doney et al., 2009) affecting also rocky benthic fauna by simplification of community structure (Hale et al., 2011), possibly leading to ecosystem simplification (Kroeker et al., 2013a). At the opposite side, primary productivity of the oceans could be enhanced by high levels of CO_2 (Riebesell et al., 2007). Nevertheless, the complex interplay of several factors influenced by climate variations may produce unforeseen impacts on the marine biota (Feng et al., 2008; Garrard et al., 2014). Concurrently with this trends, benthic harmful algal blooms (BHAB) have also increased their intensity, frequency and distribution over the last decades (Hallegraeff, 2010) causing severe impacts on coastal zones (Zingone, 2014), possibly in relation with the rise of sea temperatures (Parsons et al., 2012). In the last decade the geographic range, intensity and frequency of *Ostreopsis* blooms have increased markedly also in temperate areas (Parsons et al., 2012). *Ostreopsis* is a benthic dinoflagellate which grows on macrophytes or other biotic and abiotic substrates. Species in the genus produce palytoxin-like molecules, which can provoke severe and also lethal intoxications in humans (Tubaro et al., 2011).

3.2 Specific aims of the chapter

Microphytobenthos has received few attention in the exploring of the impacts of ocean acidification on temperate benthic ecosystems (Johnson et al., 2013) and at present the numbers of studies is very limited (Alsterberg et al., 2013; Kerfahi et al., 2014). Considering the impacts that this global-scale phenomenon may have on coastal environments and the rising concern about BHABs in temperate areas, we assessed the possible effects of ocean acidification on the toxic dinoflagellate *Ostreopsis* cf. *ovata,* sampling the species along a pH gradient in a natural CO_2 venting site that we used as a field laboratory. This study aims to verify the occurrence and abundance of *Ostreopsis* cf. *ovata* in coastal sites at variable pH comparable, in terms of acidification, to those provided in the coming decades.

3.3 Study area: Castello Aragonese of Ischia Island

Castello Aragonese is located at North-East side of Ischia island (40° 43.84’ N, 13° 57.08’ E), Tyrrhenian Sea, Mediterranean Sea. Vents occur in shallow waters of the area (Tedesco, 1996) with gas concentrations of 90-95% CO_2, 3-6% N_2, 0.6-0.8% O_2, 0.2-0.8% CH_4 and 0.08-0.1% Ar, bubbling at about 1.4×10^6 $l \cdot d^{-1}$ (Cigliano et al., 2010). Salinity and total alkalinity have relatively constant values of 38.0 and 2.5 $mEq \cdot kg^{-1}$, respectively (Hall-Spencer et al., 2008; Martin et al., 2008). The site is microtidal (0.30 – 0.50 m) and the CO_2 vents lack sulphur (Tedesco, 1996).

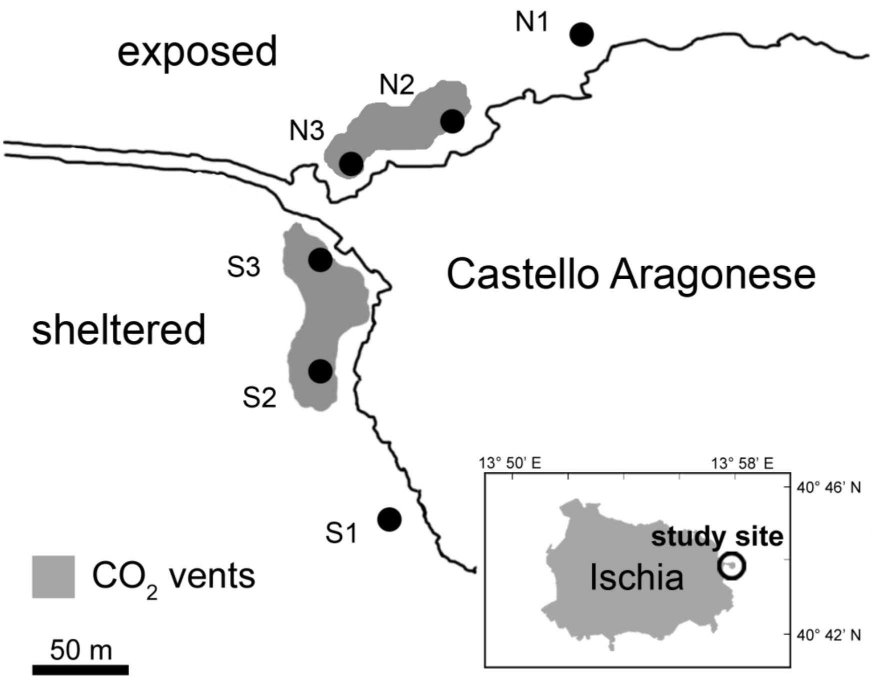

Figure 3.1. Map of the vent site at the Castello Aragonese (Ischia Island) with sampling stations, pH conditions and hydrodynamic regimes. CO_2 venting site determine a pH gradient ranging from environmental values (8.1, station N1 and S1) to very low values (ca. 6.8, N3 and S3 stations). Intermediate pH values (ca. 7.8) are found at N2 and S2 stations.

CO_2 emissions have occurred at these sites for centuries, as thermal springs in Ischia have been known since Romans (Buchner, 1965). Acidification of the seawater moves along a pH gradient from 8.17 down to 6.57 for 300 m, running parallel to the rocky shore, both on the north and south sides of the Castello Aragonese (Hall-Spencer et al., 2008); southern side is more sheltered from wave action, while the northern side is an exposed station, facing open waters of the Gulf of Gaeta and then more prone to intense hydrodynamic events. These gradients are physically separated by a bridge connecting Castello Aragonese to the main island of Ischia (fig. 3.1). N1 and S1 (fig. 3.1) are stations with ambient pH conditions (8.1), comparable to present conditions in Mediterranean Sea waters; N2 and S2 are low pH stations, with mean intermediate pH values (7.8) similar to those predicted for near-future scenario (year 2100) and high pH fluctuations (Kroeker et al., 2011), whereas N3 and S3 are characterized by extremely acidified conditions (pH 6.6 - 7.2) (Kroeker et al., 2013a), corresponding to more distant future conditions (Caldeira and Wickett, 2003). Dramatic effects of the low pH have been demonstrated on a broad range of organisms in the area, including calcareous epiphytes (Martin et al., 2008), benthic diatoms (Porzio et al., 2012), whole macroalgal community (Porzio et al., 2011) and invertebrates (Kroeker et al., 2011; Ricevuto et al., 2012; Ricevuto et al., 2014; Basso et al., 2015). Although confined to small areas and subject to high variations in pH, these sites can provide information about the ecological effects of long-term exposures to acidified conditions and evaluate feedbacks and indirect effects that occur within natural marine systems (Hall-Spencer et al., 2008; Riebesell, 2008).

3.4 Material and methods

3.4.1 Sampling method

Considering the marked seasonality of *Ostreopsis* cf. *ovata*, with peaks occurring during the early summer in the Gulf of Naples (ARPA Campania, unpublished data) and in the North Western Mediterranean Sea (Mangialajo et al., 2011), sampling activity was planned mostly in summer, when *Ostreopsis* cells are expected to reach their maximum abundances. Thirteen sampling campaigns were conducted over four years (2011-2014), from June to October. Samples were collected in the summers of 2011 (27th June and 21th July), 2012 (2nd July, 13th July and 31th July), 2013 (4th July, 26th July, 12th September, 1st October) and 2014 (16th July, 23th July, 5th August and 2nd October) from the brown alga *Dictyota dichotoma* with the exception of last two campaigns in 2013, where mixed samples of *Halopteris scoparia* and *Dictyota dichotoma* were collected. Sampling was performed according to the “Falcon method” (ISPRA, 2012) (see Appendix I of this thesis for details). Samples were collected by SCUBA diving at a depth of about 2 m, along 300 m transects on the North and South sides of Castello Aragonese at six stations (N1-3, S1-3, fig. 3.1), where significant differences in pH due to CO_2 vents have been recorded (Hall-Spencer et al., 2008). For each sampling point, samples were taken in triplicates at a distance of 5 m one from another, put into 50 ml Falcon™ tubes and promptly closed underwater.

3.4.2 Sample treatment and cell counting

Immediately after collection, macroalgal samples were fixed with neutralized formaldehyde at a final concentration of 0.7% and stored in the dark until the analyses. Falcon tubes were vigorously shaken at least 20 times to allow the detachment of benthic cells. Macroalgal thalli were rinsed with fresh water, dry-blotted with paper on a pre-weighted aluminium foil, and then weighed to determine fresh weight (g fw). The volume of the water sample containing detached cells inside the Falcon tube was measured (ml). Cell counting was performed using a Zeiss Axioskop 2 plus, at a magnification of 125 x, settling 1 ml of the water sample in Sedgewick-Rafter slides. Abundances of *Ostreopsis* cells were expressed as cells g^{-1} fw and calculated as follows:

$$\text{cells g}^{-1}\text{ fw} = \frac{counted\ cells * factor * \frac{final\ vol}{settled\ vol}}{weight}$$

where: counted cells = average number of cells per transect;
factor = ratio of Sedgewick-Rafter chamber area with explored area;
final vol = volume of seawater after the removal of the macroalga (ml);
settled vol = volume of Sedgewick-Rafter chambers (1 ml);
weight = fresh weight of the macroalgal sample.

3.4.3 Environmental variables

3.4.3.1 Temperature

Temperature values were measured with HOBO Pendant® Temperature/Light Data Logger (Onset) in July 2012 and July - October 2013 at stations S2 and N3 with a sampling frequency of 15’ and an accuracy of ± 0.53 °C.

3.4.3.2 Nutrients

Water samples for nutrient analysis (nitrates, nitrites, ammonia, ortophosphates and silicates) were collected in duplicates on 2, 13 and 31 July 2012 and 26 July 2013. Samples were stored in polyethylene bottles at –20°C and unfreezed before the analysis at 37°C in a water bath. Analysis were performed following the method reported in “Metodologie Analitiche di Riferimento” (Cicero and Di Girolamo, 2001), using a continuous flux analyzer (Flow-Sys Systea). Detection limits were 0.05 $\mu mol \cdot l^{-1}$ for ammonia and silica and 0.01 $\mu mol \cdot l^{-1}$ for nitrites, nitrates and orthophosphates.

3.4.4 Statistical analysis

Differences in the abundances of *Ostreopsis* cells, their coefficient of variation, and nutrient concentrations among site and pH zone were tested through a two-factor ANOVA model, type II with fixed effects and interactions, using log-transformed data of *Ostreopsis* abundances. Correlation between *Ostreopsis* abundances and nutrient concentrations was tested through a Spearman rank test. Normality assumption was assessed by visual check (Q-Q plot) and evaluated with the Shapiro-Wilk test. Homogeneity of variance for the two groups was assessed with Levene's test. A non-parametric Kruskal-Wallis test (K/S) was used for the variable "position" who did not meet the assumption of equal variance for abundance data. Significance level value was set for $p<0.05$. All tests were performed in R software (R Development Core Team, 2011).

3.5 Results

3.5.1 Abiotic variables

Temperature

Temperatures in July ranged from a minimum of 22.5 °C to the seasonal maxima of 29.15 °C. Average temperature at each side of Castello Aragonese was ca. 26.1 °C but higher variability was found at the Northern station. Temperatures showed also different distributions at each side of the island (K/S test; $D = 0.15$, $p < 0.05$).

