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Daniela Catania

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UNIVERSITÉ CÔTE D'AZUR - École Doctorale de Sciences
Fondamentales et Appliqués
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T H È S E de doctorat

Présentée en vue de l'obtention de grade de docteur en SCIENCES DE
L'ENVIRONNEMENT de L'UNIVERSITÉ CÔTE D'AZUR

par

DANIELA CATANIA

L'influence des macroalgues sur la prolifération et la régulation des
efflorescences du dinoflagellé benthique *Ostreopsis cf. ovata*

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The influence of macroalgae on the proliferation and regulation of
the benthic dinoflagellate *Ostreopsis* cf. *ovata* blooms

A dissertation submitted in satisfaction of the requirements for the degree Doctor of
Philosophy in Environmental Sciences

by

Daniela Catania

I dedicate this Ph.D first and foremost to my family, and to Valerie and Coco.

“Education is the most powerful weapon which you can use to change the world.”

-Nelson Mandela

In loving memory of Sammy, Nonna Suzanne and Nonno Elio.

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Résumé

Les proliférations de microalgues peuvent être nocives. L'augmentation récente de la fréquence et de l'étendue géographique des efflorescences de dinoflagellés benthiques toxiques comme *Ostreopsis* cf. *ovata* peut poser de réels problèmes de santé publique. La côte méditerranéenne Nord-Occidentale (lieu de cette étude) est l'une des nombreuses régions méditerranéennes où les proliférations d'algues nuisibles représentent une menace pour l'économie touristique. Dans les années à venir, une attention particulière devra être portée à la gestion et la prévision des proliférations de ces microalgues nuisibles benthiques et c'est dans ce contexte que cette étude a été menée.

Une étude bibliographique portant sur les proliférations d'*Ostreopsis* spp. indique un manque important de données en relation l'écologie d'*O.* cf. *ovata*, en particulier concernant les substrats biotiques, ainsi que les communautés les habitats benthiques et, par conséquent, les rôles éventuels que ceux-ci peuvent jouer en tant que stimulateurs majeurs des efflorescences d'*Ostreopsis* spp.

Durant les étés 2015 et 2016, des expériences *in situ* de courte durée ont été menées sur les récifs côtiers et ont été complétées par des expériences en laboratoire. Les résultats obtenus ont permis d'établir que quatre communautés distinctes de macroalgues abritent des abondances différentes d'*O.* cf. *ovata*. En particulier, les abondances les plus élevées ont été enregistrées dans des sites dominés par des structures communautaires peu complexes ; Turf (TUR) et Dictyotales (DIC), alors que les sites dominés par des communautés complexes de *Cystoseira* (CYS) n'ont montré aucune prolifération significative de microalgues. Ces résultats impliquent que les régions côtières dominées par les communautés composées de *Cystoseira* spp. pourraient potentiellement réduire les proliférations de *Ostreopsis* spp. Les résultats des expériences de terrain menées en 2016 ont montré une corrélation négative significative entre les abondances de *O.* cf. *ovata* et la biomasse des macroalgues au sein des communautés dominées par *Padina pavonica*. En outre, d'autres facteurs abiotiques, tels que les concentrations en nutriments et métaux traces, ne contribuent pas (ou peu) à expliquer la dynamique des populations de *O.* cf. *ovata*.

Les multiples facteurs de stress d'origine anthropique continueront à influencer le fonctionnement de l'écosystème marin. La compréhension de ces impacts et la façon dont ils influencent la dynamique des dinoflagellés benthiques toxiques est impérative pour prévoir, gérer et éventuellement réduire ces proliférations, à l'échelle de l'océan mondial.

Mots clés: *Ostreopsis* cf. *ovata*, HABs, substrats biotiques, communautés de macroalgues.

ABSTRACT

Algal blooms can be harmful. The global management and forecasting of benthic harmful algal blooms (BHABs) will be of increasing importance in the years ahead and that is what this study sets out to address. The increase over recent decades, in both frequency and geographical range, of the potentially harmful benthic dinoflagellate *Ostreopsis* cf. *ovata* can pose real problems for human health. The French Côte d'Azur, the location for this study, is just one of many Mediterranean areas where harmful algal blooms pose a potential economic threat to a tourist-based economy. A review of the existing literature on *Ostreopsis* spp. blooms shows a severe lack of information about the ecology of *O.* cf. *ovata* in relation to biotic substrates, communities and habitats and thus any possible roles these may play in fostering major *Ostreopsis* spp. blooms.

Through a series of *in situ* experiments on temperate reefs on the Côte d'Azur over the summers of 2015 and 2016 with follow-up experiments in the laboratory, this study establishes that four distinct macroalgal communities harbour different *O.* cf. *ovata* abundances. The results indicated that higher abundances were recorded in sites which were dominated by less complex community structures; Turf (TUR) and Dicyotales (DIC), while sites with *Cystoseira* spp. communities (CYS) present did not harbour significant microalgal blooms. These results imply that coastal regions with a dominance of *Cystoseira*-composed-communities could potentially be less prone to blooms or even inhibit *Ostreopsis* spp. proliferation. The results from the field experiments of 2016 showed a significantly negative correlation between *O.* cf. *ovata* abundances and macroalgal biomass, in macroalgal communities dominated by *Padina pavonica*. Although, no clear relationship was found between inorganic nutrient concentrations and *O.* cf. *ovata* abundances, it was observed that in the bloom onset period, nitrogen compounds in HAL and ROC were higher than in the rest of the study period (both in 2015 and 2016). Moreover, trace metal concentrations appear to not contribute to an important effect on local *O.* cf. *ovata* population dynamics.

Multiple human stressors will continue to impact marine vegetation, understanding these impacts and how they then influence bloom dynamics of toxic benthic dinoflagellates is imperative for the global forecasting, management and mitigation of BHABs.

Keywords: *Ostreopsis* cf. *ovata*, BHABs, biotic substrates, macroalgal communities.

LIST OF PUBLICATIONS AND COMMUNICATIONS

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POSTER COMMUNICATIONS

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CHAPTER 1

GENERAL INTRODUCTION

1.1 BACKGROUND

Phytoplankton blooms have been the subject of scientific and general interest for centuries. Blooms of microalgae are known to occasionally discolour aquatic systems red or brownish-red, commonly known as 'Red Tides'. Water discoloration in the lower Nile was noted in Biblical times (Exodus 7:20 – 21), and Charles Darwin recorded his microscopic observations of discoloured seawater during the voyage of the HMS Beagle:

“Some of the water placed in a glass was of a pale reddish tint and, examined under a microscope, was seen to swarm with minute animalculae darting about and often exploding. Their shape is oval and contracted in the middle by a ring of vibrating curved ciliae.” (The Voyage of the Beagle,: Galtsoff, 1949; Granéli and Turner, 2006).

These blooms are natural phenomena, and most are harmless, but some species of microalgae produce phycotoxins that can create various adverse effects in humans when in contact or ingested. When these phycotoxins bioaccumulate in the tissues of marine species that are consumed by humans, they may result in nausea, diarrhea, paralysis, amnesia, and even death. In cases where the aerosol is breathed in, effects include skin rashes, breathing difficulties, and/or fever.

Harmful algal blooms (HABs) refers to blooms of such harmful algae (HA), and their frequency, and the regions affected, are increasing worldwide (Rhodes, 2011). In recent years, increasing numbers of phycotoxin-producing algal species have been identified - currently 80 species, and new phycotoxins continue to be characterized chemically (Granéli and Turner, 2006; Tartaglione et al., 2016).

In addition to the deleterious impacts of phycotoxins, blooms also reduce light penetration into the water column, and the bloom decay can lead to low dissolved oxygen concentrations in subsurface waters. Both processes disrupt ecosystems and in some cases have completely destroyed benthic (seabed) communities (Hallegraeff, 1993; Anderson, 2009).

1.2 WHAT ARE BENTHIC HARMFUL ALGAL BLOOMS (BHABS)?

Of the known 5,000 named living phytoplankton species (Sournia et al., 1991), 2,000 are dinoflagellates; approximately 1,700 of these are marine and about 220 are freshwater (Taylor et al., 2008). Known HAB species comprise some 300 species that can cause water discolouration, and only some 80 species that produce phycotoxins that can cause human shellfish poisoning (Hallegraeff, 1995). Both planktonic and benthic dinoflagellates can produce HABs. Benthic dinoflagellate species occur in different types of habitats, including the interstitial spaces of marine sediments, on macrophyte surfaces (epiphytic), in tide pools.. (etc); on floating detritus; corals and pebbles. They usually grow in shallow and well-illuminated environments where nutrients are scarce or depleted (Tindall and Morton, 1998; Shears and Ross, 2009; Pistocchi et al., 2011). They are adapted to a benthic lifestyle in morphology, and behaviour (Hoppenrath et al., 2014). Most benthic dinoflagellates are small with dorso-ventrally flattened cells that may facilitate movement in interstitial habitats and easy attachment to surfaces (Hoppennath et al., 2014). Flattened cells may also increase the uptake of nutrients in oligotrophic conditions, as the surface area:volume ratio is higher than in spherical cells (Fraga et al., 2012). Blooms caused by benthic dinoflagellates, such as species from the genus *Ostreopsis*, are known as benthic harmful algal blooms (BHABs).

Different groups of BHABs in diverse oceanographic systems may be encouraged by varying forcing functions, including physical dynamics, climate change, nutrient loading, and other anthropogenic influences such as reductions or changes in the grazing community through fishing or aquaculture (Gilbert et al., 2005; Anderson, 2009; Spatharis et al., 2009).

1.3 IMPORTANCE OF STUDYING THE ECOLOGY OF BHABS

Much of the scientific interest of HABs has focused on planktonic microalgal species; less interest has been dedicated to benthic epiphytic toxic microalgae (Fraga et al., 2012). However, over the past four decades, there has been a rising interest in studying the ecology of BHABs, mainly due to the impact of ciguatera fish poisoning (CFP) from eating contaminated fish caught in tropical and subtropical waters (Litaker et al., 2010). CFP is

the leading non-bacterial illness associated with seafood consumption (WHO, 2009), with an estimation of 25,000 – 500,000 human intoxication cases per year (Parsons et al., 2012).

The economic effects of BHABs can thus be extensive, including losses due to closures of aquaculture farms and beaches (affecting recreational activities), tourism, and human health care. In Europe, such losses can amount to 862 million Euros, and in the USA 82 million dollars annually (Hoagland and Scatasta, 2006).

The study area of this thesis was based in the Provence-Alpes-Côte d’Azur (PACA) region. Several *Ostreopsis* spp. blooming events have been recorded along the French Ligurian Sea since 2008 (Mangialajo et al., 2011; Cohu et al., 2012, 2013). This region was selected because the economy of the French Riviera is strongly linked to the sea, through aquaculture, port-based trading, and tourism (Lemée et al., 2012). Tourists spend an estimated 11 billion Euros in the region?? each year, mainly during the summer. Major *Ostreopsis* spp. bloom events could result in closures of popular tourist beaches, affecting the local businesses. Even a slight drop in tourism could lead to a financial loss of tens of millions of Euros in the PACA region (Lemée et al., 2012).

1.4 OSTREOPSIS SPP.

Ostreopsis Schmidt is a genus of benthic dinoflagellate. There are currently eleven described species of *Ostreopsis*, five of which are known to be toxic; *O. cf. ovata*, *O. cf. siamensis*, *O. mascrensis*, *O. lenticularis* and *O. fattorussoi* (Yasumoto et al., 1987; Meunier et al., 1997; Lenoir et al., 2004; Scalco et al., 2012; Brissard et al., 2014, 2015; García-Altare et al., 2015; Accoroni et al., 2016).

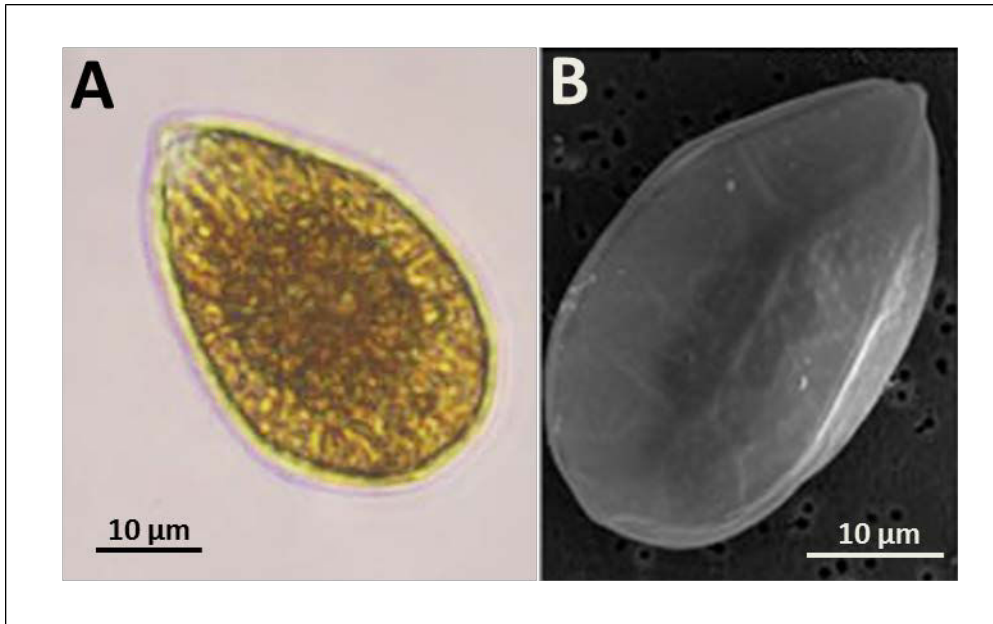


Figure 1.1. A) Light microscope image of *Ostreopsis cf. ovata* from live samples collected in the Ligurian Sea (Photo: D. Catania); B) Scanning electron micrograph of *Ostreopsis cf. siamensis* (Photo: M.Vila and J.M.Fortuño, ©ICM DIVULGA)

These species produce potent palytoxin-like compounds that include putative palytoxins and ovatoxins – a, b, c, d, e, f, l, j₁, j₂ and k (Uchida et al., 2013; Brissard et al., 2014; Tartaglione et al., 2016) and mascarenotoxins – a and c (Rossi et al., 2010; Scalco et al., 2012). Palytoxins are one of the most potent toxic marine compounds known (Usami et al., 1995; Ukena et al., 2001; Ciminiello et al., 2010; Tartaglione et al., 2016). The importance of studying *Ostreopsis* spp. has increased over the last 20 years due to its geographic expansion from (sub)tropical to temperate regions (Rhodes, 2011) and the proposed link between *Ostreopsis* spp. blooms and respiratory problems in the Mediterranean Sea shores (Vila and Masó, 2005; Mangialajo et al., 2008a; Illoul et al., 2012; Iddir-Ihaddaden et al., 2013). These toxins have been reported to cause human intoxications through inhalations in aerosol form and by contact (mainly skin irritations). Moreover, benthic communities comprised of bivalves, gastropods, cirripeds, echinoderms and fishes were reported to be affected by suffering or mass mortalities (Sansoni et al., 2003; Shears and Ross, 2009; Parsons et al., 2012). No human intoxication by the consumption of seafood has been reported (Tubaro et al., 2011), although *Ostreopsis cf. ovata* phycotoxins have been found in several marine organisms (Biré et al., 2013, 2015).

1.5 IMPORTANCE OF BIOTIC SUBSTRATES, COMMUNITIES AND ECOLOGICAL HABITAT ON THE PROLIFERATION OF *OSTREOPSIS SPP.* BLOOMS

Benthic dinoflagellates depend on a fixed substratum in order to proliferate, therefore any alterations in the benthic communities can directly or indirectly affect benthic dinoflagellate population dynamics (Fraga et al., 2012). Coastal development in Europe is among the major drivers of the loss of complex macroalgal beds, mainly due to the degradation of water quality (Airoldi and Beck 2007). In general, two of the primary controls of macroalgal abundances across all systems are the availability of nutrients, predominantly nitrogen and phosphorous, and the herbivory pressure (Fong and Paul, 2011; Clausing, 2014;). Human activity is altering nutrient content (Valiela et al., 1992; Vitousek et al., 1997; Tilman et al., 2001; Suding et al., 2005) and transforming the biodiversity, population dynamics and distribution of BHABS worldwide (Rhodes, 2011). The Ligurian Sea coastline of France, in the North-West Mediterranean Sea, is highly urbanised and consequently the composition of benthic communities is changing due to pollution, local eutrophication, and increased water turbidity (Mangialajo et al., 2008b). As a result of enhanced concentration of nutrients or rates of sediment deposition on urban coasts, complex macroalgal communities are shifted to habitats dominated by turf-forming algae (filamentous assemblages of algae <5 mm in height) in temperate regions (Gorgula and Connell, 2004). Some studies also suggest that nutrient enrichment on temperate reefs could increase the abundance and dominance of opportunistic macroalgal species instead of canopy-forming algae (Airoldi et al. 2005; Clausing, 2014).

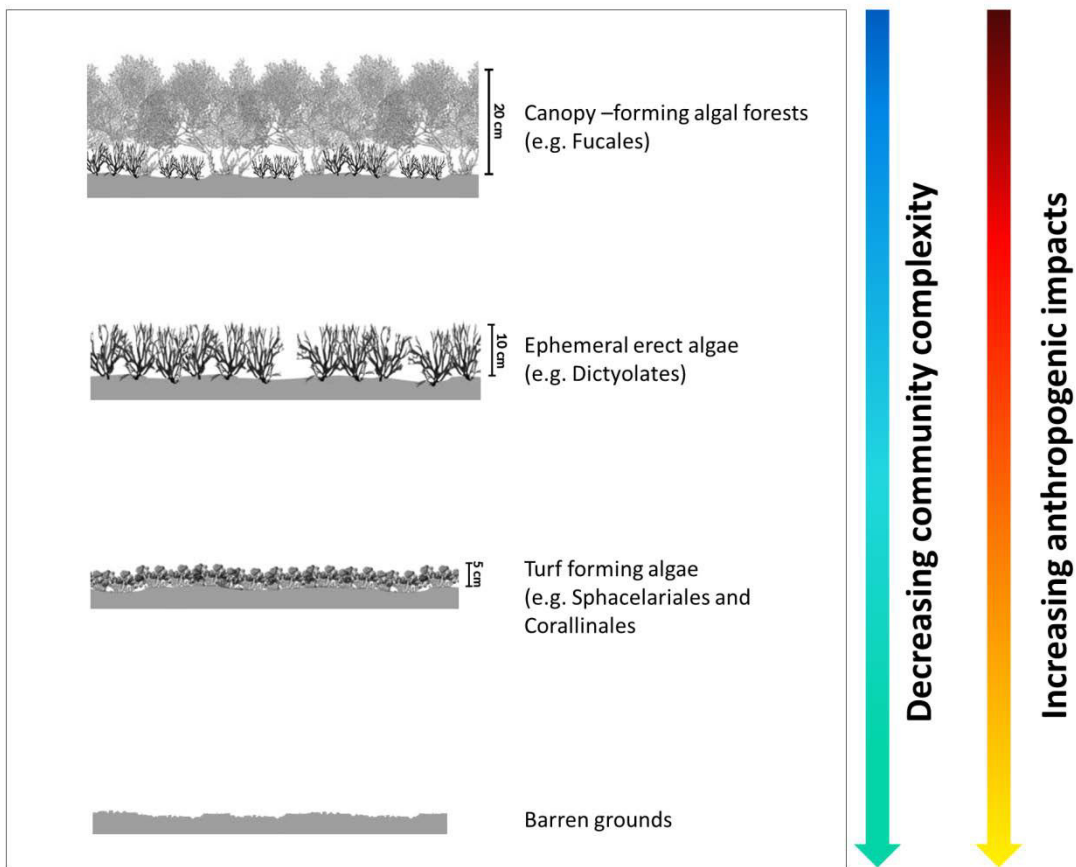


Figure 1.2. Schematic diagram to represent changes from complex canopy-forming algal forests to barren grounds along a gradient of anthropogenic impacts. (Modified from Thiriet, 2014).

Several studies in the Mediterranean have indicated the loss of canopy-forming species, such as *Cystoseira* and *Sargassum*, in urbanised areas (Thibaut et al. 2005, Arèvalo et al. 2007; Mangialajo et al., 2008a). Mangialajo et al. 2008 demonstrated that benthic understory assemblages were significantly affected by changes in the distribution of *Cystoseira* species along an urbanisation gradient. These results suggest that human activities that reduce water quality by affecting nutrient and sediment loads may be responsible for the major changes observed on urbanised coasts. However, information is lacking about the mechanisms that switch the primary sub-tidal habitat from canopy-forming algae to turf-forming algae on human-dominated coasts (Gorgula and Connell, 2004). In (sub)tropical coral reefs, the chronic impacts of overfishing and coastal pollution increased coral mortality and recruitment failure, have caused persistent shifts from the original dominance by scleractinian corals to a dominance of fleshy macroalgae or other

weeds (Done, 1992; Hughes, 1994; Scheffer et al., 2001; Hoegh-Guldberg et al., 2007; Hughes et al., 2010). A switch to macroalgal dominance constitutes a fundamental change in the benthic community structure of the reef (Done, 1992) and benthic dinoflagellate communities associated with the reef system (Clausing, 2014).

Benthic dinoflagellates in both shallow rocky shores in temperate regions and in coral reef environments go through unexpected and catastrophic macrophytobenthos phase shifts, which in turn can modify *Ostreopsis* spp. population dynamics. Thus, studies are needed that incorporate heterogeneity in the environments (e.g. stress, nutrient concentrations) while accounting the changes in *Ostreopsis* cf. *ovata* abundances that they cause, in order to understand how macroalgal modifications due to anthropogenic pressures control *Ostreopsis* spp. blooms.

1.6 AIMS OF THE THESIS

This thesis investigates how biotic substrate, particularly macroalgal community structure, influences *Ostreopsis* cf. *ovata* blooms in the Côte d'Azur. Moreover, the aim was to investigate how human stressors may indirectly impact *Ostreopsis* spp. blooms in the NW Mediterranean Sea. First, a bibliographic review was performed to summarise the general state of knowledge of the existing information on how specific macrobenthic biotic substrates, communities and/or marine habitats facilitate *Ostreopsis* spp. blooms. A systematic collation of these findings may contribute to the development of effective management policies to regulate *Ostreopsis* spp. blooms in the most-affected regions (Chapter 2).

Next, *in situ* field experiments were conducted in the summers of 2015 and 2016 on temperate intertidal reefs located between the coastal airport of Nice and the Port of Saint Jean Cap Ferrat, along the French Ligurian coastline in the south of France. In the summer of 2015 we conducted an observational field study on the different abundances of *O.* cf. *ovata* on different levels of macroalgal community complexities, manipulating macroalgal assemblages to artificially reduce the complexity. This was done to examine how different macroalgal community complexities, shaped by multiple human stressors, can influence the proliferation of *O.* cf. *ovata* blooms in the Côte d'Azur.

In the summer of 2016, we quantified the abundances of *O. cf. ovata* on different macroalgal communities dominated by specific macroalgal species, and endeavoured to identify a correlation between *O. cf. ovata* abundances and macroalgal biomass. During both sampling season (summers of 2015 and 2016), multiple environment and weather factors were measured at the sampling sites to be able to characterise the sites and to determine their effects on *O. cf. ovata* abundances across a spatial variability.

CHAPTER 2

**BIBLIOGRAPHIC REVIEW ON THE POTENTIAL INTERACTIONS BETWEEN
DINOFLAGELLATES OF THE GENUS *OSTREOPSIS* AND BENTHIC BIOTIC
SUBSTRATES, COMMUNITY ASSEMBLAGES AND HABITATS.**

2.1 ABSTRACT

Harmful Algal Blooms (HABs) have been increasing in frequency and intensity over the past two decades, with negative economic, ecological and human health impacts. Due to the challenges they pose to public health, understanding bloom dynamics of potentially harmful benthic dinoflagellates such as *Ostreopsis* spp. is becoming increasingly important. To improve understanding of *Ostreopsis* spp. bloom dynamics, this study investigated, through a review of the existing literature, the role of habitat and ecosystem characteristics in the facilitation/regulation of blooms, in particular potential biotic interactions. Quantitative assessment of Aquatic Sciences and Fisheries Abstracts (ASFA) and ISI Web of Science (WoS) databases demonstrated a sustained increase in publications on *Ostreopsis* spp. in international scientific journals from 1973 to 2016. *Ostreopsis* spp. has been expanding geographically in recent decades, especially in temperate regions, with 67 % of studies focused on these regions.

Analysis of the literature shows that 78 % of the relevant studies sampled benthic macroalgae from the field for subsequent work on *Ostreopsis* spp., showing the important role algae play in bloom dynamics. The most commonly observed biotic communities (50 % of 159 studies) are of erect shrub-like macroalgal (of heights ≤ 5 cm) assemblages in temperate rocky shores systems. This systematic review revealed that there is incomplete information concerning biotic community assemblages and ecosystems at sampling sites that support *Ostreopsis* spp. blooms: only 20 % of research articles provided a description of the macrobenthic habitat of the sampling site. Future studies should provide detailed surveys and descriptions of the surrounding community and habitat structure of the sampling sites. This would further our understanding of the interactions between *Ostreopsis* spp. bloom dynamics and benthic community structures and ecosystems. This information could assist with predictions of *Ostreopsis* spp. bloom development in coastal regions where this phenomenon is prevalent, as well as provide a basis for effective bloom management and mitigation.

2.2 INTRODUCTION

Over the last several decades, many countries throughout the world have experienced an accelerating trend of Harmful Algal Blooms (HABs) events (Anderson, 2009). At present, the majority of coastal areas are affected by HABs (Anderson, 2009; Parsons et al., 2012). A significant proportion of the scientific research in HABs has focused on planktonic microalgal species over HAB-forming benthic microalgae (Fraga et al., 2012). However, over the past few decades, interest in tropical benthic dinoflagellate ecology has increased, mainly due to the incidence of ciguatera fish poisoning (CFP) in tropical and subtropical regions (Litaker et al., 2010) and to the expansion of the geographic range of some species in temperate waters (Rhodes, 2011).

Over the last twenty years, frequent and intense *Ostreopsis* spp. blooms have been recorded throughout the Mediterranean Sea (Vila et al., 2001b; Abboud-Abi Saab et al., 2004; Penna et al., 2005; Turki, 2005; Vila and Masó, 2005; Aligizaki and Nikolaidis, 2006; Mangialajo et al., 2008a; Totti et al., 2010; Cochu et al., 2011; Ismael and Halim, 2012), Western Atlantic (Granéli et al., 2002; Nascimento et al., 2012), Sea of Japan (Selina and Levchenko, 2011), the South-West Pacific (Chang et al., 2000; Rhodes et al., 2000; Shears and Ross, 2009), and the North-West Pacific (Faust, 1999; Yamaguchi et al., 2012).

The importance of *Ostreopsis* spp. research in these regions has consequently increased due to the toxic nature of five out of the eleven identified species, and the resulting detrimental effects blooms have on coastal marine ecosystems and human health. *Ostreopsis* species are considered harmful as they produce palytoxin (PLTX), one of the most potent known toxic marine compounds (Usami et al., 1995; Ukena et al., 2001; Ciminiello et al., 2010; Suzuki et al., 2012), and its analogues. These toxic compounds can cause public health issues in temperate regions, PLTXs have been reported to cause human intoxication through inhalation and by dermal contact (Tichadou et al., 2010, Tubaro et al., 2011). However, data describing human intoxications are unreliable, particularly after oral exposure to contaminated seafood, due to the difficulty of performing analyses to confirm and quantify the toxins in leftover food or in clinical samples. This issue of uncertainty of PLTX toxicity was recently highlighted in temperate coastal waters in connection with *Ostreopsis* spp. blooms (Gallitelli et al., 2005; Durando

et al., 2007; Tubaro et al., 2011), where no reports of direct human intoxication after seafood consumption have been identified. Ecologically, benthic *Ostreopsis* spp. blooms may, in addition, harm bivalve, gastropod, cirriped, and echinoderm fecundity and communities, even causing mass mortalities (Sansoni et al., 2003; Shears and Ross, 2009; Parsons et al., 2012; Migliaccio et al., 2016).

In recent years, there has been an increase in interest in predicting the occurrence of BHABs through better understanding of preferred biotic substrates. Cell abundances of epiphytic benthic dinoflagellates have been investigated on various substrata, in both tropical and temperate regions. *Ostreopsis* species are usually described as epiphytic on macroalgae and seagrasses (Rhodes, 2001), but can also be found on dead coral, sediments, rocks, and in the water column (Bomber et al., 1989; Vila et al., 2001; Shears and Ross, 2009, 2010; Totti et al., 2010). Some studies suggest that living substrates harbour lower cell abundances of *Ostreopsis* spp. as secondary metabolites are produced by macrophytes, whereas abiotic hard substrata have no defence mechanisms and thus do not inhibit cellular growth (Cruz-Rivera and Villareal, 2006; Totti et al., 2010).

No study has yet compared abundances of *Ostreopsis* spp. between biotic and abiotic substrates or explored how macrophyte morphology and defence strategies may alter benthic dinoflagellate populations in general (Cruz-Rivera and Villareal, 2006). Several studies have, however, examined the role of substrate in bloom dynamics of *Ostreopsis* spp. populations (Aligizaki and Nikolaidis, 2006; Accoroni et al., 2011, 2016). *Ostreopsis* spp. appears to display algal host preference, similar to the benthic dinoflagellate *Gambierdiscus* (Cruz-Rivera and Villareal, 2006; Parsons et al., 2012). In Kiribati waters, *Ostreopsis siamensis* was found on the macroalgae *Hypnea* but not on *Padina*, *Caulerpa*, *Enteromorpha*, *Liagora* and *Lomentaria* (Tebano, 1984; Parsons et al., 2012). Similarly, *O. cf. ovata* in the Aegean Sea and northern Adriatic was most often found on macroalgae from the phyla Phaeophyceae and Rhodophyceae (Aligizaki and Nikolaidis, 2006; Monti et al., 2007). One paper from Tasmania reported that *O. siamensis* was common on seagrasses (Pearce et al., 2001). All of which suggest that *Ostreopsis* spp. shows selectivity in regards to hosts. However, a study in the Balearic Sea of the North-West Mediterranean did not document any host preference for *O. cf. ovata* (Vila et al., 2001b).

Biotic substrates such as macroalgae and seagrasses employ a variety of defence mechanisms, both chemical and structural, but no definitive trends have yet been demonstrated in the literature regarding their influence on *Ostreopsis* spp. growth. The eleven described *Ostreopsis* species may exhibit different host preferences, which it is thought are a function of surface area, morphology, or secondary metabolites produced by the macrophyte; further studies are needed to determine which of these variables are determining factors (Parsons et al., 2012). A small number of studies have emphasised the importance of the architecture of the macrophyte host thallus (Lobel et al., 1988, Totti et al., 2010). However, there is no evidence of the allelopathic or physical relationships between *Ostreopsis* spp., its biotic substrates, and community structures, and their effects on bloom dynamics.

Little is known about how benthic community structure influences the growth of *Ostreopsis* spp. Studies have reported that CFP episodes recurrently follow disturbances to coral reefs by natural and artificial events such as hurricanes, dredging, and shipwrecks (Bagnis, 1994; de Sylva, 1994; Rains and Parsons, 2015). Similarly, natural and anthropogenic disturbances may contribute to the risk of *Ostreopsis* spp. bloom outbreaks by increasing the amount of benthic substrate (e.g bare rock or dead coral) available to harbour epiphytic dinoflagellates (Hallegraeff, 1993; Cruz-Rivera and Villareal, 2006).

There is a reasonable body of research focused on the effects of nutrients, and salinity and seawater temperature on *Ostreopsis* spp. populations (reviewed in Parsons et al., 2012; Accoroni and Totti, 2016), however, the role of ecological habitats in the facilitation of blooms has rarely been considered. The complex habitats in which *Ostreopsis* spp. blooms occur, coupled with the variation in community composition and distribution of biotic substrates, make it challenging to understand the bloom dynamics of this species. It has been suggested that benthic community assemblages influence *Ostreopsis* spp. populations. Efficiently regulating coastal benthic blooms will be helped if it can be established which of these, and in what combination, facilitate *Ostreopsis* spp. blooms.

To ascertain whether different benthic substrates, communities and/or ecosystems facilitate the proliferation of *Ostreopsis* spp. blooms, a literature review was conducted examining; 1) whether general relationships can be made linking *Ostreopsis* spp.

abundances to characteristics of benthic substrate and/or community assemblages and 2) where habitats in which *Ostreopsis* spp. is present have been described in detail, how these systems could influence the bloom dynamics. The goal is to evaluate the utility of a more comprehensive approach, which considers the characteristics of benthic communities and the ecological habitat when monitoring and managing coastal regions where benthic HABs are prevalent. These findings could contribute to the development of effective management policies to regulate *Ostreopsis* spp. blooms in regions most affected, both in potential health risks to marine life as well as to humans. Furthermore, these findings will provide a clear picture of how the status of research on *Ostreopsis* spp. and the association with the benthos has evolved over five decades, identifying the strengths and gaps in knowledge of the subject.

2.3 MATERIALS AND METHODS

A bibliographic review assessment was performed in November 2016 using two databases: the Aquatic Science and Fisheries Abstracts (ASFA) published by Cambridge Scientific and the Web of Science (WoS) published by Thomson Reuters ISI. Two research engines were used to avoid bias and to ensure that as many scientific publications as possible were included. Search parameters were kept as wide as possible to ensure that all the relevant publications on the topic were found in the database search. The keyword used for the ASFA and WoS search was "*Ostreopsis*" and the parameters of the search were to find the word "*Ostreopsis*" 'everywhere' in the body or text of the publications, and between anytime and 2016. Both ASFA and WoS include scientific articles, reports, articles in conference proceedings, dissertations and books, but grey literature and reports or articles with restricted distribution may have not been highlighted by the search engines and thus were not included in subsequent analyses.

To quantify and compare the relationship between benthic substrate type and *Ostreopsis* spp., only articles involving field collection of *Ostreopsis* spp. were included in this review. The key information extracted from each article included, when available: first author; title of publication; year of publication; name of journal; ocean/sea of study; country of study; temperate/tropical region; description of sampling site; description of water motion (sheltered or exposed to wind and waves); substrates sampled (e.g. macrophytes,

rocks, shells, artificial substrates, others); species sampled for biotic substrates (e.g. *Dictyota dichotoma*, *Cystoseira amentacea* var. *stricta*, *Halopteris scoparia*), benthic community (i.e. if the study mentions the species composing the community); and ecological habitat of the sampling site (e.g. seagrass meadows, coral reefs, temperate reef).

Tropical regions were defined as areas between the Tropics of Cancer and Capricorn and temperate regions as those between the tropics and polar regions. A small number of the studies were conducted in two or more separate regions around the globe, including two or more seas/oceans or two or more countries. Therefore, it is possible that one article was counted twice or more during the subsequent analyses.

2.4 RESULTS AND DISCUSSION

2.4.1 Bibliometric study

At the time of analysis, the ASFA database included 268 publications spanning 1973 to 2016, and the WoS database had 312 references from 1982 to 2016. In total 580 scientific publications were identified. The bibliometric study clearly shows a sustained increase in scientific publications in national and international journals that correlates with increasing HABs incidences in recent decades (Anderson, 2009). Figure 2.1 demonstrates a rapid increase in publications after 2006; this is potentially due to significant *Ostreopsis* spp. blooming events in temperate regions such as in the Mediterranean Sea (e.g., Tognetto et al., 1995). There was a peak of *Ostreopsis* spp. publications in 2012, however this is likely to be strongly influenced by the publication of 18 articles in the Cryptogamie Algologie special issue, published after the International Congress on *Ostreopsis* Development (ICOD conference) of 2011 in France (Lemée et al., 2012).

