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EFFECTS OF ALLELOPATHIC COMPOUNDS PRODUCED BY NATIVE AND
INVASIVE MARINE MACROALGAE ON THE ASSOCIATED
COMMUNITIES

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Abstract

Micro and macroalgae produce a wide range of bioactive secondary metabolites called "allelochemicals", including alkaloids, phenols, organic acids, cyclic peptides, and polyunsaturated aldehydes (PUAs). PUAs derive from the oxidation of fatty acids and are characterized by different lengths of the carbon chains and by number and position of double bonds.

Negative effects on grazers, such as copepods, exposed to PUAs have been observed (e.g. reduction in survival, egg production and hatching success). Additionally, changes in growth rate, cell membrane permeability, and cell morphology in phytoplankton organisms exposed to PUAs have been reported. However, little information is available on the production of these compounds by macroalgae and on their effect on microphytobenthic and meiobenthic communities, as most of the studies have been performed on planktonic organisms.

Marine macroalgae are widely recognized as ecosystem engineers and/or key organisms in many habitats; in the last decades, it has been seen how the introduction of invasive species can change the benthic habitat structure. Such changes will propagate along food chains affecting lower trophic levels, such as microphytobenthic and organisms and, indirectly, higher trophic level species that feed on these invertebrates and use the algal structure as refuge. In fact, macroalgae present a great variability in their morphology and structural complexity, that is a primary factor in structuring the associated communities.

Various studies have focused on the search for chemical compounds produced by invasive macroalgae, as it could be useful for understanding their success and evaluating their impact on invaded areas.

In this context, my thesis is focused on native and invasive macroalgae in terms of production of allelochemicals (i.e., PUAs) and structural complexity and their role in influencing the interaction between benthic organisms, such as meio and microphytobenthos.

My work has been articulated in several field and laboratory studies:

- a field study at Piscinetta del Passetto (Ancona, North-western Adriatic Sea) that analysed the qualitative and quantitative production of aldehydes by two different native macroalgae species, their morphology, and their associated microphytobenthos and meiofauna over a period of several months;
- a field study at two sites in the Ancona area (Piscinetta del Passetto and Port of Ancona) that analysed the qualitative and quantitative production of aldehydes by four native and one invasive macroalgae species (*Sargassum muticum*), their morphology, and their associated microphytobenthos and meiofauna;
- a field study at Tarifa (Spain, West Mediterranean Sea) that analysed the qualitative and quantitative production of aldehydes by one native and one invasive macroalgae species (*Rugulopterix okamurae*), and their associated microphytobenthos and meiofauna;
- a microcosm laboratory study that evaluated the effect on meiofauna of the PUAs producing diatom *Skeletonema marinoi* and of a PUA standard (i.e., decadienal, C10:2);
- Laboratory toxicity tests that assessed the inhibitory effect of dilkamural (an allelopathic compound recently isolated from *Rugulopterix okamurae*) on the growth of unicellular phototrophs (diatoms and cyanobacteria).

PUAs produced by macroalgae resulted to be a fingerprint for the species, resulting in a similar aldehydes profile independently of the sampling site and time, and furthermore they showed to have a species-specific effect on the benthic community.

Morphology and structural complexity of macroalgae has been confirmed as an important factor structuring the associated communities, especially for microphytobenthos.

Moreover, invasive species (*S. muticum*, *R. okamurae*), have shown a huge effect on the native benthic communities, significantly reducing their biodiversity, and causing a more homogeneous and simpler habitat.

In particular, the production of dilkamural by *R. okamurae* has been hypothesized as an allelochemical defense and results of the laboratory toxicity tests performed testing different concentrations of dissolved dilkamural on various microalgae and cyanobacteria confirmed this hypothesis. Overall the results obtained in the present thesis through field and laboratory experiments have highlighted how allelopathic interactions and macroalgal structural

complexity, can play an important role in regulating the interactions between epibenthic communities, leading to speculation that allelochemicals and algal structure can trigger complex cascading effects in the trophic web within the benthic environment.

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Chapter 1 – General Introduction

1.1 Allelopathy

The phenomenon of allelopathy (from the Greek “allélon” = of each other or mutual, and “pàthos” = suffering), meaning the injurious effect of one upon another, has long been known and exploited by agronomists in the cultivation of terrestrial plants. The term regards the production of biomolecules, mostly specialized metabolites, by an organism that can induce suffering in, or give benefit to, another organism (e.g., plant). The concept suggests that biomolecules, namely allelochemicals, produced by an organism escape into the environment and subsequently influence the growth and development of other neighbouring organisms. The term that identifies the discipline was coined by Molisch in 1937 (Fistarol et al., 2004), including both harmful and beneficial biochemicals that are contracted between all plants, directly or through the mediation of microorganisms. There are other definitions of the term. According to the International Allelopathy Society, the term refers to any process involving secondary metabolites (allelochemical) produced by plants, algae, bacteria, and fungi that influence the development of natural and agricultural systems. This definition includes both stimulants and inhibitors, and both direct and indirect effects (Legrand et al., 2003). The term "allelopathy" has been used to refer to aquatic environments only in relatively recent times. In 1931, Akehurst developed a series of hypotheses to explain the succession of algal species in freshwater ponds, suggesting that organisms, such as diatoms, produce substances that favour the presence of members of chlorophytes, and vice versa. In the mid-1930s, it was suggested that substances released by phytoplankton may affect zooplankton species (e.g. on egg production, egg hatching success and/or *nauplii* survival).

Today we know that the production of bioactive substances is very common in phytoplankton species: some allelochemicals influence intraspecific competition and others towards consumers (Burkholder et al., 2006), although many compounds isolated or suspected of having allelopathic effects have yet to be characterized (Legrand et al., 2003).

The algal taxonomic groups known to produce allelopathic substances towards other algae, bacteria, and predators include both marine and freshwater species, such as cyanobacteria, diatoms, dinoflagellates, prymnesiophytes, and raphidophytes (Granéli et al., 2008). In the aquatic environment, studies of allelopathic interactions between phytoplankton organisms often have the donor organism as their main object, but the study of the response of the receptor organism can help to understand the role of allelopathy in competition between

phytoplankton, especially in natural environments. The proximity between donor and target organism ensures the transfer of the allelochemical from one to another, but in the aquatic environment the substances released are constantly diluted, and the advantage of the donor for the reduced competition and the increased availability of resources overlaps the capacity of the receptor to resist or not the chemical agent encountered, to coexist with the donor and perhaps to benefit from the allelochemicals (Legrand et al., 2003). For planktonic species, the allelochemicals must be somehow water-soluble to facilitate their transfer to target cells. Due to the dilution, the concentrations of the excreted molecules decrease rapidly as the distance from the cell increases, so allelopathy occurs especially during blooms when the cell density is sufficient for the allelochemicals to reach high concentrations. In the closed environment of the biofilm, lipophilic allelochemicals could become very concentrated, while they remain practically absent in the surrounding water (Allen et al., 2016). Benthic microalgae can be exposed to more concentrated allelochemicals emitted by neighbouring cells; frequent exposures to allelochemicals would cause a high selective pressure which leads to the appearance of resistant strains.

In the natural environment, the agents causing stress (e.g., nutrient depletion) affect both the donor and the receptor: the first reacts by increasing the production of allelochemical substances and the second becomes more sensitive to these substances. Some microalgae, however, cause allelopathy even in non-limiting nutrient conditions, indicating that allelopathy is not just a strategy used in stressful conditions (Legrand et al., 2003). On the other hand, many allelochemical-producing species are more allelopathic during the exponential growth phase rather than the stationary phase, highlighting the ecological importance of these compounds in interference competition (Suikkanen et al., 2004).

Even eutrophication, by altering the relationship between nitrogen and phosphorus, unbalances the availability of nutrients and can stimulate some algal species to produce more allelochemicals, including toxins (Granéli et al., 2008).

The consequences of allelopathic interactions can be various and extent, including loss of motility, cell deformation, and mortality, however many allelopathic compounds produced by algae are not so harmful and can inhibit some functions of the target cell without causing its death. For example, photosynthesis inhibition, growth rate reduction, and grazing inhibition have been observed (Granéli et al., 2008; Legrand et al., 2003).

Specifically, diatoms produce cytotoxic compounds responsible for growth inhibition and teratogenic activity, potentially sabotaging future generations of grazers by inducing poor recruitment. Diatoms not only interfered with egg maturation but also induced strong developmental aberrations in those *nauplii* that developed to hatching. Teratogenic (abnormal) *nauplii* showed a variety of birth defects such as asymmetrical bodies and malformed or reduced number of swimming and feeding appendages (Ianora et al., 2004; Poulet et al., 1995).

The secondary metabolites have multiple functions and a bioactive role in chemical defense (antigrazers, antibacterials, allelopathics). Allelopathic compounds isolated from marine organisms include low-molecular-weight peptides, phenols, alkaloids, fatty acids, and their derivatives, including oxylipins such as aldehydes (Tab.1.1).

Table 1.1- The comparative effects of some polyunsaturated aldehydes and eicosapentaenoic acid (EPA) on various organisms (Leflaive and Ten-Hage, 2009). DD, 2E,4E/Z-decadienal; EC₅₀, compound concentration giving a 50% reduction in growth rate; HD, 2E,4E/Z-heptadienal; LC₅₀, lethal dose required to kill 50% of individuals; MIC, minimum inhibitory concentration; OD, 2E,4E/Z-octadienal.

Target organism	Group	Compound	Exposure time	Effective concentration or tested concentration ($\mu\text{g ml}^{-1}$)	Effects	Reference
<i>Thalassiosira weissflogii</i>	Alga (diatom)	DD	24 h	EC ₅₀ = 0.29 0.5	Growth inhibition Interference with cell cycle progression Photosynthetic efficiency decrease Apoptosis-like cell death	Casotti et al. (2005)
<i>Thalassiosira weissflogii</i>	Alga (diatom)	DD	2 h	10	Nitric oxide generation	Vardi et al. (2006)
<i>Phaeodactylum tricornutum</i>	Alga (diatom)	DD	24 h	EC ₅₀ = 1.07	Growth inhibition	Vardi et al. (2006)
			2 min	10	Increase in intracellular calcium	
			30 min	10	Nitric oxide generation	
			2 h	10	Cell death	
<i>Dunaliella tertiolecta</i>	Alga (chlorophyte)	DD	24 h	EC ₅₀ = 0.33	Growth inhibition	Ribalet et al. (2007a)
		OD	24 h	EC ₅₀ = 0.70	DNA degradation	
		HD	24 h	EC ₅₀ = 1.18		
<i>Amphidinium carterae</i>	Alga (dinoflagellate)	DD, HD, OD	24 h	twice EC ₅₀	Growth inhibition	Ribalet et al. (2007a)
		DD	24 h	EC ₅₀ = 0.25	Chromatin fragmentation	
		OD	24 h	EC ₅₀ = 0.65		
		HD	24 h	EC ₅₀ = 0.99		
		DD, HD, OD	24 h	twice EC ₅₀		
<i>Staphylococcus aureus</i>	Bacteria	DD	18 h	MIC = 7.8	Growth inhibition	Bisignano et al. (2001)
<i>Vibrio splendidus</i>	Bacteria	DD	24 h	33.3 μg per disc	Growth inhibition (agar diffusion assay)	Adolph et al. (2004)
<i>Listonella anguillarum</i>	Bacteria	EPA	24 h	0.3	Growth inhibition	Desbois et al. (2009)
<i>Artemia salina</i>	Crustacean (brine shrimp)	DD	24 h	EC ₅₀ = 2.14	Larval mortality	Caldwell et al. (2003)
<i>Calanus helgolandicus</i>	Crustacean (copepod)	DD	72 h	1.5 (female exposure)	Teratogenesis and nauplii death - Induction of apoptosis in embryos	Ianora et al. (2004)
			1 h	5	Hatching success decrease	Romano et al. (2003)
<i>Temora stylifera</i>	Crustacean (copepod)	DD	24 h	1.5	Hatching success decrease	Miralto et al. (1999)
<i>Tisbe holothuriae</i>	Crustacean (copepod)	DD	24 h	LD ₅₀ = 1.4	Nauplii death	Ceballos & Ianora (2003)
<i>Sphaerechinus granularis</i>	Echinoderm (urchin)	DD	2-3 h	1.5-3	Cell cleavage blockage (eggs) Cell divisions blockage (embryos)	Taylor et al. (2007) Adolph et al. (2004)
<i>Asterias rubens</i>	Echinoderm (sea star)	DD	48 h	0.5	Hatching success decrease	Caldwell et al. (2002)
		EPA	4 h	25	Development inhibition	Caldwell et al. (2004)
			4 h	5	Fertilization success decrease	
			15 min	0.05	Decrease in sperm motility	
			4 h	20	No effect	

1.2 Polyunsaturated aldehydes (PUAs)

Aldehydes are among the most studied allelopathic compounds identified in the aquatic environment (Dabrowska and Nawrocki, 2013). They can originate from a variety of processes like oxidation, photochemical transformations, and decomposition of organic matter.

In particular, oxylipins are a large family of compounds comprising polyunsaturated aldehydes (PUAs), firstly identified from *Thalassiosira rotula* (Miralto et al., 1999), and other fatty acid derivatives with hydroxy-, keto-, oxo- and hydroxy-epoxy units, generically named non-volatile oxylipins and recently reported as linear oxygenated fatty acids (Ruocco et al., 2020). Several studies have suggested that, in addition to PUAs and oxygenated fatty acids, fatty acid hydroperoxides can trigger impacts on marine biota because these primary LOX products are reactive oxygen species (ROS), inducing DNA and protein damage that contribute to cell ageing (Fontana et al., 2007).

The enzymatic cascade leading to oxylipin production involves lipoxygenase (LOX)/hydroperoxidelyase (HPL) enzymes, which convert polyunsaturated fatty acids (PUFAs) into fatty acid hydroperoxides that are, in turn, converted into a plethora of compounds through mechanisms that are still largely unknown (Cutignano et al., 2006).

Diatoms are considered among the principal PUA-producers. PUAs are volatile compounds commonly released into the aquatic environment (at nanomolar concentrations) by different phytoplankton species (Wichard et al., 2005) in response to environmental stressors (e.g. wounding, grazing, or competition for nutrients) (Dittami et al., 2010; Ribalet et al., 2014), and can persist in the water even after phytoplankton bloom decline (Bartual and Ortega, 2013). Experimental works demonstrated that PUAs can induce changes in microzooplankton growth dynamic and community structure (Franzè et al., 2018), have a teratogenic effect on copepods (Romano et al., 2010) and have inhibitory effects on the reproduction and development of marine invertebrates, thus they are considered an anti-grazer defense (Miralto et al., 1999; Poulet et al., 2007; Lauritano et al., 2012; Brugnano et al., 2016). In particular, the teratogenic effect of PUAs on zooplankton grazers highlights the diatom–copepod paradox that refers to the decreased hatching success when copepods feed on diatoms (Ianora et al., 2003).

Some studies evidenced PUAs production by different benthic diatoms (Jüttner et al., 2010; Pezzolesi et al., 2017; Scholz and Liebezeit, 2012), demonstrating as benthic species could affect community composition. As for macroalgae, there are only a few reports that attest to the production of PUAs, and mostly refer to *Ulva* spp., which report the production of long-

chain aldehydes (e.g. pentadecanal, heptadecenal, decadienal) (Kajiwara et al. 1996; Akakabe et al. 2003; Alsufyani et al. 2014). In a recent study by Pezolesi et al. (2021), several Mediterranean macroalgae were investigated for PUAs production at different times and *Dictyopteris polypodioides* and *U. cf. rigida* resulted among as the main producers documenting the production of high amount of long chain compounds (such as tetradecapentaenal, hexadecatrienal). All these studies demonstrated that the amount and structural diversity of released PUAs, as well as other oxylipins, can vary depending on the species and on the environmental conditions (Barbosa et al., 2016).

1.3 Macroalgae as habitat forming

Macroalgae are key elements in the ecology of rocky shores due to their role as primary producers as well as habitat providers for a wide variety of species (Graham et al. 2017; Thornber et al. 2017). They increase substrate heterogeneity and provide both resources and shelter for microbial and algal epiphytes, sessile and mobile invertebrates (Thornber et al. 2017; Losi et al. 2018). As they add roughness to the landscape and are semi-permeable to water, light, and particles, macroalgae can modify their local environment by acting as ecosystem engineers. Associated species often show complex trophic interactions and may also act as additional secondary habitat-forming species; therefore, macroalgae initiate both trophic and habitat cascades that greatly increase coastal biodiversity (Thomsen et al. 2016; Cunha et al. 2018).

Large habitat-forming algae have been adversely affected by various perturbations, such as coast-wide urbanization, human usage, species invasions, and increases in sedimentation (Airoldi and Beck, 2007, Airoldi et al., 2008, Mangialajo et al., 2008, Perkol-Finkel and Airoldi, 2010). These diffuse and local impacts can have both short and long-term consequences for benthic communities, resulting in the loss of biogenic habitat, reduction in diversity, simplification of vertical structure, and reduction or loss of functioning such as primary productivity (Schiel and Lilley, 2011).

1.4 Non-indigenous macroalgae species

In the Mediterranean Sea, in the last decades, more than 60 macroalgae have been introduced and about 8 of them caused serious invasions (Verlaque and Boudouresque, 2002), where introduced macroalgae constitute the dominant organisms of benthic assemblages (Verlaque and Fritayre, 1994; Piazzì and Cinelli, 2003) and represent a threat for native assemblages (Verlaque 1994; Balata et al. 2004). Biological invasions are considered one of the most important drivers of biodiversity loss with both ecological and economic impacts (Clavero and García-Berthou, 2005; Bellard et al., 2016; Bacher et al., 2018; Pysek et al., 2020). Marine non-indigenous species may become invasive leading to several consequences, such as the displacement of native species, loss of native genotypes, habitat modifications, changes in the community structure, changes in food web structure and ecosystem processes, impact on human health, and substantial economic losses (Grosholz, 2002; Wallentinus and Nyberg, 2007; Molnar et al., 2008; Vilà et al., 2010). Marine macroalgae account for a substantial proportion of the known introductions of marine species globally (Schaffelke et al., 2006) and their ecological effects have been reviewed comprehensively over the last decades (e.g. Schaffelke et al., 2006; Williams and Smith, 2007). Nevertheless, impacts have been mostly documented for a few, well studied, high-profile algal species such as *Caulerpa taxifolia*, *Codium fragile*, *Sargassum muticum* and *Undaria pinnatifida* (Byers et al., 2010; Cacabelos et al., 2013, Salvaterra et al., 2013; Vaz-Pinto et al., 2014). Three main factors are listed as contributing to the species introduction and spreading: 1) heavy tourist and commercial naval traffic with extra-Mediterranean countries; 2) the import of shellfish products, and 3) aquaculture (Zenetos et al., 2012). Globally, the impacts of invasive macroalgal populations are typically expressed as community dominance through space monopolization and change in competitive relationships among native assemblages. They can outcompete native species for space, light, or nutrients, and the competition usually creates monospecific stands, less diversity and homogenized habitats (Davidson et al., 2015).

Non-indigenous macroalgae are particularly likely to become invasive because their high reproductive rates, the production of specialized metabolites (such as allelochemicals), and/or their perennial status which make them more competitive with native species (Máximo et al., 2018) (Tab. 1.2).

Table 1.2- Life traits of the Mediterranean populations of invasive macrophytes. “Vegetative reproduction” means dispersal of cuttings. ++ and + =yes, - = no,?= uncertain (Verlaque and Boudouresque, 2002).

Species	Large sized	Perennial	Without a resting stage	Sexual reproduction	Vegetative multiplication	Defence metabolites	No or few predators	Functional form group
<i>Acrothamnion pressii</i>	-	?	+	-	+	+	?	Filamentous
<i>Asparagopsis armata</i>	+	-	-	+	+	++	+	Coarsely branched
<i>Lophocladia lallemandii</i>	-	?	-	+	-	+	+	Filamentous
<i>Womersleyella setacea</i>	-	-	+	-	+	?	+	Filamentous
<i>Sargassum muticum</i>	+	+	-	+	-	+	+	Thick leathery
<i>Styopodium schimperi</i>	+	+	-	?	-	+	?	Thick leathery
<i>Caulerpa racemosa</i>	+	+	-	+	+	+	+	Coarsely branched
<i>Caulerpa taxifolia</i>	+	+	+	-	+	++	+	Coarsely branched
<i>Halophila stipulacea</i>	-	+	+	+	+	-	-	Coarsely branched
<i>Antithamnion pectinatum</i>	-	-	-	-	+	?	?	Filamentous
<i>Audouinella sargassicola</i>	-	-	-	-	-	-	-	Filamentous
<i>Chrysomenia wrightii</i>	+	-	-	+	-	?	?	Coarsely branched
<i>Pleonosporium caribaeum</i>	-	-	-	+	-	-	-	Filamentous
<i>Colpomenia peregrina</i>	-	-	-	-	-	-	-	Coarsely branched
<i>Laminaria japonica</i>	+	-	-	+	-	-	+	Thick leathery
<i>Undaria pinnatifida</i>	+	-	-	+	-	-	-	Thick leathery
<i>Caulerpa mexicana</i>	+	+	-	+	+	+	?	Coarsely branched
<i>Codium fragile</i>	+	+	+	-	+	-	+	Coarsely branched

In this context, important invasive events have occurred in the Mediterranean coastal areas in recent years, dealing with the exotic brown macroalgae *Sargassum muticum* and *Rugulopterix okamurae*.

Sargassum muticum (Yendo) Fensholt (Fig. 1.2), is known to be a highly successful alien seaweed, with many of the intrinsic traits of an invasive species, including very high growth rates of 2-4 cm per day, high fecundity, monoecious individuals with a perennial life history (Norton 1977) and multiple-range dispersal mechanisms including germling settlement and drifting fertile thalli (Sabour et al., 2013). This Japanese seaweed was accidentally introduced to Pacific North America in the 1940s and then to European coasts in the early 1970s, in both cases associated with the intentional transfer of the Pacific oyster *Crassostrea gigas* (Thunberg, 1793). In many areas it forms dense beds that replace the native vegetation. As for the Italian coasts, this species was recorded in Venice in the 1990s and early 2000s, and since 2009so also at the harbour of Ancona, probably due to transport by ships or by currents (Falace et al., 2010).

While *R. okamurae* has shown an intense proliferation in the south-western coasts of Europe (Strait of Gibraltar), becoming the most abundant species in a single year, covering over 90% of the seabed between 2 and 20 m depth. There is currently no clue as to the reasons for such a huge invasive potential, although the involvement of chemical defenses has been hypothesized. Recently, a chemical study on *R. okamurae* (Fig. 1.1) from the Strait of Gibraltar

led to the isolation of several metabolites, among which a compound named dilkamural stands out due to its high concentration.

Recently, chemical ecologists have started to consider how research on natural products might be useful in understanding marine biological invasions, assessing their impact in the invaded areas, and considering how to deal with them. Their efforts especially focused on the Mediterranean Sea, which is one of the major hotspots of marine biological invasions on earth, showing in what way marine natural products may influence (1) the ability of exotic marine organisms to invade and to get established, (2) the invaded biota, and (3) public health and the economy.



Fig. 1.1: *Rugulopteryx okamurae* © Photo El Diario



Fig. 1.2: *Sargassum muticum* in Ancona harbour (Italy)

1.5 Ecology of meio and microphytobenthos associated with macroalgae

Rocky coasts (or hard bottoms) are the most common littoral habitats and constitute both areas exposed to wave motion and more sheltered and protected ones, which descend steeply to the sea. These environments form complex ecosystems that play a crucial role in numerous marine biological and geological processes, such as seabed colonization and sedimentation phases (Thompson et al. 2002). The resulting complex ecosystems determine the presence of high density and diversity of marine organisms that have developed a multitude of strategies to adhere to and/or excavate the occupied substrate. In rocky coasts, the subtidal benthic domain is characterized by marked vertical zoning, generated by the action of physical gradients such as solar radiation and wave motion. These habitats are characterized by populations of photophilous macroalgae, which support a rich animal and plant community associated with them.

However, macroalgal structural composition and ecophysiological properties, such as morphology (Padilla and Allen, 2000), surface area (Thomas and Jiang, 1986), functional form (Steneck and Watling, 1982), and even allelochemical potential (Steinberg, 1985) can all contribute to the abundance and distribution of the associated species. Algae with a complex structure and with a greater deposit of fine sediments are mainly occupied by small-sized organisms, better adapted to move in these small spaces and to exploit the accessible resources (Gibbons, 1991). At the same time, macroalgae represent an important trophic resource for herbivorous and grazing species, facilitating the recruitment of various benthic species, including polychaetae annelids, bivalve mollusks, amphipod crustaceans and copepods. On the other hand, algae with small and simple fronds offer many meiofaunal organisms insufficient protection against predation, desiccation, and wave action. Furthermore, algae with simple structures provide an inadequate substrate for the accumulation of sediment and detritus, a potential source of food for meiofaunal organisms. A greater complexity of the algal fronds leads to a greater surface area available for colonization by organisms of the meio- and phytobenthos. The presence of epiphytic algae, in turn, further increases the complexity of the microhabitat, encouraging the settlement and development of meiofaunal communities (Chemello and Milazzo, 2002). Studies conducted to investigate the importance of complexity in habitats made up of macrophyte communities have shown that the presence of epiphytes does not affect the total number of individuals,

but rather the associated species, influencing the taxonomic composition of the community (Young & Young, 1977; Sánchez-Jerez et al., 1999).

The composition and structure of the meiobenthic communities associated with macroalgae also depend on the components of the algae themselves, for instance, the meiofauna typical of the fronds is relatively different from that of the rhizome and copepods are generally dominant in the fronds, while nematodes on the thallus and basal disc. Many of the frond-dominating copepods have developed special adaptations to adhere to this substrate and to the mucilaginous secretions produced by the algal cells (Arroyo et al., 2004), for example, they have developed structures suitable for this type of locomotion such as prehensile legs or long filamentous spines (e.g., harpacticoid copepods of the genera *Porcellidium*, *Ectinosoma* and *Thalestris*). In particular, harpacticoid copepods often represent in hard bottoms almost half of the total meiofauna both in terms of abundance and productivity (Danovaro and Fraschetti, 2002). Moreover, also the chemical composition of algae can influence the preference of the meiofauna for the habitat, both in terms of niches and availability of food (Gestoso et al., 2012). In fact, some taxa of the meiofauna feed on algae choosing them based on their components which can enrich their chemical defences, while others, such as various species of amphipods, prefer algae which defend themselves chemically and which are therefore less attractive to predators (Hicks & Grahame, 1979; Hicks, 1980, 1985). Even for microphytobenthos, macroalgae with higher structural complexity may be relevant in modulating their abundance. Furthermore, macroalgal complexity may also have an indirect effect on the richness of the microphytobenthic community under the canopy, as in addition to improving the physical environment, the complexity of a habitat can help in reducing predation and competition, thus providing suitable spaces for microphytobenthic species with particular traits (Bertness and Callaway, 1994; Grabowski, 2004). In fact, diatoms are frequent epiphytes of branching and jointed macroalgae, due to their mobile, erect and adnate forms of growth (Suzuki et al. 2001, Al-Handal and Wulff 2008, Costa et al. 2016).

Thus, the effect of various abiotic and biotic factors on macroalgal communities may in turn have a significant influence on the diversity and abundance of macroalgal-associated organisms (Wilson et al., 2014).

1.6 Research purpose

Research on marine allelochemicals has been focused mainly on their role in regulating the interactions between phytoplankton and its grazers. Most studies have investigated the relationships between planktonic crustaceans and diatoms, the latter being in fact one of the main constituents of phytoplankton in aquatic ecosystems, known to produce allelochemical compounds, such as polyunsaturated aldehydes (PUAs). These substances can reduce the reproduction of the small crustaceans that feed on diatoms, thus regulating the biological cycle of the populations and the functioning of the food web, from algae to the upper levels consumers, such as fish, birds and mammals.

Only few studies have specifically concerned the benthic environment and included the potential involvement of macroalgae both in terms of production of allelochemical compounds and regulation of interactions between the various benthic organisms, and rarely considered the morphological characteristics of the algal species.