Nutrients

Nutrient concentrations were generally similar at each sampling date, both among pH zones and between the two sides of Castello Aragonese (fig. 3.2). They all showed moderate level of variability at temporal scale. The average DIN concentration, obtained as the sum of nitrate (NO_3), nitrite (NO_2) and ammonium (NH_4), ranged from 0.30 (S1 station, 31th July 2012) to 1.54 μmol · l^{-1} (S3 station, 2nd July 2012). DIN concentrations at each sampling dates were similar among pH zones (control, 0.76 ± 0.45 μmol · l^{-1}; low pH, 0.73 ± 0.29 μmol · l^{-1}; very low pH, 0.98 ± 0.47 μmol · l^{-1}) and between each sides of Castello Aragonese (Northern, 0.84 ± 0.38 μmol · l^{-1}; Southern, 0.80 ± 0.46 μmol · l^{-1}) and their differences were statistically not significant for both factors (ANOVA "pH zones"; F-value = 0.59, $p = 0.5591$; ANOVA "position"; F-value = 1.01, $p = 0.3176$).

PO_4 concentrations ranged from 0.01 (N1 station, 26th July 2012) to 0.10 µmol · l^{-1} (N3 station, 2nd July 2012). PO_4 values at each sampling were similar among pH zones (control, 0.03 ± 0.02 µmol · l^{-1}; low pH, 0.04 ± 0.05 µmol · l^{-1}; very low pH, 0.05 ± 0.04 µmol · l^{-1}) and between each side of the island (Northern side; 0.04 ± 0.04 µmol · l^{-1}; Southern side, 0.03 ± 0.03 µmol · l^{-1}. Differences in average PO_4 concentrations were not statistically significant for both factors (ANOVA “pH zones”; F-value = 2.38, p = 0.10; ANOVA “position”; F-value = 1.65, p = 0.20).

SiO_2 concentrations ranged from 1.42 (S2 stations, 26th July 2012) to 12.31 µmol · l^{-1} (N2 station, 2nd July 2012). SiO_2 concentrations were generally higher at the beginning of July at each side of Castello Aragonese and showed decreasing value later on the season. SiO_2 concentrations showed an inverse linear relationship with the acidified gradient (control, 2.88 ± 1.71 µmol · l^{-1}; low 4.34 ± 4.35 µmol · l^{-1}; very low, 5.13 ± 5.03 µmol · l^{-1}) and higher values were found on the Northern side (4.89 ± 5.06 µmol · l^{-1}) compared to the Southern one (3.35 ± 2.44 µmol · l^{-1}). However, their differences were not statistically significant (ANOVA “pH zones”; F-value = 1.98, p = 0.15; ANOVA “position”; F-value = 2.72, p = 0.10).

No significant differences in nutrients concentrations were found between the northern and southern side (Wilcoxon signed-rank test, one-tailed; TIN, Z-value = -0.54, p = 0.29; PO_4, Z-value = -0.39, p = 0.35; SiO_2, Z-value = -1.18, p = 0.12). *Ostreopsis* abundances were not correlated with nutrients (Spearman Rank Order Correlations; DIN: p = 0.14, PO_4: p = 0.09, SiO_2: p = 0.26).

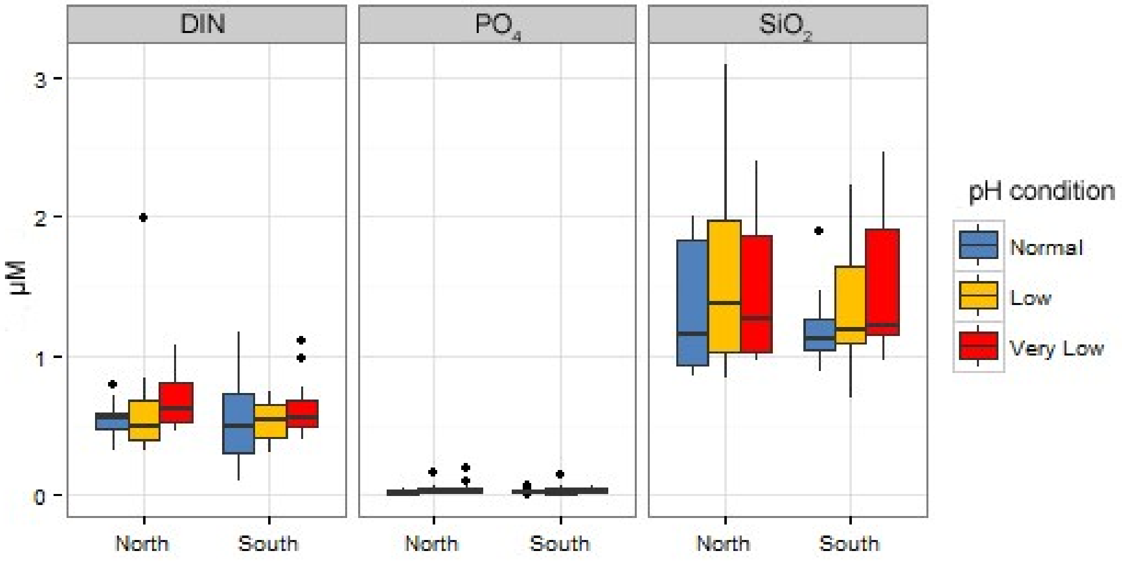

Figure 3.2. Nutrient concentrations (in logarithmic scale) among pH zones at each side of Castello Aragonese sampled on 2, 13 and 31 July 2012 and 26 July 2013.

3.5.2 Interannual variability of *Ostreopsis* abundance

A total of 234 samples were collected (13 sampling dates x 18 samples) but only 162 were analyzed and used for statistical tests, due to very poor *Ostreopsis* cells number in 4 sampling dates (21th June 2011, 31th July 2012, 12th September 2013, 2nd October 2014). The detection limit for cell counting was 21.62 cells g^{-1} fw. The average weight of treated macroalgal samples were 7.51 ± 5.08 g. *Ostreopsis* abundances ranged from 24.43 cells g^{-1} fw (S2 station, 26th July 2013, replicate "a") to $1.13 \cdot 10^6$ cells g^{-1} fw (N2 station, 21th July 2011) with different values along the years (fig. 3.3). Higher overall abundances were found in 2011 ($4.17 \pm 3.89 \cdot 10^5$ cells g^{-1} fw) compared to following years where lower values were recorded (2012, $6.96 \pm 6.26 \cdot 10^3$ cells g^{-1} fw; 2013, 3.00 ± 5.86 cells $\cdot 10^3$ g^{-1} fw; 2014, $2.65 \pm 1.48 \cdot 10^4$ cells g^{-1} fw). At morphological level, no visible difference was observed among cells collected from different pH zones.

In 2011, the highest average abundances of the entire sampling campaign were found at both sides of Castello Aragonese (21st July 2011, $4.17 \pm 3.89 \cdot 10^5$ cells g^{-1} fw), with no significant differences along the pH gradient zones (ANOVA; "pH zones": F-value = 0.12, p = 0.89).

In 2012, *Ostreopsis* cells were found in the first half of July, while rare cells were found in the third sampling day (31th July). Abundance values within the three sampling dates were much lower than the ones reported the year before (2012, 6.96 ± 6.26 · 10^3 cells g^{-1} fw). Higher abundances were found on N1 site, (2nd July; 1.42 ± 0.27 · 10^4 cells g^{-1} fw) while other stations showed similar values within the Southern side, in the same date, and among all the stations in the following sampling (13th July).

In 2013, average overall abundances among all the stations were the lowest of the entire sampling campaign (3.00 ± 5.81 · 10^3 cells g^{-1} fw). Higher abundance values were found for altered pH zones in July 2013 on the Northern side compared to normal pH stations. Lowest overall values were found in October among all the stations (1.06 ± 1.32 · 10^3 cells g^{-1} fw). Cell counting showed rare cells occurrence on 12th September (>35 cells g^{-1} fw) and hence they were not considered in the statistical analysis.

In 2014 higher overall abundances were found on the first sampling (16th July 2014, 3.57 ± 3.70 · 10^4 cells g^{-1} fw) while decreasing concentrations followed on 24th July and 5th August. Cell counting showed rare cells occurrence on 2nd October (fig. 3.4), and were not considered in the statistical analyses.

Variability among replicates

Coefficient of variation of average abundances of the replicates were generally comparable among pH zones (0.54, normal; 0.52, low; 0.60, very low) and at each sides of Castello Aragonese (0.48, Northern side; 0.63, Southern side). It ranged from a minimum value of 0.08 (N2 station, 4th July 2013) to a maximum value of 1.13 (S3 station, 23th July 2014).

3.5.3 *Ostreopsis* abundances along the pH gradient

Ostreopsis abundance among the pH zones showed low interannual variability at each side of Castello Aragonese (fig. 3.5). However, at each sampling date, some variation to the general trend were found: slightly decreasing abundances along the decreasing pH gradient occurred at the Northern side (2nd July 2012 and 1st October 2013) and in the Southern side (13th July 2012 and 4th July 2013) while, at the opposite, increasing abundances values along the decreasing pH gradient were found at the Northern side (13th July 2012, 4th July 2013 and 26th July 2013). Abundance value among pH zones showed significant differences in 3 samplings out of 9, and only in 2014: however, when considered together, their differences were not significant (ANOVA, "pH zones"; F-value = 1.471, $p = 0.233$).

3.5.4 *Ostreopsis* abundances on northern and southern side

Very high differences in *Ostreopsis* concentrations were found instead between Northern and Southern stations: abundances were generally always higher in the Northern stations at each sampling. At interannual scale, abundance were always one order of magnitude higher in the North, except in 2012 when more similar values were found (fig. 3.6). The difference in the abundances between Northern and Southern stations were significant at each single sampling dates (tab. 3.1) except in the 13th July 2012 (ANOVA: F-value = 1.154, p = 0.304) and 23th July 2014 (ANOVA: F-value = 0.279, p = 0.606). However, when comparing all the abundances from Northern and Southern stations, their differences were found significant (ANOVA, position factor; F-value = 35.242, $p < 0.05$).

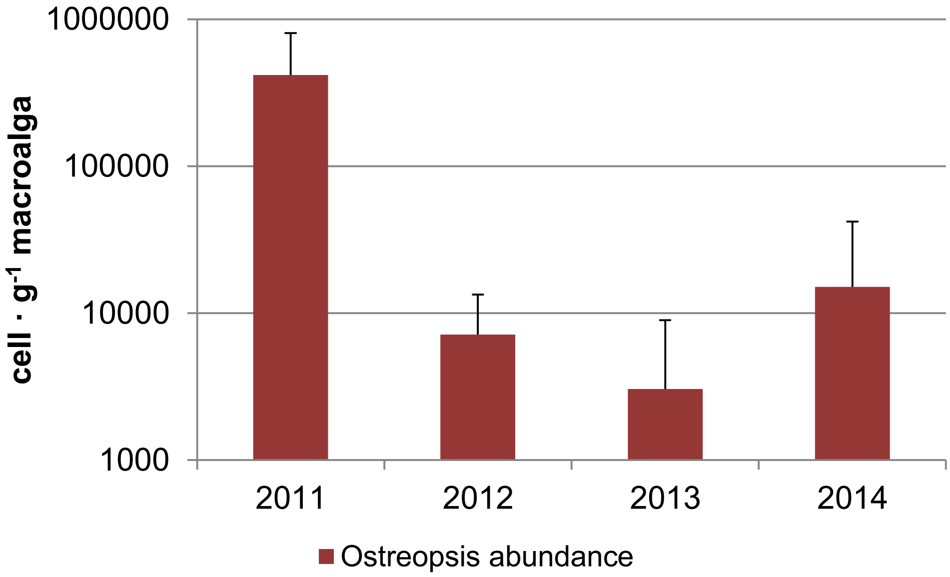

Figure 3.3. Interannual variability of *Ostreopsis* abundances at Castello Aragonese stations. Highest abundances values were found in 2011, while lower values were observed in the following years.