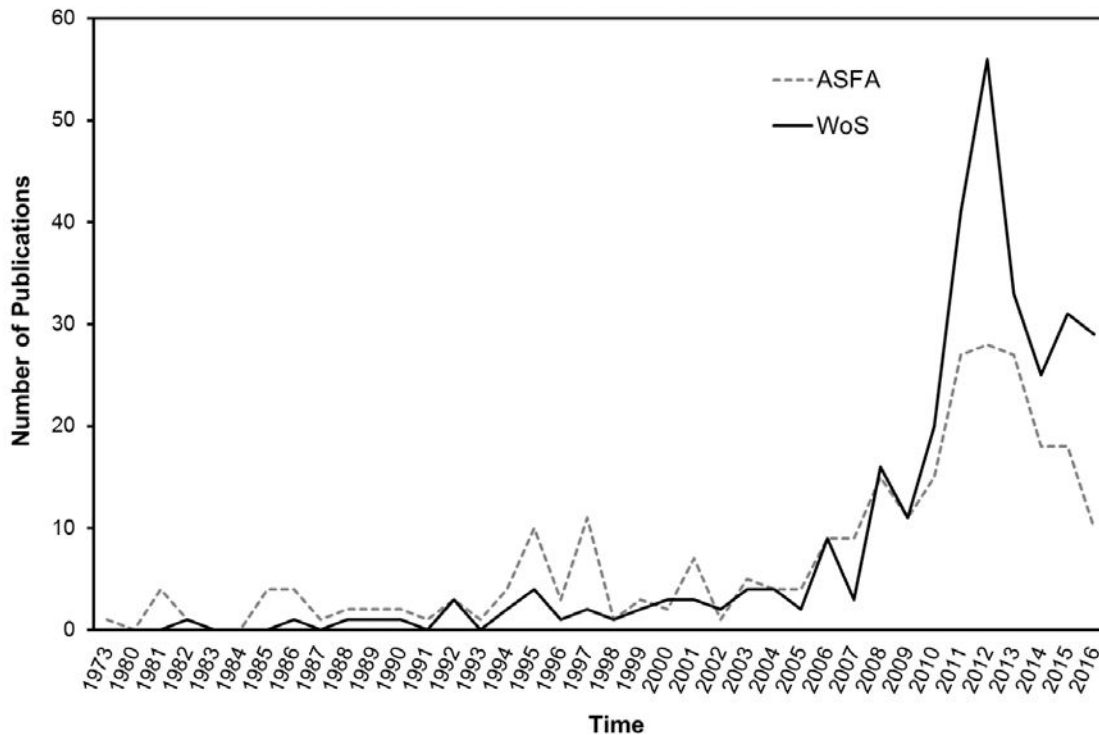


Figure 2.1. Number of publications concerning *Ostreopsis* spp. recovered from the Aquatic Science and Fisheries Abstracts (ASFA; dotted grey line) and Web of Science (WoS; solid black line) databases.

Journal articles represent 51 % of the 580 scientific publications extracted from the ASFA and WoS databases. The research also yielded 233 conference proceedings, 24 books or chapters of books, 9 reports, 5 dissertations, 5 workshops, 4 newsletters, 3 editorial materials, 2 letters and 1 note (Fig. 2.2). The search of the WoS identified more journal articles (165 vs. 129) and conference proceedings (128 vs. 105) than ASFA. On the other hand, the ASFA search produced more monographs (books or book chapters and dissertations) than WoS (Fig. 2.2). This disparity in search results highlights the advantage of using multiple databases, increasing the chances of all available *Ostreopsis* spp. scientific publications being included in this study. The resulting journal articles obtained from ASFA and WoS were combined and scrutinized to eliminate duplicate references. The combined database comprised 51 relevant journal articles from the ASFA and 108 from WoS; a total of 159.

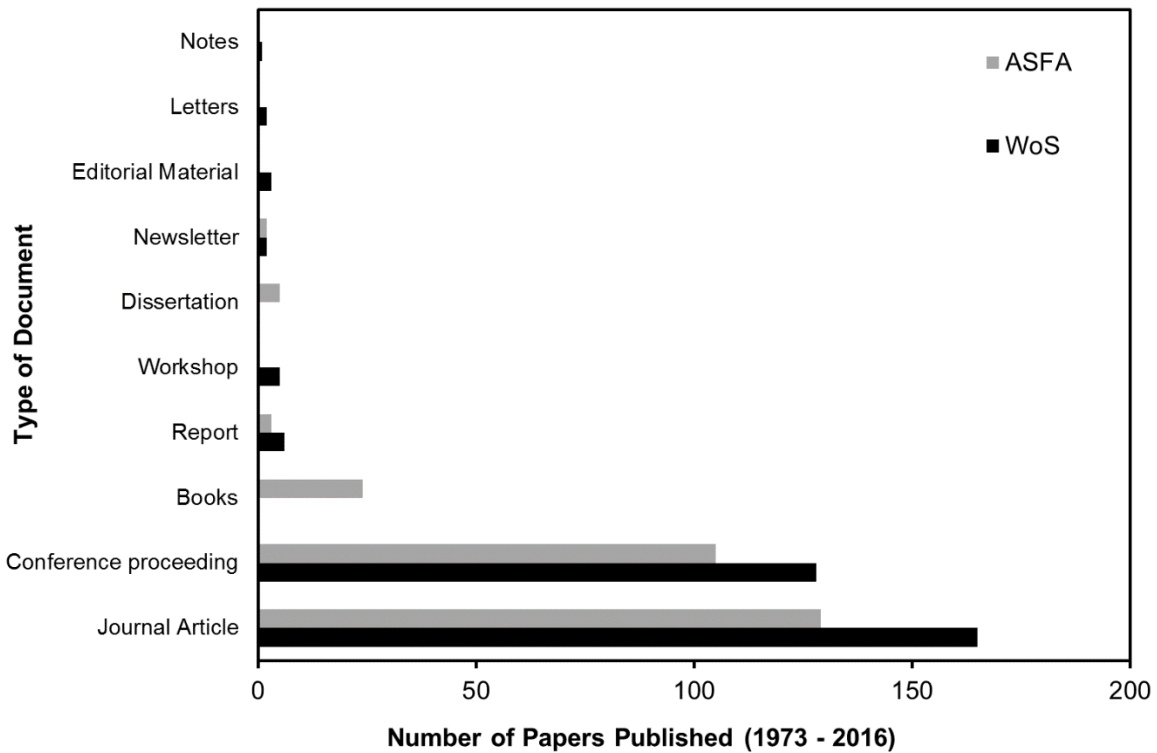


Figure. 2.2. Number of publications concerning *Ostreopsis* spp. published between 1973 and 2016 grouped per type of source document (total n = 294).

The 159 research articles pertaining to *Ostreopsis* spp. published in scientific journals are distributed among 67 international journals. Table 2.1 lists the top 10 journals publishing articles on *Ostreopsis* spp. Harmful Algae has printed the most (18 %) of all *Ostreopsis* spp. publications (Table 2.1). Cryptogamie Algologie (8 %), Biologia Marina Mediterranea (6 %) and Toxicon (6 %), are also major outlets of research on *Ostreopsis* spp.

Table 2.1. The top 10 most active journals publishing articles on *Ostreopsis* spp. during the period of 1973–2016. The table shows the total number of publications, percentage (%) of 159 journal articles, impact factor (IF) and Web of Science subject category of journals.

Journal Title	Total Number of Publications	% Out of 159	IF (2015/2016)	Subject Category
Harmful Algae	28	18	3.874	Marine and Freshwater Biology
Cryptogamie Algologie	12	8	1.487	Plant Sciences/Marine and Freshwater Biology
Biologia Marina Mediterranea	9	6	0.980	Marine Biology
Toxicon	9	6	2.309	Pharmacology and Pharmacy
Marine Pollution Bulletin	7	4	3.099	Environmental Sciences/Marine and Freshwater Biology
Journal of Phycology	6	4	2.536	Plant Sciences/Marine and Freshwater Biology
Cahiers de Biologie Marine	5	3	0.680	Marine and Freshwater Biology
Botanica Marina	4	3	1.402	Plant Sciences/Marine and Freshwater Biology
Environmental Science and Technology	4	3	5.393	Engineering, Environmental
Journal of Experimental Marine Biology and Ecology	3	2	1.866	Marine Biochemistry, Physiology, Genetics and Ecology

2.4.2. Geographic region of studies

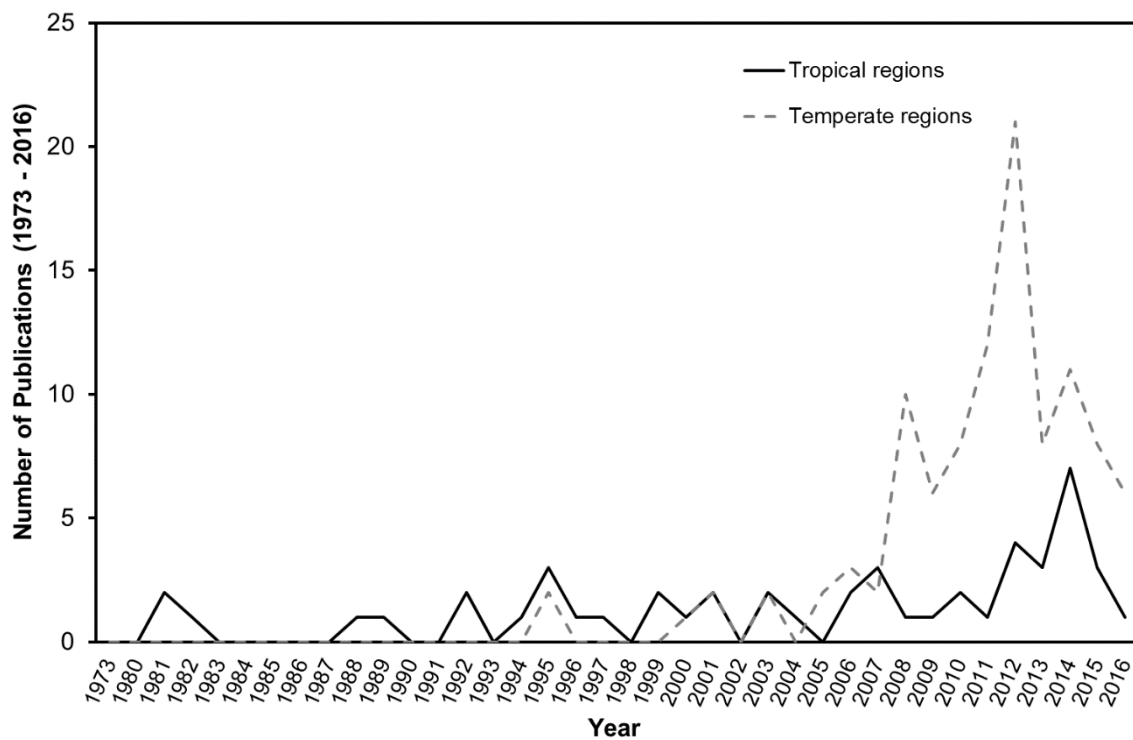


Figure 2.3. Number of publications concerning *Ostreopsis* spp. originating from tropical (solid black line) and from temperate (dotted grey line) regions during the period 1973 to 2016.

The majority of the publications from the 1980s to the early 2000s originated from tropical regions, whereas in recent years most publications have come from temperate regions (Fig. 2.3). Overall, in the last 20 years, 106 studies (67 %) were performed in temperate regions compared to tropical regions with 53 studies (33 %). Figure 2.4 reports the number of scientific studies based on *Ostreopsis* spp. that were conducted in different water basins around the world. The highest number of these studies was conducted in the Mediterranean Sea (52 %) and the lowest number of studies was performed in enclosed coastal lakes (Turki et al., 2006; Verma et al., 2016) with only 1 % reported research projects (Fig. 2.4).

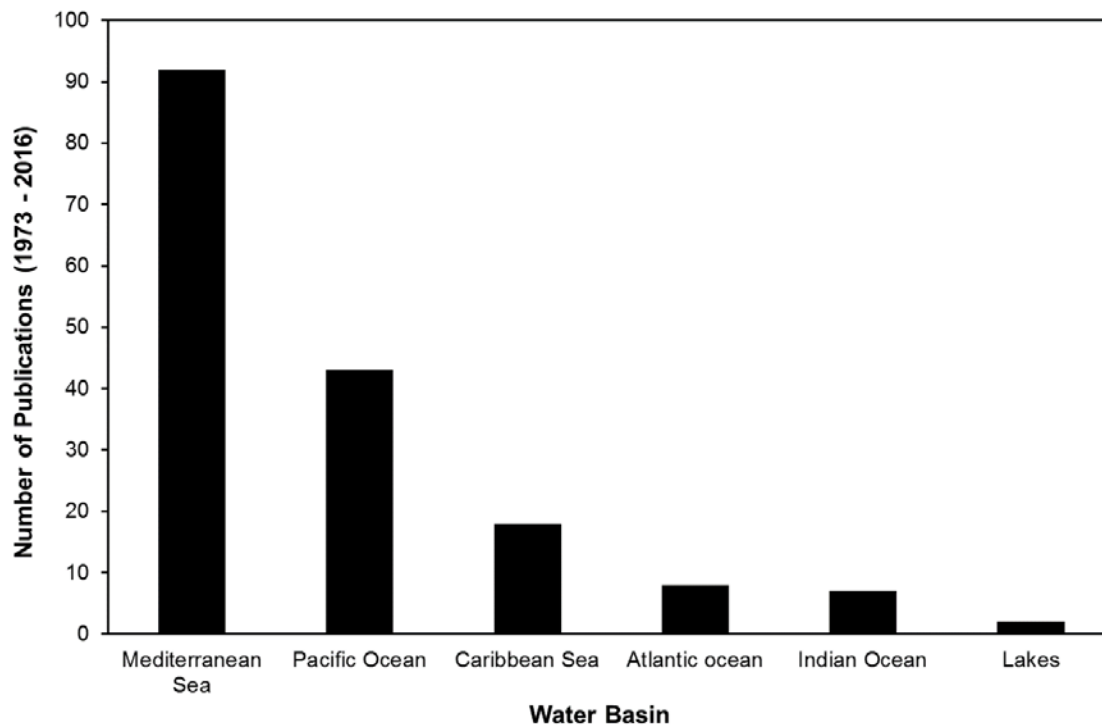


Figure 2.4. Number of publications concerning *Ostreopsis* spp. (from the 159 relevant articles) originating from different water basins around the world between 1973 and 2016. Locations were extracted from the ASFA and WoS databases. Note that some publications investigated two or more basins in the same study.

Because it occurs in both tropical and temperate regions and in the major water basins around the world, *Ostreopsis* spp. has become a global topic of interest and is researched in 44 different countries. Seventy percent of the relevant scientific articles originate from just five countries (based on the affiliation of the first authors): Italy (59 articles out of 159; 37 %), France (20 articles; 13 %), Spain (15 articles; 9 %), Belize (9 articles; 6 %) and Japan (9 articles; 6 %) (Fig. 2.5). With the first described bloom in 1994 near Naples and the first human intoxications caused by an *Ostreopsis* spp. bloom in Genoa, Italy has been a focal point of *Ostreopsis* spp. research (Tognetto et al., 1995; Brescianini et al., 2006).

Three of the five main countries where research on *Ostreopsis* spp. has been published has Mediterranean coastline, indicating strong efforts to study the ecology of *Ostreopsis* spp. in this Sea (114 out of 159; 72 %). In the Mediterranean Sea, annual *Ostreopsis* spp. blooms occur along the Catalan, Southern French, Monegasque and Italian coastlines during the summer months (Battocchi et al., 2010; Cohu et al., 2011; Mangialajo et al., 2011; Accoroni and Totti, 2016; Vila et al., 2016). Studies on

Ostreopsis spp. have also been performed in countries along the southern and eastern Mediterranean coast where blooms occur, such as Algeria, Tunisia, Egypt, and Lebanon (Turki, 2005; Ben Brahim et al., 2010; Illoul et al., 2012; Ismael and Halim, 2012; Mabrouk et al., 2014; Accoroni et al., 2016).

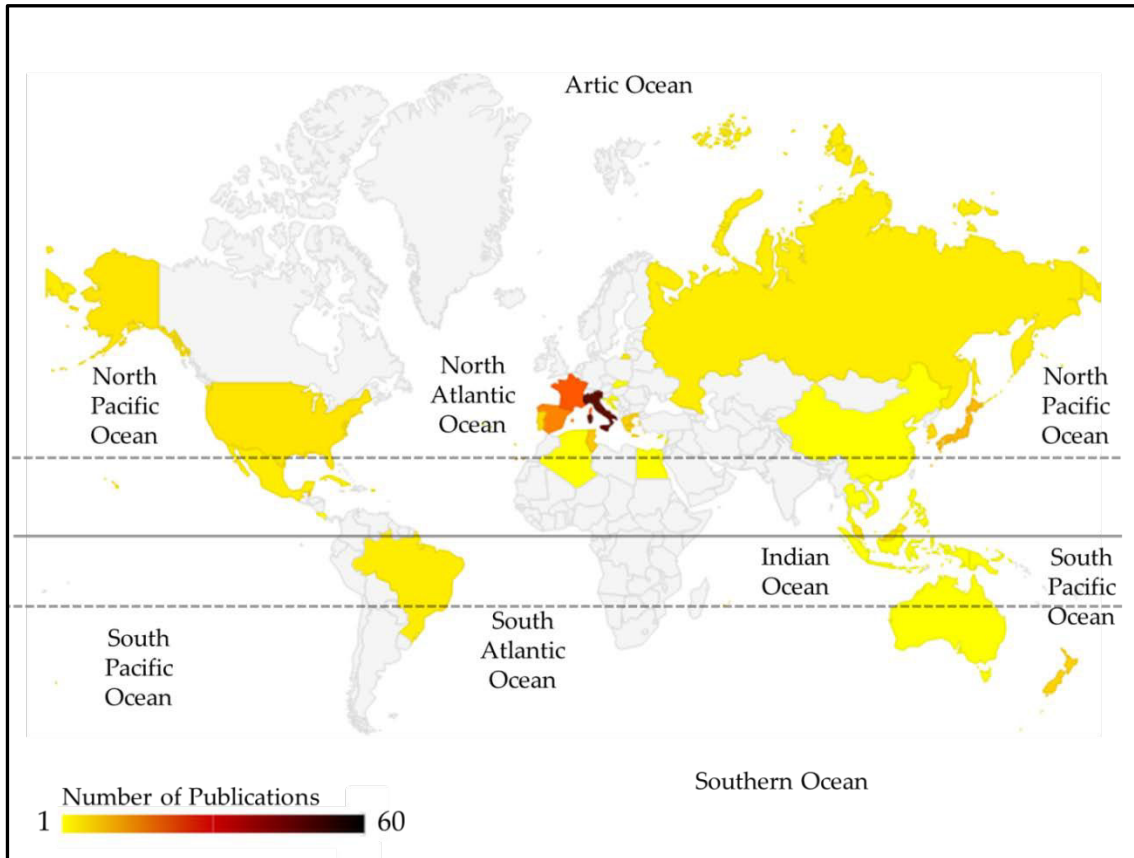


Figure 2.5. Geographical distribution of the study sites of the 159 relevant articles published from 1973 to 2016. Locations were extracted from the ASFA and WoS databases. Note that some papers investigated sites located in two regions or more. The coloured gradient scale represents the number of publications produced by the country of research.

The prevalence of *Ostreopsis* spp. studies along the NW Mediterranean coast is mostly the product of collaborations of several research laboratories in Spain, France and Italy. These collaborations have been instigated in order to gain a better understanding of *Ostreopsis* spp. blooms and to set up an efficient monitoring program during the bloom seasons in the region (Lemée et al., 2012). As an example, in the framework of an international agreement between Spain, Italy, Monaco and France known as the Accord RAMOGE, regular meetings are held during the year with microphytobenthic marine ecologists based in these countries in order to standardise

the sampling protocols and share their results (for more information: <http://www.ramoge.org>).

2.4.3 Macrobenthic substrates, communities and habitats/ecosystems

All the relevant research articles provided information on the substrate sampled. A number of different biotic and abiotic substrates were collected from coastal waters to estimate *Ostreopsis* spp. cell abundances (Fig. 2.6). Of the 159 articles, 92 % collected biotic substrates and 16 % collected exclusively abiotic substrates. The most ubiquitous biotic substrates to be sampled were macroalgae (124 studies; 78 %) and seagrasses (17 studies; 11 %). Abiotic substrates collected were sand/sediment (14 studies; 9 %) and pebble/small rocks (7 studies; 4 %) (Fig. 2.6). The majority of the studies in both temperate and tropical regions sampled macroalgae as the substrate, identified the algae to species level, and collected samples in intertidal zones. The remaining 54 studies either identified algae to the genera level or did not detail the macroalgae that were sampled. Some studies (Totti et al., 2010, Accoroni et al., 2011) have reported that *Ostreopsis* spp. are benthic organisms that are not obligate epiphytes, indicating that, *Ostreopsis* may not be restricted to a particular substrate. However, it must to be noted that these results refer to a single area in the Mediterranean Sea (NW Adriatic Sea) and cannot be applied at a global scale. The preference of *Ostreopsis* spp. for certain substrates could be species, strain or location specific. Moreover, *Ostreopsis* spp. has also been observed in the surrounding water (Turki, 2005; Spatharis et al., 2009; Ciminiello et al., 2010; Cochu et al., 2011, 2013), which reveals that while *Ostreopsis* spp. displays close preferences for some macroalgae in some studies, it may be found free-living in certain conditions or life stages.

Quantification of *Ostreopsis* spp. cell abundances in the tropics has mostly been done on fragments of dead coral; in temperate regions, non-living objects such as small rocks, pebbles and dead *Mytilus* spp. and *Patella* spp. shells have been sampled.

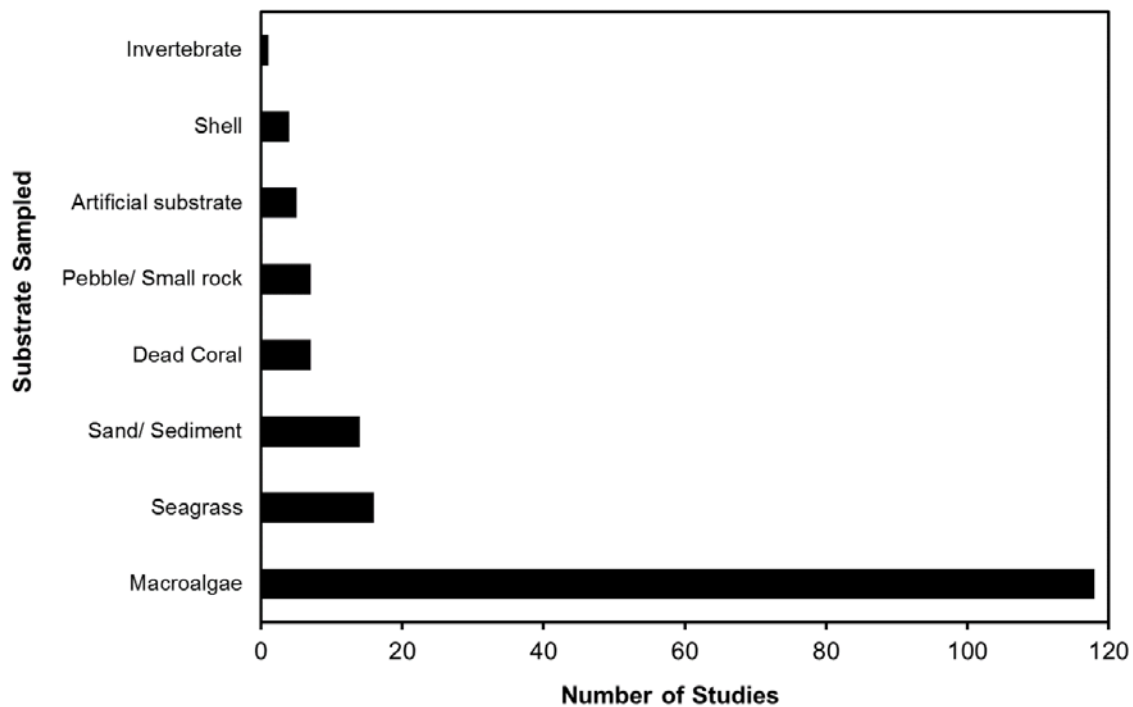


Figure 2.6. Different biotic and abiotic substrates sampled to quantify *Ostreopsis* spp. cell abundances during experimental field studies from 1973 – 2016.

Out of the 159 relevant articles, 74 % (that is 118 articles) mentioned the collection of macroalgal samples; of these, 53 % studies sampled only macroalgae as a substrate and 56 % of these identified the macroalgal samples at the species level. These studies have provided valuable insight into the diversity of algal hosts where *Ostreopsis* spp. is found, with 189 different macroalgal species yielding cell densities ranging from 2 to 8.54×10^6 cells g^{-1} FW macroalgae (Tawong et al., 2014 and Coahu et al., 2013, respectively). Forty four percent of the studies identifying the algae sampled (to species or genera level) also reported the maximum *Ostreopsis* spp. cell density (Table 2.2). There is a need for studies that provide more information on the macroalgae sampled in order to enable easier analysis of host preferences and to identify any biotic interactions between *Ostreopsis* spp. cells and their biotic substrate. The cell abundances from the different macroalgal species listed in Table 2.2 are not comparable, as cell abundances depend on the specific weight and morphology of the different macrophyte host species (Mangialajo et al., 2017).

The second most regularly sampled biotic substrates for *Ostreopsis* spp. cells were seagrasses (such as *Posidonia oceanica* and *Thalassia testudinum*) with 17 studies, and

the third were corals (such as *Acropora cervicornis* and *Montastrea annularis*) with seven studies. Results from this literature study highlights the fact that most data available on benthic substrates are from studies focusing on *Ostreopsis* spp. macrophyte assemblages and on the stock of cells that are attached to biotic substrates. Other substrates (sediments/sand, shells, pebbles/rocks and artificial substrates) accounted for only 16 % of the 159 studies.

Table 2.2. Maximum cell densities of *Ostreopsis* spp. (cells g⁻¹ FW macrophyte) on different recorded macrophyte hosts which were specified in the studies.

Macrophyte host genus	Maximum <i>Ostreopsis</i> density (cells g ⁻¹ FW)	Country of Study	Reference
Chlorophyta			
<i>Cladophora</i>	120	South Korea	Shah et al., 2013
<i>Cladophora</i>	160,000	Italy	Battocchi et al., 2010
Phaeophyta			
<i>Carpophyllum</i>	1,100	New Zealand	Chang et al., 2000
<i>Carpophyllum</i>	1,400,000	New Zealand	Shears and Ross, 2009
<i>Corallina</i>	79,000	Algeria	Illoul et al., 2012
<i>Corallina</i>	760,000	Italy, Spain, Greece	Casabianca et al., 2014
<i>Cystoseira</i>	334,306	Croatia	Pfannkuchen et al., 2012
<i>Dictyopteris</i>	150,000	Italy	Abbate et al., 2012
<i>Dictyopteris</i>	1,300,000	Italy	Accoroni et al., 2012
<i>Dictyota</i>	43,359	Puerto Rico	Ballentine, 1988
<i>Dictyota</i>	57,000	British and US Virgin Islands	Kohler and Kohler, 1992
<i>Dictyota</i>	79,000	Cuba	Moreira et al., 2012
<i>Dictyota</i>	330,333	Italy	Cabrini et al., 2010
<i>Dictyota</i>	8,540,000	France	Cohu et al., 2013
<i>Halopteris</i>	596,000	Italy	Vila et al., 2001
<i>Halopteris</i>	658,448	France	Guidi-Guilvard et al., 2012
<i>Halopteris</i>	1,570,000	France	Cohu et al., 2011
<i>Halopteris</i>	3,700,000	France	Brissard et al., 2014
Rhodophyta			
<i>Heterosiphonia</i>	1,479	U.S.A	Bomber et al., 1989
<i>Hypnea</i>	9,030	Tunisia	Ismael and Halim 2012
<i>Hypnea</i>	1,700,000	Italy	Totti et al., 2010
<i>Jania</i>	4,300	France	Cohu et al., 2013
<i>Jania</i>	1,500,000	Spain	Carnicer et al., 2015
<i>Laurencia</i>	99,000	Brazil	Nascimento et al., 2012
<i>Martensia</i>	8,660	South Korea	Kim et al., 2011
<i>Padina</i>	1,388	U.S.A	Bomber et al., 1989
<i>Pterocladia</i>	18,194	U.S.A	Parsons and Preskitt, 2007
<i>Pterocladia</i>	545,000	Italy	Ciminiello et al., 2014
<i>Turbinaria</i>	>100	Mayotte Island	Grzebyk et al., 1994
Phanerogams			
<i>Posidonia</i>	360,000	Tunisia	Turki, 2005
<i>Thalassia</i>	1,464	Belize, Malaysia	Tester et al., 2014

The highest *Ostreopsis* spp. cell abundance was recorded on the macroalgae *Dictyota*, with 8.54×10^6 cells g⁻¹ FW (Table 2.2; Cohu et al., 2013). Monitoring surveys and studies on dinoflagellate bloom dynamics predominantly collect visually obvious macroalgae as their sampling substrate as it is assumed that these algae are a readily available source of dinoflagellate densities (Cruz-Rivera and Villareal, 2006). Moreover, most studies conducted in coral reefs select macroalgae rather than dead coral fragments. This would suggest that researchers believe there are greater abundances of *Ostreopsis* spp. on macroalgae rather than on coral. Alternative methods have also

been developed and used to quantify the occurrence of benthic dinoflagellates and either express the cell concentration per volume of seawater or per surface area of substrate or seafloor (Tester et al., 2014; Jauzein et al., 2016; Mangialajo et al., 2017). The use of artificial substrates is a more recent methodology for the collection of epiphytic dinoflagellate cells and could be the new alternative to macroalgal samples as they are less ecologically destructive. Furthermore, artificial substrates standardises collection methods for benthic toxic dinoflagellates and allows for the comparison of cell abundances in time and space with other studies (Tester et al., 2014; Jauzein et al., 2016). Twenty eight percent of the articles (44 out of 159 studies) provided a description of the dominant macrobenthic community, of which 30 % were from rocky shore systems. The scarcity of information offered by research scientists monitoring *Ostreopsis* spp. cell abundances and about the biotic community structures present at their preferred sampling site is demonstrated by the fact that, of the 159 studies, only 44 (28 %) provided a description of the dominant macrobenthic community. Of these, 13 were from rocky shore systems. The information obtained from the literature can group the communities into four functional assemblages; (1) canopy-forming macrophytes (predominately *Cystoseira* and *Sargassum*), (2) erect shrub-like macroalgae (with a height ≤ 10 cm), (3) turf forming algae (including Ceramiales and Corallines) and (4) seagrass meadows (marine angiosperms).

Nine of the field studies described the dominant community to be composed predominantly by canopy-forming algae: four in the tropics (Mohammad-Noor et al., 2007, 2004; Widiarti et al., 2008; Kohli et al., 2014) and five in temperate regions (e.g. Chang et al., 2000; Shears and Ross, 2010, 2009; Ciminiello et al., 2010; Pfannkuchen et al., 2012; Fig. 2.7). Communities composed of erect shrub like algae (i.e. erect algae of ≤ 10 cm in height) are usually dominated by Dictyotales such as *Dictyota* spp., and *Padina pavonica*, and by Rhodophyceae such as *Laurencia* spp. This review found that 23 studies described their biotic assemblages dominated by erect shrub-like algae (Fig. 2.7). Ten studies had turf forming/filamentous, calcareous algae (e.g. *Jania*, *Amphiroa*, *Hypnea*, *Gelidium* and *Corallina* etc.) dominating the biotic communities of their sampling sites (e.g. Chinain et al., 2010; Totti et al., 2010; Bellés - Gurulera, et al., 2016; Vila et al., 2016). Lastly, only 10 studies described the biotic community as being dominated by seagrasses (Fig. 2.7, e.g. Faust and Morton, 1995a; Turki, 2005;

Okolodkov et al., 2007; Faust, 2009; Mabrouk et al., 2011, 2014; Zina et al., 2012; Verma et al., 2016).

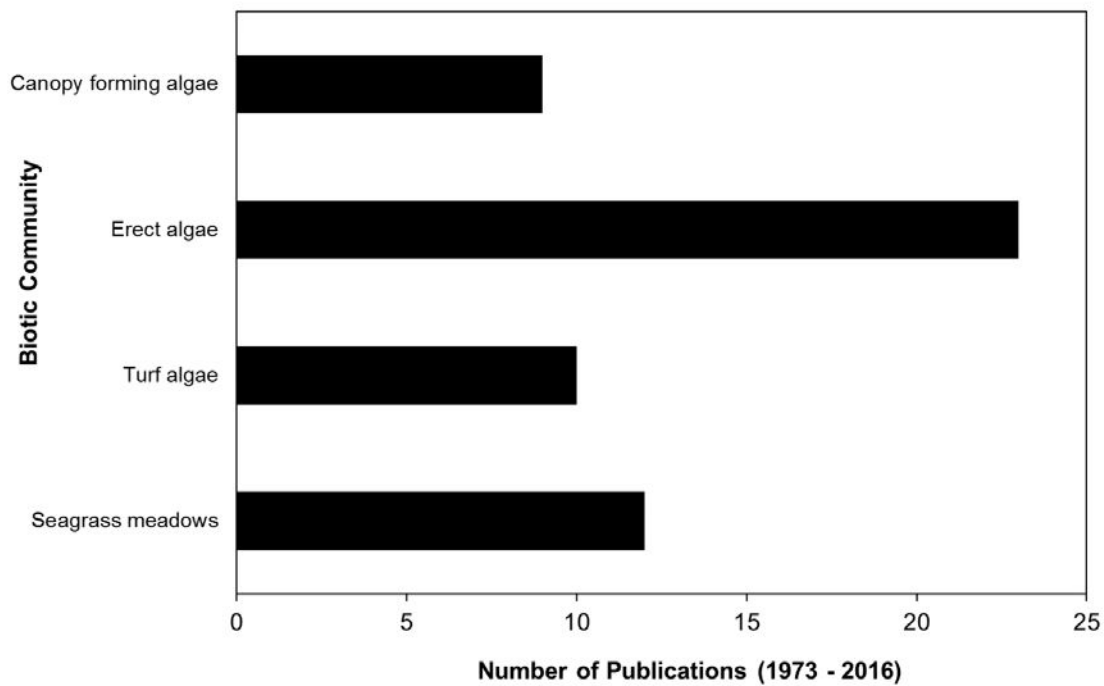


Figure 2.7. Described benthic community assemblages at the sampling sites. Descriptions provided by 44 out of 159 studies, from the period 1973 to 2016.

There is limited information in the literature on the habitat in which *Ostreopsis* spp. species are likely to be sampled. Forty percent all of the relevant studies collated for this review (64 out of 159) gave a description of the ecological habitat of the sampling sites. Of these studies, 29 % were conducted on temperate reefs, 23 % on coral reefs and 7 % in seagrass meadows; the remaining 42 % of the studies did not provide descriptions of the ecosystem of the sampling site (Fig. 2.8). Furthermore, only 20 % of the research articles (32 out of 159 articles) provided a description of the biological community as well as the physical environment associated with it (i.e. description of the ecosystem). Of these studies, 23 were based in temperate regions (e.g. Vila, 2001a and b; Accoroni et al., 2011, 2012; Shears and Ross, 2009, 2010; Cohu et al., 2012, 2013; Pfannkuchen et al., 2012; Vila et al., 2016) and the remaining 9 occurred in tropical regions (e.g. Faust, 2009, 1995; Nascimento et al., 2012; Tester et al., 2014; Verma et al., 2016). These studies provided a description of the topography, water motion and depth at which the sampling took place as well as a description of the biotic substrates sampled and the dominant biotic community present.

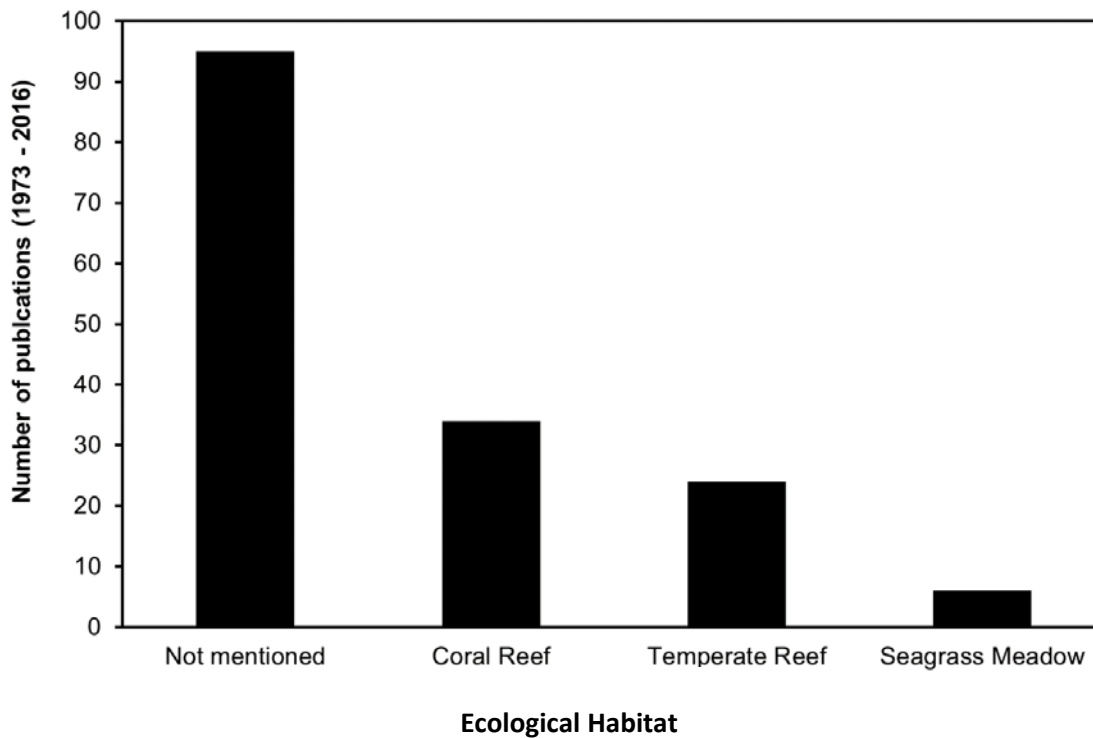


Figure 2.8. Habitats where *Ostreopsis* species can be found. Findings based on 159 relevant scientific articles from 1973 – 2016.