In the recent decades most studies on the ecological consequences of climate change have focused on native fauna and flora, while dynamics of non-indigenous and/or invasive species remained not well understood (Bailey et al., 2020). Invasive marine species often have a disproportionate influence on ecological processes (Molnar et al., 2008; Occhipinti-Ambrogi and Galil, 2010) and could produce greater shifts in species composition, diversity, and abundance. Moreover, they may cause severe ecological impacts by modifying the invaded habitat, its food webs, and community structure, as well as by displacing native species (Viard and Comtet, 2016).

As the allelochemicals production and the morphological characteristics of the algae are assumed to be among the main drivers in structuring the associated benthic communities, the general purpose of this thesis is to investigate the effect of allelochemicals (i.e., PUAs and diatoms) produced by invasive and non-invasive macroalgae and of the algal morphological structure on the composition of the associated microalgal and meiofaunal assemblages.

The work has been organized in five chapters, structured as follows:

Chapter 2: this study was performed in field, in a benthic environment of the North-western Adriatic Sea (Piscinetta del Passetto, Ancona, Italy), and the main aims of this study were to
i) analyze the qualitative and quantitative production of aldehydes by two different macroalgae (*Cystoseira compressa* (Esper) Gerloff & Nizamuddin and *Dictyopteris*

polypodioides A.P. De Candolle J.V.) Lamouroux, over a period of some months (Mediterranean spring and summer); ii) understand the relationship between meiobenthic communities, with particular interest to harpacticoid copepods, and the microphytobenthos present on the two macroalgal species, considering the structural complexity of macroalgae and the potential role of aldehydes in regulating their interaction. To our knowledge, this is the first study where macroalgal allelochemistry has been investigated to understand both the microphytobenthos and meiofauna community structure, in relation to the algal complexity. This chapter has been published in the journal Science of the Total Environment (Lenzo et al., 2022).

Chapter 3: In this study I wanted to introduce the topic of invasive species, which in the last decade have proved to be a real threat to the ecosystem. In fact, the study was performed at two rocky shore sites in NW Adriatic Sea (Piscinetta del Passetto and Port of Ancona, Italy), the first site characterized by the presence of a rich phytobenthic assemblage that includes common species, representative of the Mediterranean native phytobenthic community (Rindi et al., 2020) and the second site characterized by the presence of the invasive species *Sargassum muticum*.

The main aims of this study were to analyse: i) the production of PUAs by four indigenous macroalgae and a non-indigenous species (*Sargassum muticum*); ii) the impact of *S. muticum* on the benthic community; iii) the relationship between the meiobenthic community, and the microphytobenthos present on the different macroalgal species, considering the structural complexity of the macroalgae and the potential role of aldehydes in regulating their interaction. This chapter has been published in Water Journal (Lenzo et al., 2023).

Chapter 3 also reports a further study with same purposes as the previous one. This study was carried out in Tarifa (Spain), on the indigenous species *Halopteris scoparia* and the invasive macroalga, *Rugulopterix okamurae*.

Chapter 4: Since several environmental factors come into play in field studies, understanding the effects of allelochemicals on community structure and relationships between taxa is complicated. For this reason, this study was performed in the laboratory in more simplified systems (i.e., microcosms).

The purpose of this study is to evaluate in microcosms: i) the effect of *Skeletonema marinoi*, which is a diatom known to produce PUA, compared to *Phaeodactylum tricoratum*, a diatom that does not produce PUA, on the meiobenthic assemblages associated with the macroalga *Dictyopteria polypodioides*; ii) the effects of decadienal (PUA C10:2), as pure chemical (analytical standard) on the meiofauna associated with *D. polypodioides*, in a short-term (96 hours) test.

Chapter 5: I performed another study in the laboratory, but in this case, this study was aimed at investigating the effects of dillkamural (a compound recently extracted from the invasive species *R. okamurae*), on unicellular phototrophs (i.e., diatoms *Phaeodactylum tricoratum* and *Cyclotella cryptica*, and the cyanobacterium *Synechococcus* sp.) in a growth inhibition assays, to understand the potential role of this compound as an allelochemical. A manuscript based on this chapter to be submitted to the Journal Aquatic Toxicology is in preparation.

Chapter 6: General conclusion of my thesis work.

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Chapter 2 - Allelopathic interactions between phytobenthos and meiofaunal community in an Adriatic benthic ecosystem: understanding the role of aldehydes and macroalgal structural complexity

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Abstract

Macroalgae produce several allelopathic substances, including polyunsaturated aldehydes (PUAs), which may inhibit photosynthesis and growth rates of other algal species, and grazing. Additionally, macroalgal structural complexity is an important factor in determining abundance patterns and size structure of epiphytic organisms.

In this study the PUAs production of two Mediterranean macroalgae, *Dictyopteris polypodioides*, (DP, Phaeophyceae, Dictyotales) and *Cystoseira compressa* (CC, Phaeophyceae, Fucales), was characterized to clarify the relationships between the meiobenthic and microphytobenthic communities. Results showed a higher PUAs production and a diverse qualitative profile for DP, which reported long-chain compounds (i.e. C14-C16) as main aldehydes, than CC, with the short-chain C6:2 as main compound, as well as variability among sampling times. A clear separation of the meiofauna and microphytobenthos assemblages was found for the macroalgae, but with different temporal trends. Dissimilarities were due to five microalgal orders, namely Naviculales, Lyrellales, Gonyaulacales (i.e. *Ostreopsis*), Bacillariales, and Licmophorales, and to the meiofaunal groups nematodes, copepods, and copepod *nauplii*, which were more abundant on DP than on CC. Results indicate that macroalgal complexity is a major determinant of the meiofaunal community structure (accounting to 26% of the variation), rather than PUAs production itself (17%). PUAs effects seem species-specific, thus affecting some grazers instead of the entire community. Conversely, microphytobenthos affected the meiofauna assemblages, particularly harpacticoids, confirming the role of these organisms as primary food source of all marine food chain producers. Since PUAs are produced also by several epiphytic diatoms, the understanding of their effects on the community structure and on the relationships among taxa in the field is complicated and requires further in-depth investigations in simplified systems (i.e. microcosms).

Keywords

Brown macroalgae; PUAs; microalgae; meiofauna; harpacticoid copepods; chemical ecology.

2.1 Introduction

Chemical compounds produced by aquatic organisms and microorganisms have received increasing attention for the important role that may have in modulating the interactions in marine ecosystems (Ivanora et al., 2012; Paffenhöfer et al., 2005). Many organisms produce allelochemicals, secondary metabolites that affect survival and growth of other species and may give a competitive advantage to their producers (Legrand et al., 2003; Fistarol et al., 2004; Tillmann, 2004; Allen et al., 2016).

Several studies regarding chemical interactions between various marine organisms, including copepods, urchins, sea stars, algae, mollusks, and polychaetes, were focused on direct responses of target species to allelochemicals and have been performed mainly under laboratory conditions (e.g. Ivanora et al., 2004; Taylor et al., 2007; Adolph et al., 2004; Caldwell et al., 2004; Ribalet et al., 2007; Vardi et al., 2006).

Algae produce a variety of these allelopathic substances, such as phenolic compounds, alkaloids, peptides, oxoacids, and polyketides, among which there are toxins synthesized by several species (Agostini-Costa et al., 2012; Pistocchi et al., 2012; Snyder et al., 2003), polyunsaturated fatty acid (PUFAs) (Grima et al., 1995; Patil et al., 2007; Tonon et al., 2002) and their derivatives, such as polyunsaturated aldehydes (PUAs) (Ivanora et al., 2012; Wichard et al., 2005; Pezolesi et al., 2017).

Consequences of allelopathic interactions between different algal species can be of various nature and magnitude, including loss of motility, cell deformation (Tang and Gobler, 2010; Pichierri et al., 2017), pigmentation loss, cytoplasm aggregation, formation of vesicles and

cellular lysis (Fistarol et al., 2004). However, the majority of allelochemicals have milder effects, for example inhibition of photosynthesis, reduction of growth rate, and inhibition of grazing (Legrand et al., 2003).

The effect of PUAs have been mostly investigated for planktonic microalgae and only a few studies have evidenced PUAs production by benthic species, such as diatoms (Jüttner et al., 2010; Scholz and Liebezeit, 2012; Pezolesi et al., 2017), and macroalgae (Kajiwara et al., 1996; Akakabe et al., 2003; Pezolesi et al., 2021). Macroalgae are among the most important components of marine coastal ecosystems because they are highly productive (Pinckney and Zingmark, 1993), have a high taxonomic diversity, and may act as foundation species providing habitat for different organisms (Cacabelos et al., 2010). The shape and structural complexity of macroalgae are important factors in determining the abundance patterns and size structure of the epiphytic organisms (McAbendroth et al., 2005). The more structurally complex macroalgal species show abundant and various populations of invertebrates because they provide greater surface area for the colonization of epifaunal assemblages and epiphytic microalgae (Chemello and Milazzo, 2002). Among epifaunal assemblages, macrofauna is the most investigated, while meiofaunal communities are overlooked. Meiofauna represent the most abundant and taxonomically diversified metazoans on Earth (Giere, 2009) and on hard substrates can overcome macrofauna in terms of abundance (Gibbons and Griffiths, 1986). Meiofauna plays a dominant role in the exchange of organic matter (Sandulli et al., 2014; Semprucci et al., 2016) as part of the "small food web" (size class 45-1000 μm). Moreover, it supports most of the higher trophic levels (Giere, 2009), being an important food resource for macrofauna, small fish, juveniles and other epibenthic predators (Chardy and Dauvin, 1992). In phytal environments, harpacticoid copepods are the dominant meiofauna group (Hicks, 1977; Coull and Wells, 1983; Hall and Bell, 1993) and show high diversity (Sarmiento and

Santos, 2012). They feed mainly on diatoms and, for this reason, they have a high content of fatty acids and play a key nutritional role for fish, carnivorous crustaceans (prawns and their larvae), and polychaetes (Coull, 1999; Giere, 2009).

Indeed, few studies have focused on the benthic environment and included the potential role of macroalgae both in terms of production of allelochemical compounds and regulation of interactions between various organisms (Kajiwara et al., 1996; Akakabe et al., 2003), while only one laboratory study analyzed the effects of PUAs on a species of harpacticoid (i.e. *Tisbe holothuriae*) (Taylor et al., 2007). PUAs strongly impair the reproduction of various potential grazers in *in vitro* studies (Ivanora et al., 2004b; Poulet et al., 1994), while in the field the relationship between aldehyde production and reproductive failure of other higher-level organisms (i.e. copepods) remains unclear (Leflaive and Ten-Hage, 2009). Some studies performed to investigate the allelopathic activity of fresh thalli or extracts of *Ulva* spp. reported inhibitory effects on the growth of several microalgae, including harmful species (Tang and Gobler, 2011). The production of aldehydes and their potential consequences on biotic interactions may thus be explored, especially in macroalgae. This would help to determine whether these compounds, which are present across phylogenetic and environmental barriers, may play a role in the ecology of their producers (Leflaive and Ten-Hage, 2009), perhaps affecting the development of epiphytic organisms, such as meiofauna or microalgae.

The main aims of this study were to i) analyze the qualitative and quantitative production of aldehydes by two different macroalgae over a period of some months (Mediterranean spring and summer); ii) understand the relationship between meiobenthic communities, with particular interest to harpacticoid copepods, and the microphytobenthos present on the two

macroalgal species, considering the structural complexity of macroalgae and the potential role of aldehydes in regulating their interaction.

Specifically, this study was performed in a benthic environment of the North-western Adriatic Sea (Piscinetta del Passetto, Ancona, Italy), characterized by the presence of a rich phytobenthic assemblage and by annual blooms of a toxic dinoflagellate, *Ostreopsis cf. ovata* Fukuyo, 1981. Two macroalgal species, *Cystoseira compressa* (Esper) Gerloff & Nizamuddin and *Dictyopteris polypodioides* A.P. De Candolle J.V. Lamouroux, commonly present in this site (Rindi et al., 2020) and representative of the Mediterranean phytobenthic community were selected, based on their different complexity and PUAs composition (Pezzolesi et al., 2021). To our knowledge, this is the first study where macroalgal allelochemistry has been investigated to understand both the microphytobenthos and meiofauna community structure and their interactions, in relation to the algal complexity.

2.2 Material and methods

2.2.1 Sampling area and sampling procedure

The study was performed in a semi-enclosed and shallow (mean depth 1.5 m) inlet called Piscinetta del Passetto (Conero Riviera, Italy, northern Adriatic Sea: 43°37'09" N, 13°31'54" E), described and showed in Pezzolesi et al. (2021). Sampling was carried out at six different times from May to September 2018 (see table S2), with monthly frequency except for September (when sampling was performed twice). In the Mediterranean area, this period corresponds to late spring and summer. Surface temperature and salinity were measured with a multiparameter water probe HQ30d (Hach-Lange GmbH) and a refractometer Atago S-10, respectively.

Apical parts (i.e. tips) of the thalli (first 5-8 cm) of two macroalgae, i.e., *Cystoseira compressa* (Phaeophyceae, Fucales) and *Dictyopteris polypodioides* (Phaeophyceae, Dictyotales), were

sampled at a depth of approximately 0.5 m. At the study site, during the time of the year in which sampling was carried out, these species are present and mostly occur in a well-developed habit. For each macroalgal species, six replicates were collected by snorkeling at each sampling time using 50 mL polypropylene tubes (VWR International), avoiding the dispersion of the associated epiphytic organisms.

Water samples for assessment of dissolved aldehydes and nutrient analysis were collected in 2 L polyethylene bottles (VWR International) in the proximity of the sampled macroalgae. Water was subsequently filtered using GF/F Whatman filters (0.7 μm porosity, 47 mm) and stored at -22 °C until analysis. Macroalgae were treated to remove all associated benthic organisms as described in Pezolesi et al. (2021) using filtered seawater. Briefly, each tube containing the thallus and their storage water was vigorously shaken to separate the macroalga from the epiphytic microalgae and the meiofauna. Then the tube was rinsed with filtered seawater and vigorously washed several times until epiphytic organisms were completely removed. The seaweed thalli were dried with absorbent paper, then weighed to determine fresh weight (g fw) and photographed; finally, they were stored at -80 °C in new tubes. The total volume of washing seawater of each sample (approximately 150 mL) was measured and then divided into two aliquots, one for the microphytobenthos and the other for the meiofauna analyses. Aliquots for microphytobenthos (about 75 mL) were fixed with Lugol and stored in 250 ml dark glass bottles. Aliquots for meiofauna analysis were sieved through a 1000- μm mesh and a 45- μm mesh; the meiobenthic organisms retained on the finer mesh sieve were fixed with 70% alcohol and stored in a 50 ml falcon until subsequent analyses. Each apical part of macroalga was placed on a white surface with a reference scale and then photographed using a digital camera Canon EOS 750D. To take into account the different morphology and complexity of the two algal species, pictures were processed using the

program ImageJ v1.53u. For each image the scale was fixed and transformed to binary format with a pixel width of 1 cm. Then the area, the perimeter, and the fractal complexity (D) were measured based on the image and using a method analogous to the grid method (boundary dimension) proposed by Sugihara and May (1990). In this method, the fractal dimension is the slope of the linear fit of $\log N(s)$ versus $\log (1/s)$; where s represents the scale of analysis and $N(s)$ is the number of objects observed at that scale.

2.2.2 Aldehydes

2.2.2.1 Aldehydes (PUAs) produced by macroalgae

The extraction and quantification of PUAs produced by the different macroalgae was carried out as described in Pezolesi et al. (2021) by gas chromatography-mass spectrometry (GC-MS). Specifically, a portion of the apical part of the thallus (about 0.2-0.8 g f. wt.) was shredded with mortar and pestle, in liquid nitrogen. The powder thus obtained was transferred into 10 mL tubes. Derivatization of the polyunsaturated aldehydes was performed with O-(2,3,4,5,6-pentafluorobenzyl)hydroxylamine hydrochloride solution (PFBHA HCl) and quantification was based on the internal standard (i.e. benzaldehyde). All reagents were purchased from Sigma-Aldrich (Milan, Italy) and used without any further purification.

2.2.2.2 Dissolved aldehydes (dPUAs) in seawater

Dissolved aldehyde concentration was determined following the protocol described by Vidoudez and Pohnert (2008) with slight modifications, using PFBHA HCl in Tris-HCl 100 mM pH 7.2 as derivatizing reagent and benzaldehyde as internal standard, as described in Pezolesi et al. (2021).

2.2.3 Nutrients

Nitrate and phosphate analyses were performed on filtered seawater aliquots (GF/F Whatman filters) and analyzed spectrophotometrically according to Strickland and Parsons (1972).

2.2.4 Microphytobenthic community

The quali-quantitative analysis of the microphytobenthos associated with the two macroalgae was performed using an inverted optical microscope (Zeiss Axiovert 100) at 320x and 200x magnification. Sub-samples (3-5 ml) of epiphytic communities fixed with Lugol were settled in counting chambers after homogenization, according to the Utermöhl's sedimentation method (Utermöhl, 1958; Edler and Elbrächter, 2010). Counting was performed in different ways. The microphytobenthos community was examined at 320x magnification on 30 random fields or 4–5 transects; then a counting at 200x of the organisms present on the whole sedimentation chamber was performed to obtain a correct evaluation of uncommon taxa. The microphytobenthos was identified and recognized following various manuals and identification keys (e.g. Tomas, 1997; Kraberg et al., 2010). The identification of individuals was based exclusively on observable morphological characters (such as shape, size, number of chloroplasts); the current taxonomic status for the microalgae was confirmed following AlgaeBase (Guiry and Guiry, 2021).

2.2.5 Meiofauna and harpacticoid copepods

Meiofaunal organisms of each sample were counted and identified at higher taxonomic levels. All harpacticoids (Order Harpacticoida Sars G.O., 1903) were collected under a stereomicroscope (Nikon SMZ 1500) and stored in 70% alcohol inside 1.5 mL-Eppendorf

labeled for subsequent identification. Harpacticoids were identified to species level using Lang (1948; 1965), and Boxshall and Halsey (2004).

2.2.6 Data analyses

PUAs produced by the macroalgae and dissolved in the seawater were analyzed and expressed as $\mu\text{g g}^{-1}$ fw and $\mu\text{g L}^{-1}$, respectively. Microphytobenthos was analyzed in terms of total epiphytic cells and main orders or genera; data were expressed as the number of cells per gram of fresh macroalgae weight (cells g^{-1} fw). Meiofauna and harpacticoid communities were analysed according to total density (N), standardized towards the fresh weight of macroalgae (ind. g^{-1} fw), the total number of taxa or species (S), and Shannon diversity (H'). For each variable, the average value of the six replicates \pm standard error is reported. All univariate variables were analyzed by a 2-way crossed ANOVA; the factors considered were the macroalgal species (fixed, two levels: *Cystoseira compressa* (CC) and *Dictyopteris polypodioides* (DP)) and time (fixed, 6 levels). Cochran's test was used to check for the homogeneity of variances and data were transformed, if necessary (Underwood, 1996). If even after transformation it was not possible to obtain homogeneity of the variances, untransformed data were analysed and results were considered robust if significant at $p < 0.01$ to compensate for the increased probability of type I error (Underwood, 1996). When significant main effects or interactions were detected, the Student–Newman–Keuls (SNK) test was used for pairwise a posteriori comparisons.

The community structure of each assemblage (microphytobenthos, meiofauna and harpacticoids) was analyzed by non-metric multidimensional scaling (nMDS) based on Bray–Curtis similarity of square root-transformed data. Differences in community structures were assessed by permutational non-parametric multivariate analysis of variance (PERMANOVA)

following the same experimental design adopted for ANOVA (Anderson, 2001; 2005). When significant main effects or interactions were detected, the specific procedure provided within PERMANOVA was used for pairwise a posteriori comparison. The analyses were performed using unrestricted permutation of the raw data and 9999 permutations.

Taxa that mostly contributed to the dissimilarity/similarity among/within macroalgal species and times were identified using the SIMPER analysis (Clarke, 1993).

Relationships between macroalgae complexity, PUAs, dominant microphytobenthic taxa, and both meiofauna and harpacticoid copepod assemblages were analyzed by the distance-based linear model (DistLM) procedure in PERMANOVA+ (Anderson et al., 2008).

A total of 14 explanatory variables, grouped in three sets, were considered: macroalgae complexity descriptors (perimeter/area, D, Area), PUA concentrations (C14:5, C16:4, C16:3, C6:2), and microphytobenthic taxa abundances (*Navicula* spp. J.B.M. Bory de Saint-Vincent, 1822, *Lyrella* spp. N.I. Karayeva, 1978, *Cocconeis* spp. C.G. Ehrenberg, 1837, *Cylindrotheca* spp. L. Rabenhorst, 1859/*Nitzschia* spp. A.H. Hassall, 1845, other diatoms, *Ostreopsis* cf. *ovata*, other dinoflagellates). The relationships between meiobenthic taxa data and harpacticoid abundance (square root transformed data) and the three groups of variables were analyzed by explicitly examining the proportion of variation in the taxa data (or harpacticoid species abundance) that was explained by PUAs concentrations and microphytobenthic taxa abundances over and above the amount explained by macroalgae complexity descriptors.

The Akaike Information Criterion with correction (AICc) was used to select the model. Prior to the DistLM, a draftsman plot and correlation matrices were performed to detect possible skewness of the variables and/or strong correlation among pairs of variables (Anderson and Robinson, 2001). Variables were not strongly correlated, so all variables were entered in the analysis. Concentrations of *Lyrella* spp., and *Cocconeis* spp were square root transformed.

Finally, Pearson correlation analysis was used to test which macroalgal attribute (i.e. complexity descriptors such as D and macroalgal surface (S), and PUAs composition) explained the variation of some representative genera of microphytobenthos, of the main meiofauna taxa and of the most representative harpacticoid species. Densities of microphytobenthic genera, main taxa and harpacticoid species were square root transformed.

Significance level was set at 0.05 (5%) for all tests. ANOVA, Cochran test, SNK test, and correlations were performed by R (version 3.5.3) using packages Lme4; all multivariate analyses were conducted with PRIMER v7 (Clarke and Gorley, 2015) with the PERMANOVA + add-on (Anderson et al., 2008).

2.3 Results

2.3.1 PUAs production by macroalgae

The interpretation of the chromatograms and relative mass spectra obtained by GC-MS gave the quali-quantitative profile of the main aldehydes produced by *Dictyopteris polypodioides* (DP) and *Cystoseira compressa* (CC).

The major aldehydes production was found in T1 (May) for both macroalgae, with values of 225.5 ± 35.6 and $17.1 \pm 4.0 \mu\text{g g}^{-1}$ fw for DP and CC, respectively. Conversely, the lowest amounts were measured at times T3 (July) for DP and T2 (June) for CC, with a concentration of 44.3 ± 5.3 and $2.0 \pm 0.3 \mu\text{g g}^{-1}$ fw, respectively (Table 2.1). From a quantitative point of view, ANOVA results (Table S1) showed a higher PUAs production in DP than in CC and variability among different sampling times ($F=17.73$, $P<0.001$).

From a qualitative point of view, the main compounds detected were the short-chain PUA hexadienal (C6:2), which was present in both algae and resulted as the main compound in CC, and some long-chain PUAs, namely hexadecatrienal (C16:3), hexadecatetraenal (C16:4), and tetradecapentaenal (C14:5), which were detected exclusively in DP (Fig. S2.1). Specifically,

C14:5 was the most abundant compound in DP, with average concentrations ranging from a maximum of $115.3 \pm 20.8 \mu\text{g g}^{-1}$ fw at time T1 to a minimum of $13.2 \pm 2.4 \mu\text{g g}^{-1}$ fw at time T3, corresponding to the 50% and 32% of the total aldehydes (Table 2.1; Fig. S2.1).

C16:4 had the maximum average concentration at time T1 with $36.4 \pm 8.8 \mu\text{g g}^{-1}$ fw, decreasing gradually to a minimum of $5.9 \pm 1.5 \mu\text{g g}^{-1}$ (relative abundance of 8-29%) at time T6. C16:3 showed a similar pattern and an average maximum concentration of $30.9 \pm 9.3 \mu\text{g g}^{-1}$ fw at T1 (relative abundance of 12%); then, its amount decreased to a minimum of $8.7 \pm 3.1 \mu\text{g g}^{-1}$ fw at time T4, and it was not detected at all at T6. In DP the most variable compound was C6:2, with a maximum average concentration of $16.1 \pm 2.2 \mu\text{g g}^{-1}$ fw at time T1; this aldehyde was not detected at times T2 and T5. C6:2 was the main aldehyde in CC, accounting for 64-91% of the total amount, and its average concentration had the maximum and minimum values of 15.4 ± 3.5 and $1.0 \pm 0.1 \mu\text{g g}^{-1}$ fw at time T1 and T2, respectively. This short-chain aldehyde resulted not significantly different between the two algae at the different sampling times.

2.3.2 PUAs (dPUAs) and nutrients in seawater

The total average concentrations of dPUAs detected in the seawater in the proximity of the two macroalgae (Fig. S2.1) highlighted high values (ranging from 99.0 to $287.8 \mu\text{g L}^{-1}$, corresponding to 0.9 and $2.7 \mu\text{M}$) and temporal changes, with concentrations significantly lower at times T2 and T3, and higher (about $200 \mu\text{g L}^{-1}$) in the subsequent times (T4-T6) (Table S2.1). A maximum value was recorded at time T4 for DP and at time T6 for CC (287.8 ± 34.2 and $261.7 \pm 33.6 \mu\text{g L}^{-1}$, respectively). ANOVA carried out on dPUAs concentrations in seawater showed highly significant differences for the factor time ($F=11.8$, $P<0.001$), while no significant differences were found between the two algal species (Table S2.1). Seawater temperatures

during the sampling times ranged between 23 and 26°C, with salinity values of about 34-39 which were higher at T4-T5 (August-September). Concerning nutrients, the concentration of nitrates was generally low, with a peak of 3.26 μM in T2 (June), while phosphates were about 0.1-0.2 μM , with a peak at T5 (0.24 μM) (Table S2.2).

2.3.3 Macroalgal complexity

Surface area (S) and fractal dimension (D) were calculated to evaluate the different morphological complexity of the apical parts of the two macroalgae that were analysed in the present work (Fig. S3). Overall S and D were higher in DP than in CC. In CC the surface remained relatively constant along the sampling period (20-25 $\text{cm}^2 \text{g}^{-1}$), while in DP S was higher at T5 and T6 than in the previous times (max value 127 $\text{cm}^2 \text{g}^{-1}$). As for D, more marked differences between the two algae were found at times T5 and T6, with higher values in DP than in CC (max values 1.84 and 1.73, respectively).

2.3.4 Microphytobenthic community

In total 25 genera were identified, belonging to 18 orders and mostly to diatoms and dinoflagellates. Sporadically, cyanobacteria Cyanophyceae Schaffner, 1909 and juvenile stages of green algae Chlorophyta Pascher, 1914 were also observed.

Total density was significantly higher in DP (2608441 \pm 425527 cells g^{-1} fw) than in CC (486309 \pm 77877 cells g^{-1} fw) (Fig. 1; Table S2.3).

Results of ANOVA showed a significant interaction between algae and times (F=5.75, P<0.001) (Table S2.3) due to a different temporal trend of density between the two macroalgae. Generally, diatoms represented the most abundant component of the microphytobenthic

community, accounting for average values of 96% and 87% of the organisms in DP and CC, respectively.

Microalgal species belonging to the diatom group were classified into 22 genera and 15 orders. Some species remained unidentified, namely undetermined pennate and centric diatoms (Fig. 2.2). On both macroalgae the most represented order was Naviculales Bessey, 1907, that in DP showed an increasing trend from T1, with an average density of 198287 ± 81945 cells g^{-1} fw, to T6, with an average density of 3489032 ± 1159137 cells g^{-1} fw (Table S2.4), which represented 58% of the epiphytic diatoms (Fig. 2.2A). For Bacillariales Hendey, 1937, a constant amount of about 60,000 cells g^{-1} fw was observed in DP from T1 to T4, and a maximum at time T5 (average density of 621096 ± 213575 cells g^{-1} fw) was recorded, which corresponded to 15% of the total diatoms (Fig. 2.2A). In terms of average abundance, Lyrellales D.G. Mann and Cocconeidales E.J. Cox, 2015 represented less than 10% of the diatoms (ranging from 25,8831 to 10,865 cells g^{-1} fw), while species belonging to other orders (i.e. Surirellales D.G. Mann, 1990, Thalassiosiphales D.G. Mann, 1990, Mastogloiales D.G. Mann, Licmophorales Round, 1990, Striatellales Round, 1990, Tabellariales Round, 1990) occurred with average densities below 100,000 cells g^{-1} fw. On CC almost all genera identified had a peak at T5, while a constant average density was observed from T1 to T4, except for Licmophorales that reached a maximum at T2 (229178 ± 91470 cells g^{-1} fw), accounting for 33% of the diatoms (Fig. 2.2B).