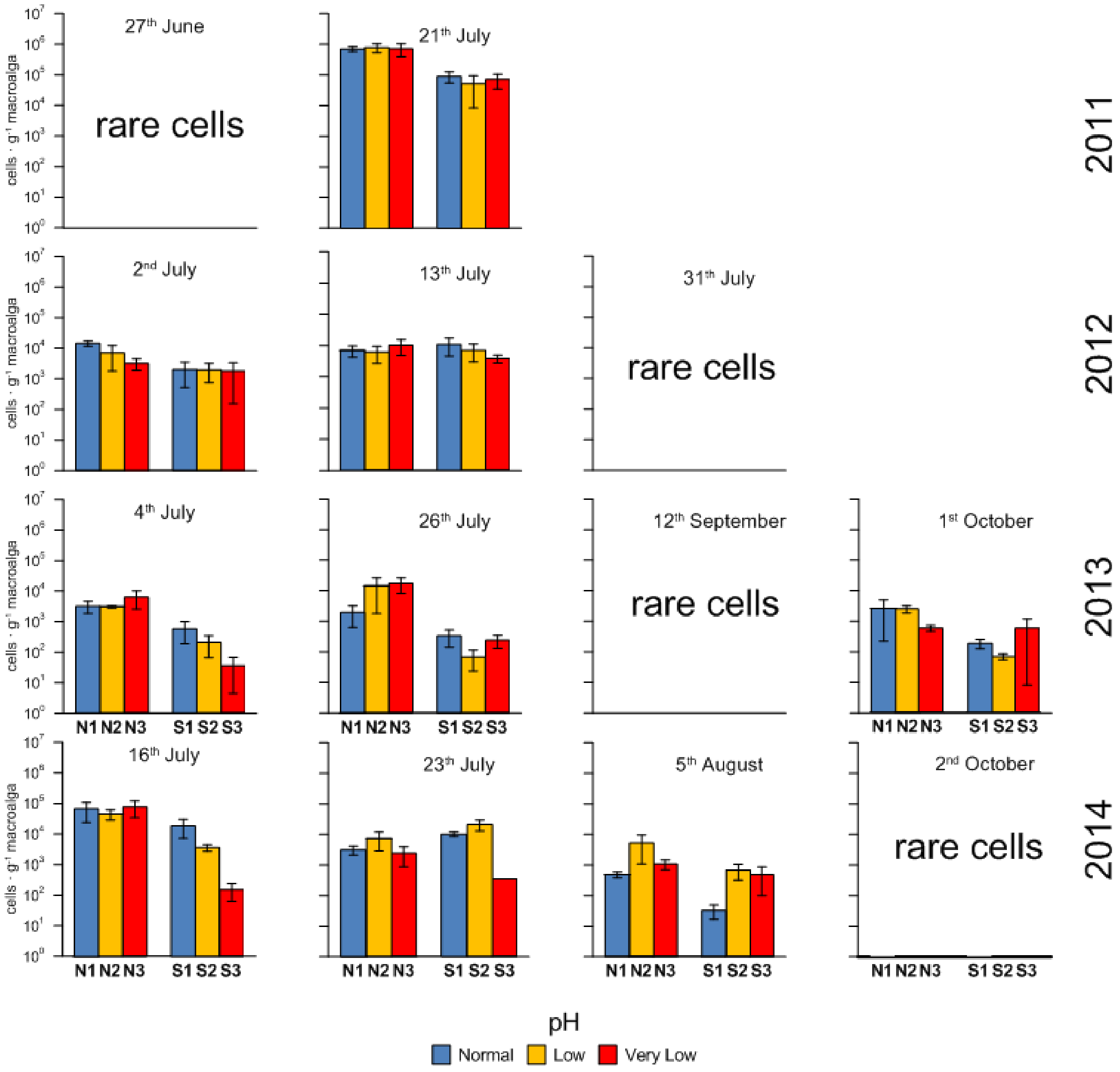

Figure 3.4. ***Ostreopsis*** **abundances at Castello Aragonese stations among pH zones (normal, low and very low) and between Northern and Southern stations (N1-N3, S1-S3). Abundance values are calculated as average of the three replicates. Despite some degree of variability among the samplings, higher abundance values were generally found at Northern stations, irrespectively to the pH values.**

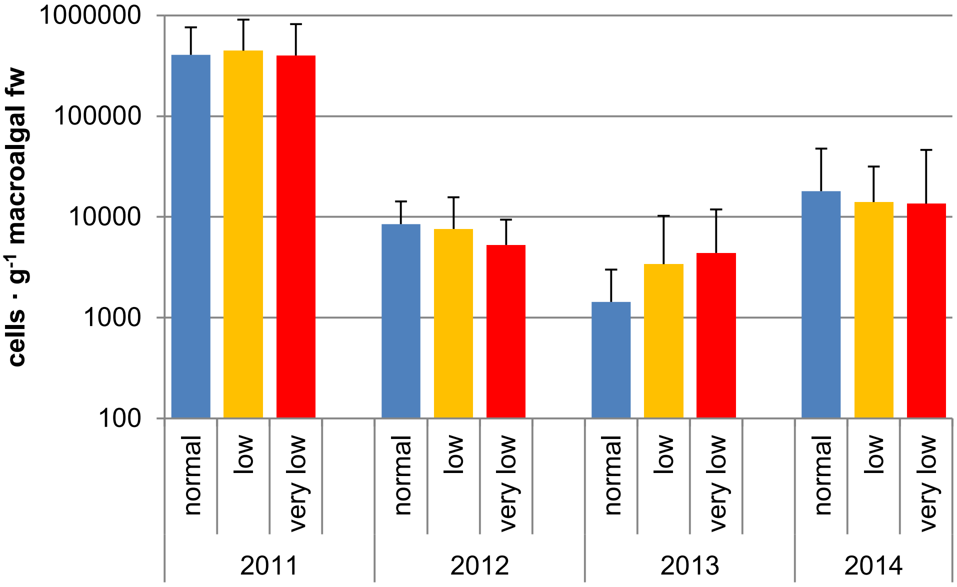

Figure 3.5. Interannual *Ostreopsis* abundances among pH zones (normal, low and very low) at Castello Aragonese stations, calculated as average abundance of the pH zone per year. Despite the wide variation of abundances observed at temporal scale, no significant difference was found among pH zones.

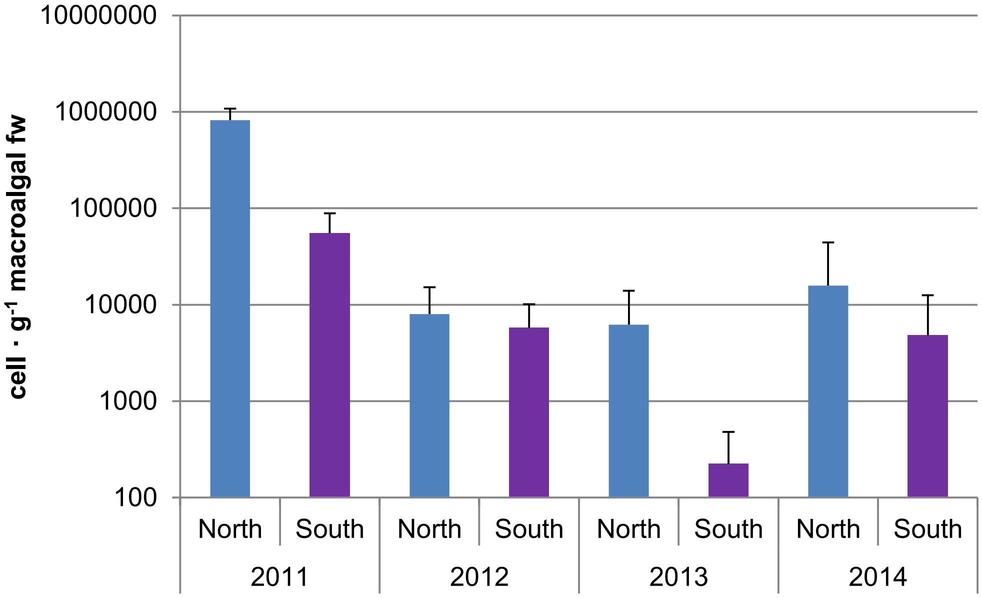

Figure 3.6. Interannual *Ostreopsis* abundances between Northern and Southern stations, calculated as average abundance at the two stations per year. Despite some degree of variability observed at temporal scale, *Ostreopsis* abundances were always higher at the Northern, exposed stations.

Table 3.1. Average *Ostreopsis* abundances (in cell · g^{-1} macroalgal fw) at Northern and Southern stations, their ratio and statistical significance of differences among pH zones and Northern and Southern stations (position). Significant values of difference are marked with X.

		***Ostreopsis* abundance**			**Factor significance**	
year	date	Northern stations	Southern stations	N/S ratio	pH	position
2011	21st July	$7.61 \cdot 10^5$	$7.27 \cdot 10^4$	10.47	-	X
2012	2nd July	$8.31 \cdot 10^3$	$1.96 \cdot 10^3$	4.23	-	X
	13nd July	$7.66 \cdot 10^3$	$7.18 \cdot 10^3$	1.07	-	-
2013	4th July	$4.12 \cdot 10^3$	$2.70 \cdot 10^2$	15.23	-	X
	26th July	$1.12 \cdot 10^4$	$2.10 \cdot 10^2$	53.66	-	X
	1st October	$1.86 \cdot 10^3$	$2.72 \cdot 10^2$	6.82	-	X
2014	16th July	$6.38 \cdot 10^4$	$7.73 \cdot 10^3$	8.24	X	X
	23th July	$4.27 \cdot 10^3$	$1.06 \cdot 10^4$	0.40	X	-
	5th August	$2.29 \cdot 10^3$	$4.00 \cdot 10^2$	5.73	X	X

3.6 Discussion

3.6.1 Seasonal variability

Ostreopsis cf. *ovata* shows seasonal peaks in the Mediterranean Sea, with blooms occurring in early summer in the northwestern Mediterranean Sea (Mangialajo et al., 2011) and in the Tyrrhenian Sea (ARPA Campania, unpublished data) while blooms occur in fall in the Adriatic Sea (Totti et al., 2010; Accoroni et al., 2012a). Data collected in our experiment are in agreement with the seasonality found in Western Mediterranean Sea: in fact, within the four years of study, the most intense *Ostreopsis* cf. *ovata* concentrations were found in July while much lower cell concentrations were found in June and September. A second bloom of *Ostreopsis* cf. *ovata* was identified also in early fall of one year, as has been reported also in the

Northern Adriatic Sea (Mangialajo et al., 2008; Totti et al., 2010; Mangialajo et al., 2011; Accoroni et al., 2012a).

3.6.2 Interannual variability

Temporal variability at multiannual scale of the abundance has already been shown for *Ostreopsis*: surveys conducted in the Northern Mediterranean Sea showed large variations among the years (Mangialajo et al., 2011; Asnaghi et al., 2012) with differences in peak intensity until one order of magnitude. Abundances values in our experiment are comparable with the variability found in other stations of the Mediterranean Sea: higher concentrations occurred in the 2011 while reduced bloom intensities, from one to two degrees of magnitude lesser (tab. 3.1), occurred in the following years. However, decreasing peak intensity at interannual scale has also been found in other sites of the Gulf of Naples (ARPAC Campania, unpublished data but see chapter II of this thesis). It is not possible to exclude that the lower intensity of peak abundances in 2012 and 2013 may correspond to initial or late phase of the bloom, likely depending on the bi-weekly frequency of sampling. Nevertheless, *Ostreopsis* cell concentrations in 2011 were comparable to maximum epiphytic abundance values recorded in the northern Mediterranean Sea (10^6 cells g^{-1} fw) (Mangialajo et al., 2011), while values attained in the following years were much lower, one order of magnitude lesser than the ones reported for North Aegean Sea (Aligizaki et al., 2008).