There are mixed conclusions on how nutrient concentrations influences population dynamics of *Ostreopsis* spp. populations in tropical waters. In the tropics, thirteen studies provided information of the physiographic characteristics of the sampling site.

Ostreopsis spp. samples have been collected in water temperatures ranging from 15.3 – 34.0 °C. *Ostreopsis* strains sampled from the tropics have exhibited a growth optima in the range 25.0 – 30.0 °C (Bomber, 1985; Morton et al., 1992; Carlson and Tindall, 1995).

Morton et al. (1992) reported that *Ostreopsis siamensis* and *O. heptagona* isolated from the Florida Keys displayed maximal growth at a salinity value of 33. In contrast, *O. ovata* isolated from Hawaii were found to be negatively correlated with salinity (Parsons and Preskitt, 2007). These results also agree with findings from ecological studies from the US and UK Virgin Islands (Carlson and Tindall, 1995).

In the Hawaiian tropical waters, dinoflagellate abundances were positively correlated with several nutrient parameters (nitrates, nitrites, phosphates and silicates) (Parsons and Preskitt, 2007). Similar results were reported in the Great Barrier Reef, where a

study confirmed a positive correlation between nitrate concentrations and *Ostreopsis* spp. growth rate in a sampling site (Skinner et al., 2013). A study based in Cuba documented that high harmful algae concentrations are related to physico-chemical conditions, influenced by large quantities of nutrients introduced by the river during the rainy season (Delgado et al., 2006). Following this observation, Ballentine et al., (1998) have hinted that the amount of rainfall may have differing effects on benthic dinoflagellate concentrations in their study sites in Puerto Rico. The study suggested that the degree of rainfall plays a role in regulating dinoflagellate populations. Moderate rainfall may increase dissolved nutrients concentrations in the seawater through terrestrial runoffs, consequently promoting dinoflagellate growth. However, heavy rainfall may result in a dilution effect and/or increase in concentrations of unfavourable substances inhibiting dinoflagellate growth (Ballentine et al., 1998). In the Gulf of Mexico, there were no significant relationships between epiphytic benthic dinoflagellate populations and physico-chemical factors observed (Okolodkov et al., 2007). While, a study on Saint Paul's rock, an uninhabited island of the Atlantic Ocean, located 1000 km away from main continental land observed high abundances of *O. cf. ovata*. These results provided an indication that eutrophication, or nutrient enrichment, does not play a significant part in stimulation blooms of this species (Nascimento et al., 2012). The inshore occurrence of *Ostreopsis* spp. has also raised the question concerning their association with nutrient enrichments due to human activities (Faust et al., 1996).

A concise summary of the effects of surface seawater temperature, salinity and nutrient concentrations on *Ostreopsis* spp. populations in temperate regions can be found in Accoroni and Totti's review (Accoroni and Totti, 2016, and references therein).

Generally, in the majority of studies reviewed, *Ostreopsis* spp. was sampled at depths ranging from 0.5 – 2 m. Most worldwide samplings have been restricted to shallow waters as it has been demonstrated in both tropical and temperate waters that *Ostreopsis* spp. cell abundance is significantly negatively correlated with depth (Richlen and Lobel, 2011; Cohu and Lemée, 2012). The mechanisms that influence *Ostreopsis* spp. populations are unclear but it is suggested that changes in the

hydrodynamic regime could be involved. Comparison of studies in exposed and sheltered sites indicates that significantly higher dinoflagellate abundances occur in sheltered conditions (Tindall and Morton, 1998; Totti et al., 2010; Richlen and Lobel, 2011; Accoroni and Totti, 2016). However, one study contradicts these conclusions indicating that *Ostreopsis* spp. is better adapted to 'slightly shaken' environments (Vila et al., 2001b).

There is currently a lack of descriptive information of both the naturally occurring flora and fauna, and the full physical aspects (bathymetry, type of hard bottom substrate, hydrographics, meteorological conditions) at the sampling sites where major *Ostreopsis* spp. blooms develop. This lack of information poses an obstacle in understanding the role of benthic community structure and composition in the population dynamics of potentially harmful benthic dinoflagellates such as *Ostreopsis* spp. It hinders not only our understanding of the bloom dynamics of *Ostreopsis* spp. but also the provision of a solid baseline for monitoring programmes and for the mitigation of the annually reoccurring potentially toxic microalgal summer blooms.

Effective management and mitigation of intense *Ostreopsis* spp. blooms can only be achieved through a thorough understanding of the ecology and bloom dynamics of these species, and thus could benefit from studies on the effect of regime shifts on *Ostreopsis* spp. populations. Benthic dinoflagellates, such as *Ostreopsis* spp., may depend on a particular substratum; therefore any alterations in the substratum, as well as the surrounding populations of other fauna and flora, will affect the epiphytic dinoflagellate populations (Fraga et al., 2012). Benthic communities of tropical and temperate reefs can change drastically over long or short periods of time, caused either by naturally occurring or anthropogenic disturbances (Hughes et al., 2003; Pandolfi et al., 2003; McManus, 2004). In the tropics areas dominated by scleratinian corals change towards areas dominated by algae following disturbances (McManus, 2004; Furby et al., 2013). In temperate regions, disturbances cause changes from macroalgal beds to turfs or to barren grounds (Bulleri et al., 2002; Guidetti, 2006) which leads to dramatic changes in benthic community structures and ecosystem functioning (Sala, 2004). Consequently, it can be hypothesised that *Ostreopsis* spp. populations can be influenced by such regime shifts (Fraga et al., 2012) as changes in

benthic community structures are likely to play a defined role in the dynamics of *Ostreopsis* spp. populations (Fraga et al., 2012).

2.5 CONCLUSIONS

There is a lack of knowledge on the benthic biotic interactions with *Ostreopsis* spp. bloom dynamics. It is apparent from this literature review that most studies did not provide detailed descriptions of benthic community assemblages in coastal regions and how marine habitats could play a role in *Ostreopsis* spp. bloom dynamics.

There is a clear need for researchers to give a thorough description of the benthic biotic substrates, community assemblages and habitats to understand the potential role benthic environments have in fostering major *Ostreopsis* spp. blooms. Understanding the role of biotic interactions with *Ostreopsis* spp. blooms is imperative in order to: prevent socio-economic damage, to reduce human health risks to beachgoers in coastal regions, and to reduce ecological damage to marine coastal ecosystems.

Despite an increase in knowledge on the ecology of *Ostreopsis* spp., the interaction of macrobenthic community structures and ecological habitats with *Ostreopsis* spp. bloom dynamics presents untapped fields of research with great potential, and identifies the challenges which still need to be addressed in the future. Future work should consider how coastal benthic community regime shifts may influence *Ostreopsis* spp. populations by measuring cell abundances on different benthic community assemblages with varying degrees of natural or anthropogenic impacts in marine coastal ecosystems.

Furthermore, in order to achieve a global and thorough understanding of *Ostreopsis* spp. bloom dynamics, it could be suggested that high ranking laboratories in various parts of the world collaborate to establish interdisciplinary research between micro- and macrophytologists interested in the bloom dynamics of epiphytic benthic dinoflagellates. This would allow us to share results in scientific outlets that are easily accessible by all and to fill in the gaps in our knowledge on the ecology of *Ostreopsis* spp.

2.6 ACKNOWLEDGEMENTS

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CHAPTER 3

**RELATIONSHIP BETWEEN ENVIRONMENTAL PARAMETERS, MARINE
COASTAL MACROALGAL ASSEMBLAGES AND THE DINOFLAGELLATE
OSTREOPSIS CF. *OVATA* IN THE CÔTE D'AZUR.**

3.1 ABSTRACT

Over the past two decades, several countries around the Mediterranean coast have reported problems related to the increasing blooms of benthic dinoflagellate *Ostreopsis cf. ovata*, which proliferations have been associated with harmful effects on human health and marine ecology.

This study aimed to elucidate the role of macroalgal communities as an ecological habitat and substrate for the formation of potentially toxic blooms. In this context, subsequent field studies along the Côte d'Azur coast were conducted on five different sampling sites in the summer of 2015 and 2016, where four levels of macroalgal community complexities were distinguished: BAR (barren grounds); TUR (communities dominated by turf-forming species, such as the ones belonging to the order *Ceramiales*); DIC (communities composed mainly of ribbon-like *Dictyotales*) and CYS (communities dominated by *Cystoseira* species) complexity. The study employed standardised methodology (BEDI, Mangialajo et al., 2017) to quantify the abundances of *O. cf. ovata* on different macroalgal community complexities to determine how habitat type influences *Ostreopsis* spp. blooms. Moreover, multiple environmental factors as well as inorganic nutrient and trace metal concentrations in ambient seawater were analysed in this study, to help identify which factors regulate *Ostreopsis cf. ovata* abundances along the French Côte d'Azur in the North-West Mediterranean Sea. The results indicated that sites with TUR communities supported the highest total *O. cf. ovata* abundances, while abundances on CYS complexities supported lower abundances. Overall, the data indicate that sites that have macroalgal communities, heavily impacted by (indirect) human stressors, may harbour greater *O. cf. ovata* abundances.

The environmental results demonstrated that environmental factors and inorganic nutrient concentrations varied by site and that there is inter-site spatial heterogeneity in *Ostreopsis cf. ovata* abundances, with Haliotis (HAL) and Rochambeau (ROC) harbouring the highest abundances. There is a potential relationship between inorganic nitrogen concentrations and *O. cf. ovata* abundances and the onset of blooms.

These findings should be considered in the management and forecasting of *Ostreopsis* spp. blooms in the NW Mediterranean Sea, and applied to the conservation of marine coastal vegetation.

3.2 INTRODUCTION

Benthic algal blooms are expanding from tropical and subtropical ecosystems to temperate environments. This global expansion represents an increasing threat to human health and coastal marine ecosystems (Rhodes, 2011; Parsons et al., 2012; Cohu et al., 2013). Blooms of the benthic dinoflagellate genus *Ostreopsis* Schmidt are becoming frequent and more intense in temperate regions (Accoroni and Totti, 2016), with some toxic species being reported in the coastal waters of the Mediterranean Sea (Tognetto et al., 1995; Vila et al., 2001; Turki et al., 2005; Aligizaki and Nikolaidis, 2006; Mangialajo et al., 2008b; Totti et al., 2010; Illoul et al., 2012; Ismael and Halim, 2012; Pfannkuchen et al., 2012), Pacific Ocean (Chang et al., 2000; Rhodes et al., 2000; Pearce et al., 2001; Shears and Ross, 2009; Verma et al., 2016), Indo-Pacific Ocean (Taniyama et al., 2003; Selina and Orlova, 2010; Kim et al., 2011; Selina and Levchenko, 2011; Hariganeya et al., 2013; Kreshchenovskaya and Orlova, 2014; Tawong et al., 2014) and the Atlantic Ocean (Granéli et al., 2002; Nascimento et al., 2012). A number of these blooms have been associated with human health consequences (Tichadou et al., 2010; Del Favero et al., 2012) and detrimental impacts to marine communities (Shears and Ross, 2009; Accoroni et al., 2011; Gorbi et al., 2012, 2013; Pagliara and Caroppo, 2012; Carella et al., 2015).

Frequent and intense *Ostreopsis* spp. blooms in temperate regions over the last 20 years have heightened the need to elucidate the drivers behind their proliferation. There is a network of factors governing and facilitating *Ostreopsis* spp. bloom dynamics (Cohu et al., 2013). However, these factors are still poorly understood, especially the role of macroalgal communities as potential substrate and habitat for the formation of the biofilm. Several environmental parameters have, however, been recognised to strongly influence bloom dynamics such as seawater temperature, salinity, hydrodynamics and nutrients (Vila et al., 2001a; Shears and Ross, 2009; Totti et al., 2010; Cohu et al., 2013; Mabrouk et al., 2011; Mangialajo et al., 2011; Accoroni and Totti, 2016). Benthic dinoflagellates have evolved various adaptive strategies to cope with environmental stressors which they can modify in response to ambient conditions (Sengupta et al., 2017). Substrate preference in benthic dinoflagellate community depends not only on specific conditions of the biotic benthic host but also

on favourable environmental factors which influence epiphytic benthic dinoflagellate populations (Widiarti et al., 2008).

Novel environmental conditions created by human activity have altered ecological habitats (Western, 2001). Examples of human stressors are deforestation, dredging, industrial and domestic discharges, construction activities and land reclamation (Airoldi, 2003; Claudet and Fraschetti, 2010). Over the past few decades, there has been a substantial increase in the terrestrial discharge of nutrients and sediments in coastal waters (Airoldi, 2003; Keith et al., 2013). Rocky shores in temperate areas are among the habitats most sensitive to increased anthropogenic pollution, causing dramatic alterations in the characteristics of the benthos (Airoldi, 2003), especially to macroalgal community complexities, due to their close proximity to discharge and the presence of pollution sensitive algal species (Smith et al., 1999; Gorgula and Connell, 2004). Such communities often disappear to degradation of water quality, increased sedimentation and/or heavy grazing by sea urchins. Consequential shifts in macroalgal communities alter the functioning of the benthos ecosystem, which could potentially have an effect on the population dynamics of benthic dinoflagellates.

Dense strands of canopy-forming macroalgae, e.g. *Cystoseira* (Fucales), are of great importance to marine coastal communities due to their high primary productivity and complex biological structure, which supports a variety of marine invertebrates (Kawamata et al., 2011) and possibly benthic dinoflagellates. Most *Cystoseira* species are sensitive to direct and indirect human impacts, and their population deterioration has been recorded across the entire Mediterranean (Gianni et al., 2013; Thiriet, 2014; Thibaut et al., 2015). *Cystoseira* forests can be modified to a lesser community complexity, dominated by shrub-like species such as Dictyotales and/or Sphacerales (Gibson et al., 2007; Mangialajo et al., 2008b). More radically, heavy commercial and recreational fishing pressures can cause a macroalgal shift from *Cystoseira* forests to barren grounds, composed of bare rocks with turf-forming algae and encrusting coralline algae and with high densities of sea urchins (Guidetti and Boero, 2004; Guidetti, 2006).

The aims of this study were to examine the biological interactions, taking into account the influence of environmental factors, on *Ostreopsis cf. ovata* abundances and to provide, through experimental field work over the summer of 2015 and 2016, a first assessment of how macroalgal community assemblages can influence *O. cf. ovata* blooms.

The field work involved the manipulation of naturally occurring macroalgal community complexities in shallow rocky shores to represent different levels of human impacts. The abundances of *O. cf. ovata* were then measured along a macroalgal shift gradient to verify whether specific macroalgal communities influence the proliferation of blooms. This could suggest management regulations to mitigation potentially toxic macroalgal blooms in coastal regions and promote the restoration and conservation of targeted macroalgal community complexities.

3.3 MATERIALS AND METHODS

In the summer months of 2015 and 2016, selected sites were chosen along the French Ligurian coast to follow the bloom dynamics of *Ostreopsis cf. ovata*. Interspersed sites, with the presence or the absence of infralittoral *Cystoseira* spp. forests, were considered. All sites were hard bottom substrates, composed of natural and/or artificial rock. Grasseuil (GRA) and Anses des Fosses (ADF) were characterised by the presence of respectively *Cystoseira brachycarpa* var. *balearica* (Sauvageau) Giaccone and *Cystoseira spinosa* (Sauvageau). Haliotis (HAL), Rochambeau (ROC) and Port of Saint Jean Cap Ferrat (PCF) were characterised by the absence of infralittoral *Cystoseira* belts (Figure 3.1). It is worth noting that in PCF small populations of *Cystoseira amentacea* (C. Agardh) Bory de Saint-Vincent were present in the upper infralittoral fringe exposed natural rocks.

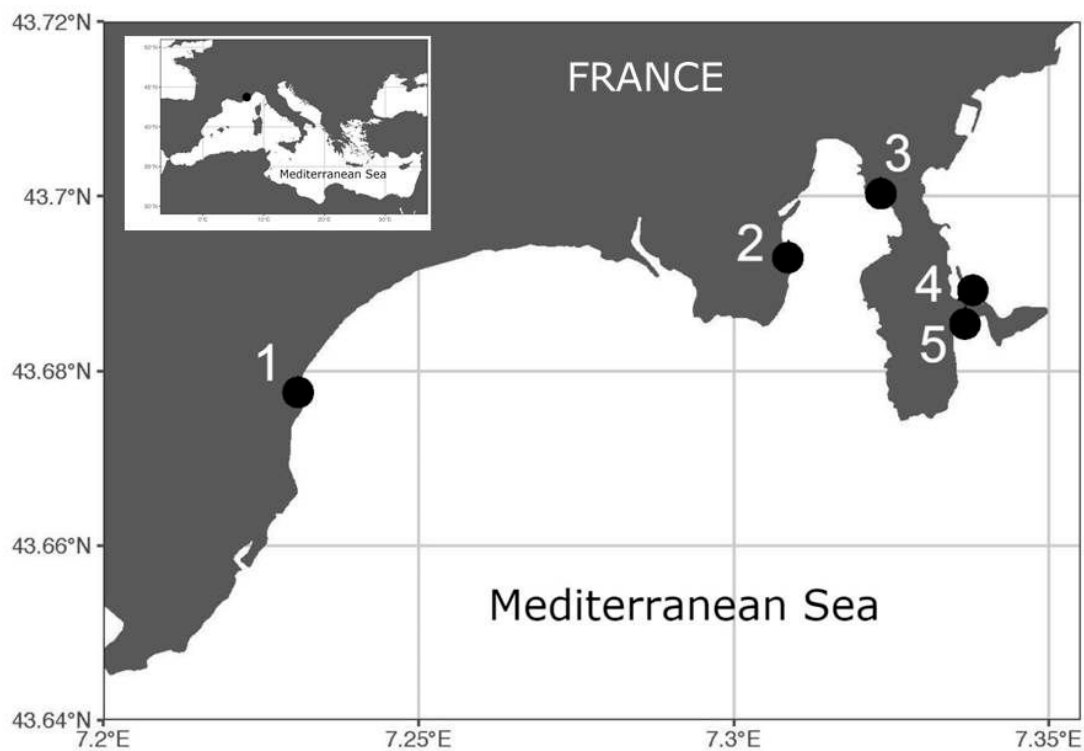


Figure 3.1. The sites studied along the French Mediterranean coast during the summer period in 2015 and 2016. 1) Haliotis (HAL); 2) Rochambeau (ROC); 3) Grasseuil (GRA); 4) Port of Saint Jean Cap Ferrat (PCF) and 5) Anses des Fosses (ADF).

Table 3.1. Sampling sites, abbreviations of the sites, wave exposure, substrate, dominant macroalgal community complexity and geographical coordinates.

Site	Abbreviation	Wave Exposure	Substrate	Dominant macroalgal community complexity type	Longitude	Latitude
Haliotis	HAL	Exposed	Artificial rock	Erect shrub-like and turf algae	43°40'39.30"N	7°13'51.17"E
Rochambeau	ROC	Semi-enclosed bay	Artificial rock	Erect shrub-like and turf algae	43°41'35.31"N	7°18'31.27"E
Grasseuil	GRA	Semi-enclosed bay	Artificial and natural rock	Canopy forming and erect shrub-like algae	43°42'01.33"N	7°19'24.08"E
Anse des Fossés	ADF	Semi-enclosed bay	Natural rock	Canopy forming and erect shrub-like algae	43°41'08.06"N	7°20'12.43"E
Port St. Jean Cap Ferrat	PCF	Exposed	Artificial and natural rock	Erect shrub-like and turf algae	43°41'20.48"N	7°20'15.77"E

Table 3.2. Field experiment sampling dates in the summers of 2015 and 2016.

2015			2016		
1	26/06/2015	T1	1	21/06/2016	T1
2	02/07/2015	T2	2	28/06/2016	T2
3	06/07/2015	T3	3	05/07/2016	T3
4	08/07/2015	T4	4	12/07/2016	T4
5	10/07/2015	T5	5	19/07/2016	T5
6	16/07/2015	T6	6	21/07/2016	T6
7	23/07/2015	T7	7	25/07/2016	T7
8	06/08/2015	T8	8	28/07/2016	T8
9	13/08/2015	T9	9	03/08/2016	T9
			10	09/08/2016	T10
			11	16/08/2016	T11
			12	23/08/2016	T12

3.3.1 Environmental factors

During the 2015 and 2016 surveys, various environmental and weather conditions were recorded. Directly on the field, for each sampling site, sea surface temperature (SST) and seawater salinity were recorded using a hand-held CTD (CASTAWAY[®] CTD, sontek, a xylem system brand, San Diego, CA, USA) as well as other relevant collection data (e.g., date, time, sea state, sample type, depth, etc.). SST was also recorded *in situ* using temperature loggers (Onset HOBO[®] Data Loggers, USA), which were left at the site for the whole duration of the summer sampling periods. Three data loggers were used in each site, each logger was attached to the hard bottom at approximately 50 cm depth, and the temperature was recorded hourly.

Meteorological conditions such as air temperature, barometric pressure, wind speed and wind direction were also recorded, and this information was taken from www.infoclimat.fr.

The water motion of the five sites sampled in 2015 was characterised by the use of the clod-cards techniques developed by Doty (1971). The method uses the dissolution rates of blocks of dried plaster of Paris (clods) affixed to a solid substrate and or surface (cards) to describe water motion. Roughly 200 g plaster of Paris blocks were cast in plastic cups following the method outlined in Doty (1971), and a small ring made from cable ties were placed just at the surface. After they were left to dry for 48 hours, the cups were removed, and each block weighed. Each clod-card was then attached to individual rocks (the size of a human hand) with cable ties and nylon rope and left in the water at a depth of 50 cm for a period of 24 hours then recovered and allowed to dry for 48 hours before re-weighing. Three clod-cards were used in each of the five sites, positioned near the area where Benthic Dinoflagellate Integrator (BEDI) samples were collected. This procedure was repeated a total of three times between 25 June and 20 August 2015 (a total of 45 clod-cards). The dissolution rate of the clod-cards was calculated as g mins^{-1} .

Additionally, water samples (250 mL, bottles) were also collected 20 cm above the macroalgae were used to measure the concentrations of nitrites (NO_2), nitrates (NO_3), phosphates (PO_4) and silicate (SiOH_4) in the seawater at each sampling site (unluckily

part of the phosphate measures of 2016 have been lost). Samples were collected in triplicates in each site. The concentration of these nutrients was measured using an automatic analysis chain (EV2-Alliance Instrument) according to Tréguer and Corre (Treguer and Corre, 1975). Concentrations of ammonium (NH_4) were also measured from the ambient seawater samples collected in 2015 (methods described in Oriol et al., 2014).

Concentrations of trace metals in the ambient seawater were also determined during the summer of 2015 at each of the five sampling sites. Five 125 mL seawater samples were collected from each site during the summer sampling period (giving a total of 25 samples). Once the samples were brought back to the laboratory, each sample was fixed with 200 μL of pure HCl. Ten-millilitre aliquots of these samples were analysed for concentrations of cadmium (Cd), cobalt (Co), chromium (Cr), copper (Cu), lead (Pb), nickel (Ni), vanadium (V) and zinc (Zn). Samples were then analysed by Inductively-Coupled Plasma Optic Emission Spectrometry (ICP-OES, Vista Pro, Agilent Technologies) and by Inductively-Coupled Plasma Mass Spectrometry (ICP-MS, Xseries2, Thermofisher Scientific). The standards solutions were prepared from a multi-elementary calibration solution (Astasol-Mix M010, Analytika, Czech Republic), in a seawater certified solution (NASS-6 from NRCC-CNRC, Ottawa, Canada). The samples were diluted in a 3% final nitric acid solution (made from a nitric acid 65% Fisher Scientific Trace Metal Grade solution) 1:2 (v/v) for ICP-OES analysis and 1:3 (v/v) for ICP-MS analysis. Quantification limit were Cd: $0.6\mu\text{g.L}^{-1}$, Co: $0.6\mu\text{g.L}^{-1}$, Cr: $1.5\mu\text{g.L}^{-1}$, Cu: $0.3\mu\text{g.L}^{-1}$, Pb: $0.6\mu\text{g.L}^{-1}$, Ni: $40\mu\text{g.L}^{-1}$, V: $6\mu\text{g.L}^{-1}$, Zn: $20\mu\text{g.L}^{-1}$.

3.3.2 *Ostreopsis cf. ovata* bloom dynamics

Regular monitoring of *Ostreopsis cf. ovata* cell abundances was carried out between June to August in 2015 and 2016. In the summer of 2015 samplings were taken a total of nine times from 25 June to the 13 August at five different sites; HAL, ROC, GRA, ADF and PCF (Fig. 3.1). In the summer of 2016 samplings were carried out a total of twelve times from the 21 June to the 23 August at three sampling sites; HAL, ROC and GRA (Fig. 3.1). Only three sites were sampled in 2016 because of logistical limitations to do all the sampling in one day. Samplings were done on a weekly basis, bi-weekly at the

peak of the bloom via shore-based snorkelling at an average water depth of 54 ± 1 cm. Three samples of the three different sampling methods were collected at each site, with each collection being made at least 10 m away from the other collections. Surrounding water samples were taken approximately 20 cm above the macroalgal assemblages in the shallow waters of the sampling site; samples were also taken of the benthic biofilm and using the benthic dinoflagellate integrator (BEDI; Mangialajo et al., 2017; Appendix 1).

To quantify cell concentrations of *O. cf. ovata* in the surrounding water, seawater samples were collected in 250 mL plastic bottles. They were taken before the benthic samples to avoid re-suspension of the epiphytic *O. cf. ovata* cells from the biofilm into the water column.

To quantify cell concentrations of *O. cf. ovata* in the biofilm, macroalgal samples were carefully picked and placed into 250 mL plastic bottles with ambient seawater.

Once the macroalgae samples were collected, the BEDI apparatus was used to collect a water sample just adjacent to where the macroalgae were sampled. For the full method on how to use the BEDI and how to quantify BEDI samples, please see Mangialajo et al., 2017 (Appendix 1).

All of the surrounding water, biofilm and BEDI samples collected were fixed with 2% acidic Lugol. For the biofilm samples, the plastic bottles containing the macroalgae were shaken vigorously for approximately one minute, and the suspension filtered through a 500 μm sieve. The macroalgae were rinsed twice with 100 mL of 0.2 μm filtered seawater to recover the maximum epiphytic microalgae (Cohu et al., 2011). The preserved samples were gently shaken, and 1 mL was analysed for benthic dinoflagellate abundance in a Sedgewick Rafter counting cell slide using a Leica DM 2500 light microscope (Wetzler, Germany) at x 100 magnification. The number of cells g^{-1} of fresh wet weight (cells g^{-1} FW) macroalgae was calculated for each sample. Subsamples (50 mL) of the surrounding water samples were settled in Utermöhl chambers (24 hours) before counting using an inverted microscope, Axiovert 40 CFL Zeiss at x 100 magnification (Utermöhl, 1958). The cell concentrations were expressed as cells per litre of seawater (cells L^{-1}).

3.3.3 Macroalgal field experiments manipulation

The five sites along the French Ligurian coast (NW Mediterranean Sea; Fig. 3.1) were chosen for this field experiment to test the effect of macroalgal community complexities on *Ostreopsis cf. ovata* abundances.

Four different levels of macroalgal community compositions were considered: high complexity characterised by canopy-forming species (such as *Cystoseira*; CYS); a medium community complexity marked by erect shrub-like algae such as ribbon-like Dictyotales (DIC); a low community complexity characterised by small Sphacelariales and Corallinales turfs (TUR); barren communities (BAR), which were artificially created removing macroalgae using a hammer and chisel.

In each site, two naturally occurring macroalgal community compositions, one of a higher composition complexity and one with a lower composition complexity, and additionally barren communities were considered (Fig. 3.2). Thus, each sampling site had a total of three different macroalgal community levels.

	Large brown algal forests	Ephemeral erect algae	Turf forming algae
HAL		✓	✓
ROC		✓	✓
GRA	✓	✓	
ADF	✓	✓	
PCF		✓	✓

Figure 3.2. Diagram representing the macroalgal community structures occurring at each sampling site (HAL – Haliotis; ROC – Rochambeau; GRA – Grasseuil; ADF – Anses des Fosses; PCF – Port of Saint Jean Cap Ferrat) in the field experiment of 2015.

In each of the five sampling sites, twelve 900 cm² (this area was chosen so that the plots were wider than the BEDI apparatus) plots were selected and marked with epoxy putty, with six of these plots were left untouched, representing triplicates of the two naturally occurring macroalgal community structures present at the site. In the remaining six plots, the macroalgal assemblage was removed (using a chisel and a hammer) to reproduce barren-like communities (Fig. 3.3). Macroalgal samples were collected in an area of 400 cm² (standard sampling area to have enough data for statistical analyses) in each of these plots for species identification and biomass assessment back in the laboratory. In order to take into account the natural evolution of macroalgal assemblages due to seasonality, 400 cm² plots were outlined in the untouched plots using a quadrat, and macroalgal samples collected from these at the end of the experiment in August 2015 (For macroalgal species identified in June and in August of 2015, please refer to Table S1 in '3.6 Supplementary Materials' on page 85).

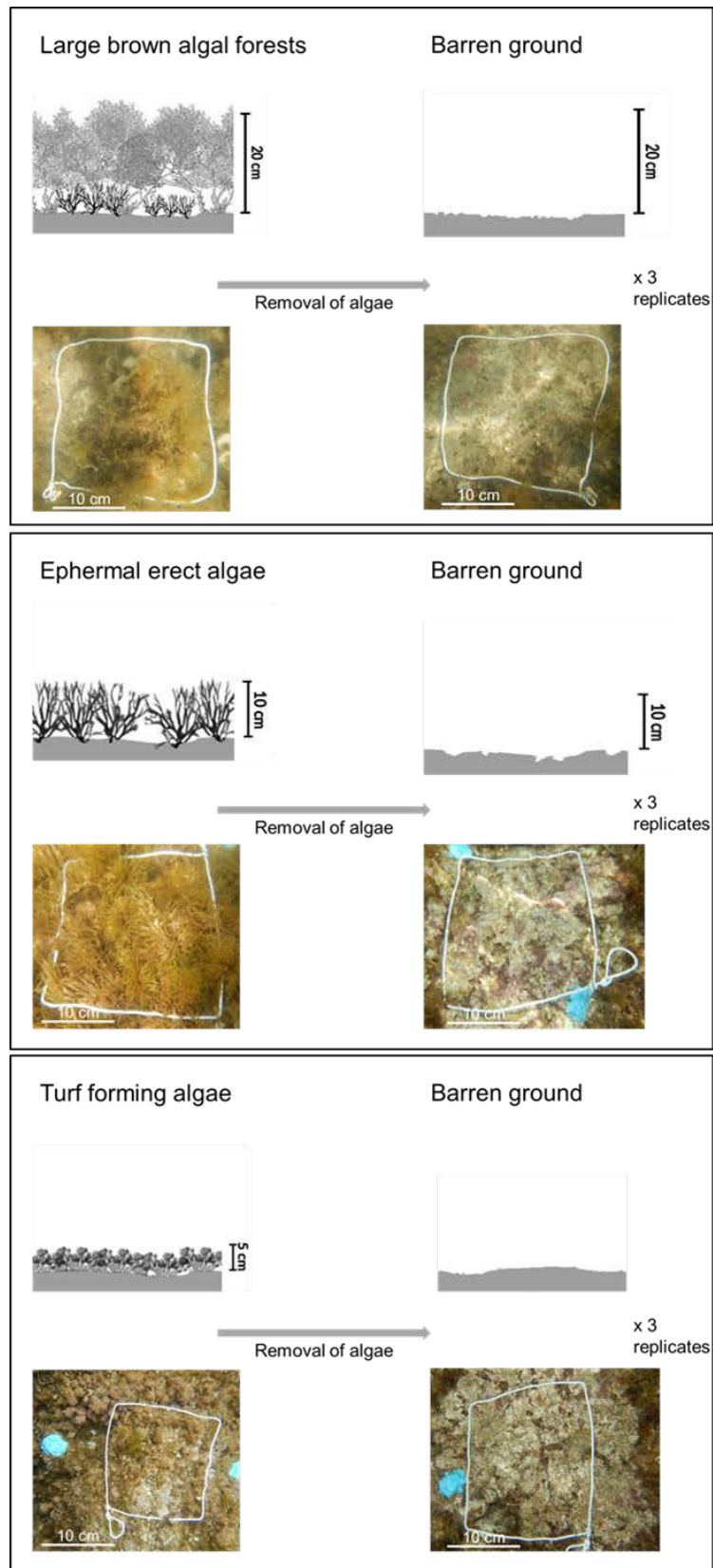


Figure 3.3. Schematic diagram representing how the naturally occurring macroalgal communities were manipulated to barren grounds in each of the five sampling sites during summer 2015 (diagrams modified from Thiriet, 2014).

Macroalgae samples collected before (May/June) and after (August) the field experiment were stored in a fridge at 4°C and sorted within 48 hours (before the macroalgae started to senesce). Or, when not possible, samples were stored in the freezer until they could be sorted. The sorting was performed with the aid of a magnifying glass. Macroalgae were identified at the lowest taxonomic level possible, and if not possible to identify at the species level, operational taxonomical units were applied. Macrophytes were identified using appropriate references (Calvo and Valdes, 1995; Abbott and Huisman, 2004; Rodríguez-Prieto et al., 2013). After identification and separation, the macroalgae were weighed (wet weight) on an Ohaus Scout Pro SPU 123 balance (Pine Brook, NJ, USA) to the nearest 0.01 g. Before being weighed, excess water from the macroalgal samples was removed by centrifuging the samples in a plastic salad spinner for 30 seconds to constant wet weight (to standardise removal of excess water after (Fong et al., 2003) and blot dried. Macroalgal biomass was measured in order to verify *a posteriori*, that the, visually selected, macroalgal community composition at each sampling site had been appropriately chosen and to describe the macroalgal assemblages present at each site.

BEDI samples were taken in each of the 12 plots delineated with epoxy putty at each of the five sampling sites in the summer of 2015. The BEDI samples collected were processed and quantified as described above in the ‘ 3.3.1 *Ostreopsis cf. ovata* bloom dynamics’ section (pages 42 – 43).

During the summer of 2016, additional BEDI samples were collected at four sites; HAL, ROC, ADF and GRA (Fig. 3.1). Three different macroalgal community assemblages were sampled at each site in triplicates. The macroalgal community sampled depended on the availability at each site. After a BEDI sample had been collected, macroalgal percentage cover within the area of the BEDI apparatus was estimated by means of the visual estimation method (method from Dethier et al., 1993). The height of each genera of macroalgal species encountered during the percentage cover was measured using a metal 15 cm ruler, accurate to the nearest mm. The sampling was performed during the *O. cf. ovata* bloom season (mid-July 2016).

Additionally, the most abundant macroalgal species from the various macroalgal community assemblages among the different sites were sampled and brought back to the laboratory. The wet weight of the macroalgae was measured to the nearest 0.01 g (using a Pb1052-S Balance, Mettler Toledo, Switzerland) for subsequent biomass calculations.

3.3.4 Statistical analyses

PCA

Environmental and weather parameters were analysed using principal component analyses (PCA) for both years 2015 and 2016. The data were normalised before the analyses, and the PCAs were calculated using the Euclidean distance dissimilarity, which allows evaluating the different environmental and weather factors between the various sampling sites.

PCO

Macroalgal biomass from the TUR, DIC and CYS plot treatments were analysed using principal coordinates analysis (PCO) before and after the field experiment in the summer of 2015.

Multivariate PERMANOVA

Macroalgal biomass data were tested for the factors site and macroalgal community complexity using a two-factor permutational multivariate analysis of variance (PERMANOVA).

Ostreopsis cf. ovata BEDI abundance data (each macroalgal community complexity and a total number of *O. cf. ovata* cells mm⁻²) were tested for the factors macroalgal community structures x sampling time x site using a three-factor PERMANOVA followed by pair-wise comparisons post-hoc tests. PERMANOVA was used as it does not assume the normality of the data (Anderson, 2001, Clarke and Warwick, 2001).

All the multivariate PERMANOVAs were run using marginal sums of the square (type III) since designs were unbalanced. PERMANOVA *p*-values were obtained based 9999 permutations of residuals under the restricted model at a confidence level of 0.05.

Pairwise tests were performed on factors or interactions giving statistically significant results. PERMANOVAs, PCAs and PCOs were computed using the PRIMER 6 & PERMANOVA + software package. Other graphical visualisations were designed in Microsoft Excel.

3.4 RESULTS

3.4.1 *Environmental factors*

The Principal Component Analyses (PCA) in Figure 3.5 illustrates how environmental and weather factors differed in each of the five sampling sites. In the summer of 2015, the first two axes of the considered environmental and weather factors explained 50.4 % of the total variance. In the summer of 2016, the different factors on the first two axes explained 65.5 % of the total variance. Both PCA representations are good because they show more than 50 % of variance.

(N.B. NH_4 measurements of 2015 were included in the PCA, whereas, since NH_4 was not measured in 2016, is it not included in the 2016 PCA).