Specifically, Naviculales, whose concentration ranged from 25 to 56% of the entire diatom community, occurred with the lowest density of $70,457 \pm 31,328$ cells g^{-1} fw at T1 and a maximum average density at T5 ($27,5598 \pm 89,219$ cells g^{-1} fw). Bacillariales were about 10% of the total diatoms and increased consistently from T1 ($8,869 \pm 2,935$ cells g^{-1} fw) to T5 ($79,071 \pm 18,396$ cells g^{-1} fw), then they decreased at T6 ($13,850 \pm 4,154$ cells g^{-1} fw).

Cocconeidales had a peak at T1 ($33,615 \pm 16,335$ cells g^{-1} fw), accounting for the 16% of the total diatoms. All other orders (i.e. Surirellales, Thalassiophysales, Mastogloiales, Licmophorales, Striatellales, Tabellariales) represented less than 5% of the entire diatom assemblage.

The average density of dinoflagellates was significantly higher on DP than on CC. This group was recorded especially from T4 to T6 (Table S2.4) due to the presence of a bloom of the toxic dinoflagellate *Ostreopsis cf. ovata*, whose concentration reached a maximum average density at T6 ($364,167 \pm 77,126$ cells g^{-1} fw).

The nMDS plot carried out on the microphytobenthic communities showed a clear separation of the assemblages associated with the two macroalgae (Fig. 2.3A), but with a different temporal trend. PERMANOVA supported this pattern resulting in a significant interaction between algae and times (pseudo-F= 2,2779, $P \leq 0.001$; Table S2.5), and the post-hoc analysis carried out between the two algae at the various sampling times confirmed the different structure of the two communities at each time.

SIMPER analysis revealed that average similarities for DP was 36% and for CC was 40%, whereas dissimilarities between the two macroalgae at each time ranged from 66% and 82% (Table S6). Results showed that the average dissimilarities between the two algae at each time were largely due to the higher abundances of five orders, namely Naviculales, Lyrellales, Gonyaulacales Taylor, 1980 (i.e. *Ostreopsis cf. ovata*), Bacillariales, and Licmophorales, that were always more abundant on DP than on CC.

2.3.5 Meiobenthic community

A total of 12 taxa belonging to the meiobenthos were identified, one represented by larval stage Copepoda *nauplii* (referred from now on as copepod *nauplii*) defined as the larvae of

the meiobenthic copepod species (mainly harpacticoids). Copepods and their *nauplii* were counted separately in view of their different ecology (Hicks and Coull 1983). Harpacticoida (33.1%) and Nematoda (31.2%) were the dominant taxa, followed by copepod *nauplii* (25.2%), Gastropoda Cuvier, 1795 (5.4%), Isopoda Latreille, 1817 (1.6%), Polychaeta Grube, 1850 (1.6%), Amphipoda Latreille, 1816 (0.7%) Halacaroidea Cunliffe, 1954 (0.5%), Ostracoda Latreille, 1802 (0.3%), Kinorhyncha Reinhard, 1885 (0.3%), Chironomidae Newman, 1834 (0.2%) and Bivalvia Linnaeus, 1758 (0.1%). Overall, total density (N) and number of taxa (S) at high taxonomic resolution resulted higher on DP than on CC (Figs 2.4 and S2.4). Total density was in mean 371 ± 46 and 90 ± 15 N g⁻¹ fw in DP and CC, respectively (Fig. 2.4), while the number of taxa was on average 5.6 ± 0.2 in DP and 4.4 ± 0.2 in CC (Fig. S2.4). ANOVA results showed a significant interaction between the factors alga and time ($F=7.06$, $P < 0.001$; $F=5.9$, $P < 0.001$) for both total density and number of taxa (Table S2.7), suggesting that the differences between macroalgae were not consistent over time. For total density (Fig. 2.5), significantly higher values occurred on DP than on CC at corresponding times, except for T1 (Table S2.7). Moreover, for DP densities increased with a peak at T6 (828 ± 126 N g⁻¹ fw), while for CC the highest density occurred at T5 (217 ± 63 N g⁻¹ fw) and then decreased at T6. The number of taxa showed the highest value on DP at T2 (7 ± 1) and the lowest on CC at T6 (3 ± 0). It resulted significantly higher on DP than on CC at times T2, T4, and T6, while was lower at T5 (5 ± 1) (Fig. S2.4; Table S2.7).

The nMDS analysis of meiofauna communities showed a clear separation between samples belonging to the two macroalgae but, for each alga, the composition of the meiofaunal assemblage changed following a different temporal pattern (Fig. 2.3B). PERMANOVA supported this pattern with a significant interaction between algae and times (pseudo- $F=2,606$, $P \leq 0.001$; Table S2.8). The assemblages on the two algae resulted significantly different

at corresponding sampling times, with the exception of T5 and, within each alga community, the structure changed among times as shown by post hoc comparisons (Table S2.8).

SIMPER analysis revealed that average similarities for each macroalga were 42.8% for DP and 43.7% for CC. Moreover, the dissimilarities between macroalgae at each time ranged from 52% to 82%. Both similarities and dissimilarities were largely due to the variations in abundance of the three dominant taxa: nematodes, copepods, and copepod *nauplii* (Table S9).

In DP average densities of nematodes (Fig. 2.5A; Table S2.10) increased from T1 ($57 \pm 22 \text{ N g}^{-1} \text{ fw}$) to a maximum in T6 ($287 \pm 46 \text{ N g}^{-1} \text{ fw}$), as well as the average values of the density of the copepods (Fig. 2.5B) that showed a similar trend to that of nematodes, with a pick at T6 ($285 \pm 69 \text{ N g}^{-1} \text{ fw}$). The densities of copepod *nauplii* (Fig. 2.5C), defined as the larvae of the meiobenthic Copepod species (harpacticoids), showed two peaks at times T2 ($215 \pm 52 \text{ N g}^{-1} \text{ fw}$) and T6 ($217 \pm 34 \text{ N g}^{-1} \text{ fw}$).

Conversely, in CC nematodes, copepods and copepod *nauplii* had lower mean densities at each time when compared with those found in DP (Fig. 2.5). Both nematodes and copepods densities were very low from time T1 (14 ± 6 and $8 \pm 2 \text{ N g}^{-1} \text{ fw}$) to T4 (10 ± 2 and $14 \pm 3 \text{ N g}^{-1} \text{ fw}$); only at T5 an increase occurred (105 ± 32 and $77 \pm 27 \text{ N g}^{-1} \text{ fw}$) and then a decrease took place at T6 (19 ± 4 and $15 \pm 7 \text{ N g}^{-1} \text{ fw}$). Instead, the mean abundance of copepod *nauplii* slowly increased until T6 ($43 \pm 15 \text{ N g}^{-1} \text{ fw}$).

2.3.6 Harpacticoid community

Twelve harpacticoid species were identified from the two macroalgae; they belonged to 11 genera and 11 families. The dominant species was *Heterolaophonte minuta* Boeck, 1873 (32.5%), followed by *Amphiascus parvulus* Claus, 1866 (11.9%), *Harpacticus gracilis* Claus,

1863 (11.6%), *Paradactylopodia brevicornis* Claus, 1866 (10.8%), *Parastenhelia spinosa* Fischer, 1860 (10.2%), and *Ectinosoma melaniceps* Boeck, 1865 (9.6%). The other species ranged from 6.8% (*Porcellidium viride* Philippi, 1840) to 0.2% (*Scutelledium longicaudum* Philippi, 1840). Results of ANOVA for total density (N), and number of species (S) are shown in table S11. Generally, total densities (N) were significantly higher in DP than in CC at T2, T3, T4, and T6 (Fig. 2.5B), while the number of harpacticoid species (S) was higher on DP compared to CC at T2 (7 vs 3) and T6 (7 vs 3) (Table S2.12).

The nMDS plot of harpacticoid species showed a clear separation between algae and a different temporal pattern of assemblages on each macroalga (Fig. 2.3C). These results were supported by the significant interaction between the factors alga and time (PERMANOVA pseudo-F=19.084; P<0.01). In particular, harpacticoid assemblages resulted significantly different between the two algae at each time, with the exception of T1 and T5 (Table S2.13). A significant gradual change in the community structure took place in DP, whereas on CC significant community structure changes were evident at T5. The details of pairwise comparisons among times for each alga are shown in Table S2.13.

SIMPER results revealed that average dissimilarity between the two algae at each time was largely due to the high abundances of five species, namely *Heterolaophonte minuta*, *Parastenhelia spinosa*, *Paradactylopodia brevicornis*, *Harpacticus gracilis*, and *Porcellidium viride*, that resulted more abundant in DP than in CC (Table S2.14).

The mean abundance over time of these six species is shown in figure 6. In DP, the densities of *Heterolaophonte minuta* showed two peaks at times T4 ($68 \pm 17 \text{ N g}^{-1} \text{ fw}$) and T6 ($82 \pm 20 \text{ N g}^{-1} \text{ fw}$), whereas on CC the densities were always low, with a peak at T3 ($17 \pm 7 \text{ N g}^{-1} \text{ fw}$). In DP the density of *Parasthenelia spinosa* increased at T5 ($17 \pm 4 \text{ N g}^{-1} \text{ fw}$) and T6 ($27 \pm 9 \text{ N g}^{-1} \text{ fw}$), while in CC there was an increase at T5 ($12 \pm 5 \text{ N g}^{-1} \text{ fw}$), then a decrease at T6 ($4 \pm 1 \text{ N g}^{-1} \text{ fw}$).

¹ fw). *Paradactylopodia brevicornis* showed higher densities at times T3 ($11 \pm 6 \text{ N g}^{-1} \text{ fw}$), T5 ($16 \pm 6 \text{ N g}^{-1} \text{ fw}$), and T6 ($31 \pm 11 \text{ N g}^{-1} \text{ fw}$) in DP, while on CC this species was almost always absent, except at T5 ($16 \pm 6 \text{ N g}^{-1} \text{ fw}$). The average densities of *Harpacticus gracilis* resulted always higher on DP. The densities of *Porcellidium viride* showed the highest values on DP at T3 ($31 \pm 11 \text{ N g}^{-1} \text{ fw}$), then decreased and finally disappeared at T6; conversely, the same pattern occurred for CC, but with lower densities. On DP the density of *Ectinosoma melaniceps* increased at T6 ($19 \pm 6 \text{ N g}^{-1} \text{ fw}$), while on CC this species was almost always absent, except at T5 ($7 \pm 3 \text{ N g}^{-1} \text{ fw}$).

2.3.7 Relationship between the meiofauna and harpacticoid assemblages with macroalgae complexity, PUAs, and microphytobenthos

The results of DistLM (Table S15) carried out to analyze the relationships between all the three sets of variables and the meiofauna assemblage showed that for the marginal test the three macroalgae complexity descriptors alone accounted for 26% of the variation in the meiofauna abundance, PUAs for 17% and microphytobenthic taxa for 38%.

After fitting complexity descriptors, PUAs and microphytobenthos taxa explained an additional 7% and 13% respectively, resulting in a total variation of 47%. These additional amounts were significant according to the sequential test.

The same analysis carried out on the harpacticoid assemblages showed that the three complexity descriptors alone accounted for 19% of the variation in the harpacticoid abundance, PUAs for 13%, and microphytobenthic taxa for 23%.

After fitting complexity, PUAs and microphytobenthos taxa explained an additional 9% and 10% respectively, resulting in a total variation of 38%. Only the additional amount of PUAs resulted significant according to the sequential test.

2.4 Discussion

Qualitative and quantitative differences were observed in the PUAs produced by the two macroalgae considered (*Dictyopteris polypodioides*, DP, and *Cystoseira compressa*, CC), in agreement with previous results obtained for the same species (Pezzolesi et al., 2021). DP produced, in fact, higher average concentrations than CC, but also a variety of long-chain compounds, such as hexadecatrienal (C16:3), hexadecatetraenal (C16:4) and tetradecapentaenal (C14:5), while *C. compressa* produced the short-chain hexadienal (C6:2) as main compound, demonstrating the ability of macroalgae to produce short, middle and/or long-chain aldehydes (Kajiwara et al., 1996; Akakabe et al., 2003; Pezzolesi et al., 2021). These results highlighted that PUAs profile could be a fingerprint for each algal species, since the same compounds were consistently detected regardless of the sampling period, while their relative and total amount may vary depending on environmental conditions or morphotypes (Alsufyani et al., 2014; Pezzolesi et al., 2021). In addition, since the apical parts of both algae are those in which growth actively takes place, they are the most metabolically active parts, and therefore presumably also those in which the greatest production of PUAs takes place. Dissolved PUAs (dPUAs) concentrations in proximity of the macroalgae was high (in the order of μM), especially when compared with results of previous studies carried out in an Adriatic planktonic community (Ribalet et al., 2014), but are in accordance with values previously recorded in the same site in other studies (Pezzolesi et al., 2021; Bartual et al., 2020) or hypothesized to occur in proximity of the PUA producers (Ribalet et al., 2008; Bartual et al., 2018). These high concentrations are reasonable considering that the sampling area is colonized by a well structured phytobenthic community (macro- and microalgal), thus dPUAs derive from the contribution of the different algal species. Additionally, the reduced

hydrodynamism of this benthic site causes a low dispersion of any secondary metabolites produced by the various organisms.

Seawater parameters (temperature, salinity and nutrients) were within the range previously reported for the Piscinetta site (e.g. Accoroni et al., 2012), and confirmed the seasonal variability associated to this shallow inlet, that is subjected to a moderate anthropic impact (mainly in the form of summer tourism, as it is a popular site for swimming in the summer months). Results showed a predominance of diatoms in the microphytobenthic community, as usually reported in the Adriatic Sea, even in planktonic communities (e.g. Accoroni et al., 2016). Dinoflagellates were present at low densities, except during the bloom of *Ostreopsis* cf. *ovata*, which was the main dinoflagellate and showed its typical blooming trend, with maximum abundances recorded in late summer (September) and within ranges previously observed (e.g. Gémin et al., 2020).

The main diatom genera found in the present study (e.g. *Navicula*, *Cylindrotheca*, *Lyrella*, *Cocconeis*, *Gyrosigma* A.H. Hassall, 1845, *Licmophora* C. Agardh, 1827, *Nitzschia*, *Mastogloia* Thwaites ex W.Smith, 1856, *Striatella* C. Agardh, 1832, *Coscinodiscus* Ehrenberg, 1839) are among the most common on Mediterranean macroalgae and in the microphytobenthos, particularly in the Adriatic Sea (Carnicer et al., 2015; Accoroni et al., 2016; Rogelja et al., 2016; Pennesi and Danovaro, 2017; Ternon et al., 2020). Species belonging to the orders Naviculales and Lyrellales on DP, to the Licmophorales on CC and, generally, to centric diatoms on both algae, showed an inverse trend (low density at T1 and high at subsequent times) in relation to the production of PUAs by macroalgae. These results could be ascribed to a negative effect of these compounds, as also demonstrated by Ribalet et al. (2007) for some planktonic species. Long-chain aldehydes, such as C14-C16, can induce a stronger growth inhibition than short-

chain PUAs, probably due to longer alkyl chains that increase the reactivity of the molecules (Adolph et al., 2003).

Since many diatom species recorded in the present study, as those belonging to the genus *Cylindrotheca* spp. and *Nitzschia* spp., are themselves among the main PUAs producers (Wichard et al., 2005a; Lavrentyev et al., 2015; Pezolesi et al., 2017; C3zar et al., 2018), they may have developed different sensitivities and/or tolerances to these compounds in a species-specific way. Studies have also shown a different susceptibility based on the life cycle, with more resistant juvenile cells and more sensitive stationary phase cells (Leflaive and Ten-Hage, 2009; Ribalet et al., 2007) and based also on other factors, such as cell size, wall properties and lipid content. In particular, species with a well-structured and mineralized cell wall, a low surface-volume ratio and a certain lipid content can limit the ability of these compounds to penetrate the cell. Centric diatoms could potentially be more sensitive to PUAs, as those found in the present study (*Chaetoceros* spp. Ehrenberg, 1844, *Coscinodiscus* spp., *Guinardia* spp. H. Peragallo, 1892 and *Rhizosolenia* spp. Brightwell, 1858) are not listed among the species able to produce these compounds (Wichard et al., 2005).

Ostreopsis cf. *ovata* does not seem to be negatively influenced, in terms of abundance, by the production of PUAs by macroalgae. Conversely, macroalgal complexity seems to explain the different abundances found on CC and DP. The presence of a rigid cell wall and the high biovolume could partially explain the apparent lower sensitivity of *Ostreopsis* cells to PUAs compared to other species, in addition to the protective role that can be offered by the mucilaginous layer produced by *O. cf. ovata*, which provides an additional barrier against the substances dissolved in the water column (Allen et al., 2016). Similarly, benthic dinoflagellates have shown a higher resistance than planktonic ones to potential allelochemicals (Ben Gharbia et al., 2017).

Regarding the meiofauna, 12 major taxa associated with the two macroalgae were found, with a total density recorded in some samples of over 1300 individuals per gram of alga and within the range found in previous studies (Jarvis and Seed, 1996). Harpacticoid copepods, together with their *nauplii*, were the most abundant taxon, comprising 58% of the total meiofauna, followed by Nematodes that were 33%, as reported in previous studies (Carlo Heip, Magda Vincx, 1985; De Troch et al., 2005).

By comparing the results obtained for the two macroalgae, the average abundances of total meiofauna and the number of taxa resulted higher on DP than CC, as well as the abundances of copepods and nematodes. As a result, the community structure resulted different between the two macroalgae and showed a different temporal pattern, although they were subjected to the same environmental characteristics in terms, for instance, of hydrodynamism and tides. DP and CC are different for life cycle, thallus structure and production of compounds. In particular, DP is formed by ribbon-like fronds, with very irregular and proliferating edges, on which numerous meiobenthic organisms can settle. In the area where sampling was conducted, the species persists in its fully developed habit for most of spring and summer. CC is a highly branched, leathery macrophyte; when fully grown, in the study area it may reach 1-1.5 m in height. For a large-sized, habitat-forming species, its growth is relatively fast, especially if compared with other Mediterranean fuclean brown algae. However, in the area of the Passetto its full development is limited to a quite restricted period of the year (from May to mid-July); by mid-summer this species loses most of its branches and persists in a more reduced habit, with a few short branches. This means that most of its fronds occur in the field for a shorter time and are not available long enough to allow the settlement of a very diverse epiphytic community. The shorter temporal availability of this substrate also means that there will be a lower accumulation of sediment and detritus, a potential source of food for

meiofaunal organisms (Hicks, 1980; Gibbons, 1988; Frame et al., 2007). Both species have apical growth, determined by divisions of a group of meristematic cells in DP and a single apical cell in CC. Therefore, the apical parts are the youngest parts of the thallus, on which sediment and epiphytes had less time to settle.

Taking into consideration all these aspects, our results suggest that the morphological complexity of DP may affect total number of individuals, but also the associated species that have evolved morphological adaptations necessary for the adhesion to a thallus of this type (Taylor and Cole, 1994; Chemello and Milazzo, 2002). Although macrophytes are not true fractal objects, estimates of complexity using tools of fractal geometry have proved to be a useful approach for quantifying and separating effects of habitat architecture from those of habitat quantity (Gee and Warwick, 1994; McAbendroth et al., 2005; Hooper and Davenport, 2006). However, fractal measures performed for not truly fractal objects are just an estimate of complexity for a given scale.

The putative role of PUAs on structuring meiofauna may be reflected by the low density of *nauplii*, copepods, and nematodes at time T1 recorded on both macroalgae, which could be related to the high concentration of PUAs in this period, as observed also for microphytobenthos. Moreover, host specificity may be supposed, according to what determined by Bates and DeWreede (2007), that found specific chemical, structural and morphological characteristics of the algal species.

Harpacticoids have a number of features that make them an attractive group of benthic organisms in which PUA toxicity responses could be investigated, such as abundance, ecological importance, and short generation cycles (Raisuddin et al., 2007). In this study, harpacticoids associated with the two macroalgae consisted of 12 species. Even if the number of species was relatively low compared to results of previous studies (Hicks, 1977b; Arroyo et

al., 2006), it is interesting to note that the 12 species belonged to 12 genera and 11 families, so showing a high taxonomic distinctness. Moreover, no species was present exclusively on a single macroalga; only the relative abundance of individual species was different between the two macroalgae, with *Heterolaophonte minuta* as the dominant species on both. Almost all species showed low abundance at T1, potentially suggesting a role of PUAs, as previously postulated for meiofauna and microphytobenthos. To our knowledge, only one study was carried out in laboratory to analyze the effects of these compounds on the harpacticoid *Tisbe holothuriae* (Taylor et al., 2007); while, other studies carried out on planktonic ecosystem have shown deleterious effects of PUAs produced by diatoms on the reproduction of calanoid copepods (e.g. *Temora stylifera*, *Calanus helgolandicus*), which feed on them (Ianora et al., 2003, 2012; Miralto et al., 1999), as well as apoptosis in maturing oocytes (Poulet et al., 2007a) during embryo development (Romano et al., 2003) and in newly hatched *nauplii* (Ianora et al., 2004b).

It has to be considered that the present study was carried out in the field, so the link between PUAs effects on the community structure is more difficult to evaluate, due to the variability of PUAs production both by diatoms (Wichard et al., 2005b) and macroalgae (Pezzolesi et al., 2021), of the copepod sensitivity (Ianora et al., 2003; Sommer, 2009) and to the detoxification ability developed by certain species of copepods (e.g. Taylor et al., 2007; Wichard et al., 2008). As attested by Taylor et al. (2007), the benthos tends to be a more stressful environment compared to planktonic, where rapid fluctuations in physical conditions occur and both natural and anthropogenic toxins can accumulate at high levels within the sediments between the algal fronds. Since benthic organisms must be highly adapted to survive in such a harsh environment, it is not unreasonable to speculate that harpacticoids may have a more developed detoxification system than planktonic calanoid copepods, thus being better

equipped to resist to the toxic effect of oxylipins. Indeed, a number of candidate detox genes were found in an analysis of 686 sequence tags expressed by *Tigriopus japonicus* (Lee et al., 2005, 2008). Therefore, it is possible that the species found during the present study have developed effective and efficient detoxification strategies to survive in an environment such as the Passetto area. The different community structure between the two macroalgae and the temporal changes could be also explained by the ecological and trophic role of the various species, and by the different morphological evolution of the two macroalgae during the sampling time. This is an important aspect to consider in the case of this study, as we sampled apical parts of thalli of CC and DP, that may be not representative of the entire thalli, as are the youngest parts on which epiphytes had less time to settle and also those most exposed to light. In particular, four species (*Porcellidium viride*, *Parastenhelia spinosa*, *Heterolaophonte minuta*, and *Harpacticus gracilis*) belonged to the phytal group sensu strictu, and two (*Paradactylopodia brevicornis*, and *Ectinosoma melaniceps*) belonged to migrator and cosmopolitan group (Mascart et al., 2015). *E. melaniceps* is a tolerant eurytopic species, which presumably is not affected by the biochemical compounds produced by macroalgae, as for example reported for the green alga *Ulva lactuca* (Hicks, 1980). *P. brevicornis* is cosmopolitan species that have a wide distribution range and was found in different habitats, therefore able to adapt to a large number of different environmental conditions (Hicks, 1980). Conversely, *P. viride*, *H. minuta* and *H. gracilis* are endemic species of the Mediterranean Sea with certain morphological characteristics that allow them to live adhering to macroalgal surfaces; therefore, they could either be affected by the effect of the various compounds produced by both macroalgae and microphytobenthos, or adapt to the various environmental conditions.

The DistLm procedure highlighted that the observed differences in the meiofauna and harpacticoid community structure between the two macroalgal species could be mainly explained by microphytobenthic main taxa, after fitting macroalgal complexity.

Microalgae, in fact, are at the base of the food web and provide energy for all the trophic levels above them; thus, these results confirm their important role as primary food source of essentially all marine food chains producers. In fact, most of the epifaunal taxa directly graze upon the macroalgae or the epiphytes for their food source, thus variations in the epiphytic algal density and composition between two macroalgal species may influence the abundance of associated epifauna (Gestoso et al., 2010). Additionally, the macroalgal complexity, rather than PUAs production alone, resulted an important component influencing the community structure. The role of the shape and structural complexity of macroalgae in determining the abundance patterns and size structure of the epiphytic organisms is supported in literature, either for epifaunal and epiphytic assemblages (e.g. Chemello and Milazzo, 2002; McAbendroth et al., 2005; Cacabelos et al., 2010). To better investigate the effect of macroalgal complexity and PUAs composition on variations of the microphytobenthos at some representative genera levels, on main meiofauna taxa, and on harpacticoid species Pearson correlation analysis was used (Fig. 2.7). Among PUAs, long-chain compounds (i.e. C14-C16), when compared with the short one (i.e. C6:2), showed higher effects on the abundances of some representative microalgae (i.e. *Navicula* spp., *Lyrella* spp., *Cocconeis* spp., *Cylindrotheca* spp./*Nitzschia* spp., and *Ostreopsis* cf. *ovata*), on the main meiobenthos taxa (i.e. Nematoda, Copepoda, copepod *nauplii*) and on harpacticoid species (i.e. *H. minuta*, *H. gracilis*, *P. viride*). Results are in agreement with laboratory studies performed to investigate the responses of allelochemicals, such as PUAs, on target organisms, including copepods and algae (Ivanora et al., 2004a; Taylor et al., 2007; Adolph et al., 2004; Caldwell et al., 2004; Ribalet et al., 2007;

Pichierri et al., 2016). Macroalgal complexity, either in terms of fractal dimension and area, is highly correlated and showed significant relationships with almost all the taxa considered, except with *P. viride*, potentially due to its ecological role, being a phytal species able to colonize a variety of macroalgae thanks to morphological adaptations that has evolved to attach to morphologically diverse thalli (Hicks, 1980).

It is important to point out that several metabolites, as well as PUAs, are known to be produced by macroalgae, including some (e.g. diterpenoids, polyphenols) that inhibit the growth of microalgae, bryozoan or other benthic species (Ternon et al., 2020), thus their contribution to the observed dynamics could not be excluded. Additionally, the epiphytic community that colonize the macroalgal surface may contribute either to the surface metabolome, adding inhibitory effects on the co-occurring species (Monti and Cecchin, 2012) or to the higher trophic levels, and directly interacting with the epifaunal, thus adding complexity to the understanding of the relationships among these organisms and to the role of PUAs. Finally, toxic microalgae such as *O. cf. ovata*, that was found to bloom during the sampling period, have been reported to affect copepods, and particularly *nauplii* (Guidi-Guilvard et al., 2012), with different sensitivities among species (Pavaux et al., 2019).

Taking into consideration all these aspects, PUAs production by macroalgae, together with their complexity, resulted one of the main factors involved in the benthic community structure dynamics, but it is not enough to explain the differences in the microphytobenthos and meiofauna assemblages.