3.6.3 Spatial heterogeneity

Spatial heterogeneity at small spatial scales is well known for *Ostreopsis* cf. *ovata* (Cohu et al., 2011; Cohu and Lemee, 2012) and it has been recorded also in our study. *Ostreopsis* cell concentrations showed a wide range in the coefficients of variation within the same station, and a high level of variability of abundances at spatial scale. The wide variations in the abundances could be due to several co-varying processes such as biological activity, gas exchange, and physical forcing influencing pH dynamics in coastal ecosystems (Price et al., 2012). It cannot also be excluded that such variations could be due to diel pH oscillation observed in these venting areas (Hall-Spencer et al., 2008; Hofmann et al., 2011; Kerrison et al., 2011; Johnson et al., 2013), although the absence of any trend in the abundances values does not bring valid evidences to this hypothesis. Nevertheless, *Ostreopsis* abundances variations among the three replicates were comparable both in different acidified conditions and at the two side of Castello Aragonese, as confirmed by the ANOVA of coefficient of variation. The diversity of biotic substrates has been also proposed as one of the factors influencing the distribution of *Ostreopsis* at small spatial scales (Totti et al., 2010): however, in our study the homogeneity of the macroalgal species sampled should have prevented such substrate variability. At mesoscale level, the intensity of the peaks observed at Castello Aragonese stations showed instead similar pattern with abundance values reported from two other sampling stations of the Gulf of Naples (see chapter II of this thesis for details).

3.6.4 Effects of the pH gradient

Our findings provides for the first time the evidence that *Ostreopsis* cf. *ovata* is not influenced by natural pH variations in a venting site. pH is an environmental factor mostly overlooked in *Ostreopsis* physiology, if compared to other physico-chemical parameters (Vila et al., 2001; Accoroni et al., 2011; Scalco et al., 2012). In our study we found the occurrence of *Ostreopsis* cells in all the stations at altered pH conditions. Moreover, variations in the pH along the acidification gradient seem to cause a null effect on *Ostreopsis* concentrations: however, it should be noted that considering single samplings, significant differences in the abundance among pH zones were sometimes found. Nonetheless, comparable values of the coefficient of variations within the pH gradient likely suggests that temporal pH variability recorded within stations (Hall-Spencer et al., 2008; Hofmann et al., 2011; Kerrison et al., 2011) exert a negligible effect on the variability of *Ostreopsis* abundances.

Low pH and microphytobenthos

The occurrence of *Ostreopsis* among zones at variable pH is consistent with other studies highlighting the presence of marine dinoflagellates in environments at high pH variability (Hansen, 2002; Hinga, 2002). Low pH tolerance is not uncommon in marine dinoflagellates: *Alexandrium* species are capable to tolerate sub-acid conditions (Kremp et al., 2012) or even thrive at low pH levels (Hwang and Lu, 2000). Experiments performed in micro and mesocosms with other phytoplanktonic species found that sub-acid conditions cause no effects on the growth rates of several coastal species of diatoms, coccolithoforids, prymnesiophytes, cryptophytes, cyanobacteria and dinoflagellates (Berge et al., 2010 and references therein; Taylor et al., 2014), though variations in diatom community composition have been also reported in some field studies (Porzio et al., 2012; Johnson et al., 2013). Benthic species may also be adapted to shallow-water macroalgal systems, which experience high variability in seawater pH (Hofmann et al., 2011) as results of multiple factors (photosynthesis, depth, seasonal and daily cycles) (Middelboe and Hansen, 2007).

Comparison with other benthic species at Castello Aragonese

Results showing no effect of pH on the distribution of *O.* cf. *ovata* diverge from those obtained for other organisms at the same vent site. Dramatic shifts in community composition have been reported for vent site areas. A reduction of community structure is known at Castello Aragonese vent sites in the most acidic pH zones (Kroeker et al., 2013a). Significant reduction was shown in coralline algal cover (Martin et al., 2008) while variations along the pH gradient were reported for invertebrate communities (Kroeker et al., 2011; Ricevuto et al., 2012). Spatial differences in other biological compartments, however, do not seem to negatively affect the intensity of *Ostreopsis* blooms along the pH gradient. The variation in sensitivity at different pH conditions amongst organisms has important implications for ecosystem responses (Kroeker et al., 2010): the absence of sea urchins in the low pH sites (Hall-Spencer et al., 2008) has been proposed as a cause of alteration in macroalgal community with the prevalence of fleshy macroalgae such as the brown alga *Dictyota dichotoma* (Porzio et al., 2011), which is a more suitable host for *Ostreopsis* (Aligizaki and Nikolaidis, 2006; Monti et al., 2007). The combined effect of enhancement of growth rates of fleshy algae (Porzio et al., 2011; Johnson et al., 2013) and mat-forming algae (Connell et al., 2013) in acidified areas, both in temperate (Kroeker et al., 2013a) and tropical (Fabricius et al., 2011) zones, will likely favour the attainment of epiphythic organisms on new available substrata in future conditions. This situation presents an analogy with the case of tropical areas, where the demise of coral reefs, as a consequence of ocean acidification and increased temperature, can lead to an increase of macroalgae, more prone to colonization by harmful benthic dinoflagellates (Hallegraeff, 2010).

3.6.5 Effects of the position in relation to environmental conditions

3.6.5.1 Hydrodynamic conditions

Hydrodynamic conditions have been proposed as important factor influencing *Ostreopsis* development but controversial results have been collected at present day. High *Ostreopsis* spp. abundances have been recorded in stable environments in different temperate areas. Similarly, in several basins of the Mediterranean Sea, intense blooms of *Ostreopsis* have been found in breakwater areas with poor water circulation (Simoni et al., 2003; Barone, 2007; Totti et al., 2010) highlighting the preference of *Ostreopsis* for sheltered site than the exposed ones. A preference for higher level of water motion was instead shown at moderate regimes along the Spanish coasts (Vila et al., 2001) for *Ostreopsis* cf. *siamensis* and in the tropics (Grzebyk et al., 1994) and at more increased wave exposure for *Ostreopsis* sp. in coral reef habitats (Carlson and Tindall, 1985; Richlen and Lobel, 2011). Considering all these differences, the preference of *Ostreopsis* spp. for different hydrodynamic regimes can be specie-specific with *Ostreopsis* cf. *ovata* preferring and better tolerate high energy waters regimes (Parsons et al., 2012). Our data are in agreement with this latter finding: very large differences in *O.* cf. *ovata* abundance were found between the northern and southern side of Castello Aragonese. *Ostreopsis* abundances values were always higher in the northern side, more exposed to hydrodynamic events, compared to the southern one, more sheltered and less prone to high energy waters. Though other physical parameters as water circulation, wind intensity and direction could influence the water motion of an area, resulting in a difficult evaluation and integration (Cohu et al., 2011), they could play a synergistic effect favouring the attainment of higher abundances in the exposed site as compared to the sheltered one.

3.6.5.2 Light

Though few literature data exist for the role played by light intensity on *Ostreopsis* blooms (but see Parsons et al., 2012), laboratory experiments have shown that irradiance could be a major driver influencing the growth of *Ostreopsis* cf. *ovata* (Scalco et al., 2012). Considering comparable daylength at both side of Castello Aragonese during each sampling, our findings seems to support data from laboratory experiments confirming that *Ostreopsis* spp. prefer low light regimes (Scalco et al., 2012). Indeed, lower irradiances are allegedly present in the northern, shadowed side of Castello Aragonese (fig. 3.1) as compared to the southern side and hence may favour more intense blooms. This is not surprising considering the epiphytic behaviour of this species: macroalgal shading has been proposed as a mechanism to reduce light intensity (Ballantine et al., 1988). In our experiment, however, the macroalga sampled was the same in all the samplings and may not account for the abundance diversity found at each side of island.

3.5.6.3 Chemical drivers

While evidences are confirming the link between coastal eutrophication and harmful algal blooms (Glibert and Burkholder, 2006) indications are also emerging (Davidson et al., 2014). Benthic dinoflagellate distributions in tropical and Mediterranean areas seem to be rather unaffected by nutrient concentrations levels (Grzebyk et al., 1994; Vila et al., 2001). There is still ambiguity on the role of nutrients in *Ostreopsis* growth: several studies pointed out that there are no clear patterns in the nutrient availability and *Ostreopsis* development (Vila et al., 2001; Shears and Ross, 2009; Accoroni et al., 2011; Cohu et al., 2011). Contrastingly, a study conducted in Pacific areas has demonstrated a direct relationship with nutrient concentration (Parson and Preskitt, 2007). Nutrients concentrations measured at Castello Aragonese were comparable with those registered in other areas where *Ostreopsis* bloom occurrence has been reported (Vila et al., 2001; Shears and Ross, 2009; Accoroni et al., 2011; Cohu et al., 2011). The relative scarcity of nutrient concentrations measurements and *O.* cf. *ovata* abundances in our experiment do not allow to establish any relationship between cell abundances and nutrient levels. However, in our study we did not find significant differences in nutrient concentrations at both sides of the island, despite the huge variability in *Ostreopsis* abundances at Northern and Southern stations. However, coastal waters at the two sides of Castello Aragonese may still differ in chemical factors, as the northern side could be more prone to inputs from a nearby river mouth (Iermano et al., 2012) at smaller temporal scales: in this case the frequency of our experimental sampling scale may have overlooked such variability. Changes in CO_2 chemistry in future ocean conditions will affect several physiological processes and biological responses in primary producers such as growth, photosynthesis and survival (Kroeker et al., 2013b) as well as toxin production (Brodie et al., 2014). Though our experiment showed no significant changes in *Ostreopsis* abundances at different pH values, it is not possible to determine whether such variations exert a null effect on the physiology of *Ostreopsis* or a shift in physiological responses is undergoing. Further studies are needed to clarify changes in the physiology under the predicted pH of future ocean conditions.

3.6.6 Future scenarios

Along with temperature, pH is an environmental factor that will surely determine future oceanic conditions (Orr et al., 2005) causing severe impacts on tropical and temperate coastal systems (Guinotte and Fabry, 2008). It is still unknown whether changing CO_2 availability and altered pH conditions will enhance growth for most species. However, pH changes will affect physiology and toxicity of some HAB species (Fu et al., 2012). Our data show that spatial distribution of *O.* cf. *ovata* is not affected by low pH conditions, indicating that this species can occur and reach bloom concentrations at pH values definitely lower than the ones predicted for 2100 (Riebesell, 2004). Considering that benthic species display higher resilience to changes in pH and CO_2 levels than planktonic ones (Nielsen et al., 2010), our findings suggest that *Ostreopsis* cf. *ovata* may be capable to tolerate changes likely occurring in future ocean conditions.

3.7 Chapter summary

Several samplings conducted along a natural vent site of CO_2 at Castello Aragonese di Ischia from 2011 to 2014 have allowed to establish:

- the occurrence of *Ostreopsis* cells also in the stations at altered pH conditions;
- a null effect on the development of this species at lower pH;
- no direct relationship between its bloom with temperature and nutrient concentrations;
- a significant effect of the “position” factor on its distribution, suggesting hydrodynamism and light intensity as important physical variables to be considered in further studies;
- the potential development of *Ostreopsis* blooms in the expected future low pH ocean conditions.