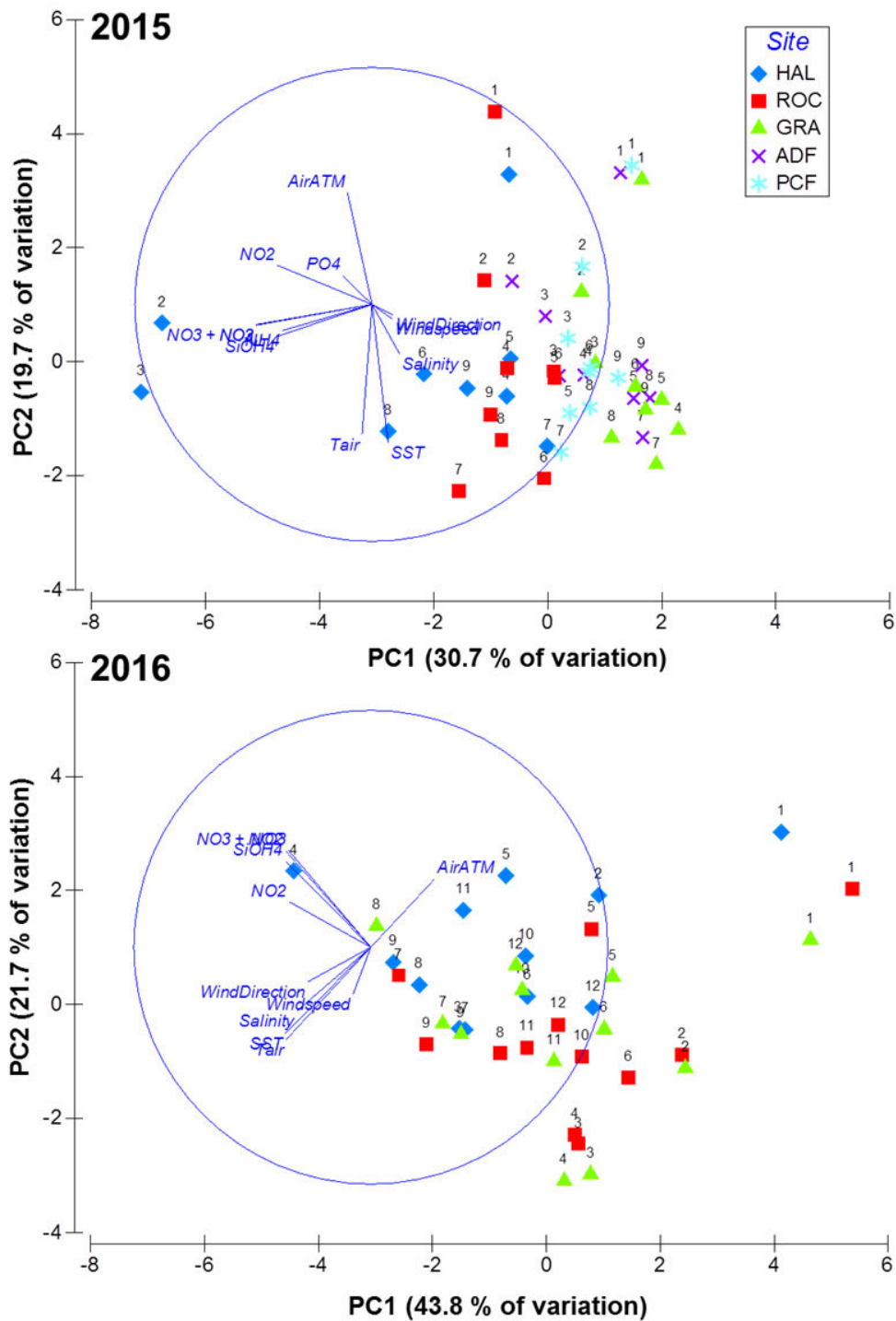


Figure 3.4. Principal component analysis (PCA) with environmental and weather variables between the different sampling sites. Salinity, Tair: air temperature, wind direction, wind speed, SST: sea surface temperature, PO4: phosphates, SiOH4: silicates, NH4: ammonium, NO3: nitrates, NO2: nitrites, NO3 + NO2: nitrates and nitrites. A) 2015 and B) 2016. PC1 and PC2 axes with % of explained variance between brackets. The different numbers represent the sampling day during the sampling periods (for the sampling dates refer to Table 3.2).

In 2015 the five sites tend to be segregated along axis 1 (Fig. 3.4; 2015). Wind direction and wind speed have very short vectors and thus are not very well represented in that plane. The variables affecting the distribution of the points are the nutrients concentrations, they are almost parallel to the axes and are well represented. The atmospheric pressure (AirATM), sea surface temperature (SST) and by the air temperature (AirT) are parallel to axis 2 and therefore are more representative of the segregation of the sampling dates (numbers above the symbols). Similarly, to 2015, in 2016 inter-site variation can be explained by AirATM, SST, AirT. In addition, the difference can also be partially explained by the seawater salinity (Fig. 3.4; 2016). Moreover, HAL differs from the other sampling sites due to nutrient concentrations (more specifically by; $\text{NO}_2 + \text{NO}_3$, NO_2 , NO_4 and SiOH_4).

Concentrations of dissolved inorganic nutrients (DIN) were measured in the summer of 2015 and 2016 (Fig. 3.5). The nutrients concentrations measured (NO_3 , NO_2 , SiOH_4 , PO_4 and NH_4) showed a high spatio-temporal variability, resulting in a significant interaction between the factors Site and Sampling Date (Table 3.3; Fig. 3.5). Generally HAL is the site showing the highest number of significant differences in space and time (24 significant differences out of the 36 possible differences with the other four sites in 2015, five out of 22 possible differences with the other two sites in 2016; Tables 3.4 and 3.5). In general, nitrogen compounds (nitrates, nitrites and ammonium) were more abundant in HAL leading to the onset of *O. cf. ovata* blooms (Fig. 3.5). Elevated concentrations of nitrogen compounds were also recorded in ROC, the other sampling site supporting an important bloom in both years. A similar trend was observed for silicates in HAL, but not in ROC (high values were recorded in ADF, where no important bloom was recorded, in 2015), and for phosphates (but only in 2016). It is worth noting that phosphates data of the peak of the bloom and of the decreasing phase are missing for 2016.

Table 3.3. PERMANOVA table of results for the nutrient concentrations at each sampling site during the sampling season in the summers of 2015 and 2016. **Bold:** significant p values; $p < 0.05^*$ significant; $p < 0.01^{**}$ highly significant.

2015						
Source	df	SS	MS	Pseudo-F	P value	Unique permutations
Site	4	276.820	69.206	29.518	0.001***	997
Sampling day	8	91.040	11.380	4.854	0.001***	999
Site x Sampling day	32	217.950	6.811	2.905	0.001***	999
Residuals	88	206.320	2.345			
Total	132	792				
2016						
Source	df	SS	MS	Pseudo-F	P value	Unique permutations
Site	2	47.319	23.659	8.431	0.001***	998
Sampling day	10	165.900	16.590	5.912	0.001***	998
Site x Sampling day	20	84.078	4.204	1.498	0.049*	997
Residuals	64	179.610	2.806			
Total	96	480				

Table 3.4. Pairwise Post Hoc tests to identify the differences in inorganic nutrients concentrations between sampling sites x sampling time. Data from the summer of 2015. **Bold:** significant p values; $p < 0.05^*$ significant; $p < 0.01^{**}$ highly significant. For sampling times (T1. T2...etc) refer to Table 3.2.

2015																		
Groups	T1		T2		T3		T4		T5		T6		T7		T8		T9	
	t	P - value	t	P - value	t	P - value	t	P - value	t	P - value	t	P - value	t	P - value	t	P - value	t	P - value
ADF, GRA	2.994	0.014*	1.824	0.083	2.262	0.053	2.715	0.026*	1.922	0.066	2.322	0.066	6.249	0.001**	2.008	0.078	2.101	0.048*
ADF, HAL	1.923	0.074	2.878	0.027*	5.923	0.004**	2.172	0.042*	2.832	0.02*	2.277	0.030*	4.311	0.01*	2.558	0.034*	3.573	0.014*
ADF, PCF	1.211	0.298	1.854	0.106	2.123	0.050	1.417	0.184	1.144	0.314	2.558	0.035*	1.416	0.193	1.992	0.063	2.545	0.011*
ADF, ROC	2.669	0.020*	1.104	0.346	1.399	0.196	1.645	0.115	1.323	0.229	2.382	0.032*	2.163	0.074	2.880	0.009**	3.695	0.013*
GRA, HAL	2.859	0.037*	3.693	0.011*	5.327	0.006**	4.954	0.005**	3.142	0.009**	3.634	0.01*	4.130	0.002**	2.230	0.055	4.176	0.011*
GRA, PCF	2.160	0.070	0.783	0.610	1.610	0.117	4.211	0.013*	1.191	0.268	1.735	0.111	2.464	0.048*	0.949	0.456	1.268	0.218
GRA, ROC	3.468	0.012*	1.890	0.097	0.732	0.629	5.375	0.007**	1.917	0.103	3.017	0.017*	2.208	0.079	1.802	0.134	4.126	0.004**
HAL, PCF	1.752	0.112	4.033	0.011*	7.068	0.001**	2.900	0.018**	1.120	0.322	3.280	0.022*	1.677	0.109	2.028	0.091	3.718	0.010*
HAL, ROC	0.573	0.759	2.955	0.038*	4.242	0.006**	2.967	0.028*	1.823	0.086	1.708	0.117	1.182	0.300	1.313	0.233	1.015	0.385
PCF, ROC	2.014	0.056	2.232	0.060	0.722	0.611	1.294	0.254	1.211	0.300	2.530	0.029*	1.259	0.255	1.736	0.124	3.564	0.012*

Table 3.5. Pairwise Post Hoc tests to identify the differences in inorganic nutrients concentrations between sampling sites x sampling time. Data from the summer of 2016. **Bold:** significant p values; $p < 0.05^*$ significant; $p < 0.01^{**}$ highly significant. For sampling times (T1. T2...etc) refer to Table 3.2.

2016																						
Groups	T1		T2		T3		T4		T5		T7		T8		T9		T10		T11		T12	
	t	P - value	t	P - value	t	P - value	t	P - value	t	P - value	t	P - value	t	P - value	t	P - value	t	P - value	t	P - value	t	P - value
HAL, ROC	2.159	0.092	5.272	0.002**	2.311	0.032*	5.217	0.004**	0.810	0.479	1.217	0.294	0.909	0.450	0.763	0.584	1.249	0.272	1.619	0.143	0.553	0.718
HAL, GRA	2.642	0.038*	7.803	0.001**	2.879	0.010*	5.881	0.003**	1.580	0.171	1.188	0.282	0.325	0.853	0.716	0.538	0.257	0.896	2.020	0.097	0.888	0.465
ROC, GRA	0.739	0.553	0.796	0.565	1.050	0.385	1.597	0.125	0.654	0.583	1.543	0.177	1.052	0.393	0.729	0.566	1.081	0.355	0.854	0.543	0.873	0.453

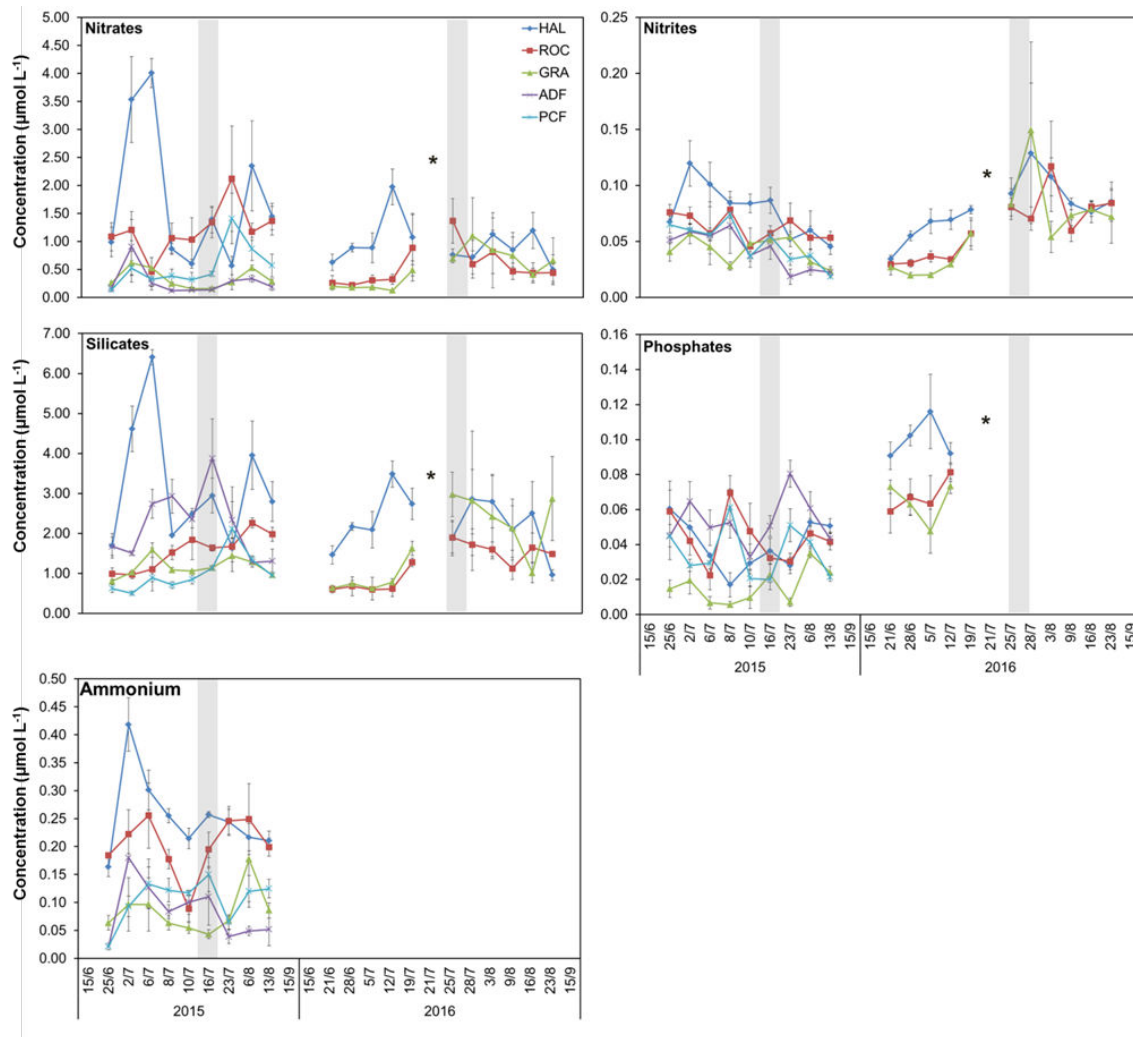


Figure 3.5. Inorganic nutrient concentrations (nitrates, nitrites, silicates, phosphates and ammonium) measured at the different sampling sites in 2015 and 2016. Ammonium concentration was not measured in 2016. * = samples were not collected on the 21 July 2016. The grey bars indicate the maximum abundance of *Ostreopsis cf. ovata*.

The N:P ratios were calculated (measured by dividing N concentrations ($\mu\text{mol L}^{-1}$) by P concentrations), based on the Redfield model (Redfield, 1958), for each sampling site for both sampling periods (summer 2015 and beginning of summer 2016), and are illustrated in Figure 3.6. If the N:P ratio > 16 , it indicates that the site evolves towards P limitation, whereas, if the N:P ratios < 16 , the site evolves towards N limitation (Béthoux et al., 2002). Working on this principle, HAL, ROC, and GRA can be considered as sites where P is a limiting factor during the onset of the *O. cf. ovata* bloom in the summer of 2015. In contrast, the sites ADF and PCF had N as the limiting factor (Fig. 3.6A). In 2016 only three sites were monitored, and unfortunately, we only have data for the first four sampling dates for phosphates concentrations. Nevertheless it is possible to determine that before the peak of the *O. cf. ovata* bloom, HAL started to have a higher N:P ratio, indicating that P was becoming limiting in this site, whereas in ROC and GRA, the N:P was relatively low, showing potential N-limitation (Fig. 3.6B).

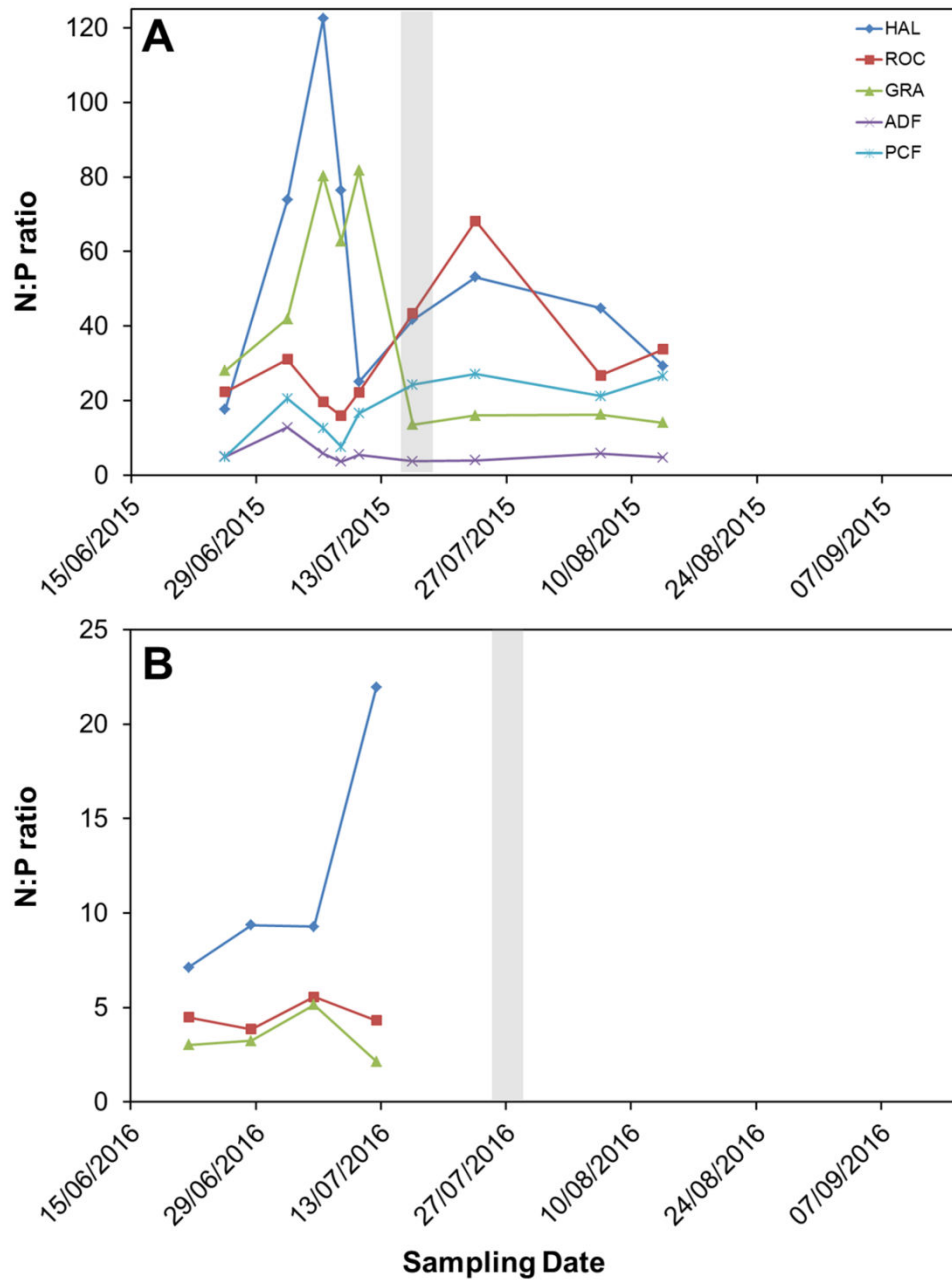


Figure 3.6. N:P ratios calculated for the different sampling sites in 2015 and 2016. If $N:P > 16$, P considered as limiting; if $N:P < 16$, N considered as limiting. The grey bars indicate the peak of the *Ostreopsis cf. ovata* bloom. The grey bars indicate the maximum abundance of *Ostreopsis cf. ovata*.

It is clear from Figure 3.7 that the clod-cards deployed in HAL and PCF had the fastest dissolution rates compared to the other sites. This indicates that these two sites have more water movement and small-scale turbulence than GRA and ADF that can be considered as more sheltered sites. Compared to the previous sites, ROC has an intermediate clod-card dissolution rate, thus, can be described as a semi-enclosed site (Fig. 3.7). However, the differences in the dissolution rates were not statistically significant and so it cannot be defined with certainty that HAL and PCF are exposed sites, ROC a semi-sheltered site and GRA and ADF as sheltered sites. To really characterise the sites more clod card replicates would be needed for each site and, there should be more repetitions carried out during the sampling period.

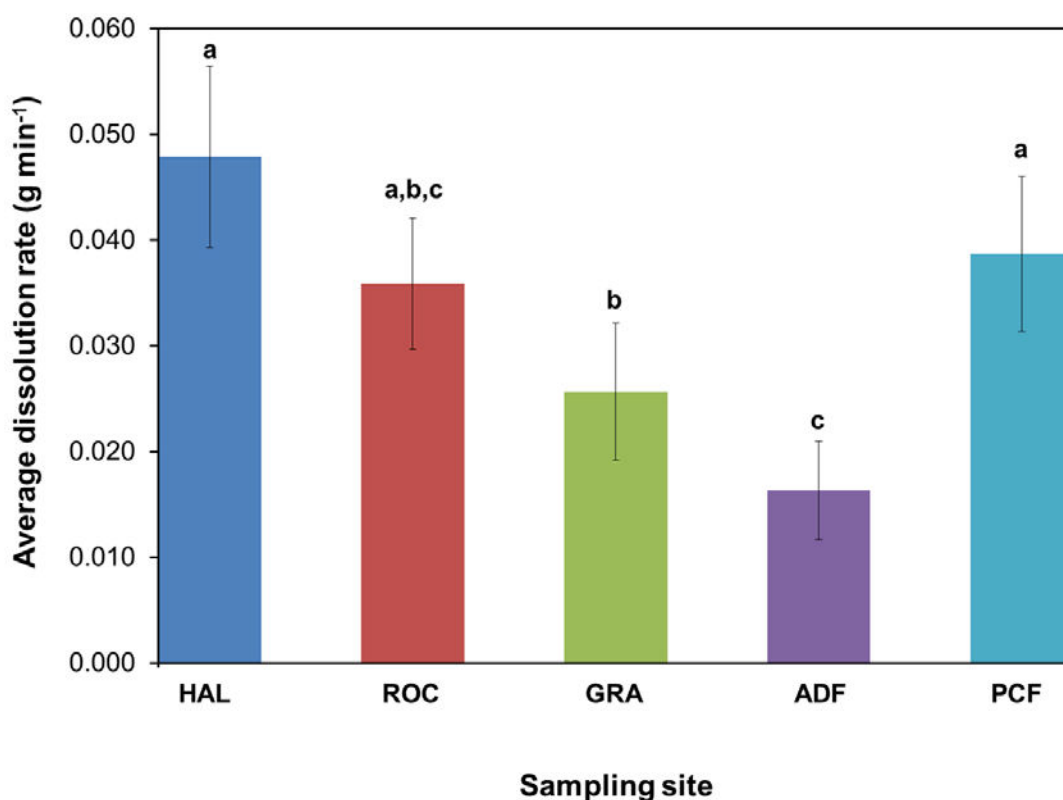


Figure 3.7. Dissolution rate (g mins⁻¹) of the clod-cards at the five different sampling sites at three replications during the summer months of 2015. Letters indicate significant differences in the PERMANOVA pair-wise tests ($p < 0.05$).

Only three out of the eight trace metals analysed had concentrations above their detection limit; Cu, Pb and Zn. Figure 3.8 demonstrates the copper (Cu), lead (Pb) and zinc (Zn) concentrations detected in the seawater samples collected during the summer period of 2015, at each of the five sampling sites. All of the sites, apart from HAL, had a high Cu concentration detected ($> 0.3 \mu\text{g L}^{-1}$) during the bloom period (Fig. 3.8). The highest concentration recorded was $2.79 \mu\text{g L}^{-1}$ (nine times above the detection limit) at GRA on the 6 August.

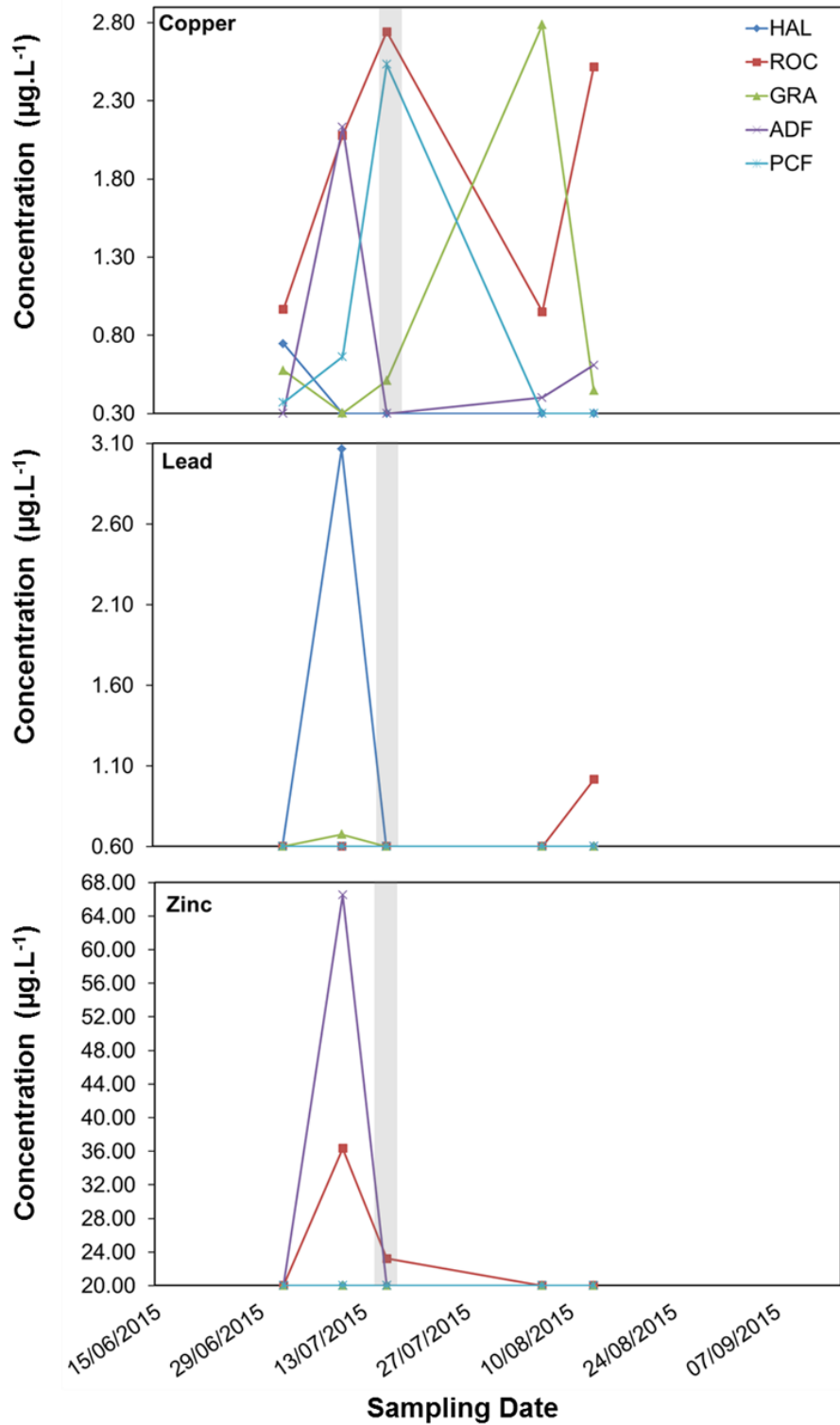


Figure 3.8. Copper (Cu), lead (Pb) and zinc (Zn) concentrations in the seawater at the five different sampling sites during the summer months of 2015. (n = 25). The grey bars illustrate the *Ostreopsis cf. ovata* bloom on the 16 July 2015.

HAL had the highest recorded [Pb] with $3.07 \mu\text{g L}^{-1}$ (five times above the detection limit) on the 10 July, and ROC had a slight peak in [Pb] on the 13 August with $1.02 \mu\text{g L}^{-1}$.

The highest Zn concentrations were measured in ADF (66.50 $\mu\text{g L}^{-1}$) and ROC (36.35 $\mu\text{g L}^{-1}$), both values were recorded on the 10 July.

The highest trace metal concentrations for Cu, Pb and Zn were recorded before and after the 16 July, the peak of the *O. cf. ovata* bloom. However, the elevated concentrations of these detected trace metals are not concomitant with the peaks of *O. cf. ovata* cell abundances during the bloom period in the summer of 2015. A two-way PERMANOVA revealed that there was no significant difference between the trace metal concentration and the sampling sites ($p = 0.166$), and between the sampling day ($p = 0.366$). Thus, it is very unlikely that trace metal concentrations influence *O. cf. ovata* blooms in the NW Mediterranean Sea.

3.4.2 *Ostreopsis cf. ovata* bloom dynamics

Between 25 June and 13 August 2015, 135 benthic biofilm, surrounding water and BEDI samples were collected (making a total of 405 samples). Between 21 June and 23 August 2016, 108 benthic biofilm, surrounding water and BEDI samples were collected (a total of 324 samples). During the summer of 2015, maximum *O. cf. ovata* cell abundances were recorded on the 16 July ($1.6 \times 10^6 \text{ g}^{-1}$ *Halopteris*; $3.7 \times 10^4 \text{ cells L}^{-1}$; $541 \text{ cells mm}^{-2}$). During the summer of 2016, maximum cell abundances were recorded on the 27 July ($2.3 \times 10^6 \text{ g}^{-1}$ *Dictyota*; $1.6 \times 10^5 \text{ cells L}^{-1}$; $717 \text{ cells mm}^{-2}$). In 2015, GRA, ADF and PCF had lower cell concentrations compared to HAL and ROC (Fig. 3.9). Moreover, the bloom observed in HAL lasted longer compared to the other sites. Accordingly, in 2016, GRA had comparatively lower *O. cf. ovata* abundances using the three sampling methods compared to HAL and ROC (Fig. 3.9). Figure 3.9 clearly demonstrates that the bloom period in 2016 started later than in 2015, lasted longer and was more intense in terms of abundances recorded.

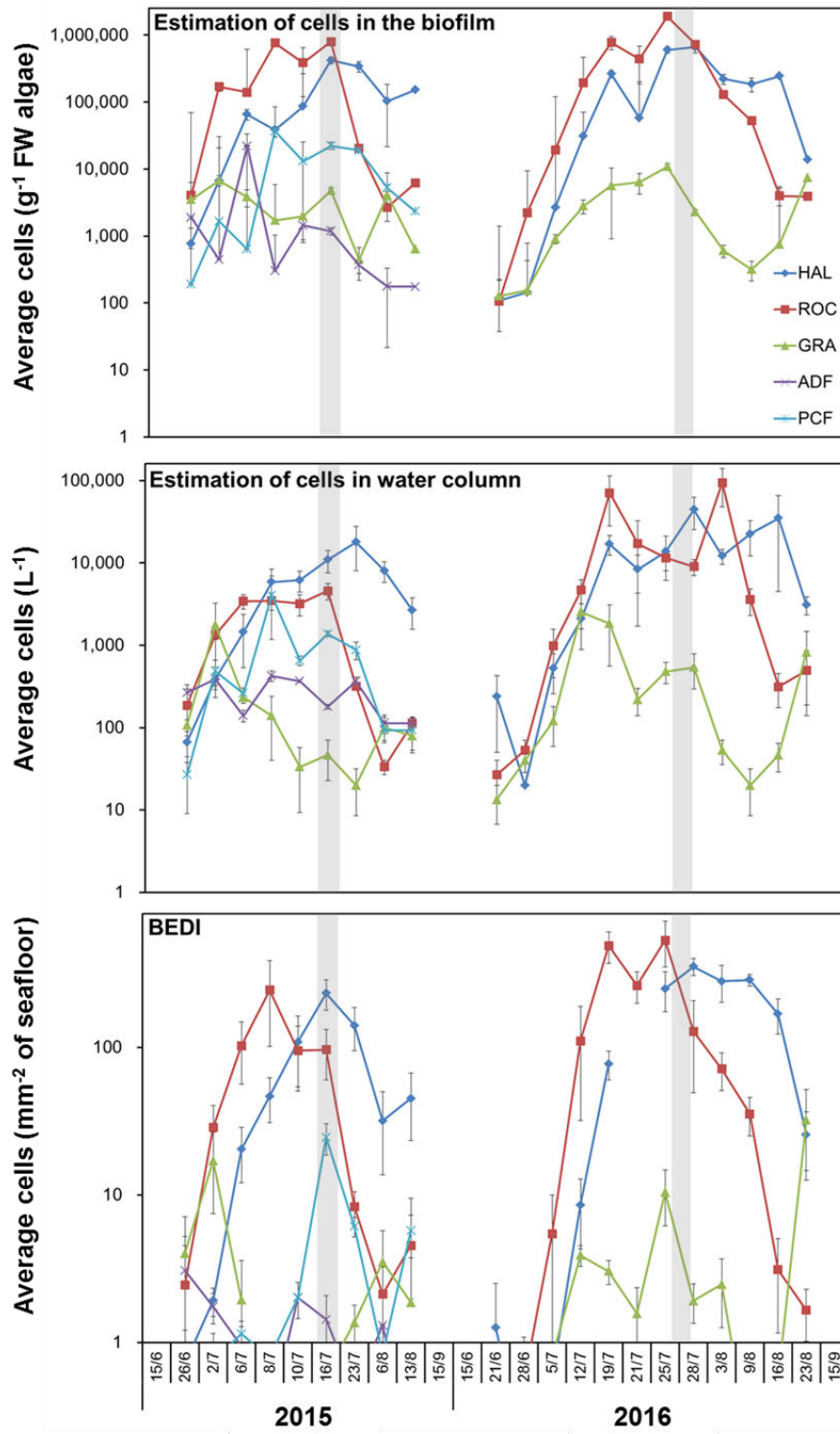


Figure 3.9. Average *Ostreopsis cf. ovata* cell abundances from the monitoring survey of 2015 and 2016 at each of the sites. A) Estimation of benthic abundances, cell concentrations expressed as g^{-1} fresh weight of macroalgae; B) Estimation of abundances from the surrounding water column, cell concentrations expressed as cells L^{-1} ; C) Estimation of cell abundances expressed as cells per mm^2 of the seafloor. Grey bars represent peaks of *O. cf. ovata* blooms. ($n = 135$ for each sampling method). The y-axes are in the Log scale.

3.4.3 Macroalgal field experiments manipulation

A total of 90 macroalgal samples (60 before and 30 after the field experiment) were sorted, weighed (to the nearest 0.01 g) and used for the subsequent analyses.

The principal coordinates analyses (PCOs; Fig 3.10) shows that TUR and CYS tend to be separated, while the DIC (intermediate complexity level) is generally positioned at an intermediate position both before and after the experiment (and therefore the *O. cf. ovata* bloom).