2.5 Conclusion

Epiphytic communities on the two macroalgae highlighted a clear separation of the meiofauna and microphytobenthos assemblages with different temporal trends. The average

dissimilarities were due to several microalgal orders, namely Naviculales, Lyrellales, Gonyaulacales (i.e. *Ostreopsis* cf. *ovata*), Bacillariales, and Licmophorales, and to the three meiofauna dominant taxa (nematodes, copepods, and copepod *nauplii*) that were always more abundant on DP than on CC. Particularly, average dissimilarities of harpacticoid copepods were largely due to the abundances of five species, namely *Heterolaophonte minuta*, *Parastenhelia spinosa*, *Paradactylopodia brevicornis*, *Harpacticus gracilis*, *Porcellidium viride*, and *Ectinosoma melaniceps*. Generally, variations in the meiofauna and harpacticoid abundances were mainly due to macroalgal complexity variables and microphytobenthos, while a minor contribution was due to PUAs. Results documented that i) microphytobenthos resulted to affect the meiofauna population dynamics, in particular the harpacticoid assemblages, attesting the role of these organisms as primary food source of essentially all marine food chains producers, being at the base of the food web and providing energy for all the trophic levels above them, ii) the macroalgal complexity rather than PUAs production alone could be a major trigger of the community structure. PUAs effects, in fact, resulted species-specific, thus affecting some grazers instead of the entire community structure, as demonstrated also by Pearson's ρ correlations between taxa abundances and several macroalgal parameters. Among PUAs, long-chain compounds (i.e. C14-C16), with respect to the short one (i.e. C6:2), showed higher effects on the abundances of some representative microalgal genera, harpacticoid species and on the main meiobenthos taxa. Since several of the epiphytic diatom species found, in addition to macroalgae, can produce PUAs, the understanding of the effects of these compounds on the community structure and on the relationships among taxa in field studies are complicated, thus opening to further in-depth investigations in simplified systems (i.e. microcosms).

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Table 2.1: Relative abundance (%) of identified and unknown polyunsaturated aldehydes (PUAs) and total concentration of PUAs ($\mu\text{g g}^{-1}$ fw) in *Dictyopterus polypodioides* (DP) and *Cystoseira compressa* (CC) at the different sampling times (T1-T6).

Time	Alga	C6:2	C16:3	C16:4	C14:5	Unknown	Tot ($\mu\text{g g}^{-1}$ fw)
T1	DP	10%	12%	15%	50%	13%	225.5
T2	DP	0%	39%	20%	29%	14%	73.9
T3	DP	7%	21%	29%	32%	11%	44.3
T4	DP	7%	8%	14%	60%	11%	100.3
T5	DP	0%	12%	19%	61%	7%	82.1
T6	DP	6%	0%	8%	79%	7%	87.4
T1	CC	91%	0%	0%	0%	9%	17.1
T2	CC	64%	0%	0%	0%	36%	2.0
T3	CC	81%	0%	0%	0%	19%	2.3
T4	CC	88%	0%	0%	0%	12%	3.4
T5	CC	83%	0%	0%	0%	17%	5.3
T6	CC	68%	0%	0%	0%	32%	4.8

Figure captions

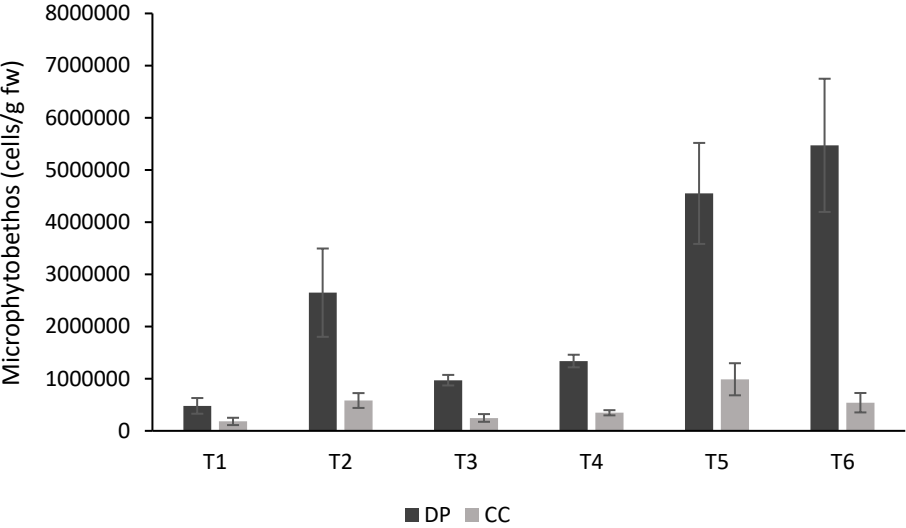


Fig. 2.1 – Total density of microphytobenthos community (cells g⁻¹ fw) in *Dictyopteris polypodioides* (DP) and *Cystoseira compressa* (CC) at the different sampling times (T1-T6).

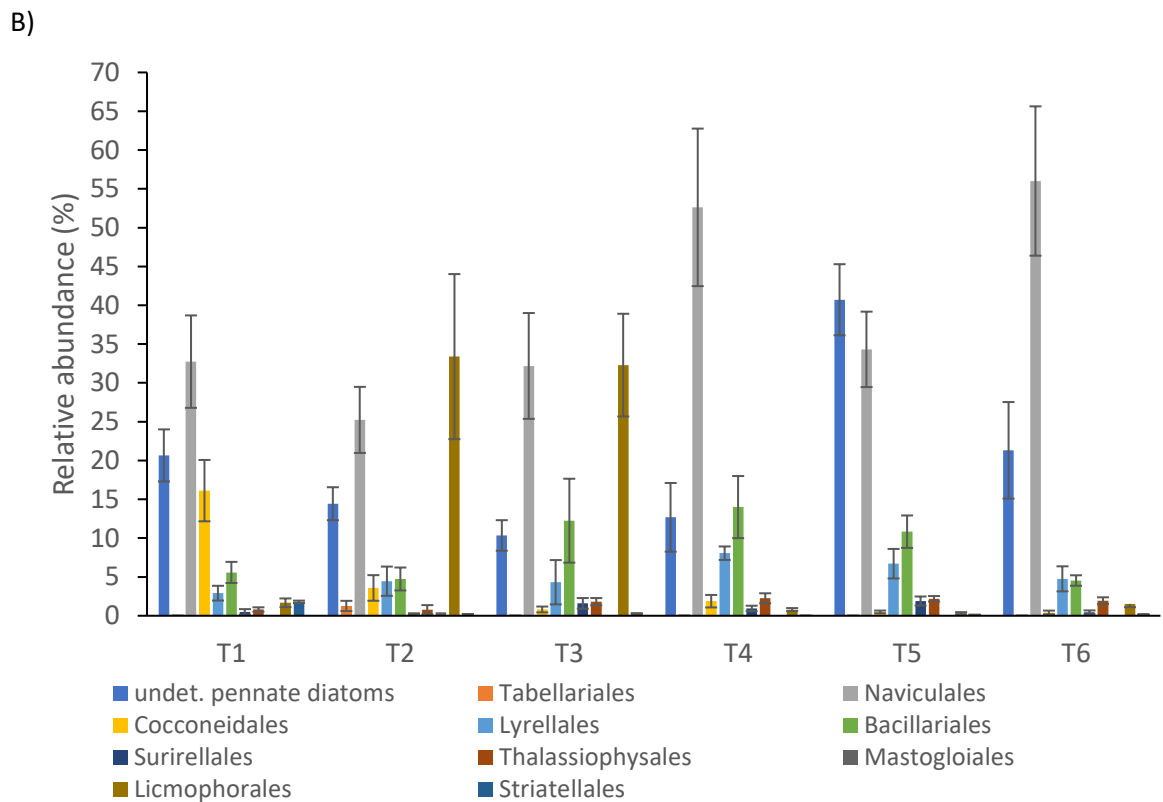
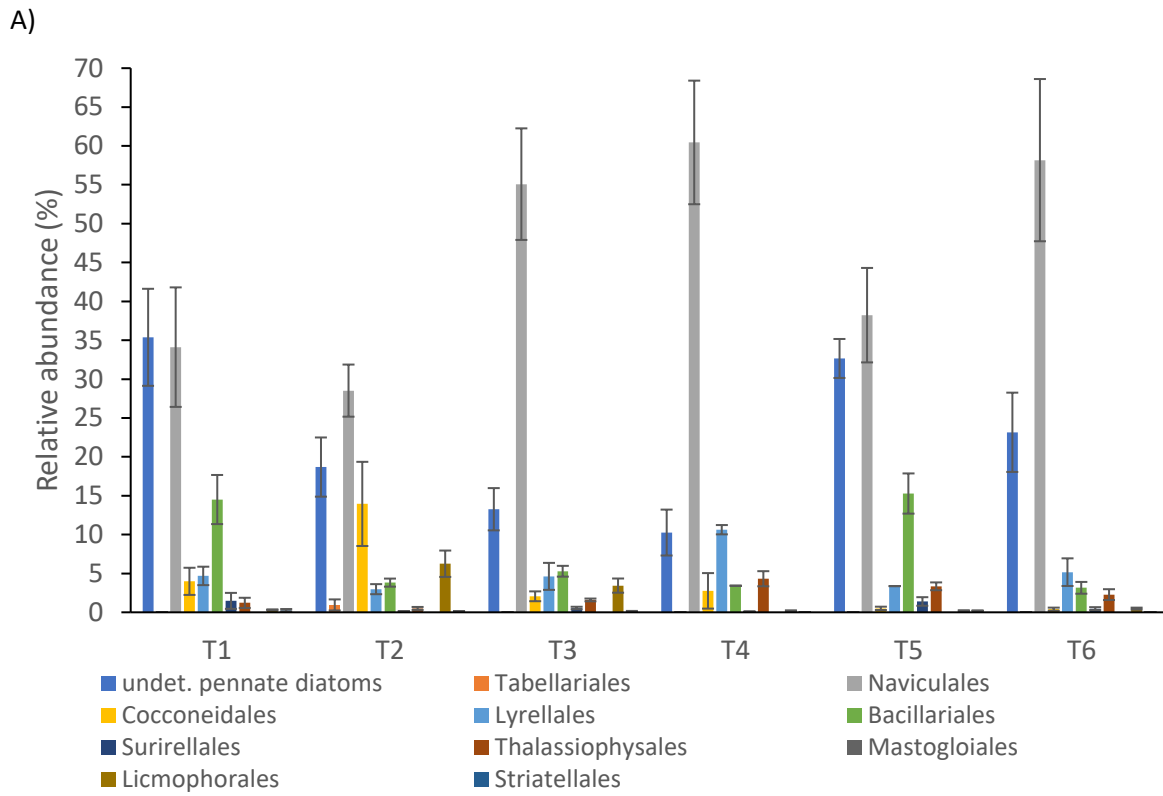


Fig. 2.2 – Relative abundance (%) of species belonging to the Bacillariophyceae orders in A) *Dictyopteris polypodioides* (DP) and B) *Cystoseira compressa* (CC) at the different sampling times (T1-T6)

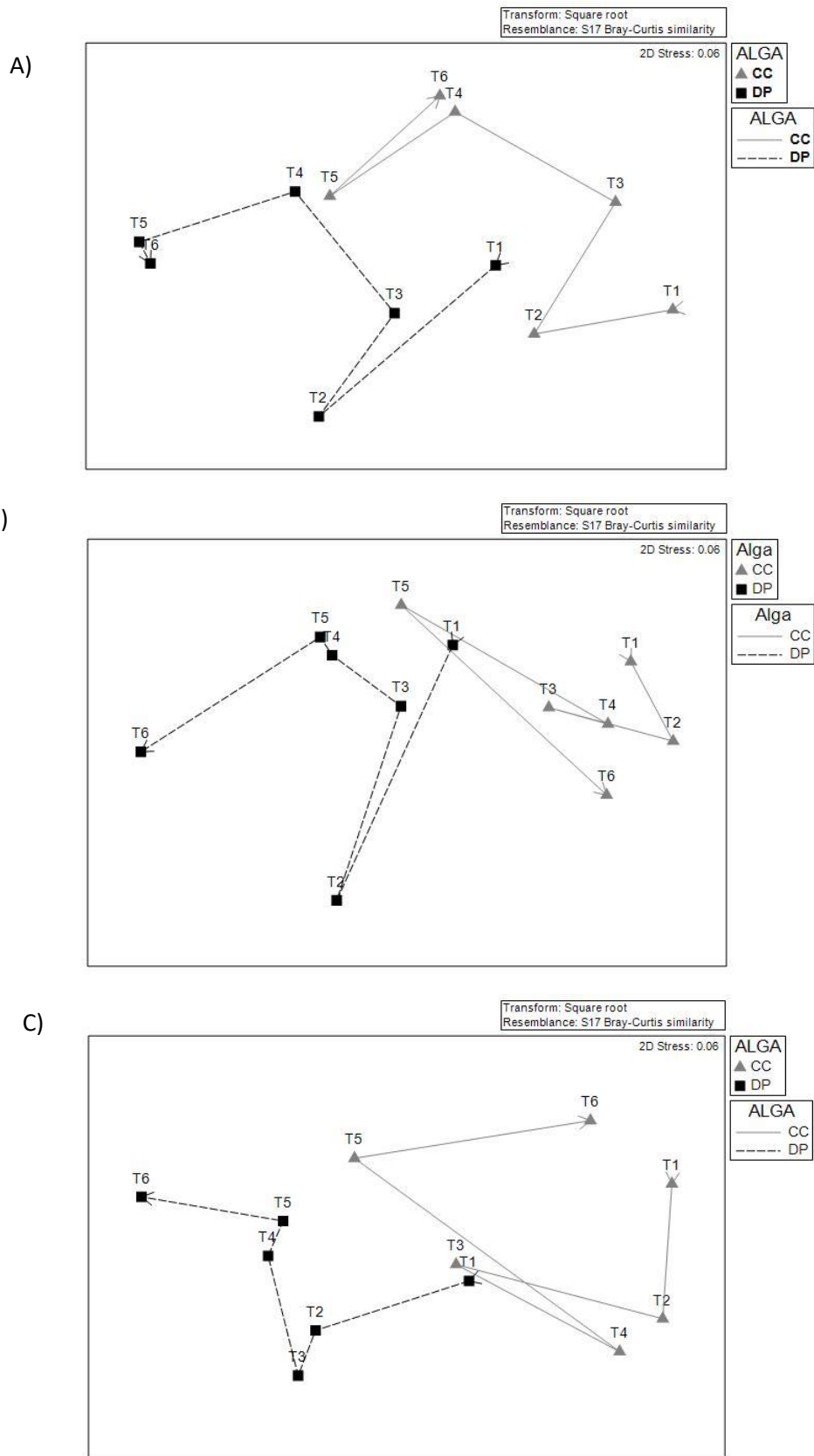


Fig. 2.3 - Two-dimensional nMDS of centroids for A) microphytobenthos orders, B) meiofauna, and C) harpacticoid community on the two algae *Dictyopterus polydoides* (DP) and *Cystoseira compressa* (CC) at the different sampling times (T1-T6).

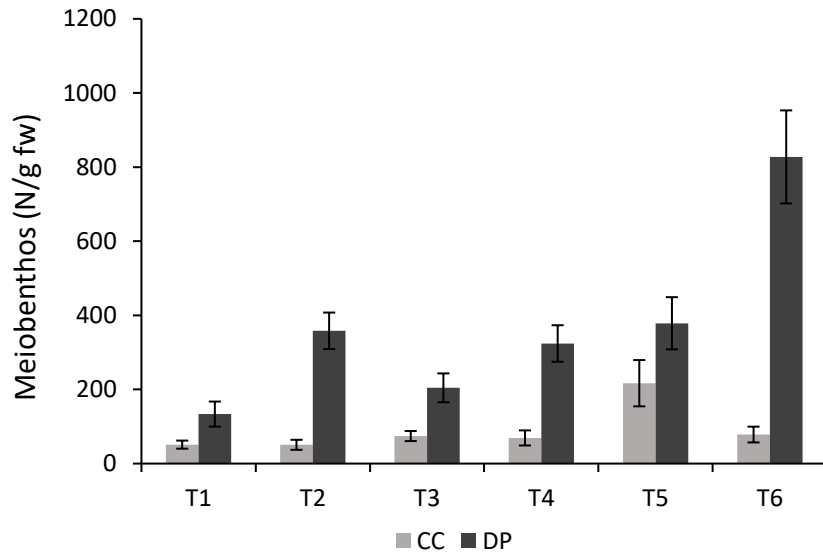


Fig. 2. 4 – Total density (N g⁻¹ fw) of meiobenthos in *Dictyopterus polypodioides* (DP) and *Cystoseira compressa* (CC) at the different sampling times (T1-T6).

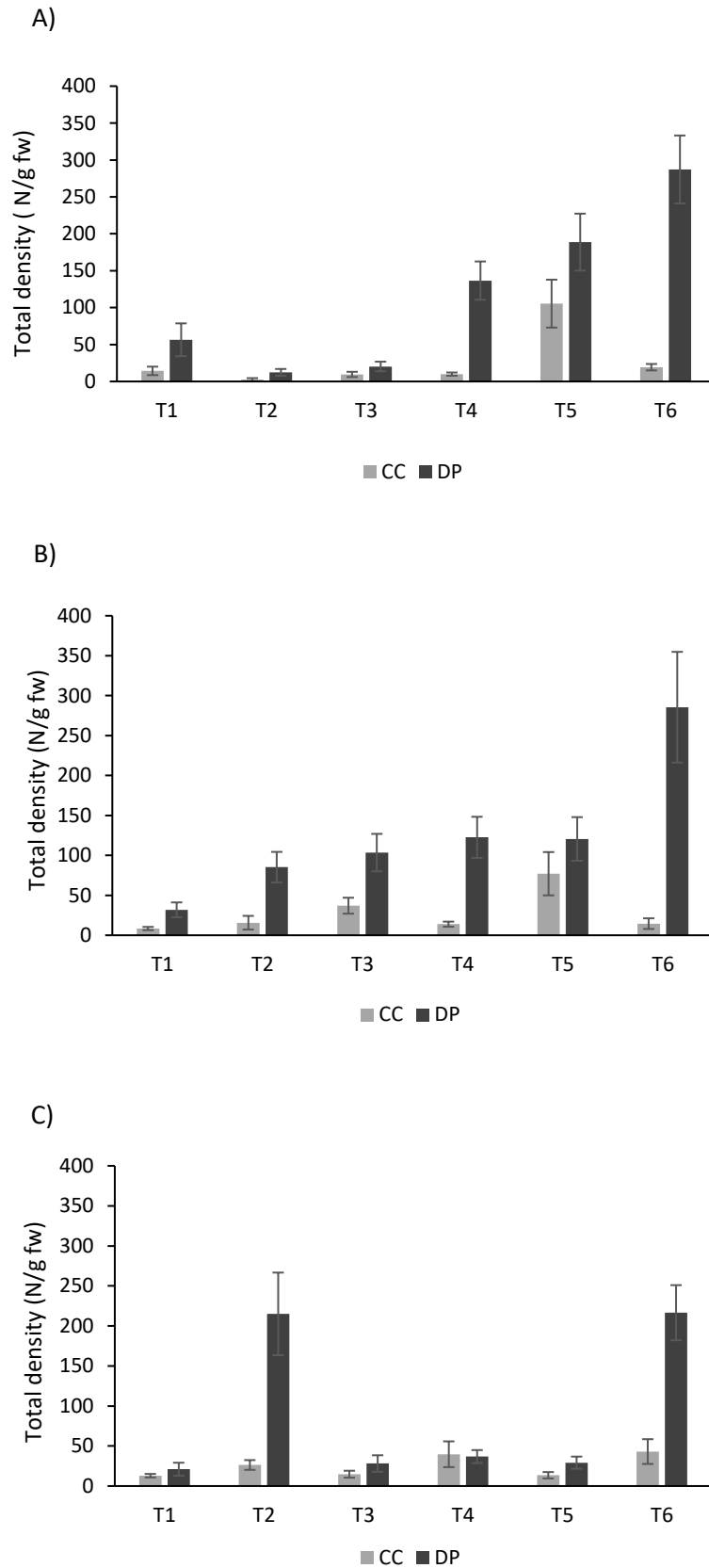
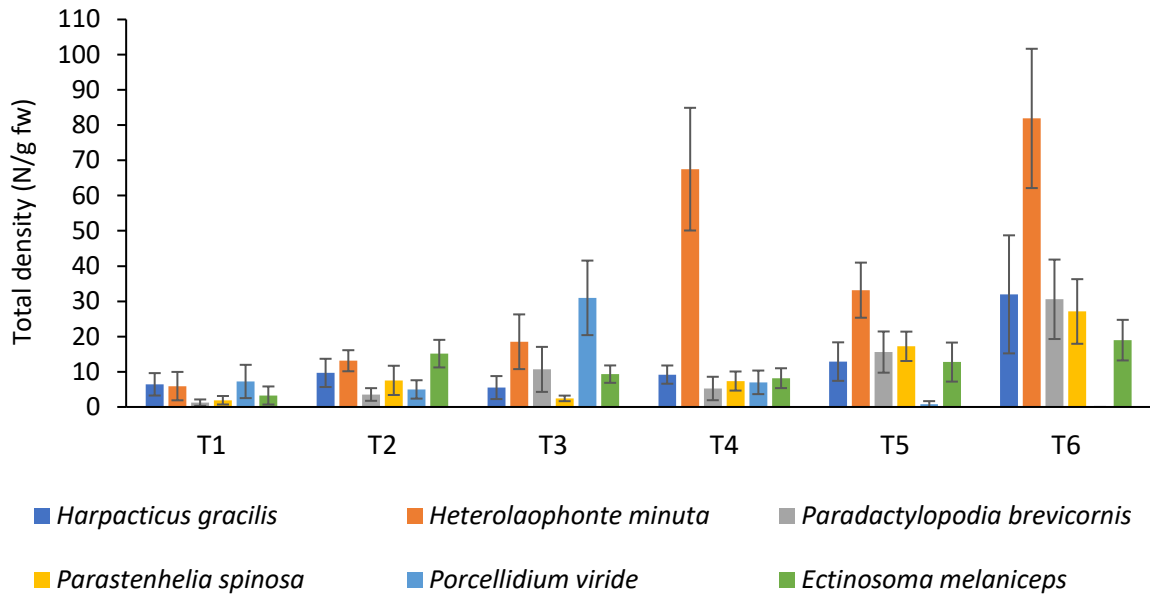


Fig.2.5 – Total density (n° organisms g⁻¹ fw) of A) nematodes, B) copepods and C) copepod *nauplii* in *Dictyopterus polypodioides* (DP) and *Cystoseira compressa* (CC) at the different sampling times (T1-T6).

A)



B)

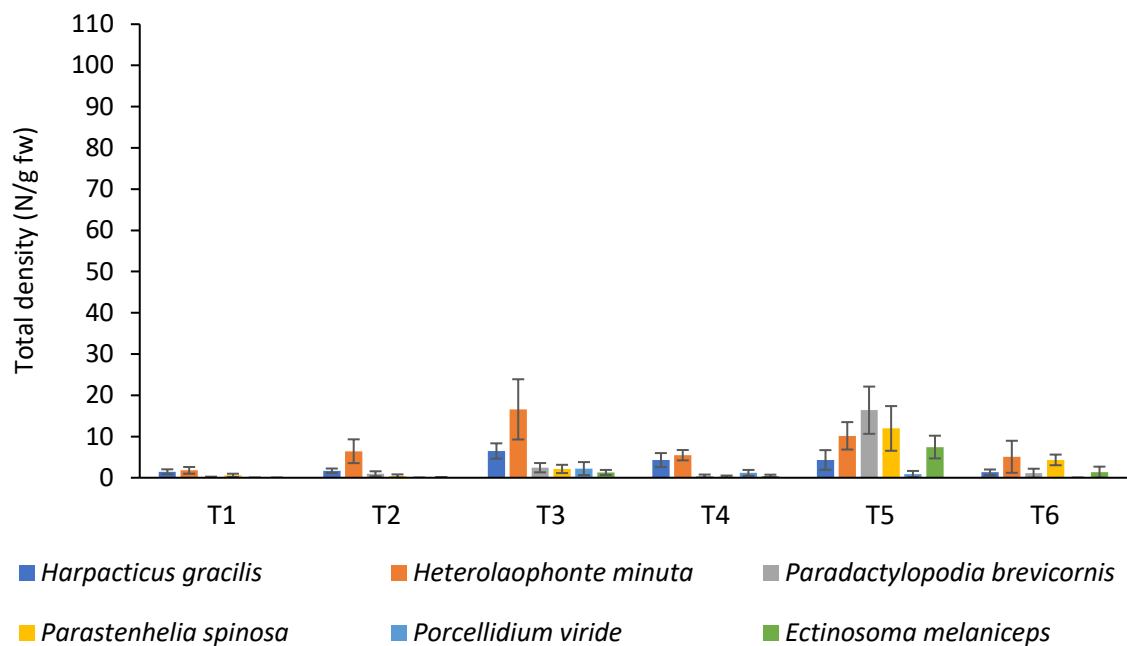


Fig. 2. 6 – Total density (N g⁻¹ fw) of harpacticoids in A) *Dictyopterus polypodioides* (DP) and B) *Cystoseira compressa* (CC) at the different sampling times (T1-T6).

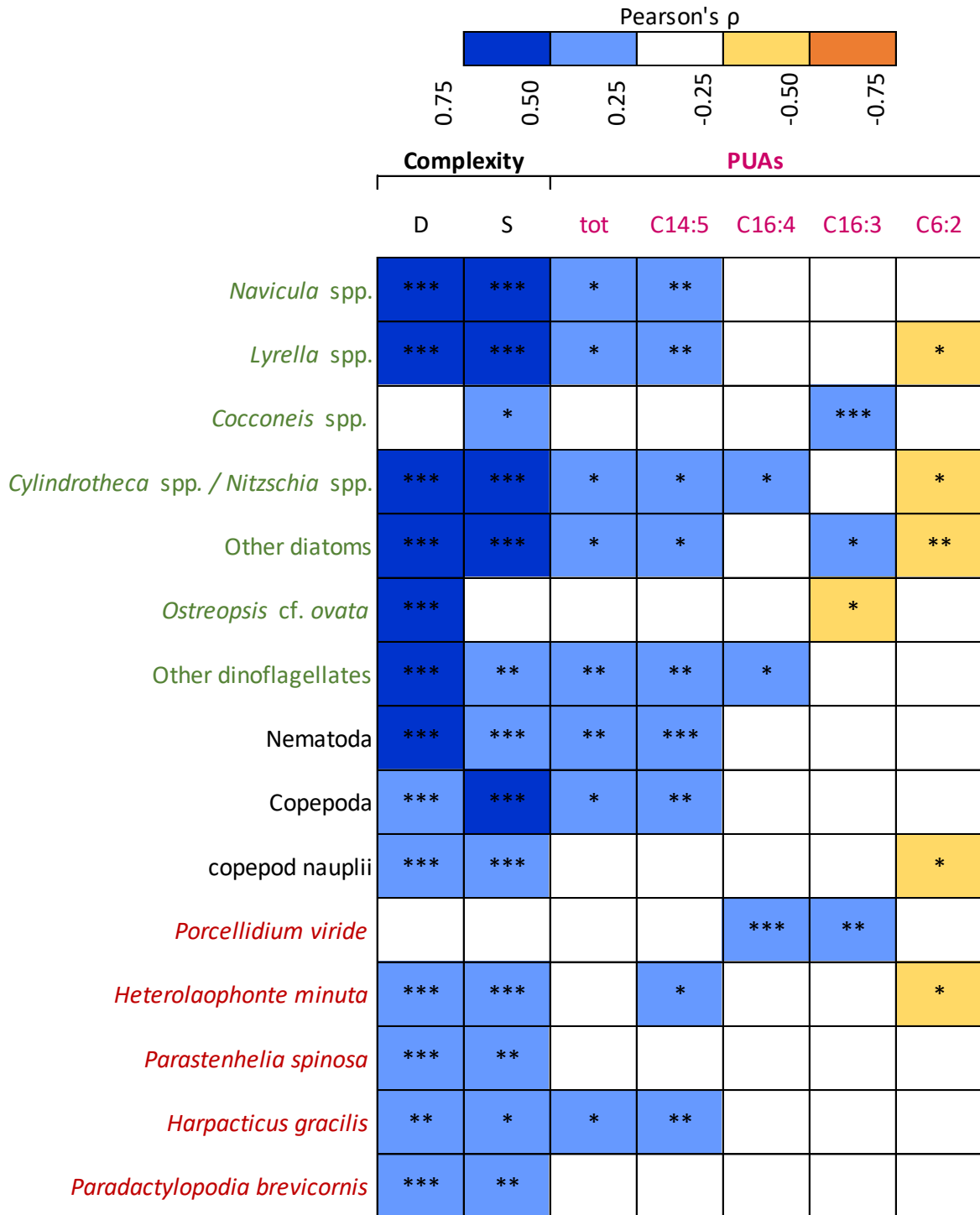


Fig. 2.7 – Results of Pearson correlation analysis between substrate attribution (D and S) and PUA composition and densities of more representative genera of microphytobenthos, main meiofauna taxa and harpacticoid species, after square root transformation. Non-significant relationships are indicated by white squares. Signif. codes: *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$. D, fractal dimension; S, macroalgal surface; tot, total PUA; C14:5, tetradecapentaenal; C16:4, hexadecatetraenal; C16:3, hexadecatrienal; C6:2, hexadienal.