Chapter 4

Spatial distribution of *Ostreopsis* cf. *ovata* in the Gulf of Salerno

ABSTRACT

The distribution of *O.* cf. *ovata* along the sandy coasts of the Gulf of Salerno was assessed to evaluate the potential influence of artificial structures for the management of the coastal erosion of future construction in this area. A targeted macroalgal sampling was performed during the maximum development of this species in July 2013 at 5 sampling stations, in correspondence of *Cymodocea nodosa* meadows, using an improved method for the detection of microalgal cells. In the Gulf of Salerno, the occurrence of *Ostreopsis* cf. *ovata* was reported, along with another potentially harmful dinoflagellate *Dinophysis caudata*, though both at low concentrations. The results of this study demonstrate that the construction of anti-coastal erosion systems in this area may elicit macroalgal settlement, which could host toxic microalgae, currently absent or scarce in the area. Planktonic blooms of existing or introduced potentially harmful microalgae may also be promoted by the reduction of water exchange within the artificial structures.

4.1 Introduction

The distribution of *O.* cf. *ovata* along the Italian coasts (fig. 4.1) has recently been summarised in the documents of the Institute for the Protection and Environmental Research, ISPRA (ISPRA, 2010; ISPRA, 2011; ISPRA, 2012; Funari et al., 2014). Since 2010, a monitoring survey of potentially toxic microalgae in bathing waters is carried out by regional agencies for the protection of the environment (ARPA), in line with art. 3 of ministerial decree of 30 march 2010 of the Italian Ministry of Health. In addition to the institutional programs mentioned, many studies have been conducted in Italians universities and research institutes, further contributing in to the knowledge of the ecology and distribution of *O.* cf. *ovata* and other toxic microalgae in the Italian seas. Based on the monitoring data and studies conducted so far, the presence of *O.* cf. *ovata* was found in the summer or the summer-autumn in all coastal regions except for Veneto, Emilia Romagna and Molise (fig. 4.1).

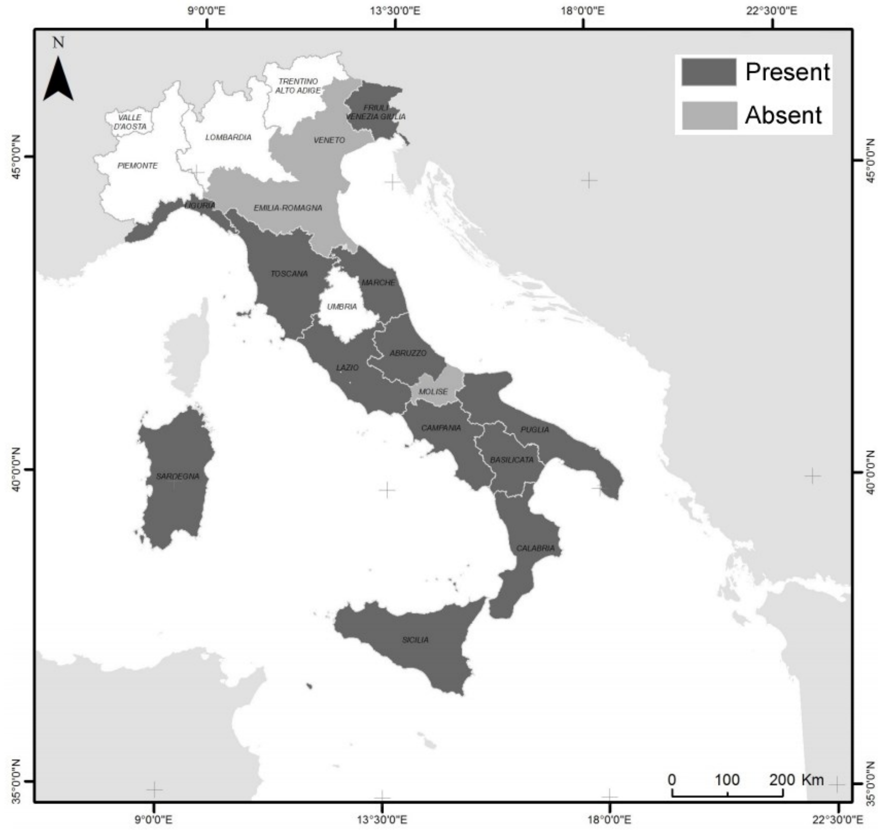

Figure 4.1. Occurrence of *Ostreopsis* cf. *ovata* along Italian coasts (modified from Funari et al., 2014).

The presence of *O.* cf. *ovata* in the waters of the Campania region is known since 2004 (Zingone et al., 2005; Zingone et al., 2006), when the species was found accidentally during macroalgae sampling. After the massive outbreak of *Ostreopsis* cf. *ovata* occurred along Genoa coasts in 2005 and 2006 (Ciminiello, 2006), in 2007 the Stazione Zoologica Anton Dohrn began a targeted sampling of macroalgae potentially colonized by *O.* cf. *ovata*, which has led to the identification of high concentrations of this toxic species in some areas, thus representing a real risk to public health. The first *Ostreopsis* monitoring campaign in August 2007 along the coasts of Campania, from the Gulf of Gaeta to Cilento coasts, showed the presence of *O.* cf. *ovata* in almost all the stations sampled, except in the sandy shore stations of the Gulf of Gaeta and the coastal region in the southern area of Salerno. In fact, in these areas, natural and/or artificial fixed structures are absent or rare, not allowing settlement of macroalgae suitable for the development of *O.* cf. *ovata*. *Ostreopsis* cf. *ovata* is instead present in the rocky coasts both in southern and northern areas mentioned above. *Ostreopsis* cf. *ovata* generally grows at high salinity (37 - 38), in relatively transparent waters, under conditions of medium or low hydrodynamic regimes and at temperature ranging between 20 and 28 °C. It is commonly thought that *O.* cf. *ovata* does not grow in areas where no or very few rocky substrates are available, such as the coasts of the region of Veneto and Emilia Romagna. Therefore, since 2008, the sandy areas of the coast of Campania have been deliberately excluded from the monitoring plan in the Region, while sampling was intensified in areas that had higher abundances of benthic microalgae. In reference to the project "Interventi di difesa e ripascimento del litorale del Golfo di Salerno" (POR Campania 2007/2013), the '*Ostreopsis*' Coordination Group of the Campania Region reported that the presence of submerged barriers and T-head groynes can promote macroalgal settlement, even of the invasive species *Caulerpa racemosa* or *Asparagopsis taxiformis*, which could provide an ideal substrate for the development of blooms of *Ostreopsis* cf. *ovata* and other benthic dinoflagellates, known or suspected to produce toxic substances, such as *Prorocentrum lima* and *Coolia monotis*. The proliferation of these microalgae would be much more likely if the species were already present in the area, even in low amounts.

4.2 Specific aims of the chapter

The purpose of this study was to verify the presence of *Ostreopsis* cf. *ovata* and other benthic toxic or potentially harmful microalgae on the existing macrophytic communities along the southern coast of Salerno in the area where management intervention for the defence from coastal erosion are planned. The aim was to verify what had been found in a previous survey in the area in 2007, i.e. that the species was absent from the area, through sampling of macrophytes not considered in that study and applying a counting method with a lower detection limit.

4.3 Study area: Gulf of Salerno

The Gulf of Salerno is the widest gulf of the Campania region. It is bounded at north by the Costiera Amalfitana and in the south by the Cilento coast. Different topographical features can be recognised along its coastline, with two major morphological typologies: a rocky coast in the northern part and a sandy coast in the South, corresponding to the “Piana Del Sele” coastal plain, named after the Sele River. This river is the most important water course of the area being larger than the other two rivers of the GOS (Irno and Tusciano). In the very final part of the Gulf, along the Cilento hill formations, the coast resumes a rocky morphology, in correspondence to the Area Marina Protetta Santa Maria di Castellabate, where pristine waters are present. The GOS is characterized by less hydrographic and biological gradients as compared to the GON. The rivers and some secondary outlets represent the main source of nutrients, draining the cultivated fields and carrying the urban sewage discharges. Nutrient concentrations in the Gulf of Salerno are generally lower than those reported for the GON (Marino et al., 1984).

4.4 Material and methods

4.4.1 Sampling strategy

The sampling was carried out on 26th July 2013 at 5 sampling stations (T1 - T5, fig. 4.2, tab. 4.1) located in the proximity of artificial structures of future construction (barriers and t-head groynes). The sampling sites were chosen in relation to the existing macrophytic population and the minimum distance possible to reach by the motorboat used in sampling.

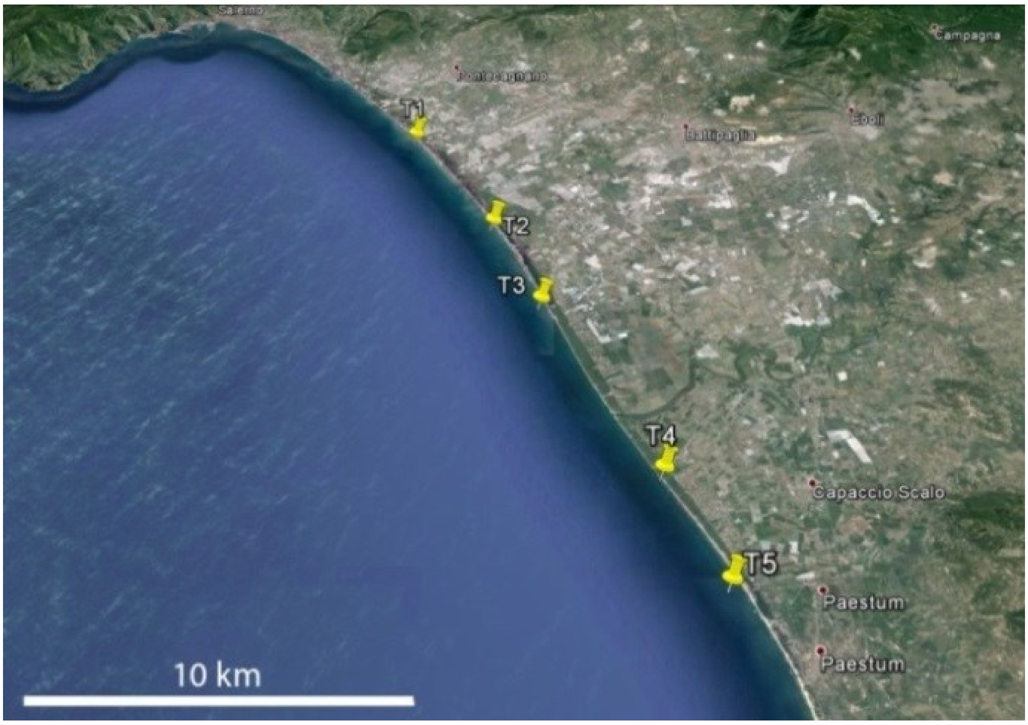

Figure 4.2. Sampling stations on the Gulf of Salerno. T1-T5 stations correspond to sites where anti-erosion coastal system (submerged barriers or t-head groynes) is planned.