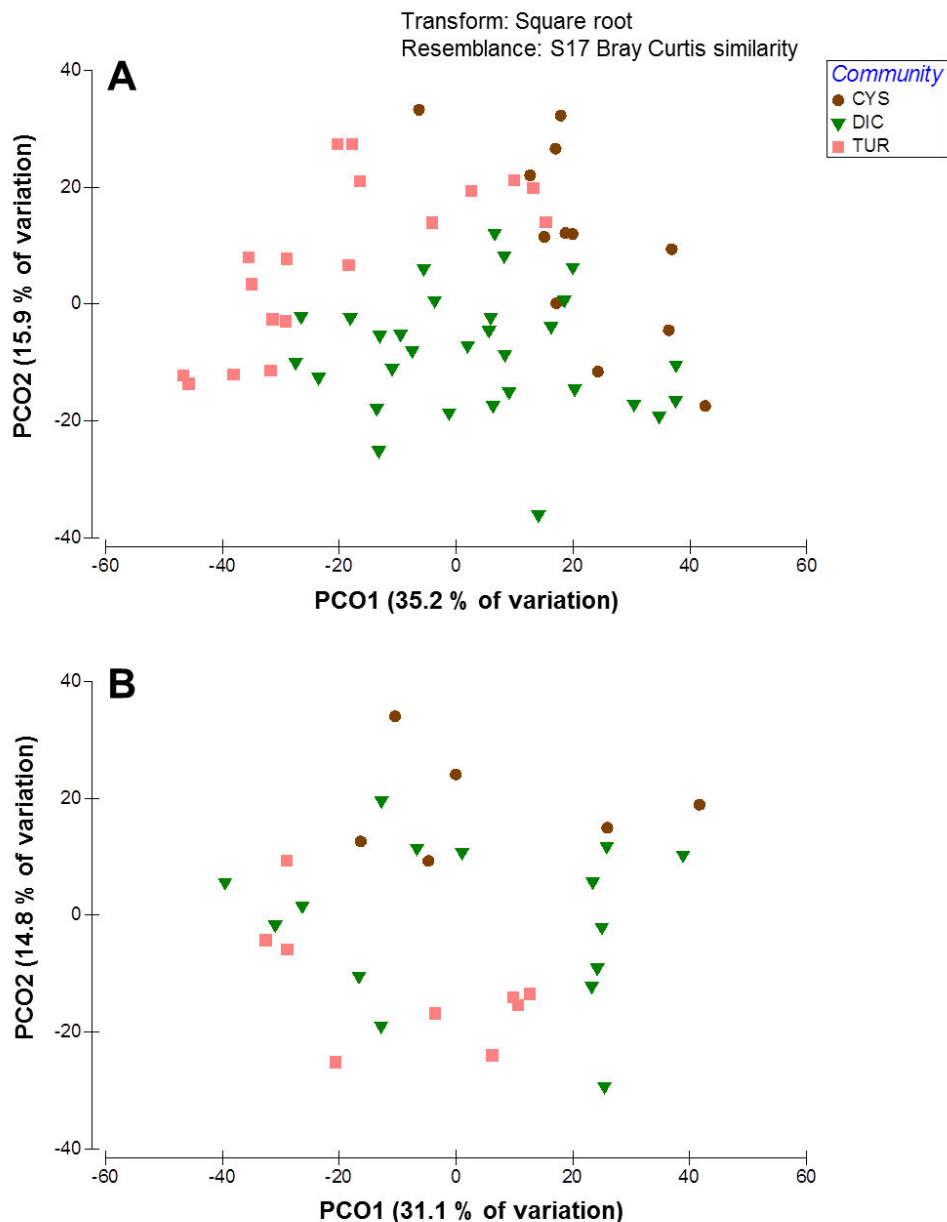


Figure 3.10. Principal coordinates analysis (PCO) of the different macroalgal community composition plot treatment A) before and B) after the field experiment. PCO1 and PCO2 axes with % of explained variance between brackets.

Corresponding two-factor PERMANOVAs (community x site) confirm this pattern only for the first sampling (before the experiment $p = 0.001$; Table 3.6) while no significant pattern at the community level was detected after the experiment ($p = 0.139$; Table 3.6). Indicating that the differences in community structure and composition between the different macroalgal community assemblages are statistically significant (Table 3.6). For both times of the experiment (i.e. before and after), high spatial variability at the site scale was detected ($p = 0.001$; Table 3.6).

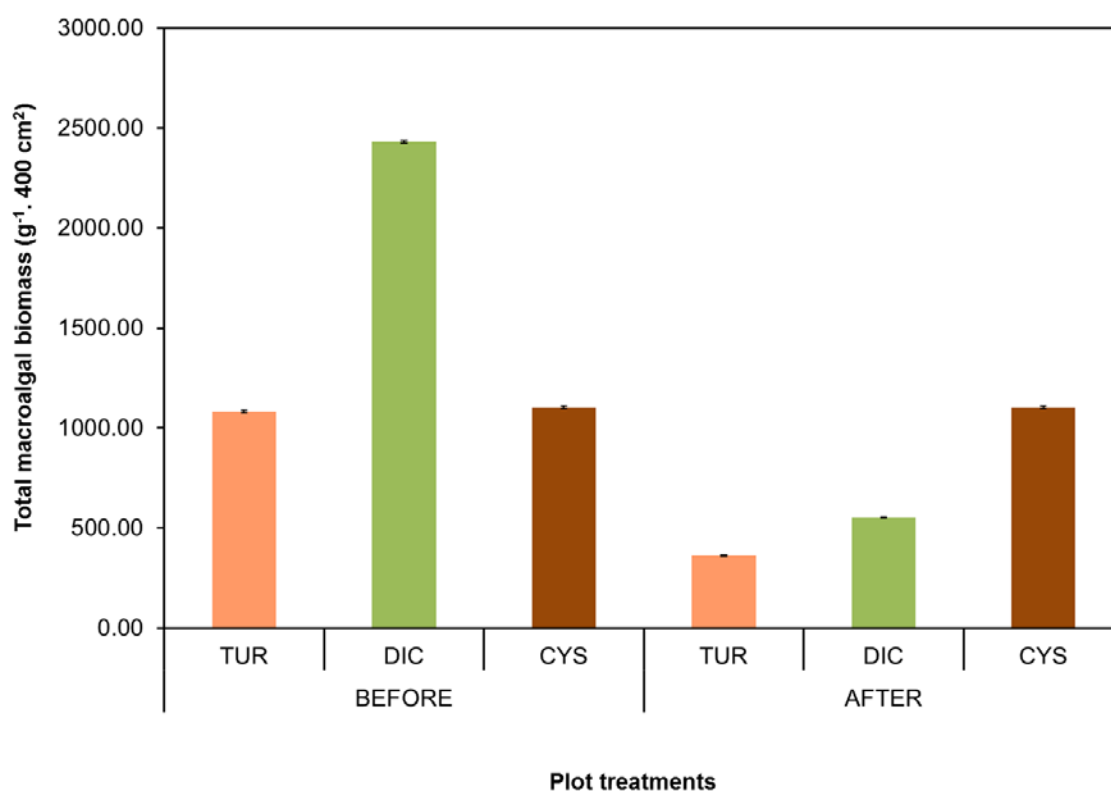


Figure 3.11. Total macroalgal biomass (g) in each different plot treatments before and after the field experiment in summer 2015. Error bars represent ± 1 SD of the mean.

The total biomass associated to the different communities shows an unexpected pattern (Fig. 3.11) before the experiment, with a largely higher value in the DIC communities (intermediate complexity level) and a comparable value for TUR and CYS. After the experiment, the biomass of the sampled communities reflects the complexity degree, with a lower value corresponding to TUR, and intermediate value corresponding to DIC and a higher value corresponding to CYS.

Table 3.6 Two-factor PERMANOVA results, macroalgal community x site, before and after the macroalgal experiment of the summer of 2015. **Bold:** significant p values; $p < 0.05$ * significant; $p < 0.01$ ** highly significant.

BEFORE						
Source	df	SS	MS	Pseudo-F	P value	Unique permutations
Site	4	23329.000	5832.300	73848.000	0.001**	997
Community	2	10754.000	5377.200	68085.000	0.001**	998
Community x Site	3	3032.700	1010.900	1.280	0.189	997
Residuals	50	39489.000	789.780			
Total	59	88198.000				
AFTER						
Source	df	SS	MS	Pseudo-F	P value	Unique permutations
Site	4	16295.000	4073.700	33967.000	0.001**	998
Community	2	2692.800	1346.400	11227.000	0.313	998
Community x Site	3	3059.400	1019.800	0.850	0.676	998
Residuals	20	23986.000	1199.300			
Total	29	49534.000				

A total of 540 BEDI samples were collected in the summer of 2015. In each of the five sampling sites, BEDI samples were collected from four different levels of macroalgal community assemblages; two naturally occurring community complexities depending on which one was available at the site, and the artificially created barren community. Average cell abundances were calculated for each community complexity for the summer of 2015 (Fig. 3.12). Figure 3.12 clearly illustrates that the highest average abundance of *Ostreopsis cf. ovata* cells was measured on the TUR and DIC plot treatments.

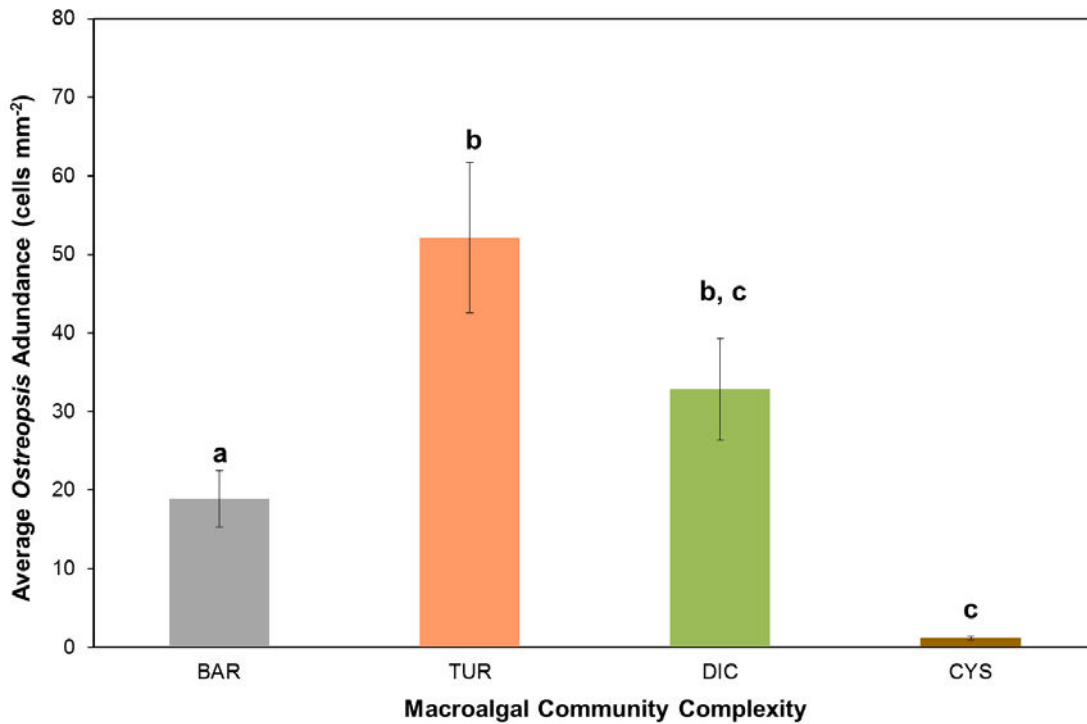


Figure 3.12. Average *Ostreopsis cf. ovata* cell abundances for each macroalgal community composition in the summer of 2015 ($n = 540$). Letters indicate differences using PERMANOVA pair-wise tests, where different letters specify significance at $p < 0.05$. Error bars represent ± 1 SE of the mean.

Subsequent three-factor PERMANOVA used to test for the factors macroalgal community complexity x sampling day x site revealed that there was a significant difference in the mean *O. cf. ovata* cell abundances (cells mm⁻²) between the different macroalgal community plot treatments ($p = 0.003$) and between the sampling sites ($p = 0.001$; Table 3.7). Pairwise post hoc analyses indicated that there was a significant difference in *O. cf. ovata* cell abundances between BAR and TUR ($p = 0.001$); BAR and DIC ($p = 0.013$); BAR and CYS ($p = 0.002$). No statistical difference was observed between TUR and DIC ($p = 0.925$); DIC and CYS ($p = 0.337$), and no test was performed between TUR and CYS because there was no sampling site with these two plot treatments at any one time (Table 3.7).

Table 3.7. PERMANOVA table of results of the *Ostreopsis cf. ovata* cells abundances (BEDl; cells mm⁻²) on the different community assemblages between the five different sampling sites. Results from the summer of 2015 only. **Bold:** significant p values; $p < 0.05$ * significant; $p < 0.01$ ** highly significant.

Source	df	SS	MS	Pseudo-F	P value	Unique permutations
Community	3	179.700	59.902	4.514	0.003**	999
Site	4	2635.100	658.780	12.027	0.001**	999
Sampling day	8	826.330	103.290	2.234	0.037*	999
Community x Site	7	82.842	11.835	3.351	0.004**	998
Community x Sampling day	24	66.866	2.786	0.775	0.757	997
Site x Sampling day	32	1752.800	54.776	11.549	0.001**	997
Community x Site x Sampling day	56	197.760	3.532	0.745	0.890	998
Residuals	405	1920.800	4.743			
Total	539	9084.900				

Figure 3.13 illustrates the statistical differences of the mean *O. cf. ovata* cell abundance between the different community complexities within each of the five sampling sites. Furthermore, in HAL, ROC, GRA and ADF it can be seen that *O. cf. ovata* cell abundances were always highest on DIC community complexities, whereas in PCF, the highest cell abundances were measured on TUR community complexities. (Fig. 3.13).

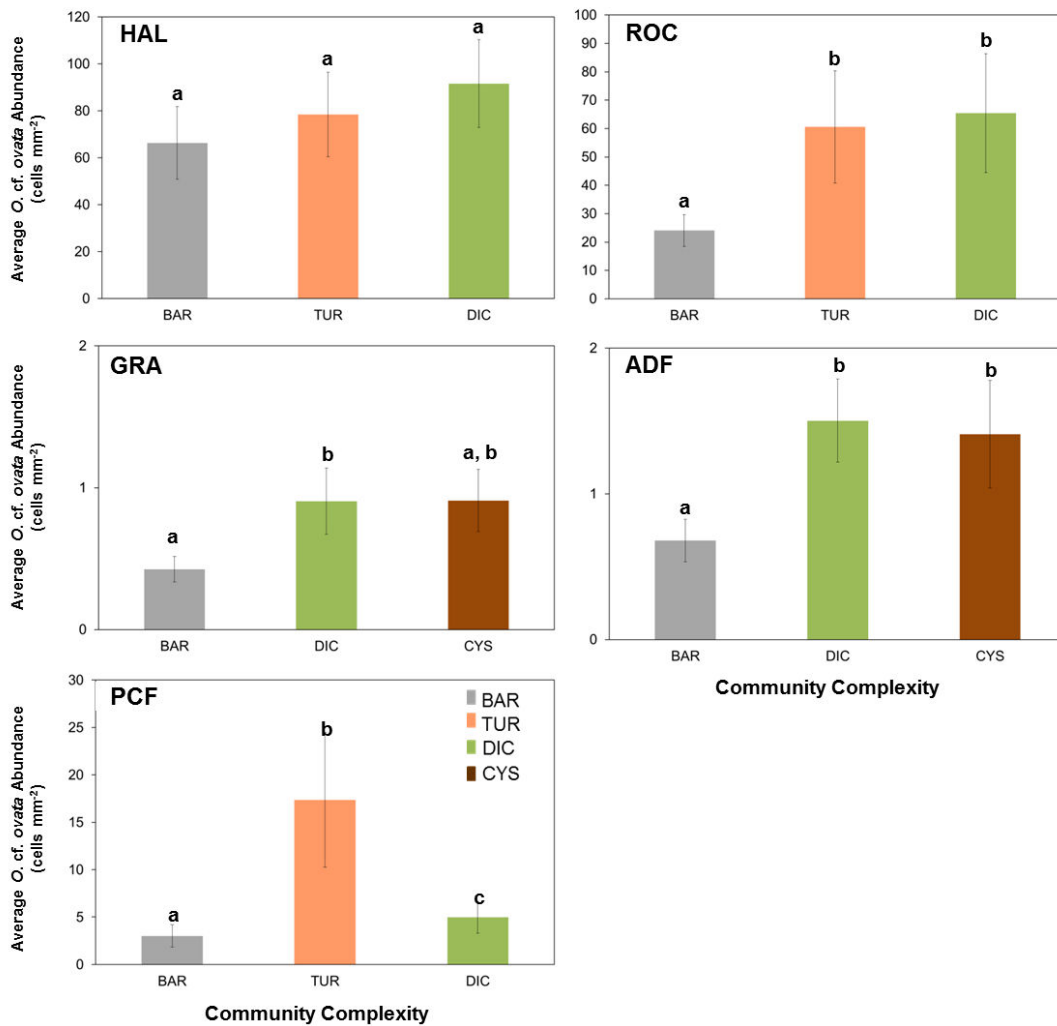


Figure 3.13. Average *Ostreopsis cf. ovata* cell abundances for each macroalgal community composition within each of the five sampling sites in the summer of 2015. Letters indicate differences using PERMANOVA pair-wise tests, where different letters specify significance at $p < 0.05$. Error bars represent ± 1 SE of the mean.

These results indicate that, in general, higher *Ostreopsis cf. ovata* cell abundances are measured on macroalgal assemblages of low and medium complexity, TUR and DIC, respectively, in sites which do not have natural *Cystoseira* spp. populations. Very low cellular abundances were measured on BAR and CYS community complexities, which could be hypothesised that these macroalgal assemblages restrict *Ostreopsis cf. ovata* blooms in these regions during the summer months. Intense blooms were observed in HAL and ROC, both sites characterised by TUR and DIC, and the results have indicated that these community complexities harbour the greatest number of *O. cf. ovata* cells. Thus, higher *O. cf. ovata* abundances were observed in sites with TUR community complexities and lower *O. cf. ovata* abundances were observed in sites with CYS

community complexities. These results are further supported by Figure 3.14, where it illustrates that the maximum *Ostreopsis cf. ovata* cell abundances measured using the BEDI method were observed in HAL and ROC, the two sites harbouring major blooms in the summer of 2015. A modest *O. cf. ovata* bloom also occurred in PCF, with maximum cell abundances reaching 150 cells mm⁻². These three sites were characterised by the absence of infralittoral *Cystoseira* spp. belts. Conversely, sites where *Cystoseira* spp. species are present (i.e. in GRA and ADF), maximum *O. cf. ovata* abundances are observed below 10 cells mm⁻². It is worth noting that in PCF, where intermediate values of *O. cf. ovata* abundances were observed, some populations of *Cystoseira amentacea* were present in the upper infralittoral fringe.

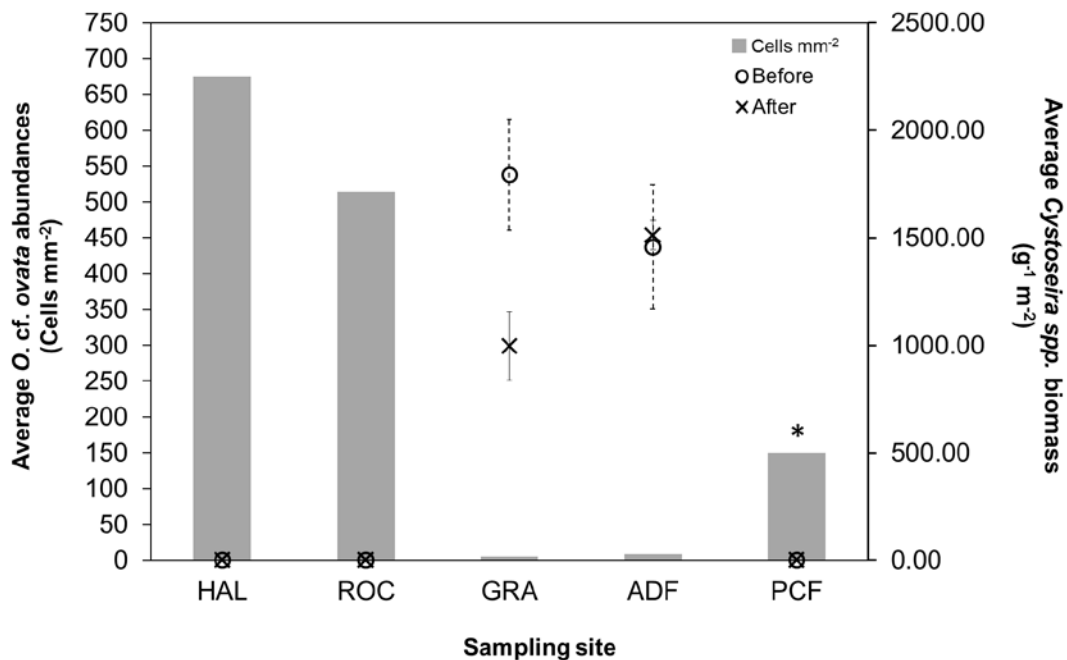


Figure 3.14. Maximum *Ostreopsis cf. ovata* cell abundances (cells mm⁻²) recorded at each sampling site in the summer of 2015 with the average *Cystoseira* spp. biomass (g⁻¹ m⁻¹) before (represented by the circles) and after (represented by the crosses) the macroalgal field experiment. Error bars represent ± 1 SE of the mean. * = In PCF small populations of *Cystoseira amentacea* (C. Agardh) Bory de Saint-Vincent were present in the upper infralittoral fringe exposed natural rocks.

The results from the 2016 field experiments indicated macroalgal biomasses were significantly different among the four sampling sites ($p = 0.001$). Within each site, there were statistical differences between biomass and the different macroalgal

community complexities (Fig. 3.15). Interestingly, in all of the sampling sites, TUR communities had the highest average biomass values.

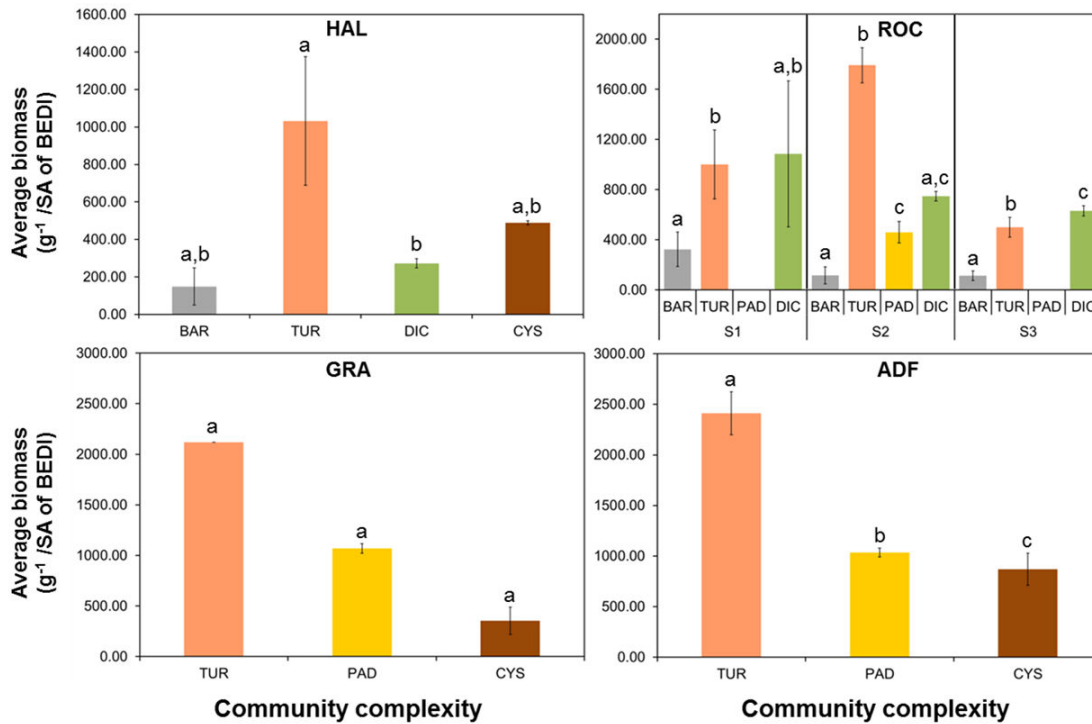


Figure 3.15. Average macroalgal biomass calculated for each community complexity in each of the sampling sites. Biomass is expressed as grammes per surface area of the BEDI apparatus. Letters indicate differences using PERMANOVA pair-wise tests, where different letters specify significance at $p < 0.05$. Error bars represent ± 1 SE of the mean. (N.B. ROC was sampled a total of three times; S1, S2 and S3).

Subsequent correlation coefficient analyses investigated the relationship between *Ostreopsis cf. ovata* abundances and the total macroalgal biomass (Fig. 3.16), and for each site (Fig. 3.17) and each macroalgal community complexity (Fig. 3.18). The correlation between *O. cf. ovata* abundances and total macroalgal biomass in an area of 491 cm² is not statistically significant ($p = 0.697$).

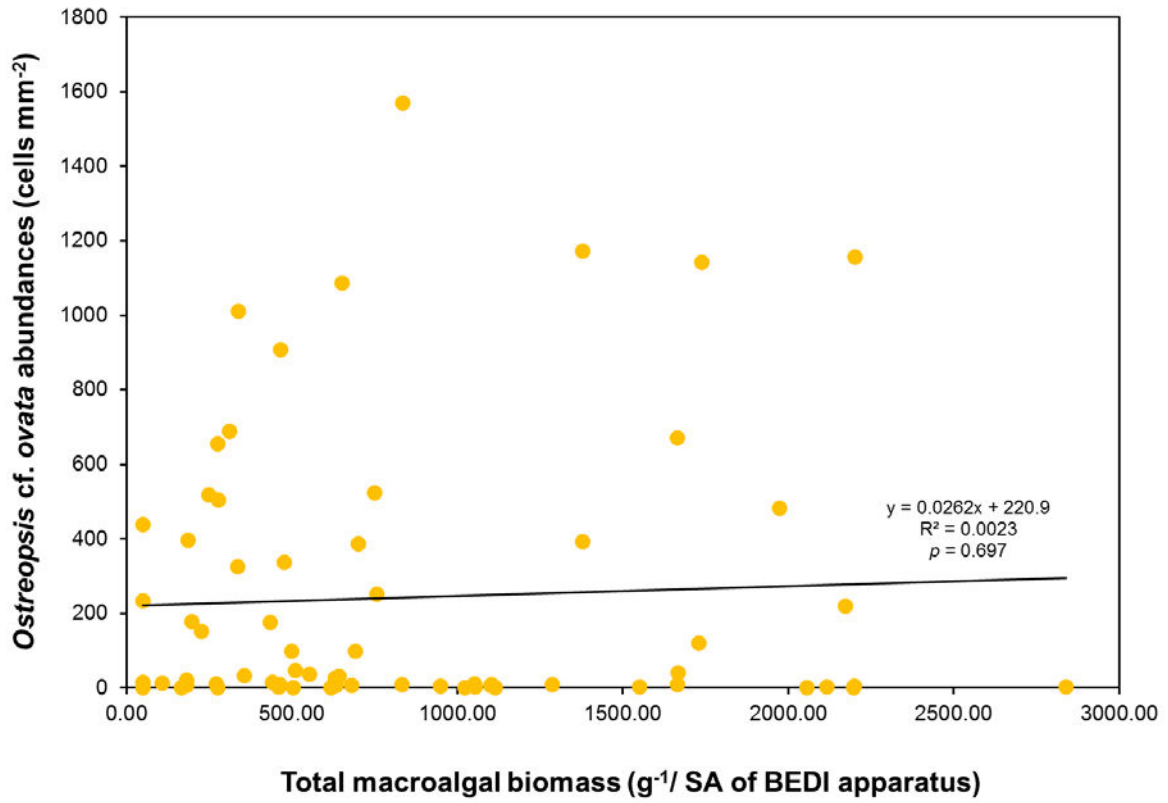


Figure 3.16. Correlations coefficient between *Ostreopsis cf. ovata* abundances and total macroalgal biomass (g) in an area of 491cm². *P* values is at the 95% confidence level.

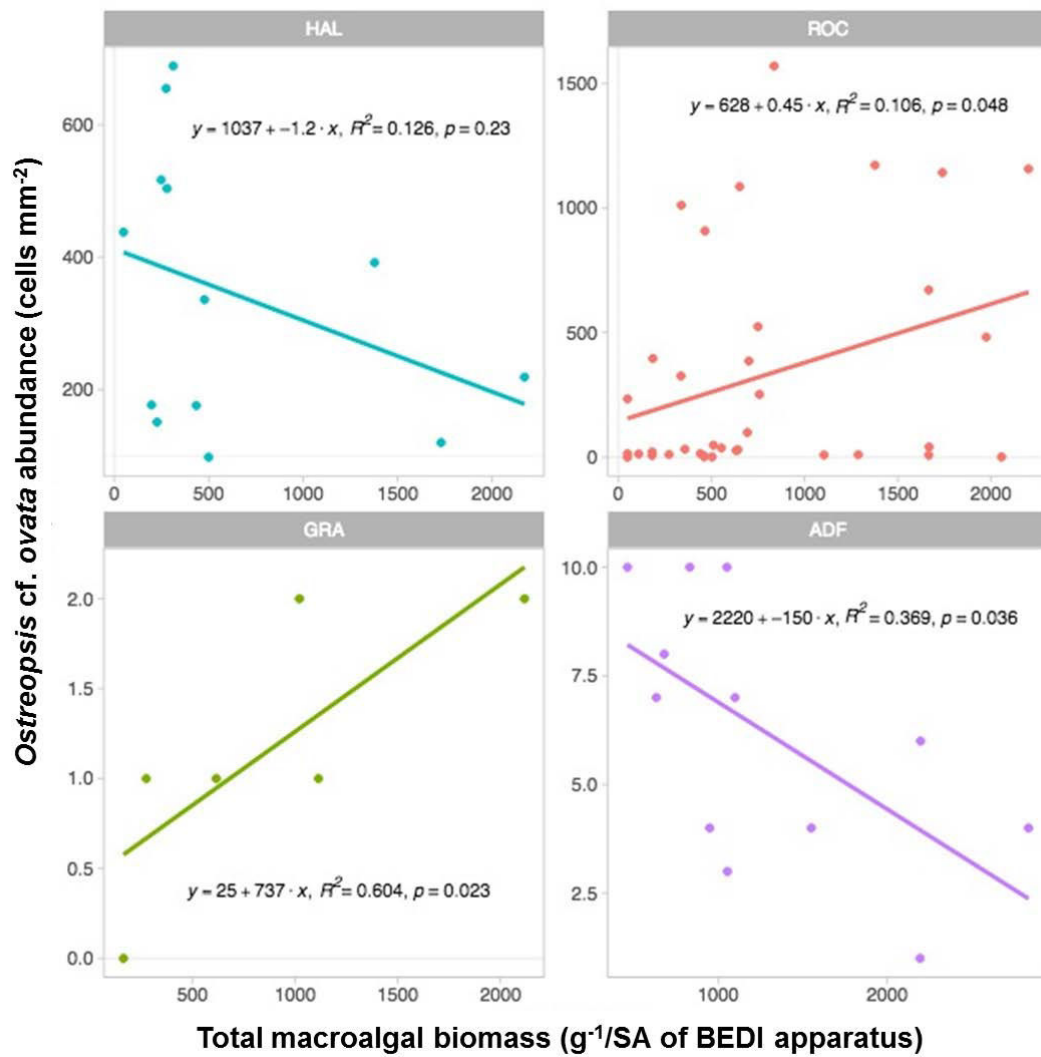


Figure 3.17. Correlations between macroalgal biomass and *Ostreopsis cf. ovata* abundances (cells mm⁻²) in the different sampling sites (HAL, ROC, GRA and ADF) in the summer of 2016. Significant *p* values for *p* < 0.05.

The results in Figure 3.17 revealed that the correlations are different in each site. There was a statistically significant correlation between dinoflagellate abundance and macroalgal biomass in ROC, GRA and ADF; the correlation was statistically significant in all of the sites apart in HAL (*p* = 0.234). ADF exhibited a negative correlation between *Ostreopsis cf. ovata* abundances and total macroalgal biomass; there were lower cell abundances with increasing macroalgal biomass. Conversely, the results from ROC and GRA indicated the opposite; *O. cf. ovata* abundances increased with increasing macroalgal biomass (Fig. 3.17).

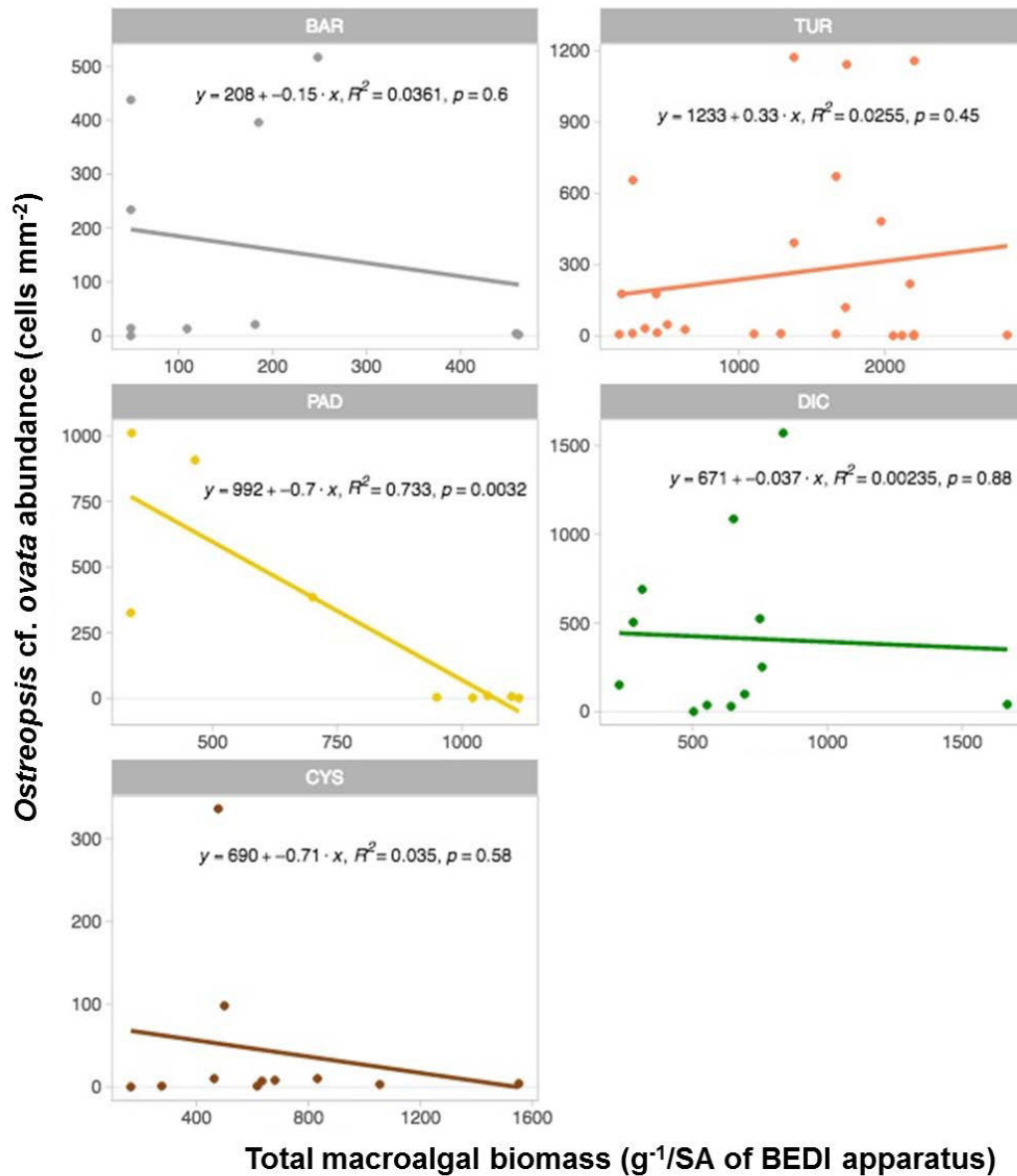


Figure 3.18. Correlations between macroalgal biomass and *Ostreopsis cf. ovata* abundances (cells mm⁻²) in the different community complexities (BAR, TUR, PAD, DIC and CYS) in the summer of 2016. Significant *p* values for *p* < 0.05.

Different correlations were also detected between *Ostreopsis cf. ovata* abundances and total macroalgal biomass for each community complexity (Fig. 3.18). From our results the correlations between macroalgal communities biomass and *O. cf. ovata* cell abundances is generally not statistically significant apart from PAD (communities dominated by *Padina pavonica*), where a negative correlation was calculated (Figure 3.18).

3.5 DISCUSSION

This study examined the population dynamics of *Ostreopsis cf. ovata* and the relation with macroalgal communities in a number of sites along the French Riviera during two consecutive summers, 2015 and 2016. Major blooms occurred at two sites along the Côte d'Azur, HAL and ROC (for definitions of *Ostreopsis* spp. blooms, please refer to Table 4 in Mangialajo et al., 2017; Appendix 1). In the sites GRA and ADF, *O. cf. ovata* abundances remained low during the sampling season (GRA in 2015 and 2016; ADF in 2015 only). The study also highlighted variations in *O. cf. ovata* abundances amongst the sites, which supports the hypothesis that there is a spatial and temporal heterogeneity in the distribution of *Ostreopsis* spp. in the Côte d'Azur (Cohu and Lemée, 2012; Cohu et al., 2011, 2013). Inter-site variations of epibenthic dinoflagellate abundances were also observed on a large spatial scale in temperate and tropical regions (Grzebyk et al., 1994; Chang et al., 2000; Aligizaki and Nikolaidis, 2006; Parsons and Preskitt, 2007; Richlen and Lobel, 2011; Cohu et al., 2013). Spatial heterogeneity could suggest a preference for specific areas (Cohu et al., 2013). The differences in cell abundances between each site could be explained by potential differences in morphology (surface area) of the macroalgal community assemblages present and/or anthropogenic pressures near the sampling sites. HAL is situated next to the Nice Côte d'Azur airport and to Nice Haliotis, a wastewater treatment plant. This treatment plant receives polluted water from several cities and towns; Nice, Aspremont, Cantaron, Castagniers, Colomars, Falicon, Levens, La Roquette sur Var, Saint André de la Roche, Saint Blaise, la Trinité, Tourrettes Levens, Saint Martin du Var, and has a capacity of 650,000 inhabitant equivalents (EqH), but treats 50 % on a daily basis. One inhabitant-equivalent uses an average of 150 liters of water and disposes of 60 g of organic matter per day per person. This therefore means that over 48 million liters of polluted water is treated on a daily basis ([url: goo.gl/JYqptx](http://goo.gl/JYqptx)).