Supplementary data

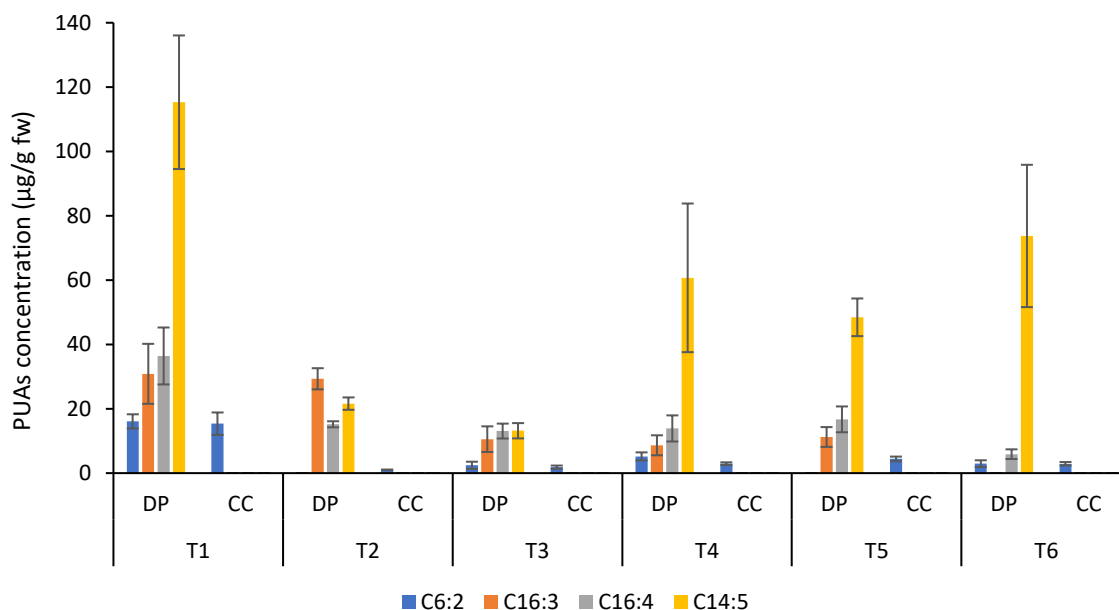


Fig. S2.1 - Concentration ($\mu\text{g g}^{-1}$ fw) of each polyunsaturated aldehydes in *Dictyopteris polypodioides* (DP) and *Cystoseira compressa* (CC) at the different sampling times (T1-T6).

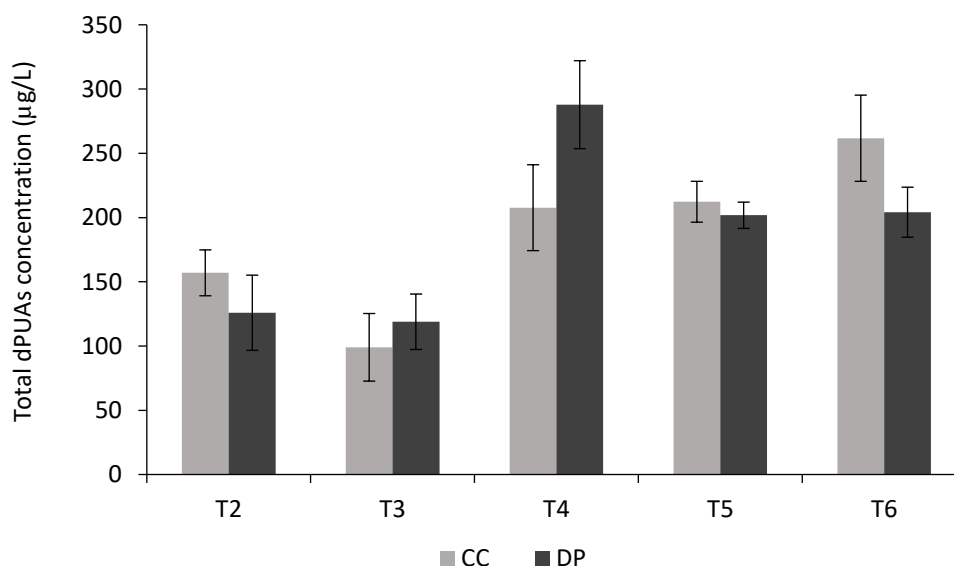


Fig. S2.2 – Total concentration ($\mu\text{g L}^{-1}$) of polyunsaturated aldehydes (dPUAs) in seawater collected in proximity of *Dictyopteris polypodioides* (DP) and *Cystoseira compressa* (CC) at the different sampling times (T2-T6). At time T1 the presence of some contaminants (i.e. surfactants) in seawater interfered with PUAs analysis and precluded their correct quantification.

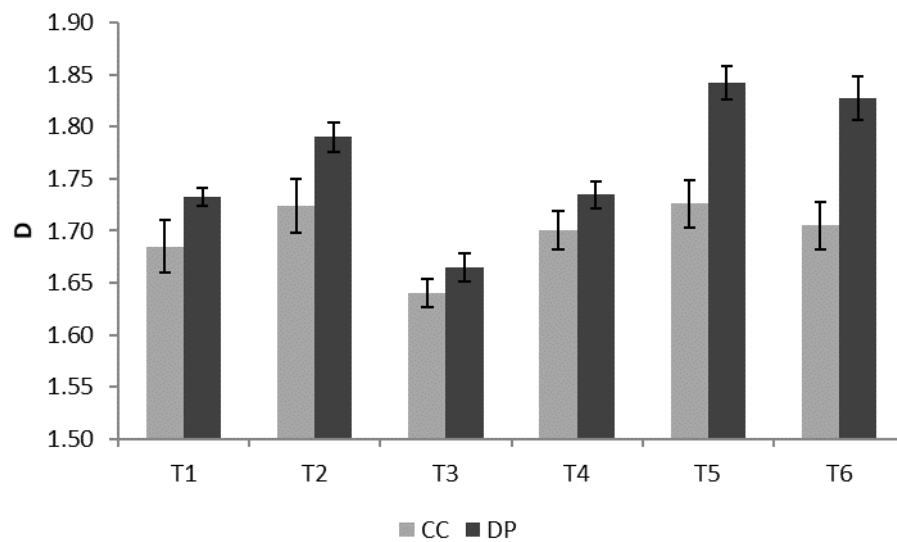
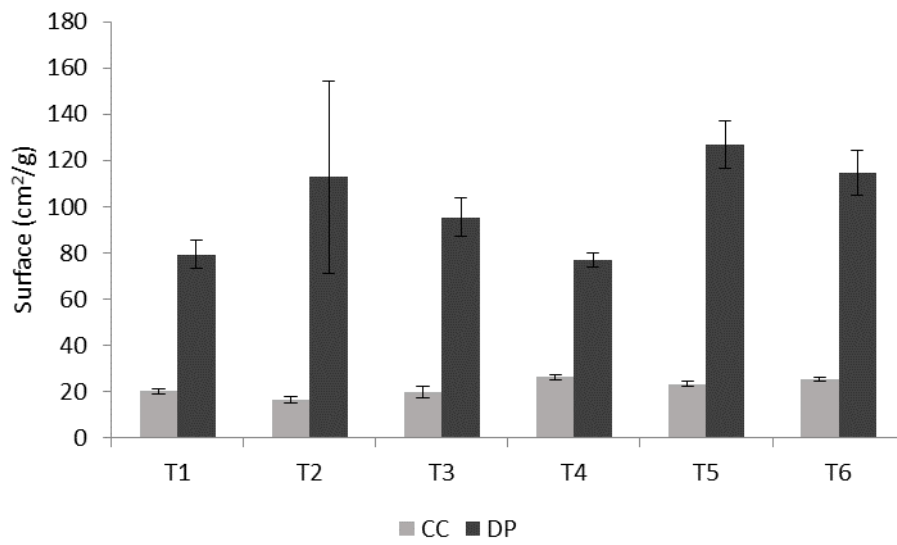


Fig. S2.3 - Surface area and fractal dimension (D) calculated for the apical parts of *Dictyopteria polypodioides* (DP) and *Cystoseira compressa* (CC) thalli at the different sampling times (T2-T6).

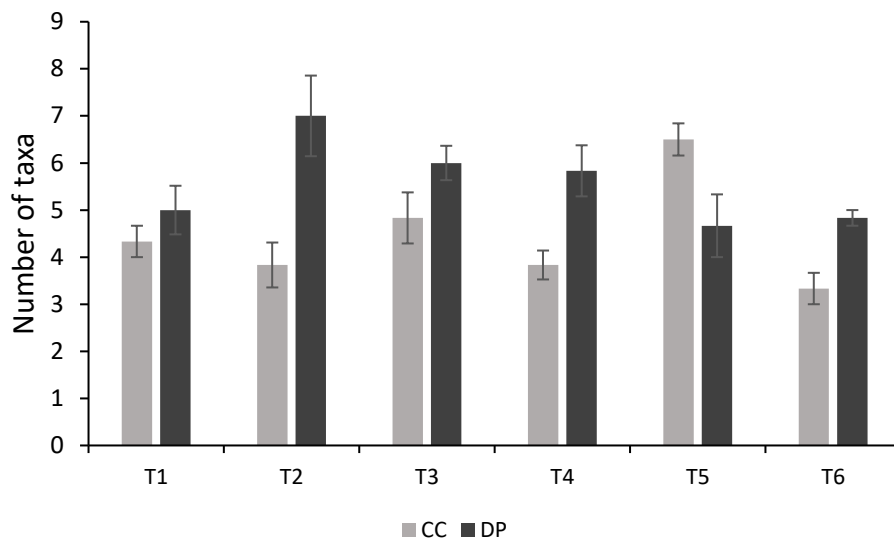


Fig. S2.4 – Number of taxa of the meiobenthic community in *Dictyopteris polypodioides* (DP) and *Cystoseira compressa* (CC) at the different sampling times (T1-T6).

Table S2.1 - Results of 2-way ANOVA and Pair-Wise comparison tests for the total concentration of PUAs in macroalgae (PUAs) and in seawater (dPUAs). TI: time; AL: macroalga.

Signif. codes: *** p < 0.001, ** p < 0.01, * p < 0.05.

		PUAs algae			PUAs seawater			
Source	df	MS	F	P	df	MS	F	P
TI	5	3.6686	17.73	0	4	43106.39	11.8	0
AL	1	1.41E+02	683.29	0	1	5.15E-01	0	0.9906
TixAL	5	0.2054	0.99	0.43	4	8407.163	2.3	0.0715
Res	60	0.2069			50	3653.751		
Total	71				59			
		C < 0,01 Ln(x+1)			C > 0,05 NS			
		Within Algae		Within Time	Within Time			
Pairwise		DP < CC **		T1>T3 **	T2=T3			
				T3<T5*	T2<T4**			
				T1>T2**	T2<T5*			
				T3<T6*	T2<T6**			
				T2=T5	T3<T4**			
				T1>T4**	T3<T5**			
				T3<T4*	T3<T6**			
				T2=T6	T4=T5			
				T4=T5	T4=T6			
				T1>T6**	T5=T6			
				T2=T3				
				T2=T4				
				T4=T6				
				T5=T6				
				T1>T5**				

Table S2.2 – Nitrates (N-NO₃), phosphates (P-PO₄), temperatures and salinities measured in seawater at the different sampling times (T1-T6).

Time	Date	N-NO ₃ (μM)	P-PO ₄ (μM)	Temperature (°C)	Salinity
T1	21st May 2018	1.15	0.04	24	34
T2	29th June 2018	3.26	0.12	24	35
T3	24th July 2018	0.85	0.10	26	37
T4	30th August 2018	0.11	0.17	26	38
T5	14th September 2018	0.63	0.24	26	39
T6	28th September 2018	1.09	0.05	23	37

Table S2.3 - Results of 2-way ANOVA for the total abundance (N) and Pair-Wise comparison tests of microphytobenthos. TI: time; AL: macroalga.

Signif. codes: *** p < 0.001, ** p < 0.01, * p < 0.05.

Source	df	MS	F	P
TI	5	1.59E+13	9.04	<0.001
AL	1	8.11E+13	46.04	<0.001
TIxAL	5	1.01E+13	5.75	0.0002
Res	60	1.76E+12		
Total	71			
		C < 0.01		
Pairwise	Within algae		Within time	
	CC: T1<T2	DP: T1<T2 *	T1: CC=DP	
	CC: T1<T3	DP: T1=T3	T2: CC<DP **	
	CC: T1<T4	DP: T1=T4	T3: CC=DP	
	CC: T1<T5	DP: T1<T5 **	T4: CC=DP	
	CC: T1<T6	DP: T1 <T6 **	T5: CC<DP**	
	CC: T2=T3	DP: T2=T3	T6: CC<DP**	
	CC: T2=T4	DP: T2=T4		
	CC: T2=T5	DP: T2<T5*		
	CC: T2=T6	DP: T2<T6**		
	CC: T3=T4	DP: T3=T4		
	CC: T3=T5	DP: T3<T5 **		
	CC: T3=T6	DP: T3<T6 **		
	CC: T4=T5	DP: T4<T5**		
	CC: T4=T6	DP: T4<T6**		
CC: T5=T6	DP: T5=T6			

Table S2.4 - Microphytobenthos community (cells g⁻¹ fw) identified in *D. polypodioides* (DP) and *C. compressa* (CC) at the different sampling times (T1-T6).

TIME	T1		T2		T3		T4		T5		T6	
MACROALGA	DP	CC	DP	CC	DP	CC	DP	CC	DP	CC	DP	CC
Density (cell g ⁻¹ fw)												
Diatoms												
<i>Achnanthes</i> spp.	-	-	2215	3698	627	37	94	-	338	113	1993	-
<i>Amphora</i> spp.	5106	1208	10980	6513	14706	4940	52599	5341	122165	20211	77735	6053
<i>Ardissonea</i> spp.	3348	9463	594	4759	549	269	82	56	-	96	-	-
<i>Bacillaria</i> spp.	-	-	169	-	-	-	-	35	-	-	-	-
<i>Biddulphia</i> spp.	-	-	-	-	-	-	-	-	-	98	-	-
<i>Chaetoceros</i> spp.	49	108	-	-	176	-	-	39	-	-	-	170
<i>Cocconeis</i> spp.	14039	33615	258831	11964	18484	1565	34047	3467	13454	5890	10865	758
<i>Coscinodiscus</i> spp.	-	193	-	-	-	-	1738	-	7506	395	11117	2093
<i>Cylindrotheca</i> spp.	22509	2033	18381	5487	11543	13768	13492	11291	157444	34135	44653	4628
<i>Diploneis</i> spp.	1423	57	687	66	307	274	188	374	554	49	406	17
<i>Entomoneis ornata</i>	608	14	1167	61	1984	672	259	854	22239	12835	6251	610
<i>Entomoneis paludosa</i>	1162	81	138	73	274	89	-	111	205	81	62	-
<i>Entomoneis</i> spp.	3553	207	968	654	2782	2620	658	906	14758	8835	9017	297
<i>Guinardia</i> spp.	-	75	619448	12389	5435	-	-	37	144	-	-	-
<i>Gyrosigma</i> spp.	319	235	967	135	2058	1202	9670	2939	102847	42050	64321	2062
<i>Licmophora</i> spp.	1615	2119	190992	229178	34911	67572	2132	2004	4894	3244	25379	6258
<i>Lyrella</i> spp.	22666	4712	99484	31974	52039	8366	130962	19563	115316	79456	163838	10254
<i>Navicula</i> spp.	192661	68543	825878	125251	552710	86155	747042	148306	1772081	233016	3423701	191020
<i>Nitzschia longissima</i>	3781	513	10066	1593	10889	11517	13707	5861	61177	17820	11354	940
<i>Nitzschia palea</i>	7843	1134	9488	10089	4510	954	694	1035	1257	-	3375	1109
<i>Nitzschia</i> spp.	28334	5135	53730	15240	23706	10176	9503	10826	396893	25858	85390	6549
<i>Plagiotropis</i> spp.	3884	1622	2660	1380	65	167	-	19	4253	484	603	33
<i>Pseudo-nitzschia</i> spp.	-	54	2237	231	33	91	1831	2945	4326	1259	1435	625
<i>Rhizosolenia</i> spp.	-	42	-	-	-	-	-	71	103	296	62	-
<i>Striatella unipunctata</i>	1054	1297	4083	1272	917	543	23	-	8183	409	352	677
<i>Toxarium</i> spp.	861	1988	591	4810	170	270	23	75	103	-	133	-

undetermined centric diatoms	12865	12919	136922	34324	109961	7068	101538	19247	110243	23111	182468	17644
undetermined pennate diatoms	144005	32523	395359	77553	121500	29000	128354	31065	1311295	352514	968052	51133
<u>Dinoflagellates</u>												
<i>Ostreopsis cf. ovata</i>	-	-	-	-	-	-	84569	78630	293530	123839	364168	235838
<i>Prorocentrum lima</i>	2276	89	377	1334	250	8	47	-	922	329	2686	68
<i>Prorocentrum micans</i>	104	554	857	539	-	-	-	112	524	346	195	34
<i>Prorocentrum minimum</i>	-	-	-	190	-	-	-	-	380	112	62	36
<i>Prorocentrum</i> spp.	-	-	-	27	-	-	-	-	380	151	-	-
undetermined Dinoflagellates	1015	102	640	484	-	78	3745	1172	20703	1448	12240	367
<u>Xanthophyceae</u>												
<i>Meringosphaera</i> spp.	3292	27	304	107	570	110	950	401	2038	242	39	221

Table S2.5 - Results of PERMANOVA conducted on the density values of the entire microphytobenthos community at the order level. TI: time; AL: macroalga.

Source	df	SS	MS	Pseudo-F	P(perm)
TI	5	34120	6824	10,075	0,0001
AL	1	17518	17518	25,864	0,0001
TIxAL	5	7714,3	1542,9	2,2779	0,0001
Res	60	40639	677,32		
Total	71	99991			
Pairwise	Within algae		Within time		
	CC: T1<T2	DP: T1<T2	T1:CC<DP		
	CC: T1<T3	DP: T1<T3	T2:CC<DP		
	CC: T1<T4	DP:T1<T4	T3:CC<DP		
	CC: T1<T5	DP:T1<T5	T4:CC<DP		
	CC: T1<T6	DP:T1<T6	T5:CC<DP		
	CC: T2>T3	DP: T2>T3	T6:CC<DP		
	CC: T2>T4	DP: T2>T4			
	CC: T2<T5	DP:T2<T5			
	CC: T2>T6	DP:T2>T6			
	CC: T3< T4	DP:T3<T4			
	CC: T3<T5	DP: T3<T5			
	CC: T3<T6	DP: T3<T6			
	CC: T4<T5	DP:T4<T5			
	CC:T4< T6	DP: T4=T6			
	CC:T5=T6	DP:T5>T6			

Table S2.6 - SIMPER analysis conducted on microphytobenthic assemblages: a) between macroalgae considering each time; b) and c) among sampling times in each macroalga. DP: *Dictyopteris polydoides*; CC: *Cystoseira compressa*; T1-T6: sampling times; Avg diss.: average dissimilarity; Contr. %: contribution %.

Contrast	Avg diss.	Taxa contribution	Contr. %	Contrast	Avg diss.	Taxa contribution	Contr. %	Contrast	Avg diss.	Taxa contribution	Contr. %				
DP vs CC (T1)	68.0	<i>Navicula</i> spp.	33.0	T1 vs T2 (CC)	70.5	<i>Navicula</i> spp.	25.1	T1 vs T2 (DP)	71.6	<i>Licmophora</i> spp.	36.9				
		undet. pennate diatoms	27.6			<i>Guinardia</i> spp.	18.9			<i>Navicula</i> spp.	19.7				
		<i>Cocconeis</i> spp.	7.8			<i>Cocconeis</i> spp.	14.4			undet. pennate diatoms	11.1				
		<i>Cylindrotheca</i> spp./ <i>Nitzschia longissima</i>	6.2			undet. pennate diatoms	13.4			undet. centric diatoms	7.4				
DP vs CC (T2)	72.0	<i>Navicula</i> spp.	24.2	T1 vs T3 (CC)	57.2	<i>Navicula</i> spp.	50.2	T1 vs T3 (DP)	71.8	<i>Navicula</i> spp.	26.9				
		<i>Guinardia</i> spp.	18.2			undet. centric diatoms	14.4			<i>Licmophora</i> spp.	26.7				
		undet. pennate diatoms	15.4			undet. pennate diatoms	10.3			undet. pennate diatoms	9.8				
		<i>Cocconeis</i> spp.	13.6			T1 vs T4 (CC)	65.4			<i>Navicula</i> spp.	49.3	T1 vs T4 (DP)	71.4	<i>Cocconeis</i> spp.	9.1
<i>Licmophora</i> spp.	10.3	<i>Lyrella</i> spp.	9.5	<i>Navicula</i> spp.	33.2										
DP vs CC (T3)	71.3	<i>Navicula</i> spp.	52.5	T1 vs T5 (CC)	81.6	undet. pennate diatoms	8.8	T1 vs T5 (DP)	77,20	<i>Ostreopsis</i> cf. <i>ovata</i>	23.2				
		undet. centric diatoms	13.7			<i>Navicula</i> spp.	31.1			undet. pennate diatoms	8.8				
		undet. pennate diatoms	11.8			undet. pennate diatoms	27.8			<i>Cocconeis</i> spp.	7.1				
DP vs CC (T4)	65.7	<i>Navicula</i> spp.	52.7	T1 vs T6 (CC)	82.4	<i>Ostreopsis</i> cf. <i>ovata</i>	9.4	T1 vs T5 (DP)	77,20	undet. pennate diatoms	32.9				
		<i>Lyrella</i> spp.	10.1			<i>Navicula</i> spp.	53.6			<i>Navicula</i> spp.	21.0				
		undet. pennate diatoms	9.7			undet. pennate diatoms	18.9			<i>Ostreopsis</i> cf. <i>ovata</i>	13.2				
DP vs CC (T5)	66.1	<i>Navicula</i> spp.	34.2	T2 vs T3 (CC)	54.7	<i>Ostreopsis</i> cf. <i>ovata</i>	7.8	T1 vs T6 (DP)	73.8	<i>Lyrella</i> spp.	5.9				
		undet. pennate diatoms	25.6			<i>Navicula</i> spp.	23.1			<i>Ostreopsis</i> cf. <i>ovata</i>	40.4				
		<i>Nitzschia</i> spp.	7.4			<i>Guinardia</i> spp.	21.7			<i>Navicula</i> spp.	28.0				
		<i>Ostreopsis</i> cf. <i>ovata</i>	7.2			<i>Cocconeis</i> spp.	14.7			undet. pennate diatoms	9.6				
DP vs CC (T6)	81.5	<i>Navicula</i> spp.	53.8	T2 vs T4 (CC)	56.8	undet. pennate diatoms	14.2	T2 vs T3 (DP)	59.9	<i>Licmophora</i> spp.	36.4				
		undet. pennate diatoms	21.2			<i>Navicula</i> spp.	24.1			<i>Navicula</i> spp.	18.0				
		<i>Ostreopsis</i> cf. <i>ovata</i>	5.9			<i>Guinardia</i> spp.	19.3			undet. pennate diatoms	11.1				
		T2 vs T5 (CC)	64.9			<i>Navicula</i> spp.	26.1			T2 vs T4 (DP)	67.1	<i>Licmophora</i> spp.	32.0		
												undet. pennate diatoms	12.3	<i>Navicula</i> spp.	18.0
												undet. pennate diatoms	12.2	<i>Ostreopsis</i> cf. <i>ovata</i>	14.4
undet. pennate diatoms	19.3	undet. pennate diatoms	19.3	undet. pennate diatoms	8.7										

		<i>Guinardia</i> spp.	11.0	T2 vs T5 (DP)	68.1	undet. pennate diatoms	23.7
		<i>Ostreopsis</i> cf. <i>ovata</i>	7.8			<i>Licmophora</i> spp.	22.3
T2 vs T6 (CC)	64.9	<i>Navicula</i> spp.	47.0			<i>Navicula</i> spp.	13.1
		undet. pennate diatoms	12.2			<i>Ostreopsis</i> cf. <i>ovata</i>	10.8
		<i>Guinardia</i> spp.	10.4	T2 vs T6 (DP)	68.8	<i>Ostreopsis</i> cf. <i>ovata</i>	28.5
T3 vs T4 (CC)	40.3	<i>Navicula</i> spp.	37.4			<i>Licmophora</i> spp.	27.8
		undet. pennate diatoms	12.9			<i>Navicula</i> spp.	16.8
		<i>Lyrella</i> spp.	9.8	T3 vs T4 (DP)	65.1	<i>Navicula</i> spp.	28.9
		undet. pennate diatoms	9.4			<i>Ostreopsis</i> cf. <i>ovata</i>	21.3
T3 vs T5 (CC)	69.9	undet. pennate diatoms	29.7			<i>Licmophora</i> spp.	17.7
		<i>Navicula</i> spp.	29.0	T3 vs T5 (DP)	72.8	undet. pennate diatoms	32.5
		<i>Ostreopsis</i> cf. <i>ovata</i>	9.6			<i>Navicula</i> spp.	18.5
T3 vs T6 (CC)	67.4	<i>Navicula</i> spp.	51.9			<i>Ostreopsis</i> cf. <i>ovata</i>	12.8
		undet. pennate diatoms	21.0			<i>Licmophora</i> spp.	9.8
		<i>Ostreopsis</i> cf. <i>ovata</i>	8.6	T3 vs T6 (DP)	69.5	<i>Ostreopsis</i> cf. <i>ovata</i>	38.0
T4 vs T5 (CC)	61.7	undet. pennate diatoms	31.2			<i>Navicula</i> spp.	23.4
		<i>Navicula</i> spp.	30.5			<i>Licmophora</i> spp.	13.7
		<i>Ostreopsis</i> cf. <i>ovata</i>	7.5			undet. pennate diatoms	8.4
		<i>Nitzschia</i> spp.	7.4	T4 vs T5 (DP)	56.6	undet. pennate diatoms	37.0
T4 vs T6 (CC)	59.1	<i>Navicula</i> spp.	53.5			<i>Navicula</i> spp.	20.8
		undet. pennate diatoms	22.1			<i>Ostreopsis</i> cf. <i>ovata</i>	11.0
		<i>Ostreopsis</i> cf. <i>ovata</i>	7.1			<i>Lyrella</i> spp.	6.5
T5 vs T6 (CC)	46.8	<i>Navicula</i> spp.	55.0	T4 vs T6 (DP)	46.2	<i>Navicula</i> spp.	33.9
		undet. pennate diatoms	14.7			<i>Ostreopsis</i> cf. <i>ovata</i>	32.4
		<i>Nitzschia</i> spp.	6.8			undet. pennate diatoms	10.5
						undet. centric diatoms	6.1
				T5 vs T6 (DP)	56.1	undet. pennate diatoms	31.0
						<i>Ostreopsis</i> cf. <i>ovata</i>	20.2
						<i>Navicula</i> spp.	20.1
						<i>Lyrella</i> spp.	6.2

Table S2.7 - Results of 2-way ANOVA for the total density (N), number of taxa (S) and Pair-Wise comparison tests of meiobenthos. TI: time; AL: macroalga.