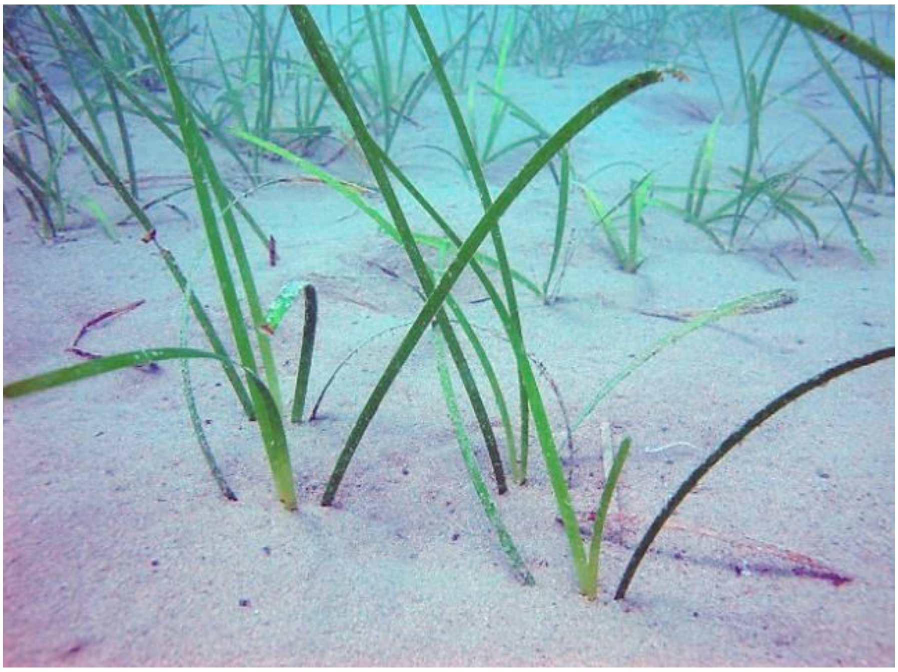

Figure 4.3. *Cymodocea nodosa* in the Gulf of Salerno. Picture credit: dott. Francesca Russo.

Table 4.1. Sampling sites along the Gulf of Salerno and relative coordinates (WGS 84).

Area	Station	Latitude (N)	Longitude (E)
Salerno	T1	40° 36’ 08’’	14° 51’ 33’’
	T2	40° 33’ 27’’	14° 53’ 41’’
	T3	40° 31’ 20’’	14° 54’ 55’’
	T4	40° 27’ 31’’	14° 57’ 29’’
	T5	40° 25’ 28’’	14° 58’ 37’’

4.4.2 Sampling method

Sampling was carried out according to the Guidelines of the Italian Ministry of Health “Gestione del rischio associato alle fioriture di *Ostreopsis ovata* nelle coste italiane” which consists of the following operations:

- enveloping a macrophyte (macroalgae or, in our case, seagrasses) with a plastic bag, cut the macrophyte at the base and quickly close the bag, in order to pick the macroalgae along with a portion of adjacent water;

- in case of scarce availability of macrophytes, scratching the bottom and collecting small macroalgal turfs and the adjacent water in the plastic bag.

In all the stations, samples of the aquatic plant (phanerogams) *Cymodocea nodosa* (fig. 4.3) were taken in triplicate at a variable depth of 6 - 6.5 m. At T4 station (fig. 4.2), closely located to a quay and characterized by low depth (2 m), it was not possible to sample of *Cymodocea*, as occurred at other stations. Three different types of substrate were sampled (tab. 4.3):

- *Ulva*, a macrophyte belonging to the group of green algae;
- mussels;
- a ferrous concretion.

Sample treatment and microalgae fixation was performed on board of the Prismambiente s.r.l. motorboat. The substrate (macrophyte or other) and the water collected were transferred into a plastic jar, which was vigorously shaken in order to allow the detachment of microalgal cells. The substrate was then removed and placed on an aluminium foil for the estimation of the weight (carried out in the laboratory with precision scale). The volume of the water collected was therefore measured with a graduated cylinder and a sub-sample of 250 ml was taken, placed in a plastic container and immediately fixed with formalin to a final concentration of 0.7% formaldehyde.

4.4.3 Cell counting

Ostreopsis cf. *ovata* cells and other potentially harmful phytoplankton organisms present in the samples obtained from the washing of the substrate were counted at the inverted microscope (Zeiss Axiovert 200). For this purpose, aliquots of 100 ml of the sample were filtered with a filter of 20 µm mesh-size and the aid of a crystallizer. The portion of the sample retained on the filter (34 ml) was placed in Utermöhl chambers (3 ml) in variable volumes (from 3 to 8 ml) and observed after ca. 16 hours. Six transects of an Utermöhl chamber at a magnification of 100X were observed, staining the samples through Calcofluor stain solution. The average detection limit of this method was approximately 35 cells per gram of substrate. Microalgal abundance values were calculated taking into account the weight of the substrate and the total volume of water sampled with it, and expressed as number of cells per gram of substrate (see formula in paragraph 3.4.2)

4.4.4 Environmental variables

During the macrophytes (and other substrates) sampling, temperature and salinity data were acquired at all the sampling stations using a multiparametric YSI CastAway™ probe.

4.5 Results

4.5.1 Environmental variables

Temperature values registered during the sampling campaign (tab. 4.2) ranged between a minimum value of 25.46 °C (T1 station) and a maximum of 27.72 °C (T4 station). This latter value differs from the mean value recorded in other stations (about 25.5 °C) because of the shallow depth of the T4 station (0.8 m). The salinity values were rather homogeneous across the sampling stations (average: 37.76 ± 0.06), which deliberately excluded the areas in proximity to the river outlets and other freshwater discharges.

Table 4.2. Temperature, salinity and sampling depth at five sampling stations of Gulf of Salerno.

station	temperature (°C)	salinity	depth (m)
T1	25.46	37.65	6
T2	25.76	37.80	6
T3	25.52	37.79	6
T4	27.72	37.77	0.8
T5	25.32	37.79	6.5

4.5.2 Benthic abundances

Microphytobenthic analysis of samples from the Gulf of Salerno confirmed the absence or very low concentration of benthic epiphytic microalgae at all sampling stations. The microalgae *Ostreopsis* cf. *ovata* was initially found only in the southernmost stations (T4 and T5), with abundance values of less than 50 cells per gram of substrate (tab. 4.4). Analysis of samples from T1 - T3 stations, performed by increasing the power of detection of the method used, has confirmed the presence of

Ostreopsis, even where no microalgae had initially been found. Given the extremely low number of cells counted and the resulting error in the calculation of the relative abundance, data reported here are only qualitative (tab. 4.4).

The microalgae *Prorocentrum lima*, known for the production of okadaic acid, associated with diarrhetic syndromes in humans (diarrhetic shellfish poisoning, DSP), was detected in the sampling stations T2 and T5 with very low values (10 - 100 cells per gram substrate). *Coolia monotis* was not found at any sampling stations (tab. 4.4). Although the analysis was conducted on benthic samples, samples analysis of station T5 has revealed the occasional presence of the planktonic dinoflagellate *Dinophysis caudata* associated with diarrhetic syndromes.

Table 4.3. Substrate type sampled and relative weight (3 replicates).

station	replica	substrate sampled	substrate weight (g)
T1	a	*Cymodocea nodosa*	8.48
	b	*Cymodocea nodosa*	9.15
	c	*Cymodocea nodosa*	8.04
T2	a	*Cymodocea nodosa*	5.22
	b	*Cymodocea nodosa*	9.18
	c	*Cymodocea nodosa*	6.91
T3	a	*Cymodocea nodosa*	8.57
	b	*Cymodocea nodosa*	8.29
	c	*Cymodocea nodosa*	7.47
T4	a	*Ulva lactuca*	7.35
	b	Mussels	33.79
	c	Iron concretion	18.10
T5	a	*Cymodocea nodosa*	11.99
	b	*Cymodocea nodosa*	18.51
	c	*Cymodocea nodosa*	7.81

Table 4.4. Average concentration of epiphytic microalgae in all the sampling stations. Values expressed as cell for gram of substrate. n.d. = absence of cell or present in amount below the detection limit of the used method.

	Ostreopsis cf. ovata	*Prorocentrum* spp.	*Coolia monotis*
T1	**present**	n.d.	n.d.
T2	**present**	present	n.d.
T3	**present**	n.d.	n.d.
T4	**present**	n.d.	n.d.
T5	**40.7**	95.0	n.d.

4.6 Discussion

The exploratory sampling for the evaluation of the presence of *Ostreopsis* and other potentially toxic microalgae in the Gulf of Salerno assessed the presence of *Ostreopsis* in all the sampled stations, although at very low concentrations. This result is completely new, since previous analyses ruled out the presence of the species throughout the sandy coasts in the southern area of the Gulf of Salerno. Indeed, in 2007 macroalgae sampling was not performed on the seagrass *Cymodocea nodosa (*which grows on sandy bottoms of the area) or on the macroalga *Caulerpa racemosa* (an invasive species known to have colonized some areas of the Salerno coast in recent years). The abundance values found at the T4 station, in correspondence of a wharf, are close to the limit of detection and highlight the low concentration of the microalgae investigated. In the sampling station T5, concentrations were very low (about 40 cells per gram of substrate) and just above the limit of detection used on this survey. In fact, given the low number of cells actually seen in the samples, the error associated with the concentration values provided is very high. These values are about 750 times lower than the threshold of concern provided by the new guidelines of the Ministry of Health (30000 cell · g^{-1} macroalgal fw), and much lower than those found in some stations in the Gulf of Naples during sampling conducted in this study in the same period (see chapter 2). For example, in the Gaiola station (Naples) on the 24th July 2013 the highest seasonal values were recorded (36603 cell · g^{-1} macroalgal fw), and the seasonal abundance peak occurred on 31st July (144134 cell · g^{-1} macroalgal fw).

Given its rarity and low numbers in the samples collected, it cannot be ruled out that *Ostreopsis* cf. *ovata* could also be present in the stations where it was not found. Previous investigations in Campania and in other areas (Totti et al., 2010) confirmed a high spatial and temporal variability in the distribution of *O.* cf. *ovata*, whose concentrations in other periods or in other areas may be much higher. However, the sampling period in the present study was selected in order to maximize the chances of finding the species, characterized by a typically summer seasonality, with peaks generally in July or August. The sampling strategy also took into account the knowledge on the ecology of the species in terms of salinity and light conditions, thus excluding from sampling stations next to freshwater outlets and giving preference to shallow areas with coverage of macrophytes.

The presence of the species and the concurrent extremely low concentrations in the sampling area are not easily explained if not in reference to the absence of a suitable substrate to its development. The ecology of *Ostreopsis* cf. *ovata*, and in particular the conditions that favour its massive growth, are still the object of research, but it is commonly accepted that the nature of the substrate, salinity, light conditions, hydrodynamics and temperature are the key environmental factors influencing its distribution. *Ostreopsis* cf. *ovata* generally grows at high salinity, in relatively transparent waters, under conditions of medium or low hydrodynamics and a temperature ranging between 20 and 29 °C. It can be assumed that in the sampled area temperature, salinity, time of the year and light availability conditions were those suitable for the development of the species; however, though apparently transported from neighbouring areas by currents, *O.* cf. *ovata* fails to develop massively due to the absence of a suitable substrate.

In the literature there are extensive and detailed case studies on the consequences of artificial structures on the spread of *Ostreopsis* and benthic toxic dinoflagellates. Two studies specifically provide relevant data. On the north Tyrrhenian Sea, along the Apuan coast (Tuscany) and Riviera di Levante (Liguria), in correspondence to anti-erosion structures similar to those planned for this project, high *Ostreopsis* cf. *ovata* concentrations have been documented from 1998 to 2001, coinciding with the occurrence of gelatinous flakes suspended in the water column, hypoxia / anoxia of the seabed and the presence of exoskeletons of Balanidae barnacles (Sansoni et al., 2003). In contrast, in the presence of similar artificial structures along the sandy beaches of the Northern Adriatic Sea (Conero Riviera, Marche), the presence of *O.* cf. *ovata* was not detected at all (Totti et al., 2010).