For the *in situ* field experiments carried out in 2015 and 2016, a new sampling method was used, the BEDI (Mangialajo et al., 2017; see Appendix 1) which reduced complications related to the mass of different macroalgal species, problems of potential macrophyte preference by BHAB dinoflagellates and allowed data to be compared across different sampling stations (Mangialajo et al., 2017). Previously, abundances of BHAB dinoflagellate have most commonly been quantified by collection

of macrophytes, and the cells are then concentrated and enumerated using standard microscopy methods (Litaker et al., 2010). A few studies have highlighted the need for new methodologies to standardise sampling and enumeration procedures when quantifying epibenthic dinoflagellates (Bomber et al., 1989; Lobel and Anderson., 1988; Tester et al., 2014). The BEDI can be used across multiple spatial and temporal scales in any environment independent of the availability of macroalgae or other biotic substrates.

The interaction/correlation of *Ostreopsis* spp. abundances on varying macroalgal community complexities has not been studied before. Although *Ostreopsis* spp. have been observed on many different types of substrates, including a variety of algal species, seagrasses, dead biotic substrates, rocks and sediments (Totti et al 2010; Richlen and Lobel, 2011; Cohu et al., 2013). The results of the present study demonstrate an important variability in the abundance of *Ostreopsis* cf. *ovata* depending on macroalgal community complexity, and community biomass at the sampling site. In the summer of 2015, higher abundances of *O.* cf. *ovata* were recorded in sites which were dominated by less complex community structures; TUR and DIC, while sites with *Cystoseira* communities (CYS) did not harbour significant microalgal blooms. These results imply that coastal regions with a dominance of *Cystoseira*-composed-communities could potentially inhibit *Ostreopsis* spp. blooms. The coast along the Côte d'Azur is witnessing a typical regime shift from macroalgal forests to less structured assemblages dominated by macroalgae such as *Halopteris scoparia*, *Padina pavonica*, *Laurencia complex*, ribbon-like Dictyotales, erect Corallinales and small filamentous algae (such as Ceramiales or Sphacelariales), or by barren grounds dominated by encrusting algae and sea urchins (Scala et al., 1998; Thibaut et al., 2015). These phase shifts are reasonably a consequence of increasing urbanisation and multiple human-induced impacts. The modifications in macroalgal assemblages due these impacts are indirectly influencing and potentially facilitating *O.* cf. *ovata* blooms in the Côte d'Azur. How local urbanization and eutrophication modify macroalgal community assemblages could also provide an explanation for the spatial heterogeneity in *O.* cf. *ovata* blooms along the Côte d'Azur, the area of the research of this study. It is, however, important to note that in our study, the sites that harboured significant blooms did not have *Cystoseira* spp. natural populations, and thus it cannot

be concluded with certainty that *Cystoseira* spp. algal communities have an active role in regulating the proliferation of *O. cf. ovata* blooms in the Côte d'Azur.

Ostreopsis cf. ovata abundances were positively correlated with macroalgal biomass in four chosen sites along the Côte d'Azur. This correlation is to be expected, the greater the biomass, the more surface area there is available for *O. cf. ovata* cells to attach on. However, when observing the correlation by site and by macroalgal community complexity, the results indicate heterogeneity in *O. cf. ovata* abundances. These results imply that *O. cf. ovata* blooms are site specific. The patterns observed could reflect preferences for a particular algal type (Accoroni et al., 2016; Accoroni and Totti, 2016), or even a particular type of structure/ morphology (Taylor, 1985; Lobel et al., 1988; Bomber et al., 1989; Vila et al., 2001b).

Previous studies have speculated that variability seen in the benthic dinoflagellate *Gambierdiscus*-host interactions was due to structural or chemical defences of the host (i.e. macrophyte) against herbivory (e.g. Anderson and Lobel, 1988; Cruz-Rivera and Villareal, 2006). Submersed macrophytes in infralittoral zones face severe competition especially for light with epiphytes and phytoplankton, and also with other macrophytes for space and nutrients. Limited spatial distance between competing organisms in benthic and littoral habitats makes allelopathy a powerful strategy enhancing survival (Gross, 1999). Macrophytes produce secondary metabolites against pathogens, parasites, or predators to enhance their survival in their tough environment. Furthermore, the secondary metabolites may inhibit the growth or survival of competing species (Núñez-Ponz and Avila, 2015). Inhibitory allelochemicals seem broad in their specificity toward certain target organisms (including epiphytic benthic dinoflagellates) or cyanobacteria (Gross et al., 1991) and provide a wide range of defensive mechanisms in order to survive in their environment (Núñez-Ponz and Avila, 2015). For example, brown algae of the family Dictyotaceae (e.g. *Dictyota*) produce diverse classes of diterpenes that function as defences against marine herbivores and competitors (Puglisi et al., 2014). This could be true for *Ostreopsis* spp. – host interactions. A study conducted in the Conero Riviera (Northern Adriatic Sea) highlighted negative interactions that occurred between *Dictyota dichotoma* (brown algae) and *Ostreopsis cf. ovata* (Accoroni et al., 2015b). The results of the study indicated that *D. dichotoma* inhibited the growth of *O. cf. ovata* and induced the

formation of cysts, suggesting that these inhibiting effects could be of allelopathic nature (Accoroni et al., 2015b).

The heterogeneity in cell abundances between the different macroalgal community structures observed at the sampling sites in this study could be linked to a less desirable substrate from the perspective of *Ostreopsis* spp. The surface area/morphology of individual macrophyte species could be considered for the discrepancies in the results as well as the overall macroalgal community composition. Furthermore, these discrepancies could reflect differences in physical/environmental conditions (factors that have not been considered in this study) among sites.

The environmental parameters measured in this study varied by site. Compared to other temperate seas, the Mediterranean Sea is characterised by extremely low nutrient concentrations (Pasqueron de Fommervault et al., 2015). The median nitrate and phosphate concentrations in the present study are in line with the median inorganic nutrient concentrations measured in the sea surface of the Ligurian Sea (Pasqueron de Fommervault et al., 2015), confirming the oligotrophic regime in the sampling region in the summer months with high values of N:P ratios. This suggests an incomplete removal of nitrate once all phosphate sources are consumed by microalgae (i.e. typical of P-limited oligotrophic configurations; Pasqueron de Fommervault et al., 2015), such as benthic dinoflagellates like *Ostreopsis* spp.

Concerning the inorganic sources of nutrients, there is very limited information on the relationship between nutrient concentrations and the stimulation of *Ostreopsis* spp. blooms (Cohu et al., 2011; Accoroni and Totti, 2016; Berdalet et al., 2017). Previous studies have mentioned that there are no associations observed between inorganic nutrient (nitrogen, phosphorous and silicon) concentrations and benthic dinoflagellate distributions in tropical and temperate regions (Grzebyk et al., 1994; Vila et al., 2001). A study in Brazil by Nascimento et al., (2012) concluded that the high abundance of *O. cf. ovata* at the St Paul's rocks (a small inhabited island located 1000 km away from the Brazilian mainland) is an indication that eutrophication is not a main driver in influencing blooms of this species in this particular region (Nascimento et al., 2012). Conversely, there are studies that have demonstrated a positive correlation between benthic dinoflagellates and inorganic nutrients (e.g. Delgado et al., 2006; Parsons and Preskitt, 2007; Skinner et al., 2013), this is in accordance with the results from the

present study, whereby elevated concentrations of nitrogen compounds (nitrates, nitrites and ammonium) were measured in the two sites (HAL and ROC) that had significant blooms in the summers of 2015 and 2016. The extent of mixotrophy among benthic dinoflagellate taxa is still unknown. However, a recent study confirmed the strong affinity of *O. cf. cells* for ammonia (NH_4^+) in the Bay of Villefranche-su-Mer, South of France (Jauzein et al., 2017). This could be a plausible explanation for the success of this species in some of the selected sites in our study in the Côte d'Azur. For most mixotrophic benthic dinoflagellates, information on functional response to irradiance, dissolved organic and inorganic nutrients and prey quality and quantity are unavailable (Stoecker, 1999). This information is also fundamental to understanding the ecology of benthic dinoflagellates, such as *Ostreopsis* spp., to efficiently mitigate annual harmful blooms in coastal regions.

Several trace metals are essential, at very low concentrations, for living organisms, but most are toxic at elevated concentrations and have direct and adverse effects on various physiological and biological processes (Rhodes et al., 2006; Debelius et al., 2009). One of the most studied toxic effects of metals on microorganisms is in growth inhibition (Debelius et al., 2009). Trace metal concentrations in the Mediterranean Sea are homogeneous, with higher concentrations in surface waters than those found in oceanic waters (Morley et al., 1997; Heimbürger et al., 2011). Trace metal enrichments, particularly in coastal regions, have been attributed to external outputs such as run-offs, atmospheric deposition or eolian effects, and vertical mixing and insufficient assimilation or recycling by organisms (Elbaz-Poulichet et al., 2001). Our results show that Cu concentrations varied in all the sites and that the concentrations were quite elevated, which is in agreement with Heimbürger et al., (2011), who demonstrated that during the summer oligotrophic regime on the Ligurian coast, Cu always presents surface enrichment profiles (Heimbürger et al., 2011). The high Cu concentration observed in the present study could indicate that the concentrations were above the threshold for the ability of phytoplankton to assimilate Cu and to therefore transfer it to deeper waters (Heimbürger et al., 2011). The Mediterranean distribution of Cu concentration is dominated by lateral transport and vertical mixing processes (Morley et al., 1997). Surface water enrichments of Cu can be clearly linked to terrestrial inputs (e.g. riverine, agricultural run-offs, atmospheric deposition; Morley

et al., 1997) and from copper-based antifouling paints used on vessel hulls (Claisse and Alizieu, 1993; Schiff et al, 2004)

Since the mid-1980's, the Pb concentrations in the atmospheric fallout have decreased (Migon et al., 2008) due to reductions of Pb in petrol. A peak in Pb concentrations was observed in HAL just before the *O. cf. ovata* bloom in 2015, but the other sites that supported *O. cf. ovata* blooms in the same summer did not sustain elevated Pb concentrations. Thus, it is not clear whether Pb concentrations influence *O. cf. ovata* bloom dynamics in the Côte d'Azur.

Zinc is mainly of urban origin, and the Ligurian Sea is subject to significant emissions from the large urbanised area between Toulon and Genoa (Heimbürger et al., 2011). Moreover, significant amounts of Zn are emitted by waste incineration (Nriagu and Pacyna, 1988). Only one major peak in Zn concentration was observed in this study in ADF, a site that did not support an *O. cf. ovata* bloom in the summer of 2015. Thus, this proposes that Zn is not linked with *O. cf. ovata* cellular growth rates.

The results from this study indicated that in the Côte d'Azur, there are greater abundances of *O. cf. ovata* cells in sites with slightly elevated water motion. Intense blooms (i.e. cell concentrations $\geq 1,000,000$ cells g^{-1} FW algae) were observed in HAL and ROC, and a slight bloom was observed ($\geq 100,000$ cells g^{-1} FW algae) in PCF. Interestingly, *O. cf. ovata* blooms were not prominent in the two sheltered sites, GRA and ADF. These results are not in line with studies that have stated that higher dinoflagellate abundances occur in sheltered sampling sites (Tindall and Morton, 1998; Totti et al., 2010; Accoroni and Totti, 2016). However, they concur with those observed by Vila in 2001, concluding that *Ostreopsis* spp. is better adapted to slightly turbulent environments (Vila et al., 2001). A study conducted by Richlen and Lobel in 2011 determined that calm lagoon/ channel sampling sites supported significantly higher total benthic dinoflagellate abundance compared to reef sites that were subject to wave activity (Richlen and Lobel, 2011). However, the study also concluded that *Ostreopsis* spp. abundances were present in greater proportions at high energy reef sites, similar to the observations of the spatial variability recorded in Vila et al's study in 2001 (Vila et al., 2001a; Richlen and Lobel, 2011).

Therefore, from the results, it can be suggested that water motion is a mechanism that could shape species fitness. Turbulence is a common physical factor in the ecology of phytoplankton, including benthic dinoflagellates, and is involved in the seasonal succession and bloom formation of phytoplankton species (Margalef, 1978; Smayda, 2001). It is still unclear as to why certain dinoflagellate taxa such as *Ostreopsis* spp. are more resistant to wave motion, whereas others seem to be highly sensitive (Richlen and Lobel, 2011). One explanation could be that *Ostreopsis* spp. adheres to different substrates by producing mucopolysaccharide filaments and mucous layers (Vila et al., 2001a; Honsell et al., 2013; Berdalet et al., 2017). The ability of *Ostreopsis* spp. to produce an effective holdfast appears to allow them to persist in 'shaken' areas and perhaps provide an advantage to these species in the competition for space in the benthic microbial community (Richlen and Lobel, 2011).

Many hydrological, chemical and biological processes interact to control distributions of trace metals in coastal surface waters (Heimbürger et al., 2011), especially in the Mediterranean surface waters. The trace metal concentrations detected in the present study do not seem to be linked with *Ostreopsis* spp. cellular growth rates and cannot be used to explain *O. cf. ovata* bloom dynamics in the Côte d'Azur.

Overall, unfavourable conditions such as nutrient limitations, heavy metals concentrations in the seawater and strong turbulence can cause physiological stress and reduced growth, affecting the species fitness and survival (Sengupta et al., 2017). This is also true for benthic dinoflagellate species, such as *O. cf. ovata*. This study could not highlight a specific environmental factor that could explain the variability of *O. cf. ovata* abundances in the sampling sites used for the field studies. There are numerous environmental factors that are strongly linked together and it is very difficult to identify a sole factor as the reason for spatial variability in *O. cf. ovata* abundances at the chosen sampling sites for this study. The need for better understanding of the causes of these blooms, and why they have been increasing in number and geographical spread, is therefore becoming increasingly urgent.

3.6 SUPPLEMENTARY MATERIALS

Table S1. Taxa found in the macroalgal sorting of the field experiment of 2015. Algal species were identified in the laboratory.

	BEFORE (May/June)	AFTER (August)	
Taxon	Species or genera		Acronym
Chlorophyta			
	<i>Acetabularia acetabulum</i>	<i>Acetabularia acetabulum</i>	ACE
	<i>Caulerpa</i> spp.	<i>Caulerpa</i> spp.	CAU
	<i>Caulerpa racemosa</i>	<i>Caulerpa racemosa</i>	
	<i>Caulerpa taxifolia</i>	<i>Caulerpa taxifolia</i>	
	<i>Cladophora</i> spp.	<i>Cladophora</i> spp.	CLADOPH
	<i>Valonia</i> spp.	<i>Valonia</i> spp.	
	<i>Flabellia petiolata</i>		
		<i>Halimeda tuna</i>	
	<i>Ulva</i> spp.	<i>Ulva</i> spp.	ULV
Phaeophyta			
	<i>Cladostephus</i> spp.		CLAD
	<i>Colpomenia sinoussa</i>	<i>Colpomenia sinoussa</i>	COLP
	<i>Cystoseira</i> spp.	<i>Cystoseira</i> spp.	CYS
	<i>C. amentacea</i>	<i>C. amentacea</i>	
	<i>C. brachycarpa</i>	<i>C. brachycarpa</i>	
	<i>C. compressa</i>	<i>C. compressa</i>	
	<i>C. spinosa</i>	<i>C. spinosa</i>	
	<i>Dictyopteris polypodioides</i>	<i>Dictyopteris polypodioides</i>	DIC. POL
	<i>Dictyota</i> spp.	<i>Dictyota</i> spp.	DIC
	<i>Padina pavonica</i>	<i>Padina pavonica</i>	PAD
	<i>Taonia atomaria</i>	<i>Taonia atomaria</i>	TAO
	<i>Halopteris/Sphacelaria</i>	<i>Halopteris/Sphacelaria</i>	
	<i>Halopteris scoparia</i>	<i>Halopteris scoparia</i>	HAL
	<i>Sphacelaria cirrhosa</i>	<i>Sphacelaria cirrhosa</i>	SPHA
	<i>Sargassum vulgare</i>	<i>Sargassum vulgare</i>	SAR
Rhodophyta			
	<i>Acrosorium ciliolatum</i>		ACRO
	<i>Amphiroa rigida</i>	<i>Amphiroa rigida</i>	AMPH
	<i>Asparagopsis</i> spp.		
	<i>Ceramiales</i> spp.	<i>Ceramiales</i> spp.	CERAM
	<i>Digenea simplex</i>	<i>Digenea simplex</i>	
	<i>Myriogramme tristomatica</i>	<i>Myriogramme tristomatica</i>	
		<i>Chondria</i> spp.	CHO
	<i>Corallina caespitosa</i>	<i>Corallina caespitosa</i>	COR
		<i>Gelidium spinosum</i>	
	<i>Hypnea musciformis</i>	<i>Hypnea musciformis</i>	HYP
	<i>Jania rubens</i>	<i>Jania rubens</i>	JAN
	<i>Laurencia complex</i>	<i>Laurencia complex</i>	LAU
	<i>Peyssonnelia</i> spp.	<i>Peyssonnelia</i> spp.	PEY
	<i>Schottera nicaeensis</i>	<i>Schottera nicaeensis</i>	SCHO
	<i>Titanoderma ramossissimum</i>		ECR

3.7. ADDITIONAL EXPERIMENTAL RESULTS

3.7.1 Interaction laboratory experiment

A number of studies underlined the importance of host architecture and morphology (Lobel et al., 1988; Bomber et al., 1989), whereas others emphasise the importance of macrophyte secondary metabolites (Cruz-Rivera and Villareal, 2006; Accoroni et al., 2015b) in the relationship between benthic dinoflagellates and biotic substrates.

The primary purpose of the interaction bioassay experiment was to determine how *Ostreopsis* cf. *ovata* cells interacted with various macrophyte host species, and to assess any possible allelopathic interactions. During the *O.* cf. *ovata* bloom season in the summer of 2016, different macrophyte species were collected by snorkelling from GRA (bay of Villefranche-sur-Mer, France; Fig. 3.1.) at 0.5 m depth. This was done to test the potential interactions between *O.* cf. *ovata* cells and macrophytes in the Côte d'Azur. The macrophytes for the experiment were chosen based on their frequent presence in the site and/or by their previously known associations with *Ostreopsis* spp. populations in past studies. Macrophyte species were identified to species level, to the best of our ability using taxonomic keys and descriptions (Boudouresque et al., 2006). For species identification, please refer to Table S2. In addition, 10 litres of surface seawater with high *O.* cf. *ovata* abundances were collected at ROC (bay of Villefranche-sur-Mer, France; Fig. 3.1) as this site is known to have high concentrations of *O.* cf. *ovata* cells during the summer months, on the same day as the laboratory experiments. The seawater was filtered through a phytoplankton mesh of 500 µm and then again through a mesh size of 20 µm. The residue collected on the 20 µm was added to 1 L of filtrate from the 20 µm mesh to concentrate the seawater with *O.* cf. *ovata* cells.

The filtered, *O.* cf. *ovata* concentrated, seawater was stirred on a magnetic stirrer (Stuart UC151, ©Bibby Scientific Ltd, UK) at a very low speed to avoid damaging the cells. The purpose of this procedure was to ensure a homogenous concentration of *O.* cf. *ovata* cells during the setup of the treatments. Please note that since ambient seawater was used, other benthic dinoflagellates and diatoms (e.g. *Prorocentrum lima*, *Coscinodiscus* spp., *Licmophora* spp.) species were present but were not counted. It was however ensured that *O.* cf. *ovata* was the most abundant dinoflagellate species through microscopic visual observations before setting up the experiments.

The macroalgal species collected were thoroughly rinsed with filtered seawater in order to remove any epiphytic materials that were attached to the macrophyte. Epiphytic material and detritus were also removed by hand, using tweezers and filtered seawater. Then, for each macrophyte species, three small pieces of algal tissue (fragments of approximately 0.004 g dry weight) were cut and placed into individual 50 mL TPP tissue culture flasks (Techno Plastic Products, AG, Switzerland) with a vented filter cap. Thus, there were triplicates for each algal species. Fifteen mL of the *O. cf. ovata* concentrated ambient seawater was added to each culture flask containing a fragment of macrophyte tissue (See Fig. S1 below).

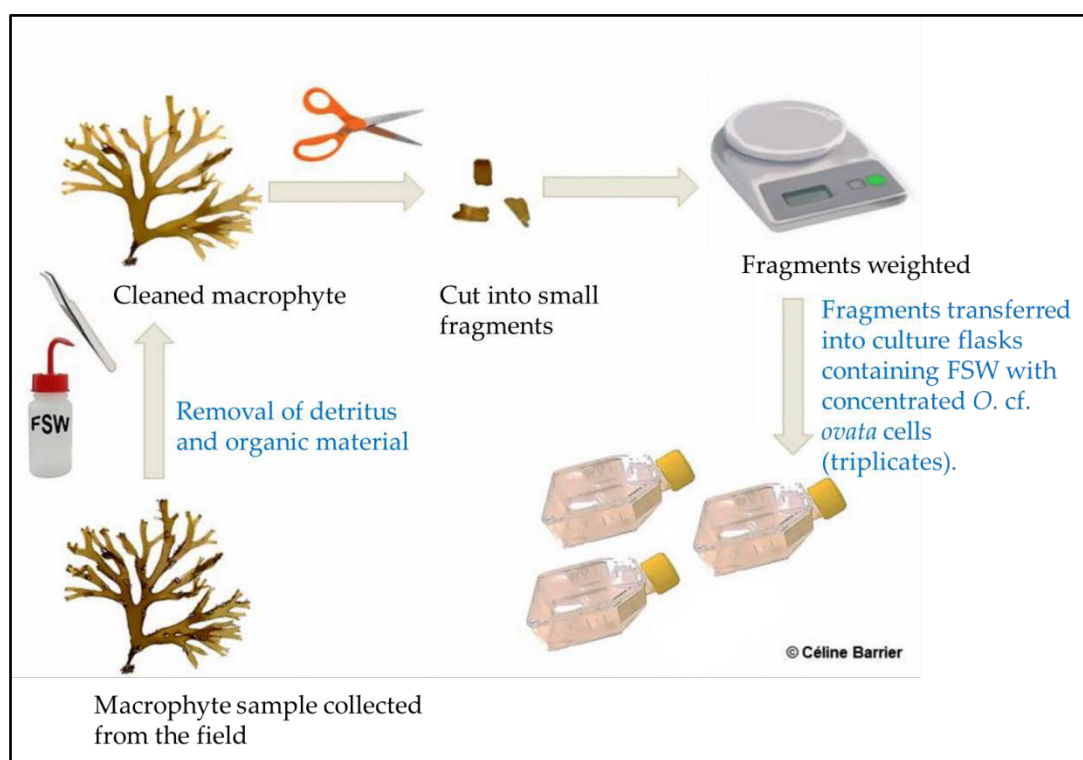


Figure S1. Schematic representation of the method used to clean, cut and weight macrophyte fragments before putting them in culture flasks for the subsequent experiment.

The basic experimental design was to place *O. cf. ovata* cells in close living association with various host macrophyte species to monitor the behaviour of the cells in response to the hosts. The tissue culture flasks were left in the laboratory at room temperature away from direct sunlight (the average room temperature was not measured, thus the exact temperature is not known) The number of moving and non-moving *O. cf. ovata* cells around the macrophyte fragment (counts were done on cells surrounding the macrophyte fragment within the optic field of the microscope, which

was a diameter of 11 mm, at a x 215 magnification) as well as cells on the macrophyte fragments were counted and recorded over the course of three days at 24-hour intervals. The samples were not 'sacrificed', the cell counts were performed on a continuous culture for the duration of the experiment. Cell counts were conducted using a binocular microscope (Carl Zeiss Stereo Discovery V12, Germany). All counts used x 215 magnification.

After the last counts on the third day, photos were taken of the macrophyte samples as well as length and width measurements in order to calculate the surface area of the samples using the software Image J[®]. The macrophyte fragments were then oven dried at 60°C for 3 hours (Memmert UNS model 100-800, Germany), and the dry weight of the macrophytes was measured to the nearest 0.001 g (AB135-S/FACT DualRange Analytical Balance, Mettler Toledo, Switzerland). The cell abundances were expressed as cells per dry weight of macrophyte (cells g⁻¹ DW). This experiment was repeated three times; T1 (12 July), T2 (25 July) and T3 (9 August) (before, during and after the *O. cf. ovata* bloom of 2016, therefore the starting cellular concentration was different for each experiment). All data were converted to relative abundances (i.e. % cells moving; % cells not moving; % cells on macrophyte). A Bray-Curtis similarity index was used to cluster (group) the different macrophyte hosts according to their resemblances in the three parameters (% moving; % not moving; % on macrophyte), to provide a preliminary assessment of cell interactions between *O. cf. ovata* and the different hosts. Only the data for the 48-hour interval were used to determine the clusters. The similarity profile permutation test (SIMPROF) procedure was used to determine which clusters were significantly different at the 95% significance level. All statistical analyses were computed using PRIMER 6 & PERMANOVA + software package.

3. 7. 2 Preliminary Results

For the following results, only the data from T2 and T3 were used as in both experiments the same macrophyte species were used. In Figure S2 A and B, the histogram illustrates that over time there is a decrease in the relative abundance of *O. cf. ovata* cells that are moving and an increase of *O. cf. ovata* cells that are not moving. Moreover, Figure S4 A and B illustrates that over time, the relative abundance of *O. cf.*

ovata cells on the macrophyte increases on selected host species; AMPH, PAD and POS.

Table S2. General description of the host macrophyte used in the study including genus, species, phyla, palatability and functional groups descriptions.

Macrophyte Host	Algal host abbreviation	Authorities	Phyla	Palatability	Functional -form Group
<i>Amphiroa rigida</i>	AMPH	J.V.Lamouroux, 1816	Rhodophyta	Palatability is variable with some tropical species being less palatable than their temperate counterparts. ³	Jointed calcareous ²
<i>Caulerpa racemosa</i>	CAU	(Forsskål) J.Agardh,	Chlorophyta	Chemically defended. ¹	Coarsely branched ²
<i>Corallina caespitosa</i>	COR	R.H.Walker, J.Brodie & L.M.Irvine, 2009	Rhodophyta	Structurally defended, low palatability. ³	Jointed calcareous ²
<i>Cystoseira amentacea</i>	C. amen	(C. Agardh) Bory de Saint - Vincent	Phaeophyta	Chemically defended, still palatable. ⁵	Coarsely branched
<i>Cystoseira brachycarpa</i>	C. brac	Var. <i>balearica</i> (Sauvageau) Giaccone	Phaeophyta	Chemically defended, still palatable. ⁵	Coarsely branched
<i>Cystoseira compressa</i>	C. comp	(Esper) Gerloff & Nizamuddin	Phaeophyta	Chemically defended, still palatable. ⁵	Thick leathery
<i>Halopteris scoparia</i>	HAL	(Linnaeus) Sauvageau	Phaeophyta	Chemically defended, low-intermediate palatability. ³	Coarsely branched ³
<i>Padina pavonica</i>	PAD	(Linnaeus) Thivy	Phaeophyta	Lightly to moderately calcified. Tropical species can be significantly less palatable than temperate counterparts. ³	Sheet ²
<i>Posidonia oceanica</i>	POS	(Med.)	Tracheophyta	Chemically defended, still palatable. ⁴	Sheet

1- Norris and Fenical, (1982); **2-** Littler et al., (1983); **3-** Cruz-Rivera and Villareal, (2006).

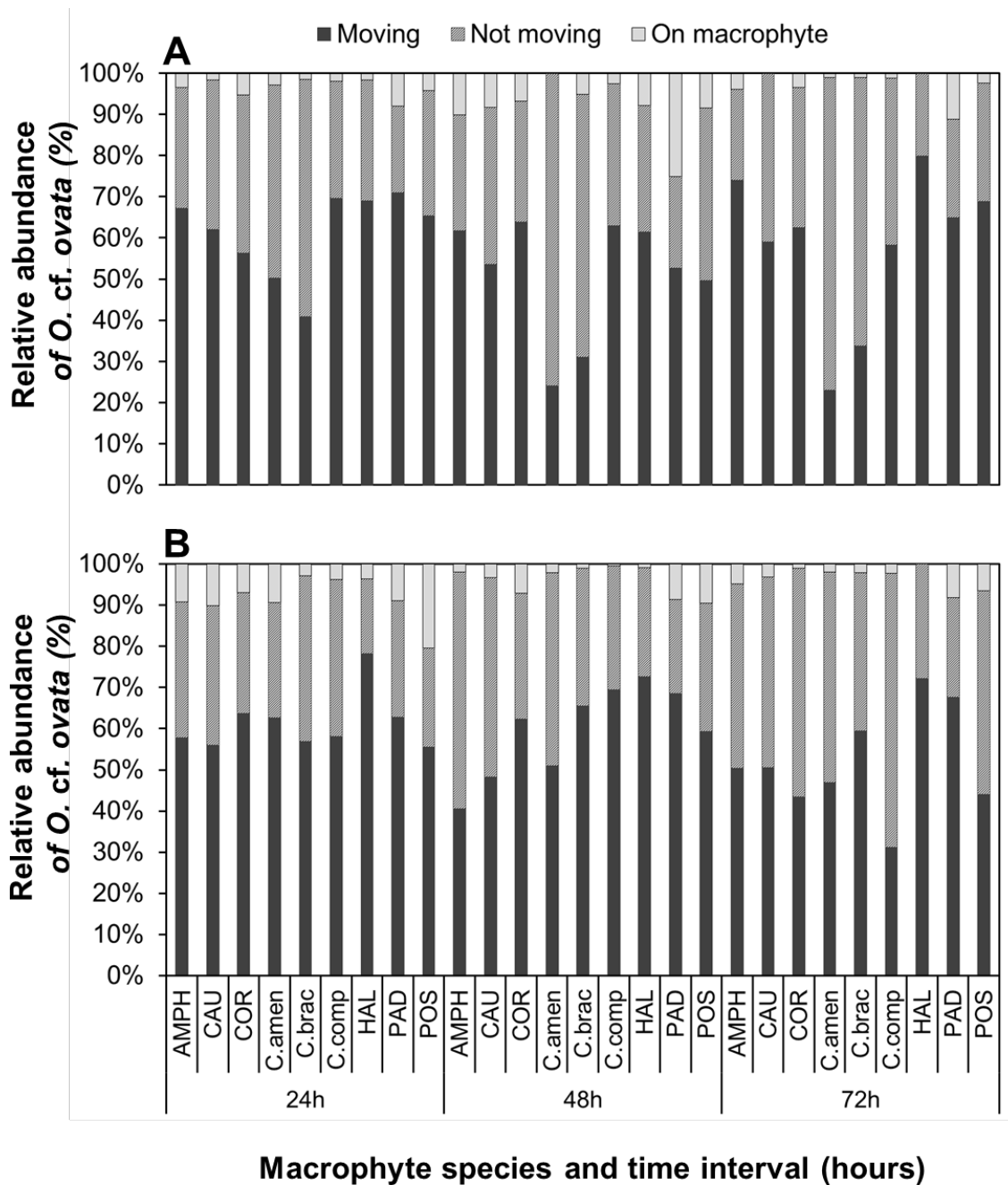


Figure S2. Histograms illustrating the relative abundances (%) of *Ostreopsis cf. ovata* cells moving around the macrophyte; cells not moving around the macrophyte; and cells on the macrophyte at the three time intervals: 24 hours, 48 hours and 72 hours. **A)** T2 and **B)** T3.

The cluster analyses results (Fig. S3) indicate that the host species in both A) T2 and B) T3 can be grouped into two significantly different groups. In T2, one group was composed by *Cystoseira amentacea* and *Cystoseira brachycarpa* and the other group was composed by the other seven macrophytes. In T3, one group was made up of *Caulerpa racemosa*, *Amphiroa rigida* and *Cystoseira amentacea* and the other group was composed of *Caulerpa racemosa*, *Cystoseira brachycarpa*, *Cystoseira compressa*, *Padina pavonica*, *Posidonia oceanica* and *Corallina caespitosa*.

Highest concentrations of *O. cf. ovata* , 1,542 and 457 cells per cm² of macrophyte, were associated with *Halopteris scoparia* and *Caulerpa racemosa*, respectively. Low concentrations of *O. cf. ovata* , lower than 100 cells per cm² of macrophyte, were found on *Padina pavonica* and *Posidonia oceanica*.

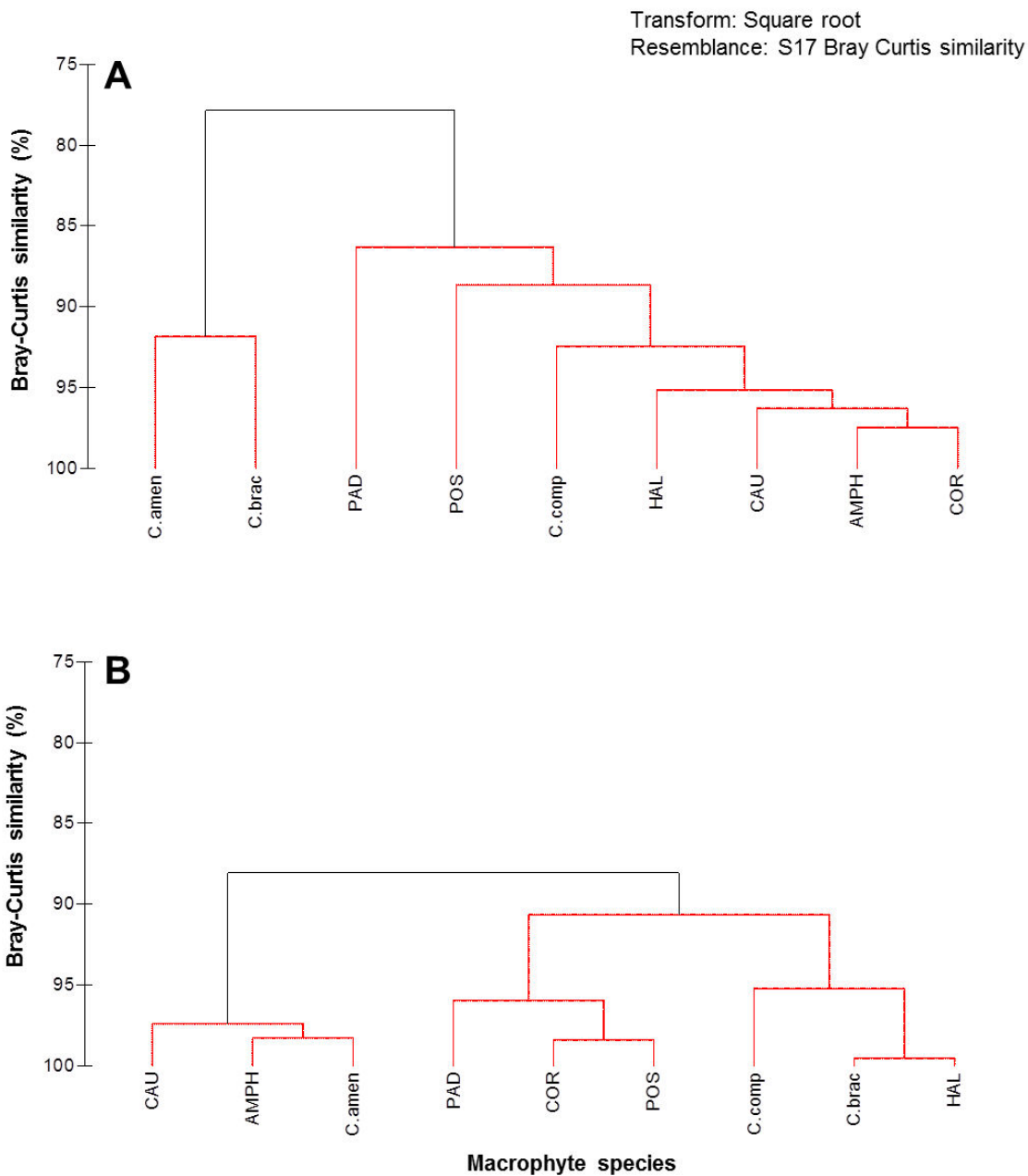


Figure S3. Hierarchical cluster analyses of the various macrophyte species according to their similarity in the three separate parameters (% cells moving /surface area; % cells not moving / surface area; % cells on macrophyte/surface area); A) T2 and B) T3. The red lines indicate significantly different samples. The macrophyte host abbreviations are shown in Table S2.

3. 7. 3 Discussion and Perspectives

The results obtained from this experiment potentially indicates that *O. cf. ovata* exhibits macrophyte substrate preferences (Fig. S3). Thus, the hypothesis that there is a possible allelopathic relationship between *O. cf. ovata* and selected macrophyte species cannot be excluded in the Côte d'Azur.