Signif. codes: *** p < 0.001, ** p < 0.01, * p < 0.05.

		n° Taxa			N		
Source	df	MS	F	P	MS	F	P
TI	1	4	2.8	0.0244	138.1374	9.17	0
AL	5	22.2222	15.56	0.0002	1570.082	104.24	0
ALxTI	5	8.4222	5.9	0.0002	106.322	7.06	0
Res	60	1.4278			15.0627		
Total	71						
		C > 0,05 NS			C<0,01 SQRT(X+1)		
		Within algae		Within time	Within algae		Within time
Pairwise		CC: T1=T2	DP: T1<T2*	T1: CC=DP	CC: T1=T2	DP: T1=T2	T1: CC=DP
		CC: T1=T3	DP: T1=T3	T2: CC<DP**	CC: T1=T3	DP: T1=T3	T2: CC<DP**
		CC: T1=T4	DP: T1=T4	T3: CC=DP	CC: T1=T4	DP: T1=T4	T3: CC<DP*
		CC: T1<T5**	DP: T1<T5	T4: CC<DP**	CC: T1<T5*	DP: T1=T5	T4: CC<DP**
		CC: T1=T6	DP: T1=T6	T5: CC>DP*	CC: T1=T6	DP: T1=T6	T5: CC<DP*
		CC: T2=T3	DP: T2=T3	T6: CC<DP*	CC: T2=T3	DP: T2=T3	T6:CC<DP**
		CC: T2=T4	DP: T2=T4		CC: T2<T5*	DP: T2=T4	
		CC: T2<T5**	DP:T2=T5		CC: T2=T6	DP: T2=T5	
		CC: T2=T6	DP: T2<T6*		CC: T3=T4	DP:T2<T6**	
		CC: T3=T4	DP: T3=T4		CC: T3=T5	DP: T3=T4	
		CC: T3<T5*	DP: T3=T5		CC: T3=T6	DP: T3=T5	
		CC: T3=T6	DP: T3=T6		CC:T4<T5	DP:T3>T6**	
		CC: T4<T5**	DP: T4=T5		CC: T4=T5	DP: T4=T5	
		CC: T4=T6	DP: T4=T6		CC: T4=T6	DP: T4>T6**	
	CC:T5 >T6**	DP: T5=T6		CC: T5>T6 *	DP: T5<T6**		

Table S2.8 - Results of PERMANOVA conducted on the density values of the entire meiobenthic community at the taxa level. TI: time; AL: macroalga.

Source	df	SS	MS	Pseudo-F	P(perm)
AL	1	18746	18746	27.031	0.0001
TI	5	18217	3643.4	5.2535	0.0001
ALxTI	5	9036.6	1807.3	2.606	0.0003
Res	60	41611	693.52		
Total	71	87612			
	Within algae		Within time		
Pairwise	CC: T1=T2	DP: T1≠T2	T1: CC≠DP		
	CC: T1=T3	DP: T1≠T3	T2: CC≠DP		
	CC: T1=T4	DP: T1=T4	T3: CC≠DP		
	CC: T1≠T5	DP: T1=T5	T4: CC≠DP		
	CC: T1≠T6	DP: T1≠T6	T5: CC=DP		
	CC: T2=T3	DP: T2≠T3	T6: CC≠DP		
	CC: T2=T4	DP: T2≠T4			
	CC: T2≠T5	DP: T2≠T5			
	CC: T2≠T6	DP: T2≠T6			
	CC: T3=T4	DP: T3≠T4			
	CC: T3≠T5	DP: T3=T5			
	CC: T3≠T6	DP: T3≠T6			
	CC: T4≠T5	DP: T4=T5			
	CC: T4=T6	DP: T4≠T6			
	CC: T5≠T6	DP: T5≠T6			

Table S2.9 - SIMPER analysis conducted on meiofauna assemblages: a) between macroalgae considering each time; b) and c) among sampling times in each macroalga. DP: *Dictyopteris polypodioides*; CC: *Cystoseira compressa*; *nauplii*: copepod *nauplii*; T1-T6: sampling times; Avg diss.: average dissimilarity; Contr. %: contribution %.

Contrast	Avg diss.	Taxa contribution	Contr. %	Contrast	Avg diss.	Taxa contribution	Contr. %	Contrast	Avg diss.	Taxa contribution	Contr. %
DP vs CC (T1)	61.8	Nematoda	36.6	T1 vs T2 (CC)	58.5	<i>nauplii</i>	30.1	T1 vs T2 (DP)	71.19	<i>nauplii</i>	53.7
		Copepoda	20.8			Nematoda	23.8			Copepoda	17.3
		Gastropoda	13.7			Gastropoda	21.4			Nematoda	13.3
		<i>nauplii</i>	13.1			Copepoda	18.2			Gastropoda	4.9
DP vs CC (T2)	76.8	<i>nauplii</i>	57.3	T1 vs T3 (CC)	56.3	Copepoda	40.2	T1 vs T3 (DP)	60.88	Copepoda	35.5
		Copepoda	23.5			Gastropoda	22.2			Nematoda	21.8
		Gastropoda	5.4			Nematoda	18.5			Gastropoda	20.3
		Nematoda	3.8			<i>nauplii</i>	13.1			<i>nauplii</i>	12.3
DP vs CC (T3)	54.5	Copepoda	43.8	T1 vs T4 (CC)	51.2	<i>nauplii</i>	40.7	T1 vs T4 (DP)	54.74	Nematoda	38.0
		Gastropoda	25.8			Gastropoda	21.8			Copepoda	35.9
		<i>nauplii</i>	14.2			Nematoda	21.1			<i>nauplii</i>	10.7
		Nematoda	9.7			Copepoda	12.6			Gastropoda	6.3
DP vs CC (T4)	72.6	Nematoda	42.9	T1 vs T5 (CC)	67.8	Nematoda	44.1	T1 vs T5 (DP)	65	Nematoda	45.4
		Copepoda	36.2			Copepoda	30.9			Copepoda	28.8
		<i>nauplii</i>	11.5			Gastropoda	10.1			Gastropoda	9.7
DP vs CC (T5)	52.0	Nematoda	44.6	T1 vs T6 (CC)	53.2	<i>nauplii</i>	7.5	T1 vs T6 (DP)	75.18	<i>nauplii</i>	8.4
		Copepoda	29.4			<i>nauplii</i>	40.9			Nematoda	32.9
		Gastropoda	10.7			Nematoda	21.9			Copepoda	32.8
DP vs CC (T6)	82.0	<i>nauplii</i>	7.7	T2 vs T3 (CC)	56.9	Gastropoda	19.1	T2 vs T3 (DP)	58.35	<i>nauplii</i>	27.2
		Nematoda	37.0			Copepoda	14.6			<i>nauplii</i>	55.8
		Copepoda	34.3			Copepoda	41.1			Copepoda	17.9
		<i>nauplii</i>	23.5			<i>nauplii</i>	24.8			Gastropoda	11.4
						Gastropoda	14.9			Nematoda	5.0
						Nematoda	12.4			<i>nauplii</i>	41.9
		T2 vs T4 (CC)	49.3	<i>nauplii</i>	45.5	Nematoda	29.2				
				Copepoda	20.1	Copepoda	16.6				
				Nematoda	18.3	Gastropoda	4.0				

T2 vs T5 (CC)	75.3	Gastropoda	10.4	T2 vs T5 (DP)	68.53	<i>nauplii</i>	38.5
		Nematoda	45.1			Nematoda	33.6
		Copepoda	28.5			Copepoda	15.3
		<i>nauplii</i>	14.8			Gastropoda	5.6
T2 vs T6 (CC)	52.3	Gastropoda	439.0	T2 vs T6 (DP)	54.44	Nematoda	43.1
		<i>nauplii</i>	39.6			Copepoda	29.0
		Nematoda	27.8			<i>nauplii</i>	16.7
		Copepoda	21.2			Polychaeta	3.2
T3 vs T4 (CC)	54.0	Gastropoda	6.1	T3 vs T4 (DP)	49.76	Nematoda	43.8
		Copepoda	35.0			Copepoda	26.3
		<i>nauplii</i>	33.8			Gastropoda	13.0
		Gastropoda	14.5			<i>nauplii</i>	10.3
T3 vs T5 (CC)	65.4	Nematoda	12.0	T3 vs T5 (DP)	58	Nematoda	48.1
		Nematoda	42.9			Copepoda	25.1
		Copepoda	33.2			Gastropoda	13.4
		<i>nauplii</i>	8.4			<i>nauplii</i>	8.2
T3 vs T6 (CC)	56.1	Gastropoda	7.7	T3 vs T6 (DP)	70.44	Nematoda	37.5
		Copepoda	34.7			<i>nauplii</i>	26.0
		<i>nauplii</i>	32.8			Copepoda	24.3
		Nematoda	16.0			Gastropoda	6.4
T4 vs T5 (CC)	68.4	Gastropoda	11.8	T4 vs T5 (DP)	40	Nematoda	42.8
		Nematoda	40.4			Copepoda	30.3
		Copepoda	28.4			Gastropoda	9.7
		<i>nauplii</i>	20.0			<i>nauplii</i>	9.4
T4 vs T6 (CC)	43.5	Gastropoda	4.1	T4 vs T6 (DP)	49.03	<i>nauplii</i>	31.7
		<i>nauplii</i>	52.5			Copepoda	29.7
		Copepoda	18.1			Nematoda	28.1
		Nematoda	18.0			Polychaeta	3.7
T5 vs T6 (CC)	67.0	Gastropoda	8.4	T5 vs T6 (DP)	47.18	<i>nauplii</i>	33.6
		Nematoda	37.6			Copepoda	30.4
		Copepoda	29.2			Nematoda	23.5

<i>nauplii</i>	21.7	Gastropoda	4.9
Gastropoda	4.6		

Table S2.10 - Meiobenthos community (N g⁻¹ fw) identified in *Dictyopterus polypodioides* (DP) and *Cystoseira compressa* (CC) at the different sampling times (T1-T6). Avg: average; S.E.: standard error.

TIME	T1		T2		T3		T4		T5		T6	
	DP	CC	DP	CC	DP	CC	DP	CC	DP	CC	DP	CC
Taxa	Avg S.E.	Avg S.E.	Avg S.E.	Avg S.E.	Avg S.E.	Avg S.E.	Avg S.E.	Avg S.E.	Avg S.E.	Avg S.E.	Avg S.E.	Avg S.E.
Nematoda	56.5±22.2	14.4±5.7	12.4±4.6	2.5±2.1	20.3±6.5	9.6±3.5	136.6±26.0	10.0±2.2	188.7±38.6	105.4±32.4	287.2±46.0	19.3±4.4
Kinorhyncha	0.5±1.9	2.8±0.5	1.2±1.2	-	1.8±1.2	0.1±0.1	1.2±1.2	-	-	0.5±0.4	-	-
Polychaeta	2.2±1.4	1.0±0.6	3.5±2.1	0.4±0.4	0.9±0.6	0.1±0.1	3.1±2.0	-	-	9.2±3.4	22.7±9.4	-
Ostracoda	-	-	3.4±1.0	-	1.7±1.7	1.1±0.8	-	-	1.7±1.7	0.5±0.5	-	-
Copepoda	31.8±9.3	8.4±2.0	85.2±19.2	15.7±8.6	103.5±23.5	37.0±10.0	122.6±25.7	13.9±3.2	120.6±27.3	77.0±27.1	285.5±69.4	14.6±6.7
copepod <i>nauplii</i>	21.0±8.1	12.9±2.2	215.1±51.6	26.3±6.1	28.1±10.3	14.8±4.3	36.8±8.1	39.7±16.1	29.2±7.6	13.5±4.0	216.5±34.4	43.1±15.5
Isopoda	6.3±3.7	-	7.1±4.8	1.9±1.3	1.4±0.9	1.1±1.0	1.8±1.4	-	7.3±3.7	3.0±1.9	13.5±5.9	0.4±0.4
Amphipoda	2.7±1.7	-	5.6±1.7	0.3±0.2	1.3±0.9	0.6±0.4	3.5±2.1	0.9±0.6	3.3±1.7	0.3±0.3	-	-
Halacaroidea	1.9±1.3	-	6.6±4.7	0.1±0.1	0.9±0.5	-	2.0±1.4	-	-	-	2.1±1.4	-
Gastropoda	8.0±5.0	13.9±7.8	15.0±7.2	3.4±2.2	44.2±17.5	9.5±5.7	16.3±3.9	4.7±2.0	27.2±15.8	6.5±2.2	-	-
Bivalvia	0.3±0.3	-	1.9±1.9	-	-	-	-	-	-	-	-	-
Chironomidae	-	-	1.3±1.3	-	0.3±0.3	0.3±0.3	-	-	0.6±0.6	0.8±0.4	-	0.9±0.9

Table S2.11 - Results of 2-way ANOVA for the α -diversity index of total density (N), number of species (S), and Pair-Wise comparison tests of harpacticoid community. TI: time; AL: macroalga.

Signif. codes: *** p < 0.001, ** p < 0.01, * p < 0.05.

		S			N			H'		
Source	df	MS	F	P	MS	F	P	MS	F	P
TI	1	15.425	4.18	0.0025	4.53E+01	6.5	0.0001	1.7728	3.75	0.005
AL	5	66.125	17.94	0.0001	544.1935	78.06	0	6.6212	14	4E-04
ALxTI	5	8.8917	2.41	0.0465	32.4973	4.66	0.0012	0.9133	1.93	0.103
Res	60	3.6861			6.9711			0.473		
Total	71									
		C > 0,05 NS			C < 0,01 SQRT (X+1)			C > 0,05 NS		
		Within algae		Within time	Within algae		Within time	TIME		
Pairwise		CC: T1=T2	DP: T1<T2*	T1: CC=DP	CC: T1=T2	DP: T1<T2*	T1: CC=DP	T1<T2*		
		CC: T1=T3	DP: T1<T3 *	T2: CC<DP **	CC: T1=T3	DP: T1<T3*	T2: CC<DP **	T1< T3 **		
		CC: T1=T4	DP: T1<T4 *	T3: CC=DP	CC: T1=T4	DP: T1<T4*	T3: CC<DP *	T1<T4*		
		CC: T1<T5 *	DP: T1=T5	T4: CC=DP	CC: T1<T5 *	DP: T1<T5*	T4: CC<DP **	T1<T5 **		
		CC: T1=T6	DP: T1<T6 *	T5: CC=DP	CC: T1=T6	DP: T1<T6**	T5: CC=DP	T1<T6 *		
		CC: T2=T3	DP: T2=T3	T6: CC<DP**	CC: T2=T3	DP: T2=T3	T6: CC<DP**	T2=T3		
		CC: T2=T4	DP: T2=T4		CC: T2=T4	DP: T2=T4		T2=T4		
		CC: T2=T5	DP: T2=T5		CC: T2=T5	DP: T2=T5		T2=T5		
		CC: T2=T6	DP: T2=T6		CC: T2=T6	DP: T2<T6**		T2=T6		
		CC: T3=T4	DP: T3=T4		CC: T3=T4	CC: T3=T4		T3=T4		
		CC: T3=T5	DP: T3=T5		CC: T3=T5	CC: T3=T5		T3=T5		
		CC: T3=T6	DP: T3=T6		CC: T3=T6	CC: T3<T6**		T3=T6		
		CC: T4=T5	DP: T4=T5		CC: T4=T5	CC: T4=T5		T4=T5		
		CC: T4=T6	DP: T4=T6		CC: T4=T6	CC: T4<T6**		T5=T6		
		CC: T5=T6	DP: T5=T6		CC: T5=T6	CC: T5<T6**				

Table S2.12 - Harpacticoid community (N g⁻¹ fw) identified in *Dictyopterus polypodioides* (DP) and *Cystoseira compressa* (CC) at the different sampling times (T1-T6). Avg: average; S.E.: standard error.

TIME	T1		T2		T3		T4		T5		T6	
	DP	CC	DP	CC	DP	CC	DP	CC	DP	CC	DP	CC
MACROALGAE	Avg S.E.		Avg S.E.		Avg S.E.		Avg S.E.		Avg S.E.		Avg S.E.	
Porcellidiidae												
<i>Porcellidium</i>												
<i>Porcellidium viride</i>	7.2±4.7	-	4.9±2.6	-	30.9±10.6	2.2±1.6	6.9±3.3	1.1±0.7	0.8±0.8	0.9±0.8	-	-
Ectinosomatidae												
<i>Ectinosoma</i>												
<i>Ectinosoma melaniceps</i>	3.2±2.6	-	15.1±3.9	0.1±0.1	9.3±2.5	1.2±0.6	8.1±2.8	0.4±0.3	12.7±5.5	7.4±2.8	18.9±5.8	1.3±1.3
Ameiridae												
<i>Ameira</i>												
<i>Amphiascus parvulus</i>	5.9±2.4	2.0±1.4	15.9±3.9	1.9±1.4	14.6±5.1	4.1±1.3	9.6±3.3	1.6±0.5	12.45±4.6	3.9±1.5	23.6±6.0	1.2±0.6
Miraciidae												
<i>Amphiascopsis</i>												
<i>Amphiascopsis cinctus</i>	-	-	0.7±0.5	-	1.4±1.0	0.8±0.6	1.5±1.1	-	4.9±2.3	7.3±3.9	22.0±3.0	-
Laophontidae												
<i>Heterolaophonte</i>												
<i>Heterolaophonte minuta</i>	5.9±4.0	1.7±0.8	13.1±3.0	6.4±2.9	18.5±7.8	16.5±7.3	67.5±17.8	5.4±1.3	33.1±7.8	10.1±3.3	81.9±19.8	5.0±3.9
Parastenheliidae												
<i>Parastenhelia</i>												
<i>Parastenhelia spinosa</i>	1.9±1.2	0.6±0.4	7.5±4.2	0.4±0.4	2.4±0.8	2.1±1.0	7.3±2.7	0.2±0.3	17.2±4.2	11.9±5.4	27.1±9.2	4.3±1.3
Harpacticidae												
<i>Harpacticus</i>												
<i>Harpacticus gracilis</i>	6.4±3.2	1.4±0.6	9.6±4.0	1.6±0.5	5.5±3.3	6.4±1.9	9.1±2.6	4.2±1.7	12.8±5.5	4.3±2.4	31.9±16.8	1.3±0.7
Dactylopusiidae												

Paradactylopodia

Paradactylopodia brevicornis 1.2±0.9 - 3.5±1.8 0.9±0.6 10.6±6.4 2.4±1.1 5.2±3.3 - 15.5±5.9 16.3±5.7 30.5±11.3 1.0±1.1

Thalestridae

Parathalestris

Parathalestris harpacticoides - - 0.4±0.4 - - - - 0.1 0.2 - 0.6±0.6 2.2±2.2 -

Dactylopusiidae

Diarthrodes

Diarthrodes ponticus - - 3.7±2.7 0.1±0.2 2.2±1.6 - 0.5±0.4 - - 0.3±0.0 1.3±1.4 -

Tisbidae

Scutellidium

Scutellidium ligusticum - - 0.2±0.3 - 0.8±0.8 - - - - - 1.1±1.1 0.4±0.4

Scutellidium longicaudum

longicaudum - - 0.5±0.6 0.1±0.2 - - 1.1±1.1 - - - - -

Table S2.13 - Results of PERMANOVA conducted on the density values of the entire harpacticoid community at the species level. TI: time; AL: macroalga.

Source	df	SS	MS	Pseudo-F	P(perm)
AL	1	20595	20595	15 232	0.0001
TI	5	20314	4062.8	30 048	0.0001
ALxTI	5	12902	2580.4	19 084	0.0044
Res	60	81127	1352.1		
Total	71	1.35E+09			
	Within algae		Within time		
Pairwise	CC: T1=T2	DP:T1=T2	T1: CC=DP		
	CC: T1=T3	DP: T1=T3	T2: CC<DP		
	CC: T1=T4	DP: T1<T4	T3: CC<DP		
	CC: T1< T5	DP: T1<T5	T4:CC<DP		
	CC:T1=T6	DP: T1<T6	T5: CC=DP		
	CC: T2=T3	DP: T2=T3	T6: CC<DP		
	CC:T2=T4	DP:T2=T4			
	CC: T2< T5	DP: T2=T5			
	CC: T2=T6	DP: T2<T6			
	CC: T3=T4	DP:T3< T4			
	CC: T3=T5	DP:T3< T5			
	CC:T3=T6	DP:T3< T6			
	CC: T4<T5	DP:T4< T6			
	CC: T4<T6	DP: T4=T6			
	CC:T5> T6	DP:T5=T6			

Table S2.14 - SIMPER analysis conducted on harpacticoid assemblages: a) between macroalgae considering each time; b) and c) among sampling times in each macroalga. DP: *Dictyopteris polypodioides*; CC: *Cystoseira compressa*; T1-T6: sampling times; Avg diss.: average dissimilarity; Contr. %: contribution %.

Contrast	Average dissimilarity	Taxa contribution	Contribution %	Contrast	Average dissimilarity	Taxa contribution	Contribution %	Contrast	Average dissimilarity	Taxa contribution	Contribution %
DP vs CC (T1)	83.0	<i>Harpacticus gracilis</i>	26.0	T1 vs T2 (CC)	65.0	<i>Heterolaophonte minuta</i>	41.3	T1 vs T2 (DP)	67.8	<i>Ectinosoma melaniceps</i>	18.3
		<i>Amphiascus parvulus</i>	18.6			<i>Amphiascus parvulus</i>	20.1			<i>Amphiascus parvulus</i>	17.7
		<i>Porcellidium viride</i>	15.5			<i>Harpacticus gracilis</i>	17.3			<i>Heterolaophonte minuta</i>	16.5
		<i>Heterolaophonte minuta</i>	14.8			<i>Parastenhelia spinosa</i>	8.6			<i>Harpacticus gracilis</i>	15.1
DP vs CC (T2)	79.3	<i>Amphiascus parvulus</i>	21.8	T1 vs T3 (CC)	76.3	<i>Heterolaophonte minuta</i>	37.0	T1 vs T3 (DP)	69.7	<i>Porcellidium viride</i>	29.7
		<i>Ectinosoma melaniceps</i>	19.9			<i>Harpacticus gracilis</i>	17.6			<i>Heterolaophonte minuta</i>	19.5
		<i>Harpacticus gracilis</i>	14.7			<i>Amphiascus parvulus</i>	12.2			<i>Amphiascus parvulus</i>	11.4
		<i>Heterolaophonte minuta</i>	13.2			<i>Parastenhelia spinosa</i>	11.0			<i>Paradactylopodia brevicornis</i>	10.9
DP vs CC (T3)	66.4	<i>Porcellidium viride</i>	31.4	T1 vs T4 (CC)	65.6	<i>Heterolaophonte minuta</i>	32.1	T1 vs T4 (DP)	75.3	<i>Heterolaophonte minuta</i>	53.6
		<i>Heterolaophonte minuta</i>	20.3			<i>Harpacticus gracilis</i>	27.3			<i>Porcellidium viride</i>	8.7
		<i>Amphiascus parvulus</i>	11.2			<i>Amphiascus parvulus</i>	16.6			<i>Amphiascus parvulus</i>	8.3
		<i>Paradactylopodia brevicornis</i>	11.1			<i>Porcellidium viride</i>	9.1			<i>Harpacticus gracilis</i>	8.1
DP vs CC (T4)	79.4	<i>Heterolaophonte minuta</i>	56.1	T1 vs T5 (CC)	83.0	<i>Paradactylopodia brevicornis</i>	21.1	T1 vs T5 (DP)	78.2	<i>Heterolaophonte minuta</i>	27.9
		<i>Amphiascus parvulus</i>	8.8			<i>Heterolaophonte minuta</i>	16.5			<i>Parastenhelia spinosa</i>	14.7
		<i>Ectinosoma melaniceps</i>	8.1			<i>Parastenhelia spinosa</i>	16.0			<i>Paradactylopodia brevicornis</i>	12.3
		<i>Harpacticus gracilis</i>	6.6			<i>Ectinosoma melaniceps</i>	13.5			<i>Harpacticus gracilis</i>	12.2
DP vs CC (T5)	62.2	<i>Heterolaophonte minuta</i>	26.6	T1 vs T6 (CC)	70.0	<i>Parastenhelia spinosa</i>	29.6	T1 vs T6 (DP)	82.1	<i>Ectinosoma melaniceps</i>	10.9
		<i>Parastenhelia spinosa</i>	15.2			<i>Heterolaophonte minuta</i>	24.9			<i>Heterolaophonte minuta</i>	33.1
		<i>Paradactylopodia brevicornis</i>	15.2			<i>Amphiascus parvulus</i>	17.1			<i>Paradactylopodia brevicornis</i>	12.1
		<i>Ectinosoma melaniceps</i>	11.9			<i>Harpacticus gracilis</i>	16.1			<i>Parastenhelia spinosa</i>	11.9
DP vs CC (T6)	88.5	<i>Heterolaophonte minuta</i>	33.2	T2 vs T3 (CC)	70.7	<i>Heterolaophonte minuta</i>	38.1	T2 vs T3 (DP)	56.1	<i>Amphiascopsis cinctus</i>	11.8
		<i>Harpacticus gracilis</i>	12.9			<i>Harpacticus gracilis</i>	17.1			<i>Harpacticus gracilis</i>	11.3
		<i>Paradactylopodia brevicornis</i>	12.0			<i>Amphiascus parvulus</i>	11.0			<i>Porcellidium viride</i>	26.8
		<i>Amphiascopsis cinctus</i>	11.9	<i>Parastenhelia spinosa</i>	10.2	<i>Amphiascus parvulus</i>	13.3				
		<i>Parastenhelia spinosa</i>	11.7	T2 vs T4 (CC)	60.1	<i>Heterolaophonte minuta</i>	44.3			<i>Heterolaophonte minuta</i>	12.7

		<i>Amphiascus parvulus</i>	10.5			<i>Ectinosoma melaniceps</i>	11.3
		<i>Ectinosoma melaniceps</i>	10.0			<i>Harpacticus gracilis</i>	10.8
		<i>Harpacticus gracilis</i>	8.8			<i>Paradactylopodia brevicornis</i>	10.4
		<i>Parastenhelia spinosa</i>	7.7	T2 vs T4 (DP)	60.1	<i>Heterolaophonte minuta</i>	44.3
T2 vs T5 (CC)	78.0	<i>Paradactylopodia brevicornis</i>	21.5			<i>Amphiascus parvulus</i>	10.5
		<i>Heterolaophonte minuta</i>	19.3			<i>Ectinosoma melaniceps</i>	10.0
		<i>Parastenhelia spinosa</i>	15.2			<i>Harpacticus gracilis</i>	8.8
		<i>Ectinosoma melaniceps</i>	12.2	T2 vs T5 (DP)	58.3	<i>Heterolaophonte minuta</i>	20.3
		<i>Amphiascopsis cinctus</i>	11.1			<i>Parastenhelia spinosa</i>	14.3
T2 vs T6 (CC)	71.4	<i>Heterolaophonte minuta</i>	36.0			<i>Ectinosoma melaniceps</i>	13.6
		<i>Parastenhelia spinosa</i>	24.5			<i>Amphiascus parvulus</i>	13.0
		<i>Harpacticus gracilis</i>	12.1			<i>Harpacticus gracilis</i>	12.4
		<i>Amphiascus parvulus</i>	11.7			<i>Paradactylopodia brevicornis</i>	12.4
		<i>Paradactylopodia brevicornis</i>	8.3	T2 vs T6 (DP)	94.9	<i>Heterolaophonte minuta</i>	31.5
T3 vs T4 (CC)	62.7	<i>Heterolaophonte minuta</i>	37.3			<i>Paradactylopodia brevicornis</i>	12.5
		<i>Harpacticus gracilis</i>	18.3			<i>Parastenhelia spinosa</i>	12.1
		<i>Porcellidium viride</i>	10.7			<i>Amphiascopsis cinctus</i>	11.9
		<i>Amphiascus parvulus</i>	9.6			<i>Harpacticus gracilis</i>	11.4
		<i>Parastenhelia spinosa</i>	9.3	T3 vs T4 (DP)	60.5	<i>Heterolaophonte minuta</i>	39.6
T3 vs T5 (CC)	69.3	<i>Heterolaophonte minuta</i>	23.9			<i>Porcellidium viride</i>	20.2
		<i>Paradactylopodia brevicornis</i>	19.4			<i>Amphiascus parvulus</i>	9.1
		<i>Parastenhelia spinosa</i>	14.1			<i>Paradactylopodia brevicornis</i>	8.6
		<i>Harpacticus gracilis</i>	11.0	T3 vs T5 (DP)	64.3	<i>Porcellidium viride</i>	23.4
		<i>Ectinosoma melaniceps</i>	9.3			<i>Heterolaophonte minuta</i>	17.8
T3 vs T6 (CC)	71.5	<i>Heterolaophonte minuta</i>	37.0			<i>Parastenhelia spinosa</i>	12.1
		<i>Harpacticus gracilis</i>	16.9			<i>Paradactylopodia brevicornis</i>	11.8
		<i>Parastenhelia spinosa</i>	12.6			<i>Amphiascus parvulus</i>	10.2
		<i>Amphiascus parvulus</i>	9.3	T3 vs T6 (DP)	68.2	<i>Heterolaophonte minuta</i>	27.1
		<i>Porcellidium viride</i>	8.2			<i>Porcellidium viride</i>	14.2
T4 vs T5 (CC)	75.8	<i>Paradactylopodia brevicornis</i>	21.3			<i>Parastenhelia spinosa</i>	11.4
		<i>Heterolaophonte minuta</i>	16.4			<i>Harpacticus gracilis</i>	11.3

		<i>Parastenhelia spinosa</i>	15.0			<i>Paradactylopodia brevicornis</i>	11.2
		<i>Harpacticus gracilis</i>	12.7	T4 vs T5 (DP)	53.8	<i>Heterolaophonte minuta</i>	35.9
T4 vs T6 (CC)	72.4	<i>Heterolaophonte minuta</i>	31.7			<i>Parastenhelia spinosa</i>	11.7
		<i>Parastenhelia spinosa</i>	20.2			<i>Paradactylopodia brevicornis</i>	11.3
		<i>Harpacticus gracilis</i>	20.0			<i>Ectinosoma melaniceps</i>	10.1
		<i>Amphiascus parvulus</i>	8.3	T4 vs T6 (DP)	72.4	<i>Heterolaophonte minuta</i>	31.7
T5 vs T6 (CC)	77.0	<i>Paradactylopodia brevicornis</i>	21.2			<i>Parastenhelia spinosa</i>	20.2
		<i>Heterolaophonte minuta</i>	18.2			<i>Harpacticus gracilis</i>	20.0
		<i>Parastenhelia spinosa</i>	17.6			<i>Amphiascus parvulus</i>	8.3
		<i>Ectinosoma melaniceps</i>	12.2	T5 vs T6 (DP)	51.9	<i>Heterolaophonte minuta</i>	27.8
		<i>Amphiascopsis cinctus</i>	10.9			<i>Paradactylopodia brevicornis</i>	13.9
		<i>Harpacticus gracilis</i>	9.0			<i>Harpacticus gracilis</i>	13.9
						<i>Parastenhelia spinosa</i>	12.5
						<i>Amphiascopsis cinctus</i>	11.1

Table S2.15 - Results of DistLM fitting set of predictor variables to (A) meiofauna and (B) copepod harpacticoid assemblages.