This difference can be interpreted considering that, while in the former case the surrounding areas of the north Tyrrhenian coasts, characterized by hard substrates, are inhabited by with dense populations of macroalgae hosting *Ostreopsis*, the anti-erosion structures in the Marche Region is under the influence of north-south currents and is adjacent to a long stretch of sandy coastline (Emilia Romagna and Veneto) where *Ostreopsis* had never been found.

It can be assumed that the construction of submerged barriers and t-head groynes in the infralittoral zone, may elicit the settlement of invasive macroalgae, as *Caulerpa racemosa* or *Asparagopsis taxiformis*, resulting in the spread of a suitable substrate for the development of blooms of the toxic microalga *O.* cf. *ovata*. The implementation of systems reducing wave action and the water exchange could be an additional factor leading to the development of benthic and planktonic blooms. The presence of *O.* cf. *ovata* in all stations sampled, bring support to the fact that this microalga can be easily transported from neighbouring rocky areas.

The presence of *O.* cf. *ovata* in the southern part of the area investigated in this study suggests a transport from areas further south, such as the rocky shores of the Cilento coasts, compatible with the main north-south direction of Tyrrhenian currents in the season. In those areas, *Ostreopsis* was found in previous surveys. For example, in August 2007 *O.* cf. *ovata* the concentrations were around 900 cell · g^{-1} macroalgal fw along Agropoli coasts and around 7000 cells · g^{-1} in Pioppi and Marina di Camerota (ARPAC, 2008). Currents of opposite direction in the areas close to the coast may instead carry *O.* cf. *ovata* also from the north, where in 2007 at concentrations of ca. 7000 cell · g^{-1} macroalgal fw was detected ("Salerno Piazza della Concordia" station, see the above-mentioned report).

In the future, sampling after the completion of the defence and beach nourishment work planned along the coast of the Gulf of Salerno could provide local authorities and public health manager with a useful tool to prevent phenomena related to the occurrence of toxic algal blooms. Another output could be to highlight ecological aspects related on the development of phytobenthic community dynamics on artificial rocky substrates and toxic algal species occurrence.

4.7 Chapter summary

The assessment of the potential development of harmful algal blooms along the sandy coasts of the Gulf of Salerno performed through a targeted sampling and lowering the detection limit for benthic microphytes showed:

- the occurrence at very low concentrations of the dinoflagellates *Ostreopsis* and *Dinophysis caudata*, potentially able to form harmful blooms;
- the absence of *Ostreopsis* cf. *ovata* blooms;
- the dispersion capability of *Ostreopsis* species from neighbouring areas;
- the absence of suitable macroalgal substrates for *Ostreopsis*.

Planned works for the protection of the sandy coast of the Gulf of Salerno raise questions about the possible development of benthic and planktonic algal blooms in relation to:

- the formation of a favourable environment for the development of macroalgae that may host toxic microalgae, currently absent or scarce in the area;
- the reduction of water exchange in the t-barriers and in the groynes, which could promote the development and the sudden collapse of blooms of benthic microalgae, with subsequent release of toxins in the water and in the aerosol;
- the increase of the residence time of the water near the coast, with possible development of planktonic blooms of existing or introduced potentially harmful microalgae (through germination of resting stages possibly present in allochthonous sediments).

Chapter 5

General conclusions

Ostreopsis cf. *ovata* blooms along the coasts of Campania region are a stable phenomenon, occurring at regular frequency over the years in summer months. Intense blooms have been reported in the Gulf of Naples along Posillipo, Ischia Island and Sorrento coasts. The marked seasonality of the blooms in the summer months and the high intensity of the blooms may represent a risk for the human population during the bathing season, either through direct contact and aerosol or following the consumption of contaminated seafood. The occurrence of a second bloom in the autumn, though less intense and frequent in the years than the first one, may also constitute a hazard especially considering the recent extension of the bathing season to September.

The decreasing intensity of the blooms observed in this study at multiannual scale does not seem to be related to temporal variability of any environmental parameter investigated. The role of physical (temperature and light intensity) and chemical (nutrient availability and their stoichiometric ratio) seawater variables investigated in this study showed to not be relevant for the occurrence of the blooms and for the regulation of their intensity.

This study also showed that the decrease of pH, an environmental forcing which will surely impact marine ecosystems in future ocean conditions, does not seem to represent a hindrance to *Ostreopsis* blooms, at least in the ranges of variability expected in the fore coming centuries. It is possible however to hypothesise that pH changes will affect the development and/or the spatial extension of *Ostreopsis* blooms due to indirect effect, e.g. on the local populations of macroalgae and their grazers. As an example, the decrease of sea urchin populations, which are impaired by low pH (Hall-Spencer et al., 2008), could result in a higher abundance of brown algae which are used as food by these animals, and hence an increase of the kind of substrate preferred by *Ostreopsis*.

The comparison of the relative abundances of *O.* cf. *ovata* under different hydrodynamic regimes between two different coastal areas of the Gulf of Naples, Posillipo (Gaiola and San Basilio) and Castello Aragonese di Ischia (north and south sites), showed contradicting results: higher concentrations were recorded in the more sheltered areas at the Posillipo site and in the more exposed area at the Ischia site, though in both cases the well-known high spatial heterogeneity at the small scale for these benthic organisms was confirmed. These contradictory results indicate that there must be other location-specific factors, not considered in this study, which could be more important than the exposure in determining the development of *Ostreopsis* blooms. Indeed the complex relationships of *Ostreopsis* cf. *ovata* with the benthic environment (facultative epiphytic behaviour, algal host preferences, ecosystem engineering by mucus production, and toxic effects on invertebrates) may explain at least in part the differences found at these sites. Further comparison of these results with other Mediterranean areas displaying different hydrodynamic features is also needed to better understand the relationships between *Ostreopsis* cf. *ovata* blooms and hydrodynamic conditions.

The finding of *Ostreopsis* cf. *ovata* along the sandy coasts of the Gulf of Salerno required lowering the detection limit of the commonly used methods, increasing the amount of sample observed, due to the very low concentrations of the species in that area. Nonetheless, the presence of the species in those environments demonstrates its dispersal capabilities at quite long distances from rocky areas, which host macrophytes that are a more favourable substrate for *Ostreopsis*. On the other hand, this new finding highlights the potential risk of the implementation of barriers and groynes that should contrast coastal erosion, which may provide an optimal substrate, compared to soft bottoms, for the development of macroalgae hosting *Ostreopsis* cf. *ovata*. Further, by reducing wave action and water exchange, these artificial barriers would constitute an additional factor promoting the development of benthic and planktonic blooms.

Overall the results of this study support a very clear seasonality for *Ostreopsis* blooms. The monitoring strategy of *Ostreopsis* blooms from summer to fall represents hence a valid tool for the defence of public health. These studies complement the data obtained from other Institutes involved in the Regional monitoring plan, allowing a better assessment and interpretation of the existing phenomena potentially able to affect the environment, the animals used for human food and the human population.

Appendix A

Assessment of an alternative sampling method for benthic microalgae

Introduction

Benthic harmful algal blooms (BHABs) are affecting human health and activities and causing environmental issues at global scale. Once limited only to tropical areas, the human syndromes associated to these events (ciguatera and clupeotoxism) are now increasingly reported also in subtropical and temperate areas worldwide. Benthic dinoflagellates *Gambierdiscus* and *Ostreopsis* genus have been found as the causative agents of these diseases. The existing focal mismatch between harmful algal ecologists (more familiar with plankton) and benthic ecologist (mostly focused to sessile organisms) call for an increased attention for the benthic microalgae. New approaches and methodologies are hence needed to correctly cope with this lack of knowledge. Quantitative analysis studies in the benthic environment may produce blurred results due to the patchy distribution of organisms. To overcome this issue, several different sampling protocols have been proposed (vacuum collection, substrate sampling and artificial substrate). In this study we tested the reliability of a substrate sampling method, alternative to the one currently proposed by the National Institute for Health (ISS) and currently used by the local agencies for environmental protection in the monitoring surveys for *Ostreopsis* blooms along Italian coasts.

Material and methods

Sampling methods

Sampling was carried out from 2011 to 2013, from middle of June until the end of the bloom at two stations of the Gulf of Naples, Gaiola and Rocce Verdi, with the support of the SZN R/V “Federico Raffaele” or “Vettoria” and in the 2012 with the logistic support of the C.S.I. Gaiola Onlus R/V “Aquavision”. Macroalgal samples of the red alga *Asparagopsis taxiformis* and the brown alga *Dictyota dichotoma* were collected in triplicates by SCUBA divers at a depth comprised from 1.7 m to 7.4 m, within 5 m of distance. Macroalgal samples were collected following two methods: the method in the Guidelines of the Italian Ministry of Health (Di Girolamo, 2007) (referred here as “official method”) and the Falcon method (ISPRA, 2012). At Gaiola and Rocce Verdi, macroalgal samples were collected from the same thallus following both methods. The Falcon method, as proposed and specified in the Operative Procotols of ISPRA (ISPRA, 2012), consists of the following operations:

- cutting the thallus at the base and rapidly putting it into a 50 ml Falcon tube, taking care to do not shake the thallus during the collection. Close it promptly underwater. The thallus should be at least of 3 g fw and easily fitting in the 50 ml tube;

- filling the tube with seawater surrounding the sample or with filtered seawater;

- storing the labelled tubes in dark conditions and field temperature until the treatment in laboratory. Fix the samples (Lugol, formaldehyde…) if will not be treated in the same day.

A total number of 123 samples were collected in triplicates with each sampling method. Their average abundance values were compared in order to establish a relationship between the two sampling methods.

Macroalgae treatment and cell counting

Macroalgal samples were fixed at a final concentration of 0.7% neutralized formaldehyde and stored in the dark until the analyses. Falcon tubes were vigorously shaken at least 20 times to allow the detachment of benthic cells. Macroalgal thalli were rinsed with fresh water, dry-blotted with paper on a pre-weighted aluminium foil, and then weighed to determine fresh weight (g fw). The volume of the water sample containing detached cells inside the Falcon tube was measured (ml). Cell counting was performed only on *Ostreopsis* cells, using a Zeiss Axioskop 2 plus, at a magnification of 125x, settling 1 ml of the water sample in Sedgewick-Rafter slides. Abundances of *Ostreopsis* cells were finally expressed as cells g^{-1} fw. Samples obtained with the official method were treated and analysed as specified in the section 2.4.1 of this thesis.

Statistical analyses

All the analysis were performed on $\log_{10}(x+1)$-transformed data of benthic cell abundances. Normality assumption of benthic cell abundances was assessed by visual check (Q-Q plot) and evaluated with Shapiro-Wilk test. Significance level value was set for $p<0.05$. All tests were performed in XLStat. Log transformed abundance data of the average of the replicates. Since normality assumption for abundances were not satisfied, non-parametric correlation test (Wilcoxon test) was used.

Results

Abundances calculated with each method were in the same magnitude interval, ranging from 868.47 to 225587.47 and 381.40 to 158490.30 for official and Falcon method, respectively. The variation among the replicates showed similar ranges and frequency of distribution (not shown) for both methods (coefficient of variation: 0.12 - 0.82, official method; 0.05 - 1.18, Falcon method). Regression analysis (fig. A) showed linearity between the abundances calculated with the two methods but with a very low goodness of fit value (y = 0.80; $R^2 = 0.12$). No significant differences between the abundances value were found, as confirmed by correlation analysis (Wilcoxon signed-rank test: Z value = -1.186, p = 0.24).