On the first inspection, these results suggest that *Ostreopsis cf. ovata* has a preference for certain macrophyte species in the early stages of colonisation (approx. 48 h). Biochemical factors that shape dynamics of BHABs are largely unknown. Marine systems experience the highest rates of herbivory on earth (Cruz-Rivera and Villareal, 2006) and consequently macroalgae have evolved complex strategies and defences that allow them to prevail in areas where marine plant-herbivore interaction are intense (Hay, 1997; Paul et al., 2001). Past studies have concluded that there are significantly higher *O. cf. ovata* abundances on pebbles than on macroalgae (Totti et al., 2010; Accoroni et al., 2011) hypothesising that the allelochemical compounds produced by macroalgae have an inhibitory effect this species (Accoroni and Totti, 2016). Many macroalgae deter herbivores through chemical defences by the production of noxious secondary metabolites (allelochemicals) (Cruz-Rivera and Villareal, 2006). The inhibitory effect of macroalgae toward benthic dinoflagellates has been investigated previously and could be interpreted as due to various factors including nutrient and light competition, changes in pH and the production of allelochemicals (Accoroni et al., 2015b). However, contrary to this, it has also been observed that a wide variety of macroalgae producing allelochemicals are known to host intermediate to dense populations (between 1,000s – 100,000s cells g⁻¹ FW) of *Gambierdiscus toxicus* and other benthic dinoflagellates (Cruz-Rivera and Villareal, 2006).

The results obtained from the additional experiments potentially add to the experimental evidence that there is an allelopathic interaction between macrophytes and benthic dinoflagellates (Accoroni et al., 2015b) reducing the health/ motility of *O. cf. ovata* cells. The use of allelopathic compounds produced by macrophytes as algicidal products in the

prevention, control and mitigation of BHABs should not be overlooked as a potential solution for blooming events.

A number of things can be modified to improve this experiment. Firstly, the cell counts and observations should be extended in time, to maybe 10 days, to really observe the growth rate of *O. cf. ovata*; and, to increase the number of replicates to a minimum of 10 for each host species. This would allow having a statistically robust set of data. It would also be interesting to investigate the effect of irradiance, water motion and turbidity, and sedimentation on the host chemistry interaction between *O. cf. ovata* and macrophyte species. In order to ensure all of the samples are under constant experimental conditions, it can be suggested to incubate the samples in a cool light incubator, where the irradiance and temperature can be accurately regulated and monitored. Another way to improve the experimental design would be to acclimatise the macroalgae fragments before incubating them with *O. cf. ovata* cells, in similar ways described in Rains and Parsons (2015), but with slight modifications. One month before the start of the experiment, large fragments of the macrophyte species that are to be tested with *O. cf. ovata* cells should be collected in the field and placed in cultures, under the same standard growth conditions as *O. cf. ovata* cultures. Before setting the macroalgae in enriched growth media, they should be cleaned in order to remove epiphytes and detritus material. Then once the macroalgae have been left to acclimatise in the growth medium, a portion of each alga should be transferred to sterile 100 mL beakers containing 50 mL ambient filtered seawater, from the Côte d'Azur, for one week. This procedure would ensure the macroalgae were growing prior to the experiment (not stressed or dying which could influence the results) using the enriched media initially, followed by the ambient water incubation period to reduce/remove the influence that the prior enrichment would have on the experiment. Then a couple of days prior to the start of the experiment, a known weight of macrophyte fragment placed in the culture flask containing 20 mL of filtered seawater. This would allow the fragments to acclimatise in the ambient seawater under the experimental conditions for a couple of days, with frequent water changes, to allow for any harmful exudates resulting from the cutting of algal thalli (to make small fragments) to disperse and

be removed before the *O. cf. ovata* cells are added (as seen in the Methods and Materials in Rains and Parsons, 2015).

3.8 ACKNOWLEDGEMENTS

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CHAPTER 4

GENERAL DISCUSSION AND PERSPECTIVES

With benthic harmful algal blooms (BHABs) being an emerging phenomenon worldwide (Shears and Ross, 2009; Parsons et al., 2012), there is an urgent need to identify common patterns and drivers of this phenomenon at a global scale as well as understanding the factors acting locally (Mangialajo et al., 2017). Currently, understanding of the roles of the biotic substrates, macroalgal community complexities and marine habitats in facilitating or shaping population dynamics of *Ostreopsis* spp. is at a very rudimentary stage, as is data on how they interact with multiple natural and human-induced factors.

Benthic dinoflagellates such as *Ostreopsis* spp. depend on a fixed substratum; therefore the naturally surrounding population of other flora and fauna associated with the substratum, and any alterations in the substratum, may be expected to affect the epiphytic dinoflagellate populations (Fraga et al., 2012).

Chapter 2 identified those generalisations that can be made from the existing literature about preferred substrates that may predict *Ostreopsis* spp. blooms. However the bibliographic literature review revealed that field studies are not yet sufficiently comprehensive in providing detailed descriptions of benthic community assemblages and marine habitats in their sampling stations to draw robust conclusions. Despite the extensive data and conclusions that have been gathered on the ecology of *Ostreopsis* species over the last five decades it is still nowhere near sufficient to understand the role of biotic interactions in bloom dynamics. Absence of understanding of the bloom dynamics of *Ostreopsis* spp. specifically means we also lack a solid baseline for global monitoring programs that would help predict and mitigate the annually reoccurring, potentially toxic microalgal summer blooms effectively.

The lack of significant data on the ecology and bloom dynamics of harmful species revealed by the review in Chapter 2 flags up the need for comprehensive surveys of the biotic community present at field sampling sites.

The *in situ* field experiments in five sampling sites in coastal waters along the Côte d'Azur in summers 2015 and 2016, as detailed in Chapter 3, set out to address how such a solid baseline might begin to be established.

The particular field experiment along the coast of the Côte d'Azur that altered the macroalgal community complexities in the summer of 2015 demonstrated differences of *O. cf. ovata* abundances in different sampling sites. The in situ 2016 field experiments indicated that there is a correlation between *Ostreopsis cf. ovata* abundances and microalgal biomass, however this correlation was not always statistically significant and were generally negative.

The four different levels of macroalgal complexity used – CYS, DIC, TUR and BAR (*Cystoseira*, Dictyotales, Turf and Barren grounds) may be interpreted as different gradations of degradation of coastal habitats due to human impacts. The CYS plots consisting of more highly complex macroalgal assemblages were not heavily impacted by pollution or by multiple human stressors, thus, did not harbour significant *O. cf. ovata* blooms.

The proliferation of *O. cf. ovata* blooms seems to correlate with human population densities along the coast, which human pressures result in significant modifications in the marine littoral ecosystem. This suggests that areas with habitats that have been less impacted by pollution may hinder the facilitation of *O. cf. ovata* blooms. By contrast, sites composed of turf-forming, filamentous and encrusting Corallines and/ or Sphacerales (i.e. HAL, ROC and PCF) support higher abundances of *O. cf. ovata* cells.

Whereas DIC and TUR communities represented areas that were impacted by coastal eutrophication and habitat destruction, BAR grounds represented areas that have been influenced by heavy fishing pressures. The decline in populations of sea urchin predators (mainly Labridae and Sparidae) as a consequence of overfishing has caused an increase in the sea urchin population (Guidetti and Boero, 2004; Hereu, 2006; Sala, 2004; Thibaut et al., 2015), which are considered to be the most effective herbivores in temperate regions (Ling et al., 2015; Gianni et al., 2017).

Recent studies have demonstrated that native herbivorous fish and sea urchins may play an important role in shaping temperate marine communities (Vergés et al., 2014, Gianni et al., 2017). More work needs to be done, when monitoring population dynamics of

BHABs dinoflagellates on different macroalgal complexities, to monitor also the densities of local herbivores (invertebrates and fishes) in order to understand the fluctuations of their populations and how they indirectly influence the abundances of benthic dinoflagellates. Manipulating herbivory could perhaps help in regulating *O. cf. ovata* blooms. However, it is very important to note that the two significant blooms that occurred in the summer of 2015 were recorded in two sites (HAL and ROC) that did not host natural *Cystoseira*-dominated assemblages, and so it cannot yet be concluded with certainty from these whether *Cystoseira* spp. communities regulate *O. cf. ovata* blooms at a regional scale. Further studies will make correlation, if any, clearer.

The *in situ* 2016 field experiments indicated that there is a correlation between *Ostreopsis cf. ovata* abundances and microalgal biomass, however this correlation was not statistically significant. This indicated that biomass is not a factor *influencing O. cf. ovata* abundances in the Côte d'Azur. Some studies have hypothesised that dinoflagellates exhibit distinct macroalgal substrate preferences. Heil et al., in 1998 measured and compared the cell abundances of the dinoflagellate *Gambierdiscus toxicus* on five different macroalgal species collected from Heron Island, Australia. The study demonstrated that the highest concentrations of *G. toxicus* were found associated with Phaeophyceae in the genera *Colpomenia* and *Chnoospora* (Heil et al., 1998). Parsons et al., in 2011 demonstrated through a laboratory study that *Gambierdiscus* spp. proliferated in the presence of some host species (e.g., *Galaxaura marginata* and *Jania* sp.), but grew little in the presence of other species (e.g., *Portieria hornemanii*) (Parsons et al., 2011).

Certain specifics of the Côte d'Azur should be noted. The Mediterranean Fucales (i.e. *Cystoseira* and *Sargassum* species) belong to perennial species that structure temperate rocky shore habitats (Thibaut et al., 2015). Their tolerance to disturbances (ecological plasticity) varies among different species (Orfanidis et al., 2011). Certain species have higher ecological plasticity than others. Only one taxon, *Cystosira compressa*, is still common on the Côte d'Azur with the other species in decline due to their sensitivity to coastal pollution, herbivory (Thibaut et al., 2005; Serio et al., 2006; Vergés et al., 2014) and habitat destruction (Meinesz et al., 2013). A loss of complex macroalgal beds, due to

the degradation of water quality has been recorded (Airoldi and Beck, 2007) in the Mediterranean Sea along the French and Italian Ligurian coastline, which is densely populated and very highly industrialised. It is important to precise, that while there are major industrialised cities along the French Ligurian coast (e.g. Marseille and Genova), the Alpes-Maritimes and the Var have a very low the number of coastal industries, thus the Côte D'Azur is not heavily impacted by industrial pollution, more so by urban pollution and from the Port of Nice.

A study conducted in the Ligurian Sea coasts, which the Côte d'Azur forms part of, confirmed the loss, with increasing urbanisation, of canopy-forming macroalgae of the genus *Cystoseira* spp. (Mangialajo et al., 2008a). A similar study in Australia reported an increase in *Ostreopsis* spp. abundances along a human disturbance gradient (Briggs and Leff, 2007). While another investigation in the Adriatic Sea demonstrated a positive correlation between *O. ovata* abundances and faecal coliforms and Streptococci (indicators of human-induced pollution; Ungano et al., 2010). The results from the present study support the findings of those previous to suggest that the effects of macroalgal community complexities may vary depending on varying degrees of human stressors. The spatial and temporal heterogeneity of *Ostreopsis* cf. *ovata* blooms across the five sites chosen was supported by the variety of environmental and weather parameters measured in 2015.

Climate change is affecting nutrient availability in complex ways (Glibert, 2017) which must not be discounted. However, anthropogenic changes in nutrient availability are likely to have strong effects on tropical and intertidal temperate reefs, particularly due to complex environmental interactions involved shaping these systems (Clausing, 2014).

It is widely acknowledged that different algal taxa have distinct requirements and capabilities in using different forms of nitrogen and phosphorus for cell growth (N, see Xu et al., 2010; Glibert et al., 2017; P, see Ryther and Dunstan, 1971). Many dinoflagellates prefer chemically reduced forms of N, mainly organic and in the form of ammonia (Glibert et al., 2016; and references therein; Shangguan et al., 2017).

A number of physiological strategies have been identified associated with taxa that can thrive when inorganic P is low relative to cellular demands (Glibert, 2017). One strategy for success is for the facility to store N and P in cells to allow growth for prolonged periods. This has been demonstrated in marine benthic dinoflagellates (Accoroni et al., 2015).

Mixotrophy, widespread among dinoflagellates, is another strategy for success under conditions when there are nutrient imbalances. This mechanism of survival is ubiquitous among algae in both nutrient-poor and nutrient-rich habitats (Stoecker, 1999; Burkholder et al., 2008; ; Flynn et al., 2013, Glibert, 2017). Cells of *Ostreopsis cf. ovata* have been proven to be mixotrophic (Jauzein et al., 2017), they have a strong affinity for NH_4^+ in N-limiting environments. This could be a plausible explanation as to why there are intense blooms of *O. cf. ovata* in oligotrophic phases along the Côte d'Azur.

Many marine dinoflagellate HABs are known to be more toxic when N is in excess over P (Glibert, 2017), pointing to one of the mechanisms by which anthropogenic changes in nutrient availability can affect toxicity.

Biological factors, such as inter-specific competition and predation play fundamental roles in shaping marine benthic community systems. Biochemical ecology (e.g. allelopathy) is a strong survival mechanism employed by macrophytes and benthic dinoflagellates mediated by released secondary metabolites which may be inhibitory or stimulatory (Rice, 1984; Wang et al, 2012). An increased understanding of chemical aspects of species-specific organism interactions, including symbiotic relationships between *Ostreopsis* spp. and their host species, will help define the chemical and molecular processes that are crucial for the proliferation of *Ostreopsis* spp. blooms, which can be applied to the mitigation of these blooms on the Côte d'Azur and elsewhere. Moreover, many marine bacteria, fungi, blue-green algae, diatoms, and other minute macroalgae can be found on macroalgal surfaces. The epiphytic microbial communities are different among species of macroalgae and sometimes among individuals of the same population (Nakahara et al., 1996), which may explain the difference of *Ostreopsis cf. ovata* population densities

among the different sites (which had different macroalgal communities) in the present study. The results of this study also underscore the need for a more comprehensive approach that considers the characteristics of benthic dinoflagellate host algae when monitoring and managing coastal regions where *Ostreopsis* spp. blooms are frequent.

The results furthermore confirm the oligotrophic regime in the summer months in this region with high values of N:P ratios, indicating that P is limiting during this period and thus could prevent primary production in phytoplankton.

Elevated heavy metals concentrations in the seawater have been found to cause physiological stress and reduced growth, affecting *Ostreopsis* spp. fitness and survival (Sengupta et al., 2017). However, the trace metal concentrations detected in the present study do not seem to be linked with *O. cf. ovata* cellular growth rates and cannot be used to explain *O. cf. ovata* bloom dynamics in the Côte d'Azur.

Turbulence is a common physical factor in the ecology of phytoplankton, including benthic dinoflagellates, and is involved in the seasonal succession and bloom formation of phytoplankton species (Margalef, 1978; Smayda, 2001). Laboratory-based data obtained using different phytoplankton species showed that dinoflagellate cells could be affected by various degrees of small-scale turbulences (Berdalet et al., 2007). Some of these effects can be beneficial for phytoplankton, for example, by increasing nutrient uptake (Karp-Boss et al., 1996; Peters et al., 2006; Barton et al., 2014). However, turbulence is often detrimental for motile species, such as *Ostreopsis* spp., causing flagellar or cellular damage, direct effects on cell biology and decrease in cell growth. Furthermore, it has been suggested that the effects of small-scale turbulences are species-specific to different species of phytoplankton (Berdalet et al., 2007), and potentially to benthic dinoflagellates. The sensitivity of dinoflagellates to turbulence may be related to the division mechanism (dinomitosis) of their nucleus or dinokaryon (Berdalet, 1992). The results from this study indicated that, in this region at least, there are greater abundances of *O. cf. ovata* cells in sites with slightly elevated water motion. Intense blooms were observed in sampling sites

with moderate to high water motion (i.e. in HAL and ROC), whereas *O. cf. ovata* blooms were not prominent in sheltered sites (i.e. GRA and ADF).

The new sampling method used for the *in situ* field experiments in this study, the BEDI (Mangialajo et al., 2017; see Appendix 1) can be used across multiple spatial and temporal scales in any environment independent of the availability of macroalgae or other biotic substrates. This reduced complications related to the mass of different macroalgal species, problems of macrophyte preference by BHAB dinoflagellates and allowed data to be compared across different sampling stations (Tester et al., 2014; Mangialajo et al., 2017). Previously, abundances of BHAB dinoflagellate have most commonly been quantified by collection of macrophytes, and the cells are then concentrated and enumerated using standard microscopy methods (Litaker et al., 2010). Some studies have highlighted the need for new methodologies to standardise sampling and enumeration procedures when quantifying epibenthic dinoflagellates (Bomber et al., 1989; Lobel and Anderson., 1988; Tester et al., 2014).

This study has identified a need for far more information than the existing literature provides about biotic factors that may affect the proliferation of *Ostreopsis cf. ovata* blooms. A number of interesting results produced by the *in situ* field studies on the Côte d'Azur and related laboratory experiments suggest areas of study where further work in this field may profitably develop our understanding on the ecology and bloom dynamics of *Ostreopsis* spp. and the possible interactions between *Ostreopsis* spp. blooms, human stressors and the alteration of marine macrophyte assemblages. Since such better understanding may well open up prospects for better management for the forecasting and mitigation of such phenomena to the benefit of human health and local economies.

The results of the fieldwork in this study strongly suggest that it would be fruitful to conduct further, similar studies in other geographical locations. The aim should be to establish, by a series of local studies, the dynamic relationship between environmental context; macroalgal community complexity response to increasing nutrient loads; and consequent influences on *Ostreopsis* spp. abundances.

In particular, future work should focus on measuring benthic dinoflagellate cell abundances on different benthic community assemblages, in order to elucidate the degree of influence that biotic substrates have in shaping the population dynamics of these organisms.

Stronger collaborations between micro- and macrophytologists working in different laboratories around the world would fill in significant gaps in our knowledge of the ecology of *Ostreopsis* spp. and of the role of macrophytes in the facilitation of their blooms.

Further *in situ* experiments and *in vitro* culture experiments are necessary to comprehend the spatio-temporal variability of *Ostreopsis* spp. abundances at the regional and global scale. Additional field studies in varied locations could usefully investigate the effect of water turbidity and sedimentation on the growth rate of macroalgal community complexities and thus on the growth rates of BHAB dinoflagellates.

Long time series would also be essential to define conditions that control and regulate *Ostreopsis* spp. abundances in the coastal regions where they are most prevalent. To date, judging by systematic analysis of the literature, there are no observational time series that span decades which have identified or analysed any trends in *Ostreopsis* spp. abundances or linked variability to environmental factors.

For *in vitro* laboratory experiments, manipulating various concentrations of different pollutants of human origin should be undertaken. This would allow for the investigation of how pharmaceuticals and personal care products could potentially influence the growth rates of *O. cf. ovata* in culture. Active pharmaceutical ingredients, surfactants, personal care products or substances of endocrine activity are among the compounds that are not completely eliminated during waste water treatments (Zweiner et al., 2000; Herberer, 2000; Lopez de alda et al., 2003). These compounds can easily be cleaved during sewage treatment and the original pharmaceutically active compounds (PhACs) will then be released into the aquatic environment mostly by effluents from municipal sewage treatment plants (Heberer, 2002). Consequently, these PhACs could pose adverse effects

in marine systems, such as affecting bloom dynamics of benthic dinoflagellates. Some human-origin pollutants to test include: caffeine; acetylsalicylic acid (ASA; most commonly known as aspirin); active agents found in sunscreens (e.g. oxybenzone, octyl methoxycinnamate, p-aminobenzoic acid, zinc oxide and titanium oxide); synthetic hormones such as oestrogen and progesterone (found in oral contraceptive pills).

The influence of land use and agriculture on the population dynamics of benthic dinoflagellates in coastal regions is another key area for further investigation. Marine ecosystems are vulnerable to agricultural runoffs, which are rich in N and P sources. New sources of N from N-based agricultural fertilizers are associated with increased phytoplankton biomass and rates of primary production, HABs and eutrophication (Galloway et al., 2003; Beman et al., 2005). Using agricultural herbicides/ fertilizers in culture work could help establish what effects these chemicals have on the growth rates of *Ostreopsis* spp.

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APPENDICES

APPENDIX 1.

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Benthic Dinoflagellate Integrator (BEDI): A new method for the quantification of Benthic Harmful Algal Blooms



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ABSTRACT

Despite the potential negative human health, ecological and economic impact, the ecology of harmful benthic dinoflagellate blooms remains largely unknown. This is probably due to the complex interactions among biotic and abiotic drivers that influence blooms, but also to the difficulty in quantifying cell abundance in a comparable way over large spatial and temporal scales. One of the recognized priorities for bHABs (benthic Harmful Algal Blooms) assessment is developing and standardizing methods that can provide comparable data. In this context, the Benthic Dinoflagellates Integrator (BEDI), a new non-destructive quantification method for benthic dinoflagellate abundances, has been developed and tested within the present study. The rationale behind the BEDI standard assessment method is that mechanical resuspension of cells enables the quantification of abundances as cells per unit of seabed surface area (i.e. cells mm⁻²) or as Potentially Resuspended cells per unit of volume (PRcells ml⁻¹), by integrating both cells in the biofilm and those in the surrounding water. Estimations of *Ostreopsis* performed with BEDI method are independent of the substratum (i.e. macroalgal species) or the dominant ecosystem (i.e. algal forests or turfs, seagrass beds, coral reefs) and potentially allow the comparison of benthic dinoflagellate blooms over broad temporal and spatial scales. The first application of the BEDI method, presented in this study, gave encouraging results: the characterization of blooms of *Ostreopsis* cf. *ovata* at three sites in the NW Mediterranean Sea is consistent with results derived from the other commonly applied methods. Quantification of the ratio between abundances of cells in the biofilm and in the surrounding water was calculated for the first time per unit of seabed surface area, demonstrating that the highest abundances of cells (the stock), and therefore the associated risk for human health, are in the biofilm. For risk assessment purposes, conversion values for commonly used monitoring alert thresholds of Mediterranean *Ostreopsis* blooms are provided.

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1. Introduction

Potentially harmful benthic dinoflagellate blooms, well known in tropical latitudes, are increasing in distribution and frequency in temperate areas (Shears and Ross, 2009; Litaker et al., 2010; Parsons et al., 2011; Rhodes, 2011). In the tropics, the genus *Gambierdiscus* is responsible for the ciguatera fish poisoning, but

other benthic dinoflagellates, such as *Ostreopsis* and *Prorocentrum*, contain toxins and therefore have the potential to contribute to seafood poisoning (see overview in Nakajima et al., 1981; Yasumoto et al., 1987; Richlen and Lobel, 2011). The true extent and potential impact of benthic dinoflagellate-based sea-food-borne illnesses on human communities remains under-studied (Skinner et al., 2011). In temperate areas, *Gambierdiscus* is generally absent or very localized (Parsons et al., 2011), while blooms of the genus *Ostreopsis* are considered as an emergent phenomenon. They are responsible for several sporadic human health issues, including skin irritations due to physical contact (Tubaro et al., 2011), as well as outbreaks of mass respiratory symptoms in beach-goers

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exposed to marine aerosols (Gallitelli et al., 2005; Durando et al., 2007; Barroso Garcia et al., 2008; Vila et al., 2016 proceedings, Ciminiello et al., 2014). Sporadic shellfish contamination (Aligizaki et al., 2008; Brissard et al., 2014; Biré et al., 2013, 2015) and mass mortality of invertebrates (Granéli et al., 2002; Sansoni et al., 2003; Shears and Ross, 2009; Totti et al., 2010) are also associated with *Ostreopsis* blooms in temperate latitudes, with potential cascade effects at the ecosystem level (Shears and Ross, 2010).

Despite the potential human health, ecological and economic impact, bHABs (benthic Harmful Algal Blooms) ecology is still poorly understood worldwide. Climate change (Sansoni et al., 2003; Shears and Ross, 2010; Granéli et al., 2011; Accoroni et al., 2015) and human impact (i.e. nutrient levels, Sansoni et al., 2003; Litaker et al., 2010; Parsons and Preskitt, 2007; Skinner et al., 2013) have been suggested as potential drivers of blooms, but remain poorly understood. This is probably due to the complex interactions among biotic and abiotic drivers that influence these blooms (Cohu et al., 2013; Accoroni et al., 2015; Accoroni and Totti, 2016), but also to the difficulty in quantifying cell abundance in a comparable way over broad spatial and temporal scales (Mangialajo et al., 2011; Berdalet et al., 2012; Zingone et al., 2012).

Benthic dinoflagellates thrive in shallow waters, growing mostly on macroalgae and hard substrates (e.g. rocks, corals, mollusc shells), where they can form a biofilm. They exhibit host preferences (see Parsons and Preskitt, 2007 and references therein) and can detach from the substrate and swim in the surrounding water (Rains and Parsons, 2015). As benthic dinoflagellates are loosely attached to the substrate, they can also be easily resuspended by mechanical action (e.g. waves, anchoring, trampling). They can therefore be found in large quantities in the surrounding water and can form mucilaginous aggregates floating at the surface (See *Ostreopsis* cf. *ovata* bloom dynamics in Fig. 1). Different techniques for quantifying cell abundance have been applied for research, monitoring and risk assessment purposes (Berdalet et al., 2012; Tester et al., 2014 for reviews). The two most common assessment methods involve the quantification of cells in the biofilm, expressed as cells g^{-1} of collected macroalga or per surface area of different substrates (i.e. pebbles, corals, shells), and the quantification of cell concentrations in the surrounding water, usually expressed as cells l^{-1} . Alternative methods for quantifying benthic dinoflagellate cell abundances independently of their natural substrates have been tested, including the application of suction apparatus which gives representative abundances of the

cells in the biofilm (Parsons et al., 2010; Abbate et al., 2012), or the deployment of artificial substrates which integrate over time the cells in the surrounding water (Tester et al., 2014; Jauzein et al., 2016). None of these promising methods have been applied to date over wide spatial and temporal scales with the aim of finding global common patterns for bHABs.

The quantification of benthic dinoflagellates in the biofilm (calculated as cells g^{-1} of macroalgae) is commonly used for bHABs in tropical areas (see Tester et al., 2014 and references therein). This measure plays a crucial role in terms of risk assessment of ciguatera poisoning, as cells adhering to the macrophytes can enter the food web via the grazing of herbivores. The measure of cells g^{-1} of macroalgae is therefore the most widespread method, but results are species-specific (dependent on the specific weight and morphology of the different macroalgal host species). The assessment of cells in the biofilm is also a widespread method for temperate bHABs, as demonstrated in a recent synthesis of results from different research teams in the Mediterranean Sea (Mangialajo et al., 2011). Due to the variety of macroalgal species collected as substrates at the investigated scale, this synthesis could not provide direct comparisons of bloom magnitudes. In order to describe general trends of bloom dynamics, the authors decided to focus their work on the relative maximum annual value, but this prevented them from finding general patterns in absolute magnitude of blooms (Mangialajo et al., 2011). In order to overcome the issue of substrate diversity, the idea of assessing the number of cells per surface unit area of the host macroalga (therefore independent of the weight) had been suggested in the past (Bomber, 1985; Lobel et al., 1988) and has been tested more recently (Totti et al., 2010). While this standardization is promising, the calculation of macroalgal surface areas (variable as a function of the precision used for the measurement) remains a major constraint.

In temperate areas, the risk of outbreaks of skin and ear, nose, throat irritation in addition to respiratory problems is mostly associated with the abundance of *Ostreopsis* spp. cells in the water, on the surface (the characteristic floating mucilaginous aggregates) and therefore in the aerosols. Quantification of cells is therefore often also performed on water samples taken a few dozen centimeters from the macroalgal assemblages (surrounding water) and bloom magnitude is expressed as cells l^{-1} . Since this is an easier sampling procedure both in the field and in the laboratory (no macroalgal separation and identification are needed), this

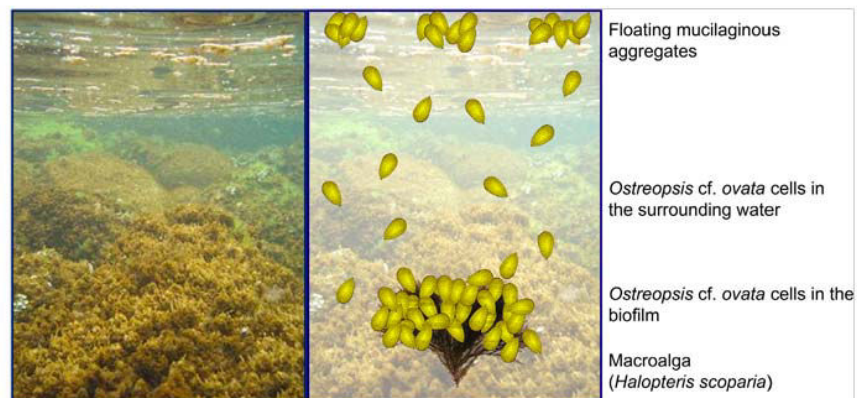


Fig. 1. Typical *Ostreopsis* cf. *ovata* bloom in the North Western Mediterranean Sea. Left: macroalgal communities covered by brownish *Ostreopsis* cf. *ovata* biofilm. Right: schematic representation of *Ostreopsis* cf. *ovata* bloom component: *Ostreopsis* cf. *ovata* cells in the brownish biofilm developing on macroalgal communities, *Ostreopsis* cf. *ovata* cells free swimming or resuspended in the surrounding water and mucilaginous aggregates floating at the surface.

measurement method is often preferred to the quantification of cells g^{-1} of macroalgae, especially in monitoring and in risk assessment studies (i.e. Funari et al., 2015). Nevertheless, the concentration of cells in the water can be highly variable during the day (Vila et al., 2001) and as a function of local hydrodynamic conditions (sea breezes, boat circulation, trampling by beachgoers) and the reliability of such measurements in the framework of risk assessment is still debated.

In conclusion, the two widespread 'biofilm' and 'surrounding water' assessment approaches present various advantages and disadvantages (Table 1). In general, scientists agree that the quantification of cells in the biofilm is more accurate than that of concentrations of cells in the surrounding water, which is dependent on local resuspension due to mechanical action and daily migration of cells. It is assumed to be more representative of the stock of cells (i.e. Mangialajo et al., 2011) and therefore of the potential risk due to cell resuspension. Unfortunately, to date it has not been possible to assess the relative abundance of cells in the biofilm and in the surrounding water at a given time, because of the lack of a sampling technique allowing a precise calculation of cell abundance in the biofilm over a given surface area of seabed. On the other hand, the quantification of cells in the biofilm requires the collection of macroalgae and is therefore an ecologically destructive method that should be avoided for large scale monitoring. Furthermore, it provides substrate-dependent measures, and does not allow the comparison of blooms over large spatial and temporal scales.

The purpose of the present study was therefore to develop and test a new non-destructive sampling method for quantifying benthic dinoflagellate blooms worldwide in a simple, reliable and comparable way that may be applied both for scientific research and for monitoring purposes, the Benthic Dinoflagellates Integrator (BEDI). The rationale behind the BEDI method is that mechanical resuspension of cells allows the quantification of abundances as cells per surface unit area of seabed (i.e. cells mm^{-2}) by integrating both cells in the biofilm and in the surrounding water. These measures are independent of the substratum (i.e. macroalgal species) and can also be expressed as Potentially Resuspended cells per unit of volume (PRcells ml^{-1}) that may be

used, at least in the case of *Ostreopsis cf. ovata* blooms, as a direct measure of the risk associated with inhalation of aerosols.

The first application of the BEDI to an *Ostreopsis cf. ovata* bloom is presented in this study. The characterization of blooms at three sites in the Ligurian Sea has been performed with BEDI and classical estimations of biofilm (macroalgal sample) and surrounding water abundances, in order to compare the results and test its appropriateness for use for both research and monitoring purposes. The application of this new method allowed the first assessment of the relative abundances of *Ostreopsis cf. ovata* cells in the biofilm and in the surrounding water. Conversions to BEDI values of both monitoring and bloom definition thresholds are also provided.

2. Materials and methods

2.1. Benthic Dinoflagellates Integrator (BEDI) device

The purpose of the BEDI device is to integrate dinoflagellate cell abundances, both in the biofilm and in the surrounding water. The results can therefore be expressed as the number of cells per seabed surface area (i.e. cells mm^{-2}), independent of the substrate (i.e. macroalgal species). The BEDI device isolates a portion of seabed (to the surface for shallow waters or to a defined height for deeper waters) and allows the mechanical resuspension of the cells in the biofilm which are naturally loosely attached to the substratum. Simple calculations, taking into account the size of the BEDI and the depth of the sampled surface, enable assessment of the integrated abundances of cells in the biofilm and in the surrounding water, corresponding to the surface area sampled.

The BEDI device tested on Mediterranean *Ostreopsis cf. ovata* blooms consists of a hollow plastic cylinder (Fig. 2a), open at both ends, with a height of 70 cm and a diameter of 25 cm (sampled area of 491 cm^2). A rubber seal is fixed to the bottom of the device, in order to avoid cell loss when sampling irregular seabed (i.e. high complexity rocky bottoms). As *Ostreopsis* blooms occur in very shallow waters in the Mediterranean Sea, the upper part of the BEDI device can be kept out of the water. In this case, the depth of each sample had to be noted, in order to calculate the total volume of water sampled and correctly estimate the abundance mm^{-1} .

Table 1

Comparison of the different sampling methods applied (BEDI, biofilm, surrounding water estimations). Various advantages (in bold) and disadvantages corresponding to each method are given for the whole procedure, from field collection to the subsequent sample treatment and counting in the laboratory.

	SURROUNDING WATER estimations (cells l^{-1})	BIOFILM estimations (cells g^{-1} of macroalgae)	BEDI estimations (cells cm^{-2})
Environmental impact	Non-destructive	Destructive	Non-destructive
Processing of samples	No treatment of samples required	Separation and weight of macroalgae required	No treatment of samples required
Assessment time	Sedimentation in 10/50/100 ml Utermöhl chambers ^a	Immediate assessment in 1 ml chambers	Immediate assessment in 1 ml chambers^b
Temporal (daily) variability	High variability linked to mechanical cells resuspension and/or migration	More consistent measures^c	No temporal variability expected^d
Substrate-dependent variability	Comparable results^e	Species-specific results ^f	Comparable results^e
Risk assessment	Not representative of the stock of cells ^g	Representative of the stock of cells^h	Representative of the stock of cells^h

^a Results are usually 24 h delayed, due to the sedimentation time (proportional to the height of the column).

^b The sedimentation is required for a precise estimation of very low cell abundances, but not for risk assessment.

^c But some variability may be observed, linked to resuspension/migration.

^d Biofilm and surrounding water cells are integrated.

^e Not dependent on substratum.

^f Dependent on substratum.

^g And therefore not representative of the potential risk.

^h And therefore representative of the potential risk.

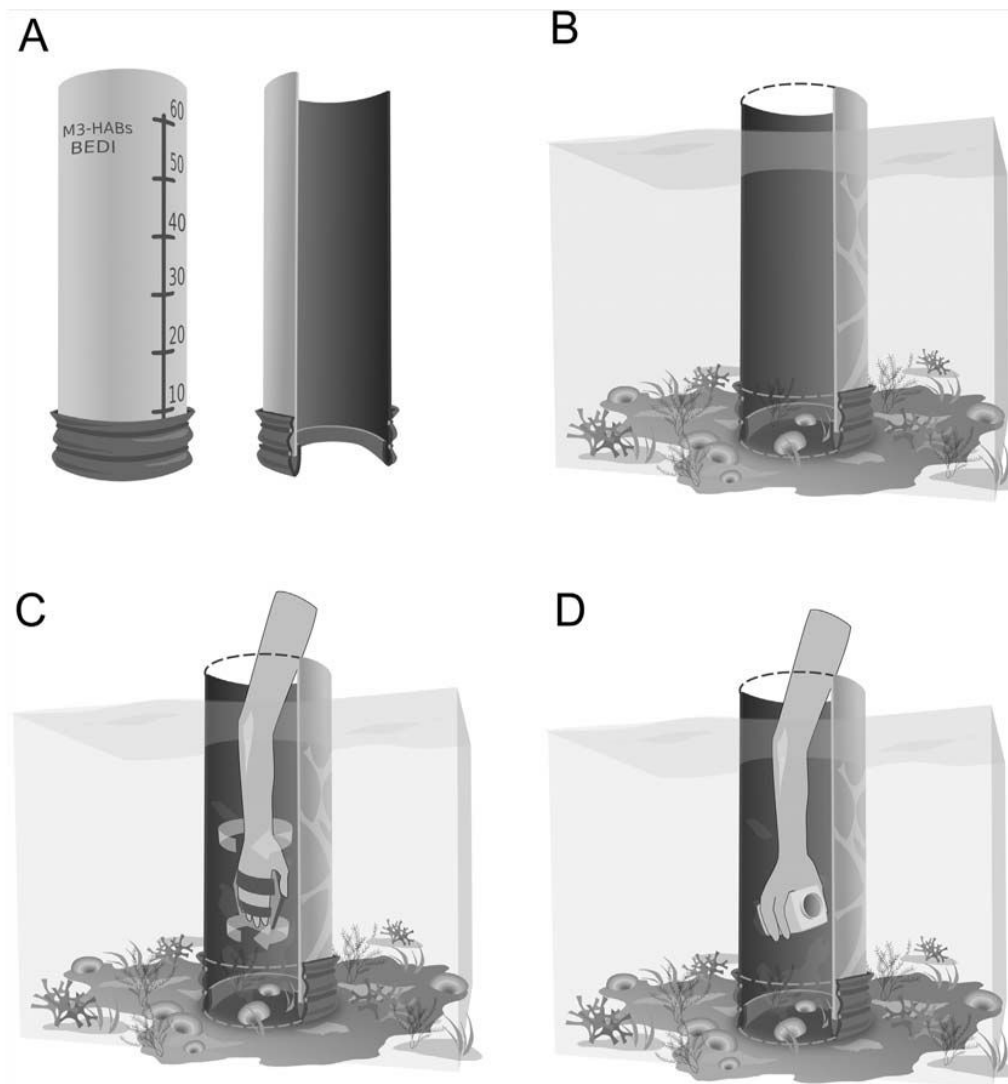


Fig. 2. Scheme of Benthic Dinoflagellates Integrator (BEDI) and its application in shallow waters (nearly 50 cm depth); a) BEDI device (70 cm in height; 25 cm diameter); b) installation over the seabed during sampling procedure; c) resuspension and homogenization of cells of the biofilm in the surrounding water by mixing; d) collection of a water sample using a 250 ml plastic flask.