(A)

MARGINAL TESTS					
Group of variables	Pseudo-F	P	Prop.		
complexity	58.346	0.0001	0.258		
PUAs	34.098	0.0001	0.169		
microphytobenthos	55.192	0.0001	0.376		
SEQUENTIAL TESTS					
Group of variables	AICc	Pseudo-F	P	Prop.	Cumul.
complexity	500.88	58.346	0.0001	0.258	0.258
PUAs	502.91	1.841	0.0233	0.078	0.336
microphytobenthos	507.7	20.187	0.0024	0.134	0.470
SEQUENTIAL TESTS					
	AICc	R ²	RSS		
complexity+PUAs+microphytobenthos	507.7	0.470	46455	3 All	

(B)

MARGINAL TESTS					
Group of variables	Pseudo-F	P	Prop.		
complexity	39.256	0.0001	0.190		
PUAs	24.328	0.0012	0.127		
microphytobenthos	26.995	0.0001	0.228		
SEQUENTIAL TESTS					
Group of variables	AICc	Pseudo-F	P	Prop.	Cumul.
complexity	538.33	39.256	0.0001	0.190	0.190
PUAs	539.93	19.483	0.0102	0.089	0.279
microphytobenthos	549.78	13.388	0.1105	0.103	0.382
SEQUENTIAL TESTS					
	AICc	R ²	RSS		
complexity+PUAs+ microphytobenthos	549.78	0.382	83337		

Chapter 3- Understanding the Role of Macroalgal Complexity and Allelochemicals Production in Invasive and Non-Invasive Macroalgae in the North-Western Adriatic Sea: Effect on the Associated Communities

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Abstract: Highly diverse microphyto and meiobenthic communities are associated with large-sized marine macroalgae. Both morphological traits and allelochemical responses of macroalgae affect the composition of these communities, but the relative importance of these factors remains incompletely understood. In this study we investigated the microphytobenthic and meiobenthic communities associated with some native macroalgae and a non-indigenous species (*Sargassum muticum*) of the north-western Adriatic Sea. These seaweeds were sampled in two coastal sites subjected to different impacts. The possible effects of the structural complexity of the macroalgae and the potential role of allelochemicals (specifically polyunsaturated aldehydes, PUAs) on the associated communities were examined using univariate and multivariate analyses. The results indicate that distinct assemblages were associated with the macroalgae collected at the two different sites. Differences in microphytobenthic communities could be ascribed to differences in the macroalgal morphological traits and in their PUAs production. Conversely, variation of the meiobenthic community seemed to be related mainly to differences in the macroalgal communities at the two sites. This apparent inconsistency between the two analyzed communities suggests that microphytobenthos and

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meiofauna were differently shaped by the environmental habitat provided by macroalgae in the two sites, that are subjected to different environmental conditions and human activities. Overall, these results indicate that interactions between organisms belonging to different trophic groups (e.g., microphytobenthos and meiofauna) should be investigated in detail to better understand the global role of macroalgae as habitat formers on coastal ecosystems, especially in the case of large-sized introduced species.

Keywords: macroalgal structural complexity; polyunsaturated aldehydes; microphytobenthos; meiofauna; non-indigenous species; chemical ecology

3.1 Introduction

In the marine environment, seaweeds are key primary producers and habitat formers, providing space, shelter, and food for a variety of associated epifaunal organisms [1] and substrate for numerous small-sized algal epiphytes [2]. The Mediterranean Sea is a global hotspot of marine biodiversity and its macroalgal flora consists of approximately 1200 species [3]. Due to its semi-enclosed nature, it is believed that human impacts are proportionally stronger in the Mediterranean than in any other sea of the world [4]. Non-indigenous macroalgae are particularly likely to become invasive because of their high reproductive rates, production of specialized metabolites which could act as allelochemicals, and perennial status that make them more competitive than native species [5]. Globally, the impacts of invasive macroalgal populations are typically expressed as community dominance through space monopolization and changes in the competitive relationships with native assemblages. Invasive aliens can outcompete native species for space, light, or nutrients, creating monospecific stands and homogenized habitats [6], causing changes in community structure, food web structure, and ecosystem processes [7].

Both invasive and native macroalgae are known to produce a variety of allelopathic substances such as phenolic compounds, alkaloids, peptides, oxoacids, and polyketides, including polyunsaturated fatty acids (PUFAs) [8–10] and their derivatives such as polyunsaturated aldehydes (PUAs) [11–13]. Among these natural products there are molecules well-known to influence the abundance and distribution of marine organisms and to play important roles in their inter and intraspecific interactions.

The brown alga *Sargassum muticum* (Yendo) Fensholt is considered one of the most invasive seaweeds in temperate ecosystems. This macroalga was introduced in Europe in the early 1970s and nowadays it is distributed from Norway to Morocco, as well as in the Mediterranean Sea [14]. Particularly, in Italian coastal areas, such as in the north-western Adriatic Sea (i.e., canals in Venice and area of Ancona) and the Mar Piccolo of Taranto, some port areas have been colonized by *S. muticum*, probably due to the intense and frequent maritime traffic [15]. Specifically, in the port of Ancona, the presence of this species has been known since 2009 [16]. Several studies have shown that, in addition to the presence of *S. muticum*, changes observed in invaded locations included a significant reduction in the abundance of previously dominant species [17,18].

Recently, chemical ecologists have also started to consider how research on natural products might be useful in understanding the dynamics of marine biological invasions, assessing their impact in the invaded areas, and evaluating the effects on other native organisms [19]. Allelopathic interactions between different algal species may have different effects on associated organisms, including loss of motility, cellular deformation [20,21], loss of pigmentation, aggregation of the cytoplasm, formation of vesicles, and cell lysis [22]. In particular, polyunsaturated aldehydes (PUAs) can inhibit photosynthesis and growth rates of other algal species, as well as grazing pressure, and can have a teratogenic effect on associated benthic organisms [23]. This was shown in a recent study [24], in which PUAs produced by two macroalgae (*Cystoseira compressa* and *Dictyopteris polypodioides*) were characterized at different sampling times to evaluate their role on structuring the meiobenthic and microphytobenthic communities and were found to affect some of the grazers rather than the entire community structure. Among PUAs, long-chain compounds (i.e., C14–C16) showed stronger effects on the abundances of some microalgal genera and meiobenthic taxa (e.g., harpacticoid species) than short-chain ones (i.e., C6:2).

Meiofauna represents the most abundant and taxonomically diverse metazoan assemblage on Earth [25] and plays a key role in the exchange of organic matter [26,27] as part of the “small food web” (size class 45–1000 μm). Moreover, it supports most of the higher trophic levels [25], being an important food resource for macrofauna, small fish, and other epibenthic predators [28]. Conversely, microphytobenthos communities consist of microalgae associated with benthic substrata. These assemblages often include settled cells or colonies of phytoplanktonic species [29]. Diatoms are usually the main component of microphytobenthos communities in temperate regions and, like macroalgae, are known to produce different PUA compounds [12]. Thus, seaweed aggregations and microphytobenthic biofilms can interact directly or indirectly, influencing the

recruitment of associated organisms. Relationship between macroalgae and epiphytic microalgae is considered as food and habitat provision for other associated assemblages. In our previous work [24], a clear separation of the meiofauna and microphytobenthos assemblages was found for the two studied macroalgae, with different temporal trends, thus confirming a seasonal dynamic; moreover, results indicated that macroalgal complexity was a major determinant of the meiofaunal community structure, rather than PUAs which showed species-specific effects.

Introduced species of marine macroalgae may be morphologically dissimilar from native species. Indeed, structural complexity of macroalgae could influence associated communities driving their diversity, abundance, and community structure [30]. If the introduced macroalga is a large-sized species, it will produce a novel habitat available for the colonization of local native species. Changes in habitat structure can be propagated to food webs by influencing invertebrates of lower trophic levels that will use the algal structure as refuge, and, as a consequence, species of higher trophic levels that feed on these invertebrates.

The main aims of this study were to analyze the following: (i) the potential production of PUAs by four indigenous macroalgae (*Cystoseira compressa* (Esper) Gerloff and Nizamuddin, *Dictyopteris polypodioides* A.P. De Candolle J.V. Lamouroux, *Dictyota dichotoma* (Hudson) J.V.Lamouroux, *Ulva* cf. *lacunculata* (Kützting) Wittrock, and a non-indigenous species (*Sargassum muticum* (Yendo) Fensholt) and their effects on the microphyto- and meiobenthic communities; (ii) the relationship between the microphytobenthos and the meiobenthos present on the different macroalgal species, considering the structural complexity of the macroalgae and the potential role of aldehydes in regulating their interaction.

3.2 Materials and Methods

3.2.1. Study Area

This study was performed at two coastal sites in the north-western Adriatic Sea in the coastal area of Ancona, a city hosting a large commercial harbor (Figure 1). The first site was a semi-enclosed and shallow (mean depth 1.5 m) inlet called Piscinetta del Passetto (43°37'09" N, 13°31'54" E). The site, hereafter referred to as Passetto (PAS), was previously described [31]. The seaweed vegetation was characterized by the co-existence of many different macroalgae belonging to the class of Chlorophyta (e.g., *Ulva* cf. *lacunculata*, *Cladophora dalmatica*, *Cladophora laeteviren*), Ochrophyta (e.g., *Asperococcus fistulosus*, *Cystoseira compressa*, *Dictyota dichotoma*, *Dictyopteris polypodioides*, *Gongolaria barbata*, *Padina pavonica*, *Scytosiphon lomentaria*), and Rhodophyta (e.g., *Alsidium corallinum*, *Callithamnion corymbosum*, *Ceramium* spp., *Chondracanthus acicularis*,

Corallina berteroi, *Gastroclonium clavatum*, *Gracilaria* spp., *Hypnea* spp., *Pterocladiaella capillacea*), the latter being the most species-diverse [15]. The second study site (43°37'32" N, 13°29'58" E) was located along a wharf (Molo Nord) within the commercial harbor of Ancona and is hereafter referred to as Porto (POR). It is characterized by the presence of concrete blocks laid over a sandy bottom (Figure 1). The most common macroalgae present at this site include the following: Chlorophyta (e.g., *Bryopsis* spp., *Ulva* cf. *lacunculata*), a few native Ochrophyta (e.g., *Dictyota dichotoma*), and Rhodophyta (e.g., *Antithamnion cruciatum*, *Chondracanthus acicularis*, *Corallina berteroi*, *Gelidium* spp., *Pyropia elongata* and *Schottera nicaeensis*). Recent studies have documented in the harbor the presence of some non-indigenous species (NIS) such as *Aglaothamnion feldmanniae*, *Grateloupia turuturu*, *Melanothamnus japonicus*, *Polysiphonia morrowii*, and *Sargassum muticum* [15]; the occurrence of most of these has been known since 2009 [16].

For the study, five different macroalgal species were sampled (Figure S1): the native *Cystoseira compressa*, *Dictyopteris polypodioides*, *Dictyota dichotoma*, and *Ulva* cf. *lacunculata*, and the non-indigenous *Sargassum muticum*. These species were selected, in order to cover a wide range of morphological complexity and a variable PUAs production, as shown by previous studies [24,31], and to compare indigenous and invasive species.



Figure 1. Sampling sites (© Google Earth).

3.2.2. Morphological Features of the Species Selected

Cystoseira compressa (Esper) Gerloff and Nizamuddin (Phaeophyceae) is a semi-perennial leathery macrophyte (i.e., most of the thallus is lost every year and the species persists in unfavorable seasons in the form of a small holdfast). The thallus consists of a basal callus arising one or a few

short axes, issuing numerous branches arranged in a radial pattern, the length of which varied depending on the time of the year. At the time of full development (late spring–early summer), the thallus has a bushy habitat and may reach up to 1 m in height. This species has been reported to produce allelochemicals, in particular the short-chain polyunsaturated aldehyde hexadienal (C6:2) [31].

Dictyota dichotoma (Hudson) J.V. Lamouroux (Phaeophyceae) is commonly found in the Mediterranean and Atlantic Seas and on rocks in calm places near the surface, and often associated with species of the closely related genus *Dictyopteris*. It has a ribbon-like corticated thallus, with regular dichotomous ramifications that end into bilobed and rounded apices. The color is olive green to yellowish brown. It measures up to 25 cm in height and tends to thrive in shallow, calm, and sheltered habitats. It has been documented by [31] that *D. dichotoma* is among the species of the class Phaeophyceae that produce long-chain PUAs, specifically eicosapentaenal (C20:5).

Ulva cf. lacinulata Wittrock (Ulvophyceae) is a leafy green seaweed with a thin thallus formed by two cell layers. The genus *Ulva* is taxonomically difficult, and the alga used for this study corresponds morphologically to the species reported until recently in the Mediterranean as *Ulva rigida* C. Agardh. In [32], however, the authors highlighted a major taxonomic and nomenclatural problem concerning the identity of this species in the Mediterranean, suggesting that *Ulva lacinulata* is probably the correct identification for most Mediterranean specimens. Several studies have shown that algal specimens identified as *Ulva rigida* could produce a wide range of medium and long-chain aldehydes (nonatetraenal C9:4 and decatetraenal C10:4) [31,33].

Dictyopteris polypodioides (De Candolle) J.V. Lamouroux (Phaeophyceae) is a very common and abundant species mainly found in the Mediterranean Sea. The fronds are corticated, ribbon-like with a central midrib, with more or less irregular and proliferating edges; the height of thallus is 10–20 cm. *D. polypodioides* is known to produce phenolic compounds [4] which are probably involved in the defense against grazers (e.g., strong deterrent to the feeding of the amphipod). *D. polypodioides* was the major PUA producer among the species analyzed by Pezzolesi et al. [31], specifically for the production of long-chain PUAs such as tetradecapentaenal (C14:5) and hexadecatetraenal (C16:4).

Sargassum muticum (Yendo) Fensholt (Sargassaceae) is a large-sized leathery brown alga native to Japan; it is well-known as one of the most invasive seaweeds at global level. The occurrence of *S. muticum* in the harbor of Ancona has been known since 2009 [16]. This species is usually 1–3 m in length but can grow up to 16 m. The long, annual branches bear numerous small (<0.5 cm) round

airbladders, making plants stand upright in the water or float on the surface, and small leaf-like branches. The alga has two distinct parts: the perennial, dark brown basal axes, and the lighter-colored annual primary laterals. The PUAs production for *S. muticum* is not yet known, but studies have reported the presence of phenolic compounds, particularly phlorotannins, making it unpalatable to grazers [34].

3.2.3. Sampling and Sample Processing

Sampling was carried out on 3 and 7 June 2021. Three macroalgal species were sampled in both sites: *Dictyota dichotoma* (DD), *Ulva* cf. *lacunculata* (UL), and *Dictyopteris polypodioides* (DP). Two species were sampled only in one site: *Cystoseira compressa* (CC) at Passetto and *Sargassum muticum* (SM) at Porto. The sampling time was chosen because in the study area this period corresponds to late spring. This is the only period in the year in which all the five species occur at the same time in their fully developed habitat, and therefore their thallus has the full morphological complexity.

Parts of the thalli (first 5–10 cm) for the branched species, and marginal fragments of *Ulva* cf. *lacunculata*, were sampled by snorkeling at a depth of about 0.5 m. For each macroalgal species, four replicates were collected using 50 mL polypropylene tubes, avoiding the dispersion of the associated epiphytic organisms. Moreover, the surface temperature and salinity of the water column were measured at each site by a multiparameter water probe HQ30d (Hachl-Lange GmbH) and a refractometer Atago S-10, respectively.

Immediately after collection, the macroalgae were carried to the laboratory and processed to remove all associated benthic organisms, as described in Lenzo et al. [24]. Each tube containing the algal tips and their storage water was vigorously shaken to separate the macroalgae from the epiphytic microalgae and the meiofauna. Then, the tube was rinsed with filtered seawater and vigorously washed several times until epiphytic organisms were completely removed.

The total volume of washing seawater of each sample (approximately 150 mL) was measured and then divided into two aliquots, one for the microphytobenthos and the other for the meiofauna analyses. Aliquots for microphytobenthos analysis (about 75 mL) were fixed with Lugol and stored in 250 mL dark glass bottles. Aliquots for meiofauna analysis were sieved through a 1000 μm mesh and a 45 μm mesh. The meiofauna retained in the latter sieve was preserved in alcohol 70% and stored in a 50 ml tube until subsequent analyses.

3.2.4. Macroalgal Morphology

To determine whether the morphology of the macroalgae influenced the structure of the associated microphytobenthic and meiobenthic assemblages, different variables were measured for each thallus.

After removing the epiphytic organisms, the volume of each thallus was measured by placing the alga in a graduated cylinder containing water and determining the volume of water displaced. The thalli were then dried with absorbent paper and weighed to determine wet weight. Then, each frond was placed on a white surface with a reference scale and photographed using a digital camera Canon EOS 750D. Finally, the macroalgae samples were stored at -80°C in new tubes.

Perimeter, area, and fractal dimensions (D), used as proxy of the habitat architecture [35], were measured processing the pictures using the program ImageJ v1.53u. The measure of fractal dimension (D) was based on the image using a method analogous to the grid method (boundary dimension) as previously proposed [36]. Moreover, for each sample volume, weight, and perimeter were standardized per area, according to the standardization made for microphytobenthos and meiofauna abundance.

3.2.5. Aldehydes (PUAs) Produced by Macroalgae

The extraction and quantification of PUAs produced by the macroalgae was carried out by gas chromatography–mass spectrometry (GC–MS) as previously described [31]. A portion of the apical part of the thallus (about 0.2–0.5 g f. wt.) was crushed with mortar and pestle in liquid nitrogen. The powder thus obtained was transferred into 10 mL tubes. Derivatization of the polyunsaturated aldehydes was performed with O-(2,3,4,5,6-pentafluorobenzyl)hydroxylamine hydrochloride solution (PFBHA HCl) and quantification was based on the internal standard (i.e., benzaldehyde). All reagents were purchased from Sigma-Aldrich (Milan, Italy) and used without any further purification.

3.2.6. Microphytobenthic Assemblages

The quali-quantitative analysis of the microphytobenthos associated with the two macroalgae was performed using an inverted optical microscope (Zeiss Axiovert 100) at 320 \times and 200 \times magnification. Subsamples (5–10 mL) of epiphytic communities fixed with Lugol were settled in counting chambers after homogenization, according to the Utermöhl's sedimentation method [37,38]. Counting was performed in different ways. The microphytobenthos community was examined at 320 \times magnification on 30 random fields or 4–5 transects; then a counting at 200 \times of the organisms present on the whole sedimentation chamber was performed to obtain a correct

evaluation of uncommon taxa. Orders and genera were identified based on various manuals and identification keys [39,40] or using data from the literature [41,42].

The identification of individuals was based exclusively on observable morphological characters (such as shape, size, number of chloroplasts); taxonomy and nomenclature for the microalgae recorded is based on AlgaeBase [43]. Abundance was expressed as number of cells per macroalgal area (cells/cm²).

3.2.7. Meiobenthic Assemblages

Meiobenthic organisms of each sample were counted and identified to major taxa under a stereomicroscope (Nikon SMZ 1500). Since harpacticoid copepods were recognized as the most sensitive group to aldehydes [24], dead copepod harpacticoids and dead *nauplii*, recognizable by the empty exuviae, were counted separately. Furthermore, since PUAs may also impact harpacticoids reproduction causing a decreased egg viability, expelled egg sacs were also counted [24]. Abundance of meiobenthic organisms was expressed as Ind/cm².

3.2.8. Data Analysis

Due to the different macroalgal species sampled at the two sites, data were analyzed considering the site–macroalgae combinations as a single fixed factor with 8 levels. PERMANOVA tests [44,45] were performed to test for differences in variables related to macroalgae complexity, PUAs concentrations, number of taxa (S) and abundance (N) for microphytobenthos and meiobenthos, and in microphytobenthic and meiobenthic assemblages by a one-way design. PERMANOVA tests of univariate analyses were based on Euclidean distances of untransformed data, while tests for differences in microphytobenthic and meiobenthic community structures was based on Bray–Curtis similarity. Pairwise comparisons were performed when the main effect resulted as significant. When the number of unique permutations available was less than 100, asymptotic Monte Carlo *p*-values (P(MC)) were considered instead of permutational ones (P(perm)). All PERMANOVA analyses were performed using unrestricted permutation of the raw data and 9999 permutations.

Principal component analysis (PCA) ordination, performed on normalized data, was used to display the relationship between morphological measures taken for each macroalgal sample, and to identify the most important variables that differentiate the habitats that they provide to microphyto and meiobenthic assemblages. Before performing the PCA analysis, the distribution and the possible correlation among variables were evaluated by draftsman plot (by applying a $|r| < 0.95$ cut-off).

Community data of microphytobenthos and meiobenthos were transformed considering the results obtained by shade plots. These routines provide a simple visual representation of abundance matrices from multivariate species assemblage, which guide the choice of the best transformation of quantitative data [46]. Microphytobenthic abundance data were fourth root transformed, while meiofauna abundance data were transformed by square root. The community structure of each assemblage (microphytobenthos and meiofauna) was analyzed by non-metric multidimensional scaling (nMDS) based on Bray–Curtis similarity.

Taxa that mostly contributed to the dissimilarity/similarity among/within macroalgal species were identified using the SIMPER analysis (60% cut-off for microphytobenthos and 70% cut-off for meiobenthos) [47].

Multivariate multiple regression, using a distance-based linear model (DistLM) procedure (in PERMANOVA+; [48]) was used to test for relationships between the set of macroalgal morphological traits and PUAs and microphytobenthic assemblages. To analyze the relationships between macroalgal complexity, PUAs, and microphytobenthic abundance with meiofauna communities, DistLM was performed on two different meiofauna matrices: all taxa and all taxa with the addition of harpacticoid and *nauplii* dead. The “BEST” procedure and Akaike Information Criterion (AICc, was used as selection criterion. Distance-based Redundancy Analysis (dbRDA) plot was used to visualize DistLM results.

Finally, to compare the response of microphytobenthic and meiobenthic assemblages to site-macroalgae, a RELATE analysis was performed.

The significance level was set at 0.05 (5%) for all tests. All analyses were conducted with PRIMER v7 [49] with the PERMANOVA + add on [48].

3.3 Results

Seawater temperature and salinity measured in the seawater during the samplings were 21.7 °C and 37 ‰ in Passetto and 20.1 °C and 38 ‰ in Porto, respectively.

3.3.1 Macroalgal Morphology

Overall, five measures were taken to evaluate the morphology of the macroalgae and three ratios were calculated to estimate the complexity of the apical parts of each species (Table 1).

Table 1. Mean value (\pm SE; n=4) of the variables measured in Passetto (PAS) and Porto (POR) on each macroalga: *Cystoseira compressa* (CC), *Dictyota dichotoma* (DD), *Ulva cf. lacinulata* (UL), and *Dictyopteris polypodioides* (DP) and *Sargassum muticum* (SM).

Site–Macroalgae	Weight (g)	Volume (mL)	Area (cm ²)	Perimeter (cm)	D	Weight/Area	Perimeter/Area	Volume/Area
PAS-CC1	0.80 \pm 0.07	1.05 \pm 0.06	9.74 \pm 0.77	185.10 \pm 14.39	1.74 \pm 0.01	0.08 \pm 0.002	19.03 \pm 0.57	0.11 \pm 0.00
PAS-DD1	0.13 \pm 0.01	0.28 \pm 0.09	5.16 \pm 0.39	68.09 \pm 9.30	1.76 \pm 0.01	0.03 \pm 0.002	13.06 \pm 0.86	0.05 \pm 0.01
PAS-UL1	0.45 \pm 0.03	1.23 \pm 0.09	25.68 \pm 2.23	49.70 \pm 4.70	1.94 \pm 0.00	0.02 \pm 0.000	1.99 \pm 0.26	0.05 \pm 0.00
PAS-DP1	0.34 \pm 0.02	0.93 \pm 0.05	15.71 \pm 1.26	56.98 \pm 2.76	1.84 \pm 0.01	0.02 \pm 0.001	3.69 \pm 0.32	0.06 \pm 0.01
POR-SM1	0.66 \pm 0.08	1.18 \pm 0.12	11.12 \pm 1.28	257.45 \pm 29.51	1.70 \pm 0.02	0.06 \pm 0.003	23.22 \pm 1.06	0.11 \pm 0.01
POR-DD1	0.32 \pm 0.01	0.50 \pm 0.00	19.59 \pm 0.52	150.48 \pm 7.08	1.78 \pm 0.01	0.02 \pm 0.001	7.68 \pm 0.30	0.03 \pm 0.00
POR-UL1	0.28 \pm 0.06	1.38 \pm 0.24	35.84 \pm 7.70	66.42 \pm 13.77	1.92 \pm 0.00	0.01 \pm 0.000	1.88 \pm 0.16	0.04 \pm 0.01
POR-DP1	0.36 \pm 0.06	0.93 \pm 0.22	18.75 \pm 2.98	68.40 \pm 11.78	1.88 \pm 0.01	0.02 \pm 0.000	3.63 \pm 0.06	0.05 \pm 0.01

The complexity of macroalgae varied among the different analyzed species. *Ulva cf. lacinulata* had the highest fractal dimension (D) in both sites, followed by *Dictyopteris polypodioides*, while the lowest values were measured in *Sargassum muticum* and *Cystoseira compressa*. An almost opposite trend was observed for the perimeter and the other measures standardized per area. The PCA analysis has been performed on D and ratios between weight, volume, and perimeter to area, in order to be consistent with the standardization carried out for microphytobenthos and meiofauna abundance.