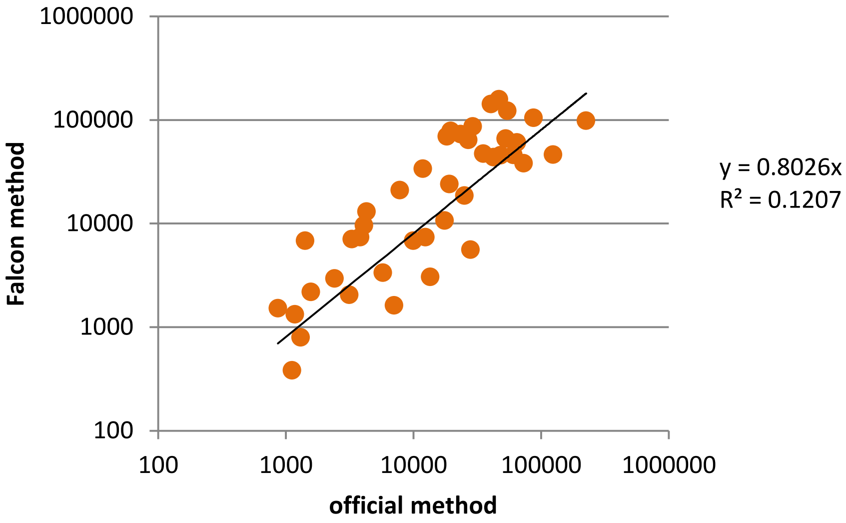

Figure A. Comparison between average abundances (in triplicates) obtained with official and Falcon sampling method. Abundances values are expressed as cell · g^{-1} macroalgal fw.

Conclusions

A comparative analysis between two methods for the sampling of benthic microalgae showed good correlation of the “official” with the Falcon method. Compared with the official method, the Falcon method shows the following advantages:

- a smaller amount of material and fixative needed;

- a more rapid sample treatment;

- a reduction of critical steps;

- a reduced sedimentation time for the counting;
- abundance differences can be obtained also with smaller macroalgal samples;
- the same measure unit of the official method.

The only disadvantage is related to a greater error in the extrapolation of the abundance of *O.* cf. *ovata* on macroalgae compared to the official one, by taking a smaller amount of material.

Appendix B

Ostreopsis species distribution in the Mediterranean Sea

Appendix B. *Ostreopsis* species distribution in the Mediterranean Sea

Table A. *Ostreopsis* species distribution in the Mediterranean Sea.

Species	Bibliographic references	Locality	Country
Ostreopsis cf. *ovata*	Abbate et al., 2007	Ligurian Sea	Italy
Ostreopsis cf. *ovata*	Amany and Halim, 2011	Alexandria coastal water	Egypt
Ostreopsis cf. *ovata*	Barone et al., 2007	Sicily, Tyrrhenian Sea	Italy
Ostreopsis cf. *ovata*	Barone and Prisinzano. 2006	Aspra, Palermo (Tyrrhenian Sea)	Italy
Ostreopsis cf. *ovata*	Battocchi et al., 2009	Conero Riviera, Adriatic Sea Riviera; Taormina, Ionian Sea; San Felice Circeo Tyrrhenian Sea	Italy
Ostreopsis cf. *ovata*	Bianco et al., 2007	Latina, Tyrrhenian Sea	Italy
Ostreopsis cf. *ovata*	Bizsel and Aligizaki, 2011	Eastern Aegean Sea	Turkey
Ostreopsis cf. *ovata*	Bottalico et al., 2002	Southern Adriatic Sea	Italy
Ostreopsis cf. *ovata*	Cabrini in: ISPRA, 2011	Trieste, Northern Adriatic Sea	Italy
Ostreopsis cf. *ovata*	Caroppo et al., 2009	Ionian Sea	Italy
Ostreopsis cf. *ovata*	Ciminiello et al. 2006	Genoa, Ligurian Sea	Italy
Ostreopsis cf. *ovata*	Ciminiello et al. 2008	Genoa and La Spezia, Ligurian Sea	Italy
Ostreopsis cf. *ovata*	Cohu et al., 2011	Larvotto, Ligurian Sea	Monaco
Ostreopsis cf. *ovata*	David et al., 2013	Iberian Peninsula, Atlantic Ocean	Portugal
Ostreopsis cf. *ovata*	Di Turi et al., 2003	Southern Adriatic Sea	Italy
Ostreopsis cf. *ovata*	Gallitelli et al., 2005	Bari, Southern Adriatic Sea	Italy
Ostreopsis cf. *ovata*	Guerrini et al., 2010	Porto Romano, Tyrrhenian Sea; Numana, Adriatic Sea	Italy
Ostreopsis cf. *ovata*	Honsell et al., 2011	Trieste, Adriatic Sea	Italy
Ostreopsis cf. *ovata*	Ingarao and Pagliani, 2009	Pescara, Ortona and Francavilla, Adriatic Sea	Italy
Ostreopsis cf. *ovata*	Mangialajo et al., 2008	Genoa, Ligurian Sea	Italy
Ostreopsis cf. *ovata*	Mangialajo et al., 2011	North Western Mediterranean Sea	Italy
Ostreopsis cf. *ovata*	Masò et al., 2003	Balearic Sea	Spain
Ostreopsis cf. *ovata*	Monti et al., 2007	Trieste and Rovinj, Adriatic Sea	Italy, Croatia
Ostreopsis cf. *ovata*	Penna et al., 2005	Northwestern Mediterranean Sea	Italy, Spain
Ostreopsis cf. *ovata*	Penna et al., 2006	Gioia Tauro, Tyrrhenian Sea	Italy

Ostreopsis cf. *ovata*	Penna et al., 2010	Aegean Sea, Adriatic Sea, Tyrrhenian Sea, Tossa del Mar (Catalan Sea), Tunisia, Balearic Sea, Canary Islands	several countries
Ostreopsis cf. *ovata*	Penna et al., 2014	Portonovo, Trieste, Bari (Adriatic Sea); Taormina (Ionian Sea); Porto Romano, Marina di Massa, Marina di Pisa, Alghero (Tyrrhenian Sea); Alghero, La Spezia, Genova, Villefranche-sur-mer (Ligurian Sea); Llavaneres, Catalan Sea; Paguera, Balearic Sea; Athens, Aggelochori (Aegean Sea); Famara, Atlantic Ocean	several countries
Ostreopsis cf. *ovata*	Perini et al., 2011	Trieste, Northern Adriatic Sea; Genoa, Ligurian Sea; Livorno, Tyrrhenian Sea; Bari, Southern Adriatic Sea; Conero Riviera, Adriatic Sea	Italy
Ostreopsis cf. *ovata*	Pfannkuchen et al., 2012	Rovinj, Adriatic Sea	Croatia
Ostreopsis cf. *ovata*	Sansoni et al. 2003	Massa Carrara, Tyrrhenian Sea	Italy
Ostreopsis cf. *ovata*	Satta et al., 2014	Sardinian coasts	Italy
Ostreopsis cf. *ovata*	Scenati et al., 2011	Monte Argentario, Tyrrhenian Sea	Italy
Ostreopsis cf. *ovata*	N. Simon, pers. comm. in Mangialajo 2011	Sant Andreu de Llavaneres, Balearic Sea	Spain
Ostreopsis cf. *ovata*	Simoni et al., 2003	Marina di Massa and Livorno, Tyrrhenian Sea	Italy
Ostreopsis cf. *ovata*	Simoni et al., 2004	Marina di Massa, Versilia, Livorno, Elba, Giannutri, Giglio, Tyrrhenian Sea	Italy
Ostreopsis cf. *ovata*	Spatharis et al., 2009	Kalloni Gulf, Lesvos Island, Aegean Sea	Greece
Ostreopsis cf. *ovata*	Tognetto, 1995	Civitavecchia, Tyrrhenian Sea	Italy
Ostreopsis cf. *ovata*	Totti et al., 2010	Conero Riviera, Adriatic Sea	Italy

Appendix B. *Ostreopsis* species distribution in the Mediterranean Sea

Ostreopsis cf. *ovata*	Ungaro et al., 2005	Southern Adriatic Sea	Italy
Ostreopsis cf. *ovata*	Vila et al., 2001	Costa Brava, North Western Mediterranean Sea	Spain
Ostreopsis cf. *ovata*	Zingone et al., 2006	Gulf of Naples, Tyrrhenian Sea	Italy
Ostreopsis cf. *siamensis*	Abdennadher et al., 2011	Gulf of Gabes	Tunisia
Ostreopsis cf. *siamensis*	Abboud-Abi Saab and El-Bakht, 1997	Lebanese coastline	Lebanon
Ostreopsis cf. siamensis	Abboud-Abi Saab et al., 2013	Lebanese water	Lebanon
Ostreopsis cf. *siamensis*	Amorim et al., 2011	Atlantic Ocean	Portugal
Ostreopsis cf. *siamensis*	Armi et al., 2009	Tunisian lagoons	Tunisia
Ostreopsis cf. *siamensis*	Bennouna et al., 2011	Atlantic Ocean	Morocco
Ostreopsis cf. *siamensis*	David et al., 2013	Iberian Peninsula, Atlantic Ocean	Spain, France, Portugal
Ostreopsis cf. *siamensis*	Ennaffah et al., 2011	South Atlantic Ocean	Morocco
Ostreopsis cf. *siamensis*	Gangemi et al., 2006	Messina, Tyrrhenian Sea	Italy
Ostreopsis cf. *siamensis*	Hamza et al., 2011	Southern coasts of Tunisia, Mediterranean Sea	Tunisia
Ostreopsis cf. *siamensis*	Mabrouk et al., 2012a	Mahdia	Tunisia
Ostreopsis cf. *siamensis*	Penna et al., 2005	Ganzirri, Northwestern Mediterranean Sea; La Fosca, Catalan Sea; Almeria, Andalucian coasts	Italy, Spain, Algeria
Ostreopsis cf. *siamensis*	Penna et al., 2010	various locations	Mediterranean Sea
Ostreopsis cf. *siamensis*	Penna et al., 2014	Sines and Cascais, Atlantic Ocean	Portugal
Ostreopsis cf. *siamensis*	Turki et al., 2006	Gulf of Gabès and the lake of Bizerte	Tunisia
Ostreopsis cf. *siamensis*	Vila et al., 2001	Costa Brava, North Western Mediterranean Sea	Spain
Ostreopsis spp.	Aligizaki & Nikolaidis, 2006	Aegean Sea	Greece
Ostreopsis spp.	Bushati et al., 2012	Butrinti lagoon	Albania
Ostreopsis spp.	Mangialajo et al., 2011	Northwestern Mediterranean coast	Italy
Ostreopsis spp.	Riobò et al., 2006	Almeria, Paguera (Western Mediterranean Sea), Canary and Madeira Islands; Sousse (Tunisian coasts)	Spain, Italy, Tunisia

Appendix B. *Ostreopsis* species distribution in the Mediterranean Sea

Ostreopsis spp.	Taylor, 1979	Villefranche-sur-mer	France
Ostreopsis spp.	Touahria & Seridji, 2011	Bou Ismail Bay, Algerian basin	Algeria
Ostreopsis sp. 1	Giussani et al., 2014	Cyprus	Cyprus
Ostreopsis sp. 1	Penna et al., 2012	Canary Islands, Greece, Crete	Spain, Greece

Chapter 6

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