Scales can be reported both inside and outside the BEDI device to help with this measure (Fig. 2a). For application in deeper environments, the BEDI device has to be sealed at the top to avoid cell loss resulting from mixing of the water and resuspension of cells. A plankton net with an elastic fastening or a hole with a rubber seal can both be used as closing systems for underwater sampling.

During BEDI sampling, the device is placed over the seabed (Fig. 2b) and the water inside is vigorously stirred using a standard hand-paddle, allowing the resuspension of cells in the biofilm and their homogenization with the cells in the surrounding water (Fig. 2c). The mixing action represents a potential source of variability and the use of a standard oval shaped hand-paddle (281 cm²) has been proposed in order to reduce the bias. As *Ostreopsis* cells (and dinoflagellate cells in general) are loosely

attached to the substrate, the variability linked to different observers should be negligible since the water is stirred vigorously inside the BEDI. The water has to be mixed for a few seconds, in order to allow complete resuspension of cells and mixing of the water, without facilitating the loss of cells from the bottom. For the Mediterranean Sea *Ostreopsis* blooms, roughly 5 s stirring was applied. Immediately after stirring, a sample is taken from the center of the BEDI column using a 250 ml plastic bottle (Fig. 2d). After sampling, the BEDI device can be immediately moved to another site and a new sample taken.

In the case of major blooms, with thick biofilms, a small amount of cells may not be resuspended and may still be found in the biofilm; however, this is difficult to measure. In that case, integrated cells per surface unit may be slightly underestimated relative to the real value.

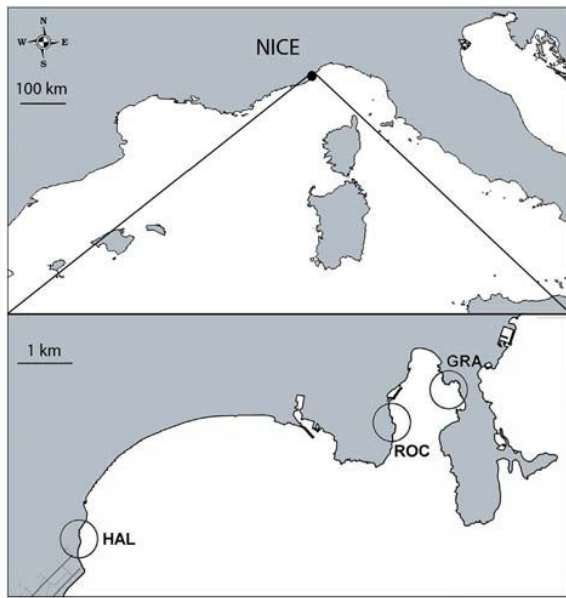


Fig. 3. Maps of sampling locations near Nice. HAL: Haliotis (43°40'38"N 7°13'51"E), b) ROC: Rochambeau (43°41'35"N 7°18'31"E) and GRA: Grasseuil (43°41'59"N 7°19'20"E).

2.2. First BEDI application

The BEDI device was tested during the 2014 *Ostreopsis cf. ovata* bloom season (mid-summer to early autumn), in the French Ligurian Sea.

Three sites were randomly chosen: Haliotis (HAL, 43°40'38"N 7°13'51"E), an artificial riprap near Nice Côte d'Azur airport, Rochambeau (ROC, 43°41'35"N 7°18'31"E) and Grasseuil (GRA, 43°41'59"N 7°19'20"E), two mixed artificial-natural zones at Villefranche-sur-mer Bay (Fig. 3). At the three sites, the macroalgal assemblages were similar, mostly lacking complex large brown algal forests and therefore being mostly dominated by ephemeral erect or turf forming algae (e.g. *Halopteris scoparia*, *Padina pavonica*, *Dictyota* spp., *Corallina elongata*, *Jania rubens*, accompanied by other small red and brown turf-forming algae).

In agreement with the temporal dynamics of *Ostreopsis* blooms in the Northwestern Mediterranean Sea (Mangialajo et al., 2011), samplings were performed on 7 dates (July 23, 28, 30; August 12, 20, September 9 and October 29, 2014).

At each site (HAL, ROC and GRA), three replicated samplings, 10 m apart, were performed at the 7 sampling times (T1–T7); in order to test the replicability of the BEDI measures, 3 replicate bottles were sampled for each measurement.

The three sites were sampled on the same day, except for the first sampling date, where the three sites were visited on two days, the 23rd and 24th of July. For logistical reasons, only two sites were sampled on 28th July (ROC and GRA) and 29th October (HAL and ROC). To avoid potential bias due to the daily variation of *Ostreopsis* abundance (Vila et al., 2001), the order of the sampling sites was interspersed on the different sampling dates.

2.3. Comparison with classical methods

To compare the BEDI abundances with other commonly used measurements, parallel biofilm and surrounding water measurements were performed. Cell concentration in the surrounding

water was estimated in a water sample taken approx. 20 cm above the macroalgae, using a 250 ml plastic bottle. Cell abundances in the biofilm were estimated by collecting 5–10 g of macroalgae. In order to minimize species-specific variability, the same macroalgal species (*Halopteris scoparia*) was always collected: this species is very common at the three sites studied and has been proven to support *Ostreopsis* development in former studies (e.g. Cohu et al., 2013).

To avoid bias due to mechanical resuspension during sampling activities, the surrounding water sample was always collected first, taking care not to resuspend cells, then the biofilm sample and lastly the BEDI sample were collected.

2.4. Sample treatment and quantification of cells

In the laboratory, samples were fixed with acidic Lugol, to a final concentration of 1% solution of Lugol:seawater. A total of 171 BEDI, 63 benthos and 63 plankton samples were collected, fixed and counted.

As no preliminary treatment is needed for BEDI and seawater samples, they were directly stored in the dark. Macroalgal samples were treated as in Cohu et al. (2013).

Cell concentrations in the water samples were assessed following the Utermöhl method (Utermöhl, 1958), counting settled cells in 50 ml sedimentation chambers under an inverted light microscope. Abundances of *Ostreopsis* in samples collected with the BEDI method were assessed without sedimentation in 1 ml Sedgewick Rafter counting chamber under standard light microscopy. In order to have precise estimations of BEDI samples abundances, when the number of *Ostreopsis cf. ovata* in the counting chamber was lower than 20 cells (82 samples, most of them from GRA), the sample was re-counted using the Utermöhl method (50 ml sedimentation chamber).

Cell abundances on macroalgae were assessed without sedimentation, in 1 ml Sedgewick Rafter counting chambers under standard light microscope.

2.5. Biofilm: surrounding water cells ratio

The ratio of cells present in the biofilm to cells present in the surrounding water per unit of seabed surface area was calculated according to:

$$R_{B/W} = \text{CELLS}_{\text{BIOFILM}} / \text{CELLS}_{\text{WATER}}$$

To estimate the cell abundances in the biofilm, the total amount of cells in the water column was subtracted from the cells in the BEDI:

$$\text{CELLS}_{\text{BIOFILM}} = \text{CELLS}_{\text{BEDI}} - \text{CELLS}_{\text{WATER}}$$

with $\text{CELLS}_{\text{BEDI}}$ the cells counted in the BEDI sample multiplied by the volume (in function of the depth) and $\text{CELLS}_{\text{WATER}}$ the cells ml^{-1} estimated in the surrounding water sample, multiplied by the volume of the BEDI sampled (in function of the depth).

The calculation was performed for all samples with >20 cells/counting chamber (in order to exclude raw approximation of abundances and zeros at the denominator). A total of 48 samples were used for this calculation, the limiting estimations being mostly the concentrations in the surrounding waters (20 cells in the 50 ml Utermöhl sedimentation chamber corresponding to 400 cells l^{-1}).

2.6. Thresholds predictions

The threshold concentrations commonly used for monitoring are based on the concentrations of cells in the water (Tichadou

et al., 2010; Lemée et al., 2012; Funari et al., 2015). Such values derive from measures performed the day after outbreaks of symptoms in beachgoers (for inhalation of aerosols and dermal exposure), or on the basis of calculations of hypothetical toxicity of ingested seawater (Funari et al., 2015). Such thresholds may potentially change in the future, after more detailed epidemiological studies, but at present they represent the reference monitoring protocols in several Mediterranean countries. Scientists usually prefer to assess magnitude of blooms on the basis of cell abundance per gram of fresh weight (FW) of macroalgae. As discussed at recent meetings (i.e. of the European Project M3-HABs), an *Ostreopsis cf. ovata* 'bloom' in the North Western Mediterranean Sea may be defined as 200,000 cells g⁻¹ of *Halopteris* (FW) and a 'major bloom' as 1,000,000 cells g⁻¹ of *Halopteris* (FW).

Based on the regression lines calculated at HAL and ROC (GRA site was excluded because the correlation between BEDI and seawater was not statistically significant), the corresponding BEDI values in both cells mm⁻³ and PRcells ml⁻¹ are predicted for both monitoring and bloom definition thresholds. On the basis of this comparative study, the thresholds commonly used by agencies for risk assessment (based on water samples) and the values associated by scientists with the definition of '*Ostreopsis cf. ovata* bloom' (based on biofilm samples) can be compared.

2.7. Statistical analyses

Variability of *Ostreopsis* abundances in space (sites) and time (sampling dates) was assessed by multifactorial ANOVAs performed on permutations (PERMANOVA, software PRIMER 6); distance matrices were based on Euclidean distances, corresponding to a classical ANOVA (but releasing the assumptions of normality and homoscedasticity of data). The pseudo-F and corresponding p values were obtained after 999 permutations and Monte Carlo p values were considered for post hoc tests when unique values were too low for correct estimation of p values.

The relationships between BEDI, biofilm and surrounding water *Ostreopsis* abundances were analyzed using Pearson linear correlation.

3. Results

The linear correlations of the *Ostreopsis* abundances estimated with the three different sampling techniques (BEDI, biofilm and surrounding water samplings) are reported, for each site, in Fig. 4. At GRA, where *Ostreopsis* abundances remained low for the whole period, only the correlation between BEDI and biofilm abundances was significant, even if weak ($R^2 = 0.2738$). At ROC, the BEDI abundances were also significantly correlated with the concentration of cells in the surrounding water (but at a lower R^2 value), while at HAL, where the bloom was greater (see below), the

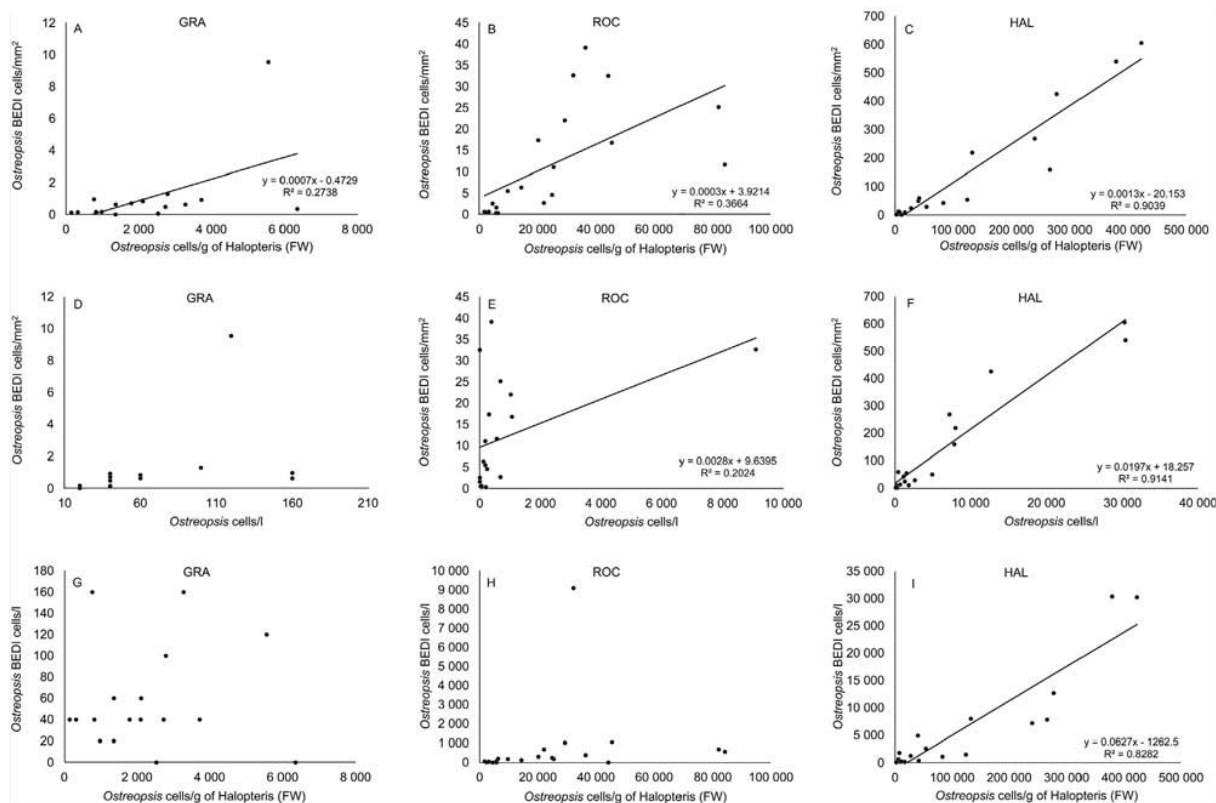


Fig. 4. *Ostreopsis cf. ovata* scatter plot and regression lines (when Pearson correlation resulted significant) between the abundances calculated with the three methods (BEDI, biofilm, surrounding water) at each site. Correlation between BEDI and biofilm cell abundances at: a) GRA, b) ROC and c) HAL; correlation between BEDI cell abundances and surrounding water cell concentrations at d) GRA, e) ROC and f) HAL; correlation between surrounding water cell concentrations and BEDI cell abundances i) GRA, h) ROC and i) HAL.

Table 2

Results of permutational multivariate analysis of variance (PERMANOVA) of abundances of *Ostreopsis cf. ovata* cells calculated with the three studied methods (BEDI abundances, cells cm⁻²; biofilm abundances, cells g⁻¹ FW of macroalga; surrounding water abundances, cells l⁻¹). In bold significant values; pairwise tests are reported in Fig. 5.

Source	BEDI cells cm ⁻²				Biofilm cells g ⁻¹ of macroalga			Surrounding water cells l ⁻¹		
	Df	MS	Pseudo-F	P (perm)	MS	Pseudo-F	P (perm)	MS	Pseudo-F	P (perm)
Site	2	20 133.0	3.7823	0.006	7.8724E10	4.4034	0.009	2.0853E8	2.8526	0.008
Time	6	4584.7	0.8761	0.652	2.1235E10	1.2053	0.284	9.7572E7	1.3603	0.241
Si x Ti	10	5339.8	5.2994	0.001	1.7734E10	23.171	0.001	7.3356E7	9.9627	0.005
Res	36	1007.6			7.6536E8			7.3631E6		
Total	54									

combinations of the three methods were significantly correlated (including biofilm and surrounding water).

The variability at the considered spatial and temporal scales was high, as shown by the significant interaction between the two major factors investigated (Sites × Time, Table 2), independently of the method applied (BEDI, biofilm and surrounding water measures). In general, post hocs demonstrate time-dependent significant differences among the sites, with a bloom at HAL that was consistently higher than at the other two sites. Here the maximum values of BEDI (980 cells mm⁻¹), biofilm (359.9 × 10³ cells g⁻¹ of FW of *Halopteris*) and surrounding water (30.4 × 10³ cells l⁻¹) abundances were recorded, all on the same sampling date, the 23rd of July. From a risk assessment point of view, these values corresponded to 2.8 × 10³ PRcells ml⁻¹ (Potentially Resuspended cells ml⁻¹). The bloom at ROC was higher than at GRA on two out of the 7 sampling dates, while at the end of the bloom (September and October samplings), no significant differences between the three sites were recorded.

Interestingly, the estimations of BEDI and of biofilm abundances gave exactly the same patterns when considering significant differences between paired sites (Fig. 5). Conversely, cells in the surrounding water gave different patterns at 3 sampling times out of seven, but still highlighting the same general trend (Fig. 5).

The analysis of repeatability of BEDI samples showed that no significant variation was observed at this supplementary level of replication (Table 3).

The use of the BEDI device allowed, for the first time, the estimation of the relative importance of cells in the biofilm and in the surrounding water per unit of sea floor surface. The ratio calculated is very high, with an average of cells in the biofilm of 66 ± 7.6-fold greater than the cells in the surrounding water per unit of surface area. This value is quite variable, with a minimum of 17 and a maximum of 106, probably due to the fleeting character of concentrations of cells in the water. A certain variability linked to the macroalgal community composition and/or to the BEDI device measures cannot be excluded. No inverse relation (more cells in the surrounding water than in the biofilm) was observed.

The guidelines proposed by Funari et al. (2015) suggest thresholds based on concentrations of cells in the water that provide a basis for planning a three-phase monitoring survey: routine (10 × 10³ cells l⁻¹), alert (30 × 10³ cells l⁻¹), emergency (100 × 10³ cells l⁻¹). As the same values are also used in other countries (Lemée et al., 2012), predictions for BEDI values (both cells cm⁻² of seabed and PRCells ml⁻¹) were calculated for these three values and are reported in Table 4. In the same table, the predictions of BEDI values for what can be defined as a bloom (200 × 10³ cells g⁻¹ of macroalga) and as a major bloom (1 000 × 10³ cells g⁻¹ of macroalga) are reported, as specified in the Materials and Methods section. Interestingly, these values correspond respectively to an intermediate value between routine and

alert thresholds and to a value that is quite close to the emergency threshold, as proposed by Funari et al. (2015).

4. Discussion

Benthic HABs are socially important at a global scale due to their potential health, ecological and economic impact. Nevertheless, the ecology of benthic dinoflagellates is poorly understood, and this is partly due to the difficulty of assessing their abundance in a reliable and comparable way (Berdalet et al., 2012; Zingone et al., 2012). The BEDI standard assessment method proposed in the present study integrates the dinoflagellate cell abundances in the biofilm and in the surrounding water, allowing the quantification of bloom magnitude over units of seabed surface area independently of the substratum (i.e. macroalgal species) or the dominant ecosystem (i.e. algal forests or turfs, seagrass beds, coral reefs).

The BEDI method was successfully applied at three sites in the NW Mediterranean Sea and allowed, for the first time, the calculation of the ratio of *Ostreopsis* cells in the biofilm and in the surrounding water per surface unit area of seabed. It was previously suggested (Mangialajo et al., 2011 and references therein) that the stock of cells is represented by the biofilm on macroalgae, not in the surrounding water. Here it is shown that this abundance, in shallow environments in the NW Mediterranean Sea, is on average 66 ± 7.6 (SE) -fold higher than the cells in the water per a given surface area. This result is particularly significant as it shows that the potential health risk linked to *Ostreopsis* is in the benthic environment: the biofilm concentrates very high abundances of cells that can be easily re-suspended in the water by mechanical action (waves or trampling) and therefore can be potentially aerosolized, causing symptoms in beachgoers. Current monitoring actions in the Mediterranean Sea are mostly based solely on the assessment of cells in the surrounding water (i.e. Tichadou et al., 2010; Lemée et al., 2012; Funari et al., 2015), which according to the results of the present study, may not be representative of the real public health risk. The mechanical action of stirring when assessing BEDI abundances could be considered a good proxy of mechanical resuspension by waves and/or trampling, and therefore the results, expressed as Potentially Resuspended cells per unit of volume (PRcells ml⁻¹), could be used as a direct measure of the potential health risk.

On the basis of the data collected in the present study, regression-based predictions have been calculated for the most recent monitoring thresholds applied in the NW Mediterranean Sea (i.e. Tichadou et al., 2010; Lemée et al., 2012; Funari et al., 2015). On the basis of ten years of *Ostreopsis* bloom observation in the NW Mediterranean Sea and coordinated research efforts (i.e. the European project M3-HABs), critical values of biofilm cell abundance are proposed for a definition of bloom and major bloom (respectively, 200 × 10³ and 1000 × 10³ cells g⁻¹ of macroalga (FW

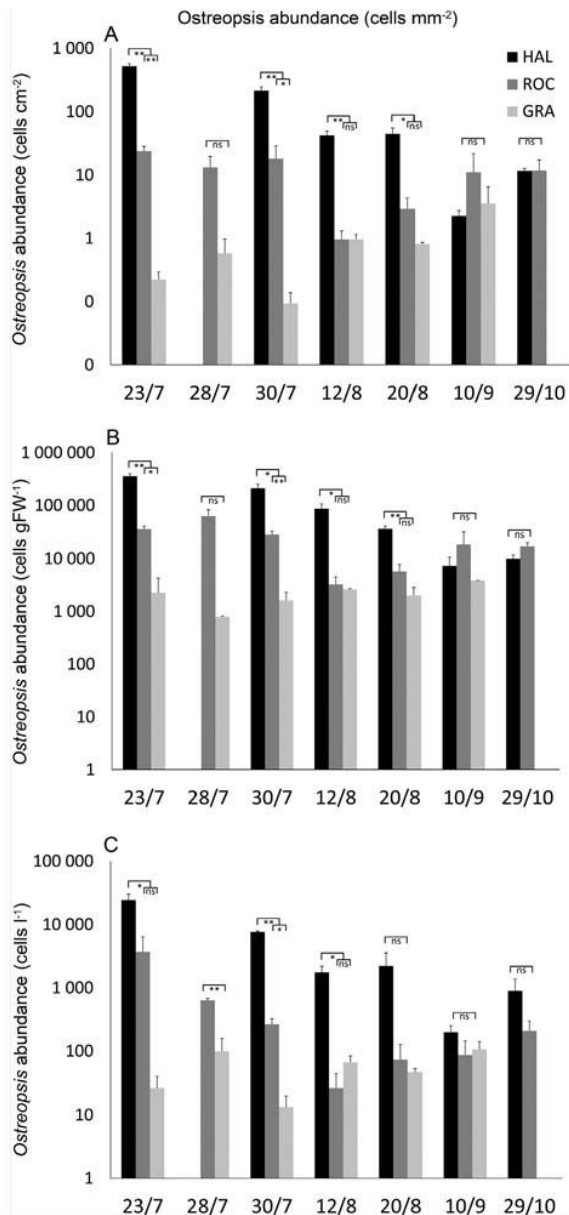


Fig. 5. *Ostreopsis cf. ovata* abundances at every site X sampling date combination; black bars: HAL; dark grey bars: ROC; light grey bars: GRA; a) BEDI abundances; b) biofilm abundances; c) surrounding water concentrations. Stars indicate different levels of significance of post hoc analyses (*: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$); ns: not significant ($p > 0.05$). In red are highlighted the results of surrounding water cell concentrations in disagreement with the patterns highlighted by BEDI and by biofilm abundances.

of *Halopectis scoparia*). Corresponding BEDI values have been calculated, and interestingly, a good relation with bloom/major bloom definitions and the thresholds proposed in management guidelines (Funari et al., 2015) is observed. Further epidemiological studies will probably improve the knowledge on these thresholds, and a wider application of BEDI will enable the refinement of the

regression line for calculations, providing better tools for the future management of this emergent phenomenon in temperate areas.

The BEDI standard assessment method is simple, easily applicable and is correlated to the classical estimations (cell abundance in the biofilm and cell concentration in the surrounding water). It is also reliable for repeated sampling, as no significant differences were detected when sampling three replicated measures of each BEDI. These findings are in agreement with the temporal patterns of *Ostreopsis* blooms observed in the North Western Mediterranean Sea: a peak in July, followed by a decrease in abundances from mid-August (Vila et al., 2001; Mangialajo et al., 2008, 2011; Coahu et al., 2013). BEDI results were consistent with results obtained with the classical methods and the site comparison performed in this study highlighted (independent of the method applied) a high variability in *Ostreopsis* abundances among sites and sampling times. Inter-site variations of benthic dinoflagellate blooms (at different spatial scales) have been observed in previous studies in tropical (Chang et al., 2000; Parsons and Preskitt, 2007) and temperate (e.g. in New Zealand, Rhodes et al., 2000; in the Mediterranean Sea, Vila et al., 2001; Coahu et al., 2013; in Australia, Skinner et al., 2013) areas. Several factors could explain this variability, including biotic substrate diversity, hydrodynamic conditions and nutrient availability, but more detailed studies would be necessary to disentangle the potential drivers of blooms.

When taking into account significant differences among the three sites investigated in the present study (HAL, ROC, GRA), exactly the same results were found with the BEDI abundances and the biofilm estimations using the macroalgal sample. When comparing BEDI abundances and surrounding water concentrations, 3 out of 7 pairwise comparisons gave slightly different results, but still highlighting the same general trend. Differences were highly significant at the peak of the bloom, however they were less evident with the decrease of cell abundances in late summer: no significant differences among sites were detected in September and October.

Compared to the classical bHABs sampling methods, the BEDI technique seem to incorporate most of the advantages of both biofilm and surrounding water quantifications (i.e. representative of the stock of benthic cells, no treatment needed in the laboratory, comparable results provided) and none of the disadvantages (i.e. non-destructive sampling, no substrate dependence and low variability expected, Table 1). Nevertheless, certain limitations concerning the sampling and the reliability of results should be highlighted:

- 1) the device is more bulky for use in the field than the bottles needed to sample biofilm/surrounding water samples;
- 2) the resuspension of cells should be better standardized than the simple use of a fixed area hand-paddle device (i.e. automatic mixing to be integrated in the device),
- 3) in the case of major blooms with thick biofilms, some of the cells may not be resuspended and therefore the integrated values may be slightly underestimated. Yet this measure, based on mimicked mechanical action of waves/trampling, still represents the best approximation of the number of cells potentially resuspended in the water that could directly or indirectly (via aerosols) affect beachgoers;
- 4) in the case of low cell abundances in the biofilm, the concentration of resuspended cells may not be high enough to allow a precise quantification of integrated abundances with the 1 ml Sedgewick-Rafter chambers. In this case, sedimentation in the Utermöhl chamber would be needed to have precise estimations of the cell abundances for research purposes, but this may not constitute a problem for monitoring and risk assessment purposes. As an example, based on the regression

Table 3

Results of permutational multivariate analysis of variance (PERMANOVA) of 3 replicates BEDI dataset (cells mm⁻²). In bold, the statistically significant values. No pairwise tests were performed as the term of interest (Station) is a random factor.

Source	Df	MS	Pseudo-F	P (perm)
Site	2	3.1976E5	2.7739	0.057
Time	6	1.1152E5	0.9806	0.477
Station (Si)	6	1 499.5	0.6935	0.715
SixTi	10	1.1514E5	53.2750	0.001
St(Si) x Ti	31	2159.4	0.7325	0.885
Residuals	110	2947.8		
Total	165			

Table 4

Ostreopsis concentration thresholds (cells l⁻¹) commonly used in monitoring (Tichadou et al., 2010; Lemée et al., 2012; Funari et al., 2015), *Ostreopsis cf. ovata* abundances used by scientists for definition of blooms (cells g⁻¹ of FW of macroalga) and corresponding predictions of BEDI values (both cells mm⁻² and PRcells ml⁻¹). Predictions were calculated from the data pooled from ROC and HAL sites, showing significant correlations (GRA site was excluded as the correlations were not always significant).

Monitoring (Funari et al., 2015)	cells l ⁻¹ of seawater	cells mm ⁻²	PRcells ml ⁻¹
Routine	10 × 10 ³	189	538
alert	30 × 10 ³	581	1656
emergency	100 × 10 ³	1953	5568

Bloom Definition	cells g ⁻¹ of macroalga	cells cm ⁻²	PRcells ml ⁻¹
bloom	200 × 10 ³	279	816
major bloom	1000 × 10 ³	1319	3856

lines provided by the first application of BEDI, 20 PRcells ml⁻¹ (cells counted in the Sedgewick Rafter chamber, corresponding to the 'Potentially Resuspended cells per millilitre) correspond to less than 7 cells mm⁻² (for an average 35 cm water depth inside BEDI), to less than 1000 cells g⁻¹ for 5 g of *Halopteris scoparia* (FW) and to less than 300 cells l⁻¹ in the surrounding water (per 50 ml sedimentation Utermöhl chamber). All these values are well below the thresholds generally used in monitoring for risk assessment (i.e. Tichadou et al., 2010; Lemée et al., 2012; Funari et al., 2015; Table 4).

Further research may lead to an improvement of the BEDI device presented here, fostering even easier and more reliable collection of data. The rationale behind the BEDI is to develop a standardized sampling method providing comparable results, a priority for a better understanding of benthic Harmful Algal Blooms worldwide (Berdalet et al., 2012; Zingone et al., 2012). Only coordinated efforts based on standardized techniques will ensure broad scale knowledge of the bHABs blooms that, when associated with local management actions, could lead to positive developments in the mitigation of blooms and/or a reduction of the potential public health, ecological and economic impact.

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APPENDIX 2.

This brochure was designed and written in collaboration with scientists within the RAMOGE Accord. The aim was to educate the communes about *Ostreopsis cf. ovata* blooms to increase public awareness of summer blooms occurring in the French Riviera, Italian Riviera and in Spanish coastlines. Below is the French version.

Quels sont les effets sur les écosystèmes ?

Par ailleurs, les différentes études menées en Méditerranée, au Brésil et en Nouvelle-Zélande ont montré que le développement de certaines espèces d'*Ostreopsis* peut être accompagné d'une mortalité chez certains invertébrés marins, comme les oursins, étoiles de mer, moules et crabes.



Cette mortalité peut être aussi bien liée à la toxicité des micro-algues qu'aux effets indirects des efflorescences (comme une diminution de la teneur en oxygène de l'eau de mer suite à la dégradation des micro-algues par les bactéries).

©M. Vila, ICM-CSIC.

Macro algues recouvertes d'un épais biofilm d'*Ostreopsis*.

Actions en cours pour mieux comprendre et gérer le phénomène *Ostreopsis*

Depuis les récents événements d'efflorescences d'*Ostreopsis* en mer Méditerranée, des programmes de surveillance ont été mis en place par les autorités et les chercheurs afin de surveiller les concentrations des micro-algues des zones de baignade les plus fréquentées pendant les périodes estivales.

Ces programmes de surveillance initiés en France, en Espagne, à Monaco et en Italie visent à réduire le risque d'intoxication et à mieux comprendre l'écologie et la dynamique d'*Ostreopsis*.

Des directives nationales ont également été édictées afin d'informer les populations locales sur les efflorescences d'*Ostreopsis* et de leur indiquer les comportements à adopter en cas d'efflorescence.

Numéro utile

En cas de symptômes pouvant être causés par une efflorescence d'*Ostreopsis*, alertez le poste de secours le plus proche sur la plage ou allez à la pharmacie.

Vous pouvez contacter le

CENTRE ANTIPOISON ET DE TOXICOVIGILANCE
de Marseille

04 91 75 25 25

L'ALGUE OSTREOPSIS



Accord RAMOGE

Prévention & Lutte contre la Pollution du Milieu Marin

Secrétariat de l'Accord RAMOGE
Le Michelangelo
7, avenue des Papeyans - 98 000 Monaco

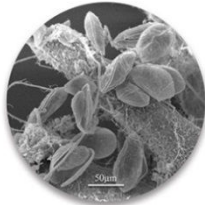
tel (+377) 98 98 42 29
fax (+377) 98 98 40 07

contact@ramoge.org

www.ramoge.org



Qu'est-ce qu'*Ostreopsis* ?

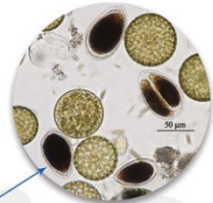


***Ostreopsis* est un micro-organisme**, c'est à dire un organisme microscopique, trop petit pour être vu à l'œil nu, constitué d'une seule cellule. Il est assimilé à une **micro-algue marine en forme de goutte d'eau** qui appartient au groupe des dinoflagellés (possède deux flagelles).

© J.M. Fortuño, M. Vila, ICM-CSIC. *Ostreopsis ovata* accrochées à une macro algue (microscope électronique).



© Paolo Moretto. Cellules d'*Ostreopsis ovata* vues au microscope.



© Paolo Moretto. Biofilm d'*Ostreopsis ovata* recouvrant une roche.

***Ostreopsis* est une espèce épibenthique** qui vit et se développe sur les algues des petits fonds rocheux, sur les feuilles de Posidonie et également sur les invertébrés mais aussi sur les rochers. Pour adhérer à ces substrats, ***Ostreopsis* peut produire du mucus**. Dans certaines circonstances, les cellules d'*Ostreopsis* peuvent se retrouver dans la colonne d'eau ainsi qu'en surface.

Où et quand est-il possible d'observer des efflorescences d'*Ostreopsis* ?



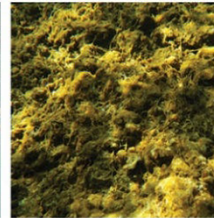
Une efflorescence d'*Ostreopsis* se produit lorsque le nombre de cellules augmente rapidement et atteint des concentrations très élevées.

Les espèces du genre *Ostreopsis* sont bien connues des régions tropicales et subtropicales du Pacifique, de l'Atlantique et de l'Océan Indien, mais elles sont également présentes dans les régions tempérées, comme en Méditerranée, où le nombre d'efflorescences a augmenté dans les dernières décennies.

Dans les régions tempérées, et en particulier sur les côtes nord-ouest de la Méditerranée, les efflorescences d'*Ostreopsis* ont un caractère saisonnier. Elles sont généralement observées durant les périodes les plus chaudes (juillet-août) et à l'automne.



© Luisa Mangialajo. Agrégats mucilagineux d'*Ostreopsis*, en surface.



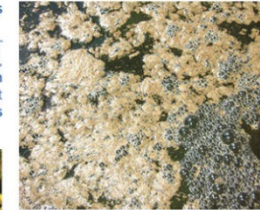
© Luisa Mangialajo. Macro algues recouvertes d'un épais biofilm d'*Ostreopsis*.

Ces fortes concentrations sont généralement favorisées par la température élevée de l'eau de mer (environ 25° C) et par un faible hydrodynamisme, c'est à dire par «mer calme». Les efflorescences d'*Ostreopsis* se produisent généralement dans les petits fonds, correspondant aux zones de baignade.

Quels sont les effets sur la santé humaine ?

En général, la prolifération d'algues est inoffensive. Cependant, lorsque les espèces qui prolifèrent sont toxiques, on parle d'efflorescences nuisibles [HABs « Harmful Algal Blooms »], qui peuvent potentiellement avoir des impacts négatifs sur la santé humaine, les écosystèmes marins et sur l'économie locale.

Certaines espèces d'*Ostreopsis* produisent une substance toxique, la palytoxine [PTX]. Ce composé toxique est aussi produit par d'autres organismes tropicaux comme certains coraux mous d'Océan Pacifique. La palytoxine est une molécule complexe qui dans les régions tropicales est à l'origine de graves intoxications en cas d'ingestion de produits de la mer contaminés [poissons et crabes].



En Méditerranée, certaines espèces d'*Ostreopsis* contiennent des dérivés de cette palytoxine, appelés ovatoxines. Les efflorescences causent fréquemment des problèmes respiratoires par inhalation d'aérosols contenant des cellules, des débris et/ou du mucilage transporté par le vent, et plus rarement des irritations cutanées et oculaires par contact direct avec des agrégats d'*Ostreopsis*.

© M. Vila, ICM-CSIC.

Agrégats mucilagineux d'*Ostreopsis* en surface.

Ces désagrégements peuvent également être accompagnés de fièvre et de toux. Dans la plupart des cas, ces problèmes de santé s'atténuent après quelques heures (2 à 6 heures) et disparaissent complètement au bout de 24 à 48 heures sans besoin de médication, juste en s'éloignant de la mer.

Bien qu'à ce jour il n'y ait pas eu de cas d'intoxication alimentaire due à des proliférations d'*Ostreopsis* en mer Méditerranée, des fruits de mer (oursins, coquillages, crabes) et poissons pourraient concentrer les toxines et ensuite présenter un risque pour la sécurité alimentaire. Il est recommandé aux personnes pratiquant la pêche de loisir d'éviscérer les poissons avant leur consommation et de ne pas consommer les coquillages, oursins et crabes prélevés dans les zones sujettes à la prolifération d'*Ostreopsis*.