The first two components accounted for 95.2% of the total variance (Figure 2). Variability along the PC1 axis was mainly explained, from left to right, by a decrease of weight/area and perimeter/area ratios while variability along the PC2 axis was mainly explained, from top to bottom, by an increase of fractal (D) (Tables 1 and S1). Accordingly, the samples were disposed more or less regularly along these gradients, with samples of *C. compressa* in Passetto and *S. muticum* in Porto grouped together in the left side of the PC1, and, in the right side, all the other macroalgae collected in both sites. The second axis detected morphological variation among macroalgae, that formed clusters depending on the macroalgal species (Figure 2). This pattern was confirmed by PERMANOVA results that showed significant differences among the site–macroalgae factor (PseudoF = 50.86, $p = 0.0001$; Table S2). Post hoc comparisons among macroalgal species within each site and between sites for each species present either in Passetto or Porto confirmed the morphological differences among macroalgae in each site, except for POR-UL vs POR-DP. Instead, morphology of *Ulva cf. lacinulata*

and *Dictyopteris polypodioides* in the two sites did not differ, suggesting morphological coherence despite the different sites.

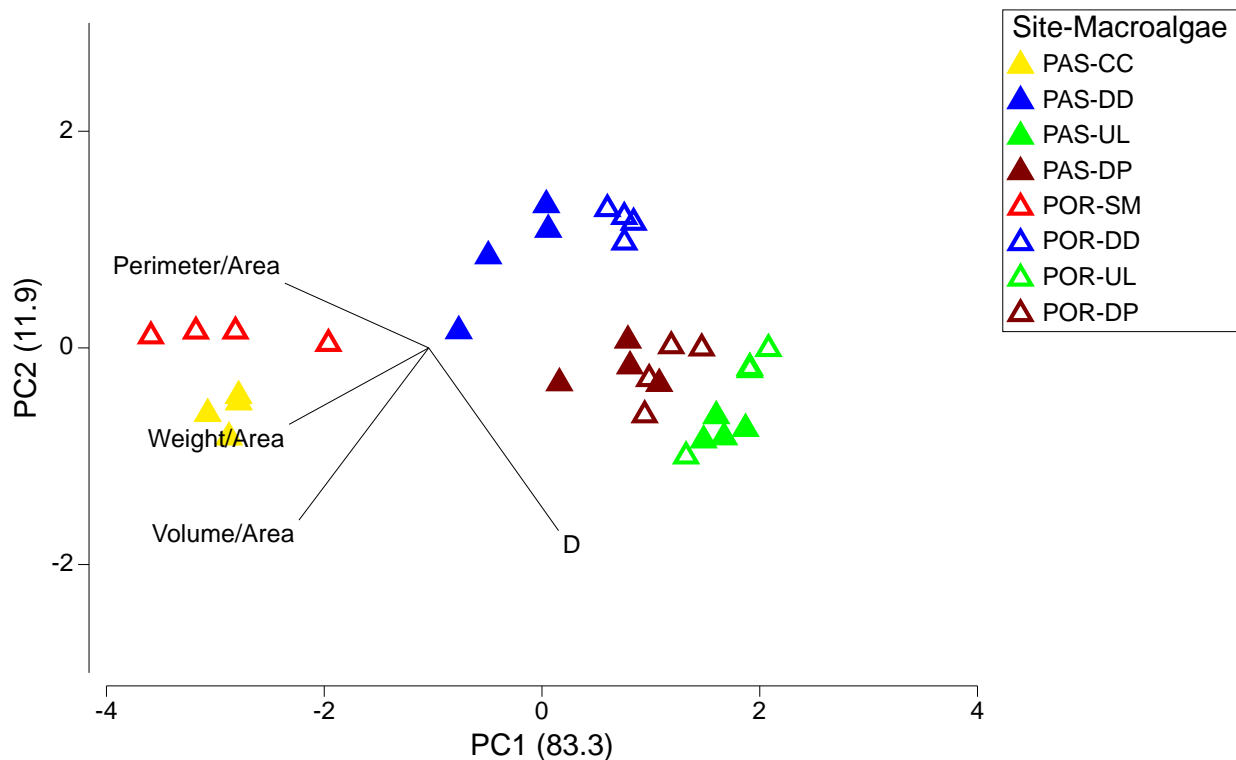


Figure 2. Principal component analysis (PCA) ordination carried out on untransformed macroalgal morphological data. PCA was performed on the normalized measures and measure ratios taken in Passetto (PAS) and Porto (POR) on each macroalga: *Cystoseira compressa* (CC), *Dictyota dichotoma* (DD), *Ulva cf. lacinulata* (UL), and *Dictyopteris polypodioides* (DP) and *Sargassum muticum* (SM).

3.3.2. Unsaturated Aldehydes (PUAs) Produced by Macroalgae

Quantitative and qualitative concentrations of the main aldehydes produced by the macroalgae sampled in Passetto and Porto obtained through GC-MS analysis highlighted significant differences among macroalgae and sites. The total average concentrations of PUAs detected in the macroalgae sampled in the two sites were shown in Figure 3a.

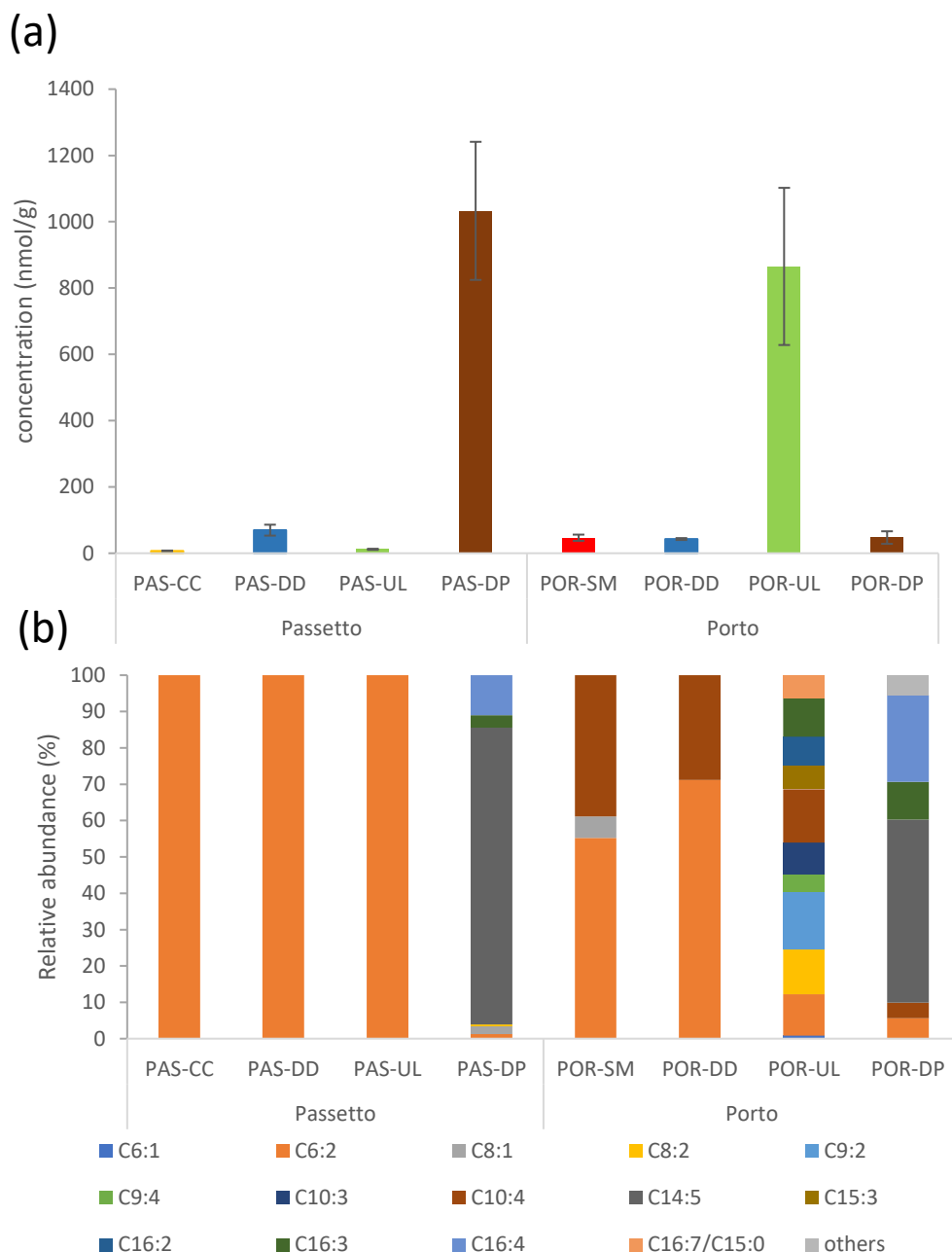


Figure 3. Mean (\pm SE; $n = 4$) of **(a)** total concentration (nmol/g) and **(b)** relative abundance (%) of polyunsaturated aldehydes in the different macroalgae collected in Passetto (PAS) and Porto (POR): *Cystoseira compressa* (CC), *Dictyota dichotoma* (DD), *Ulva cf. lacinulata* (UL), *Dictyopteris polypodioides* (DP) and *Sargassum muticum* (SM).

In the Passetto site, the highest aldehyde production was detected in PAS-DP, with a concentration of 1032.8 nmol/g. In PAS-DD, the total amount resulted as 69.7 nmol/g, while in PAS-UL and PAS-CC, PUAs concentrations were very low (<12 nmol/g). In the Porto site, POR-UL was the major PUAs producer (865.2 nmol/g), while PUAs concentrations in POR-SM, POR-DD, and POR-DP were low and very similar, ranging between 43 and 47 nmol/g. This pattern was supported by the statistical analysis (PERMANOVA, PseudoF = 14.39; $p = 0.0003$; Table S3). In Passetto, a significantly higher concentration was measured in PAS-DP compared to the concentrations measured in the other

algae; to a lesser extent a relatively higher concentration occurred in PAS-DD than in PAS-UL and PAS-CC. In Porto, post hoc results highlighted the significantly higher concentration in POR-UL than in all other macroalgae that did not show significant differences. Pairwise comparisons made between sites for each corresponding macroalga evidenced a significantly higher concentration in POR-UL than in PAS-UL and a higher concentration in PAS-DP than in POR-DP (Table S3).

From a qualitative point of view, PUAs relative abundance measured in each macroalga in the two sites showed high variability (Figure 3b). The small-chained compound hexadienal (C6:2) was the most abundant aldehyde in several macroalgal species (i.e., POR-SM and POR-DD), and reported relative abundances up to 100% in PAS-CC, PAS-DD, and PAS-UL. Conversely, PAS-DP highlighted the production of long-chained aldehydes, such as tetradecapentaenal (14:5) and hexadecatetraenal (16:4) which were the most abundant (about 82 and 11%, respectively). In Porto, decatetraenal (C10:4) was among the main aldehydes in several species, with relative abundances up to 29% and 39% in POR-DD and POR-SM, respectively. As for POR-DP, the PUAs profile was similar to the one detected for PAS-DP, although C14:5 was less abundant (50%), while relative abundances of C16 aldehydes were higher and resulted in 10% and 24% for hexadecatrienal (C16:3) and C16:4, respectively. POR-UL showed a PUAs profile completely different from the one reported for PAS-UL, characterized by a variety of small- (i.e., C6:2, octadienal C8:2), medium- (i.e., nonadienal C9:2, C10:4), and long-chained (i.e., C16:3) compounds, all present at relatively low abundances (about 10–15%).

3.3.3. Microphytobenthic Assemblages

Species belonging to at least 27 microalgal genera were identified, and some diatom species remained unknown (namely undetermined pennate or centric diatoms) (Table S4). Generally, the number of taxa (*S*) varied significantly (PERMANOVA results: PseudoF = 10.50; $p < 0.0001$; Table S5) among site–macroalgae and a higher number of taxa was observed on the macroalgae collected in Passetto (ranging between 14.25 and 17.75) compared to Porto (ranging between 8.5 and 12.25). Post hoc results showed a significantly higher number of taxa on PAS-UL and PAS-DD in comparisons to POR-UL and POR-DD, respectively, while in Porto, a significantly lower number of taxa resulted on POR-DP with respect to POR-SM and POR-DD (Figure 4a; Table S5). Results of PERMANOVA on the total microphytobenthos abundance showed a significant effect of site–macroalgae (PseudoF = 4.613; $p = 0.0026$; Table S5). Post hoc comparisons indicated that the highest abundances for each site, observed on PAS-DD (46,412 cell/cm²) and POR-SM (45,080 cell/cm²), were significantly higher

only compared to PAS-UL and POR-UL, respectively (ST-3.4). Conversely, in PAS-UL and POR-UL, microphytobenthos abundance was lower compared to other samples (i.e., PAS-CC; and POR-DD and POR-DP), reporting values of 11,606 and 2480 cell/cm², respectively. Microphytobenthos abundances on POR-DD and POR-DP were 32,814 and 14,488 cell/cm² and resulted as significantly different (Figure 4b; Table S5).

Globally, the taxonomic composition of the microphytobenthos communities associated to the different macroalgae in both sites was dominated by diatoms (Figure 5), although representatives of several algal classes were reported (Bacillariophyceae, Mediophyceae, Xantophyceae, Dinophyceae). Specifically, in PASS-CC, the relative abundance of Mediophyceae resulted higher than in all other samples due to the abundance of *Leptocylindrus* spp. (46%), while Bacillariophyceae were generally more abundant (about 80–95%) and Dinophyceae below 5% except for POR-UL (21%) in all other samples. *Cocconeis* spp. resulted more abundant in macroalgae collected in Passetto (up to 24% in PAS-DP), while *Navicula* spp. were present on each macroalgal species in the two sites, with the lowest relative abundance in POR-SM (14%) and the highest in PASS-DP (48%). *Nitzschia* spp. and *Pseudo-nitzschia* spp. mainly characterized the microphytobenthic community in POR-SM with relative abundances of 40% and 4%, respectively. Some dinoflagellate species belonging to the genera *Prorocentrum* and *Amphidinium*, but also to some known to have a planktonic behavior (i.e., *Alexandrium* spp.), were reported, although at low abundances. Macroalgae collected in Porto, and in particular POR-DD, were characterized by a high abundance of pennate diatoms whose identification remained undetermined (namely undetermined pennate diatoms). POR-UL microphytobenthic assemblage resulted less characterized by diatoms as, together with dinoflagellates, also small cells (<20 µm) were abundant (49%). These cells were characterized by observable morphological characters ascribable to species belonging to the Cryptophyceae, although further in-depth investigation would be needed to better clarify their taxonomic identity.

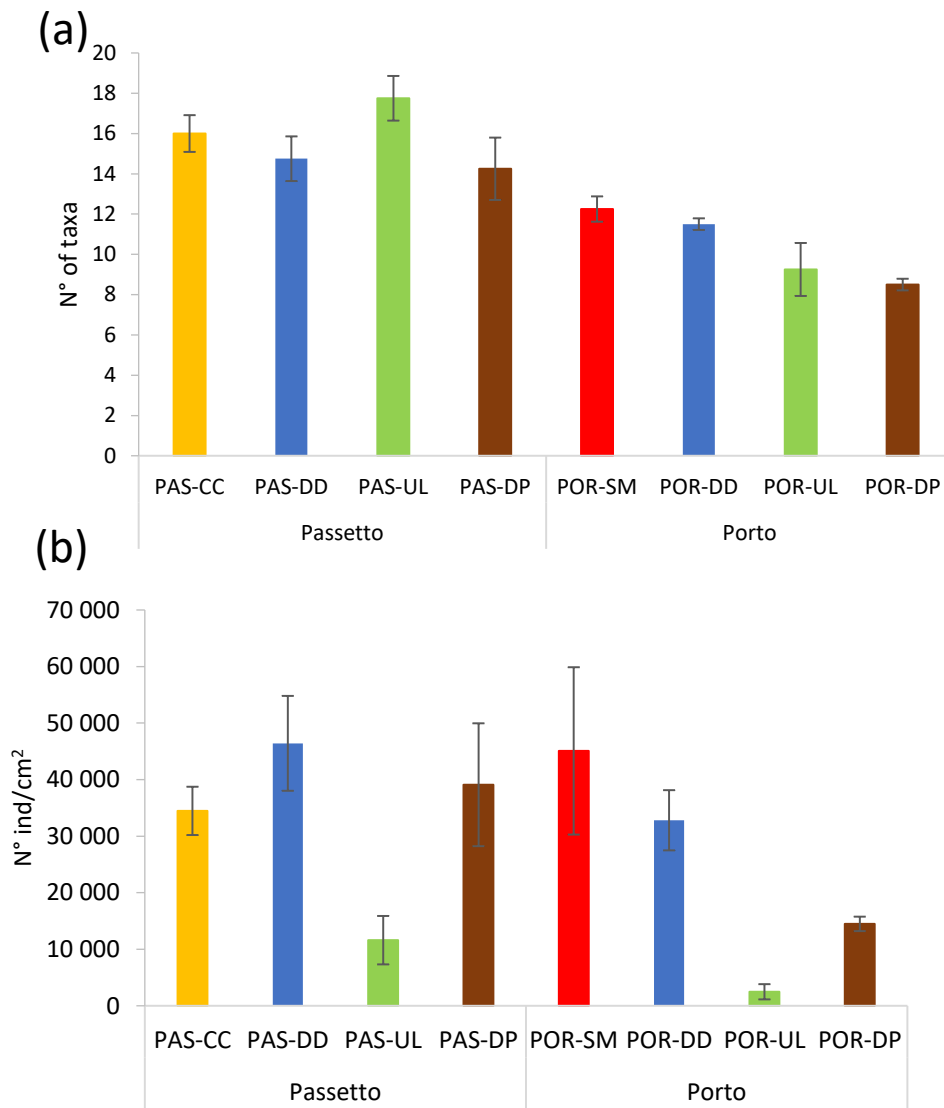


Figure 4. Mean (\pm SE; $n = 4$) of (a) number of taxa and (b) total abundance of the microphytobenthic community measured in Passetto (PAS) and Porto (POR) on each macroalga: *Cystoseira compressa* (CC), *Dictyota dichotoma* (DD), *Ulva cf. lacinulata* (UL), *Dictyopterus polypodioides* (DP), and *Sargassum muticum* (SM).

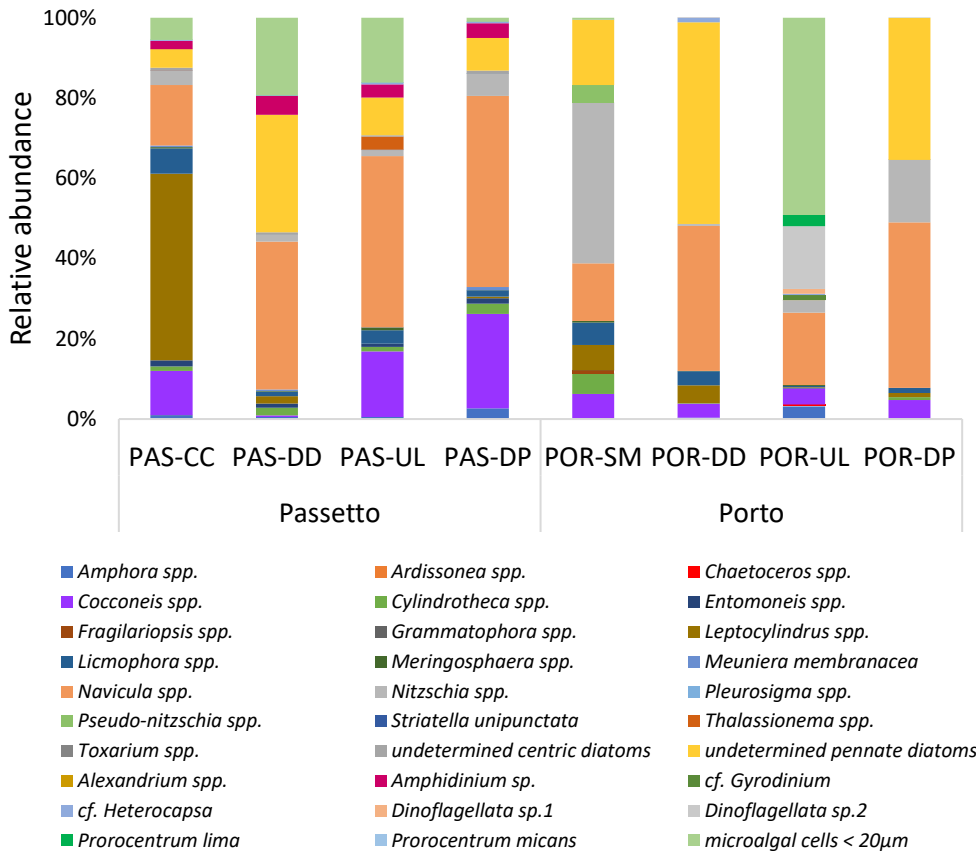


Figure 5. Taxonomic composition of the microphytobenthos community in Passetto (PAS) and Porto (POR) on each macroalga: *Cystoseira compressa* (CC), *Dictyota dichotoma* (DD), *Ulva cf. lacinulata* (UL), *Dictyopteris polypodioides* (DP) and *Sargassum muticum* (SM).

The nMDS analysis of the microphytobenthos communities showed two distinct groups, identified by samples collected at the two different sites, with POR-UL samples slightly separated and showing high dispersion (Figure 6). PERMANOVA carried out on taxonomic structure showed significant differences among site–macroalgae (PseudoF = 8.59, $p = 0.0001$; Table S6). According to the results of the pairwise comparisons, the community structure was significantly different on the same macroalgal species collected in the two sites. Within each site, the differences among macroalgal species were all significant, except for PAS-DP which was not significantly different from any of the other species sampled at Passetto.

SIMPER analysis revealed that average dissimilarities between macroalgae sampled in Passetto ranged from 30.19% between PAS-C and PAS-DD to 40.37% between PAS-UL and PAS-DP. In general, dissimilarities were largely due to the variations in abundance of some diatoms (e.g., species belonging to the genera *Leptocylindrus* and *Cocconeis*, or undetermined pennate diatoms), without a clear pattern. On the other hand, in Porto, average dissimilarities between macroalgae were higher than in Passetto, ranging between 31.50% (POR-DD and POR-DP) and 69.46% (POR-DD and POR-UL), and mainly due to abundance variation of *Navicula spp.*, *Pseudo-nitzschia spp.*, *Nitzschia*

spp., or undetermined pennate diatoms which resulted generally abundant in POR-DD and POR-SM (Table S7).

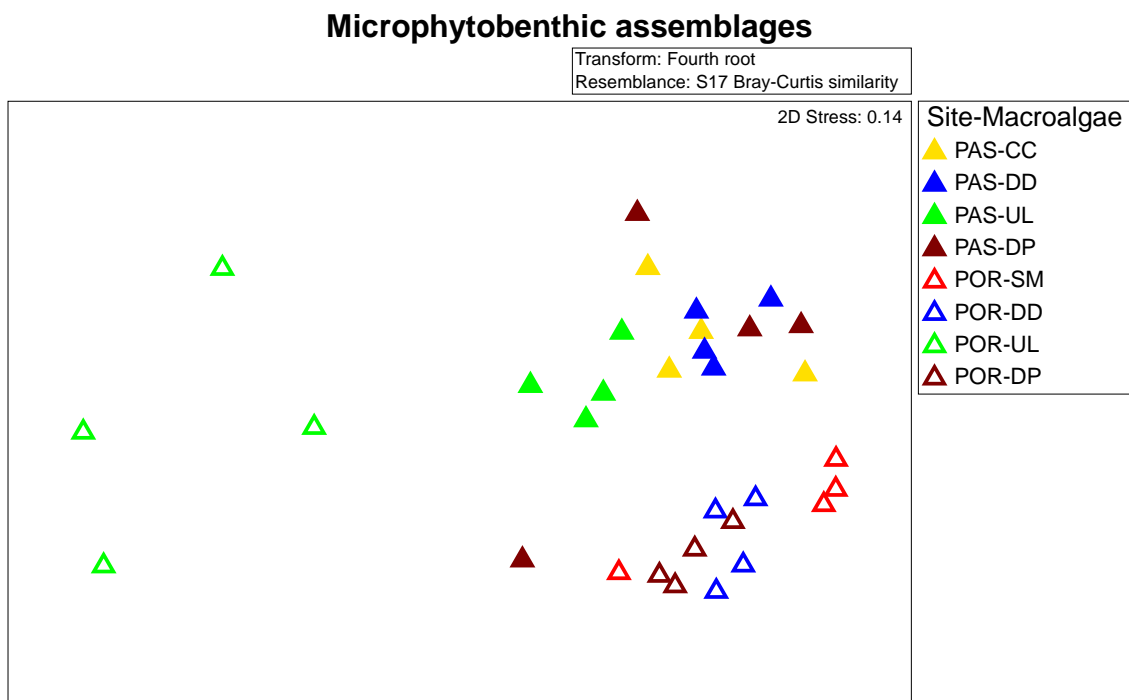


Figure 6. nMDS ordination of a sample of microphytobenthic assemblages based on fourth-root transformed abundance (cell/cm²) and Bray–Curtis similarity in Passetto (PAS) and Porto (POR) associated with each macroalga: *Cystoseira compressa* (CC), *Dictyota dichotoma* (DD), *Ulva cf. lacinulata* (UL), *Dictyopteris polypodioides* (DP), and *Sargassum muticum* (SM).

The comparison among microphytobenthos communities found on the same macroalga in the two sites showed the highest dissimilarity between PAS-UL and POR-UL (58.36%) and was mainly due to the higher abundance of undetermined pennate diatoms, and species belonging to the genera *Navicula*, *Amphidinium*, and *Cocconeis* in PAS-UL (Table S7).

3.3.4. Meiobenthic Assemblages

In total 13 major meiobenthic taxa were identified across samples (Figure 7; Table S8). Copepod *nauplii* have been considered a taxon, separate from later copepod stages [24]. Globally, *nauplii* and harpacticoids dominated in both sites on all macroalgal species. In Passetto on PAS-DP, a relatively higher diversity of taxa was found where amphipods, isopods, and gastropods represent a small percentage of the total. Moreover, in Passetto, all the four macroalgae hosted a greater variability of taxa in comparison with those hosted on macroalgae in Porto, where more than 70% was represented by copepod *nauplii* followed by adult harpacticoids. (Figure 7).

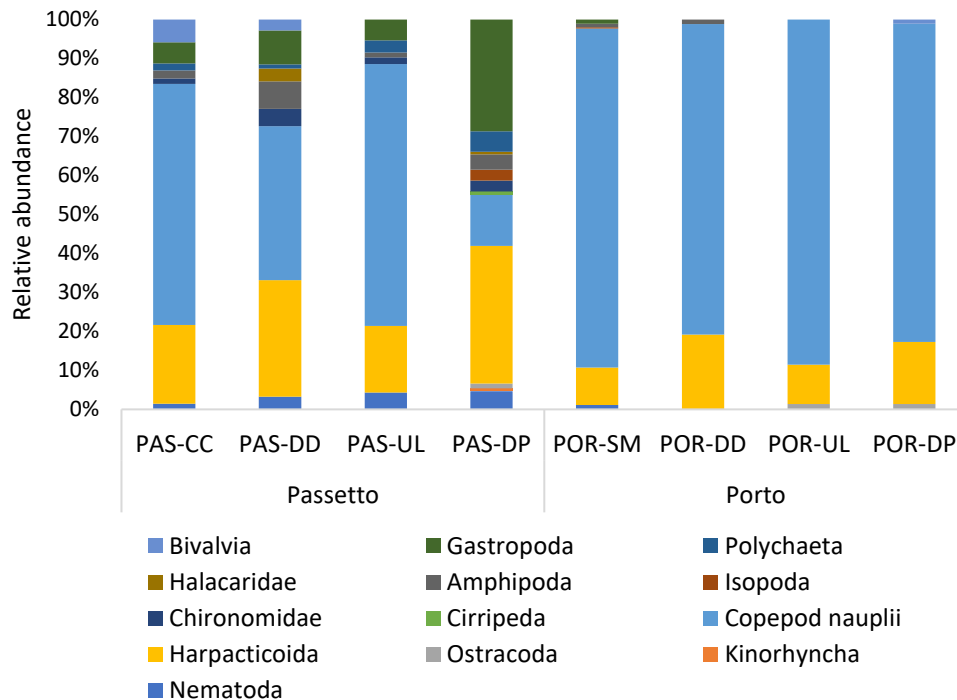


Figure 7. Relative abundance of major meiobenthic taxa in Passetto (PAS) and Porto (POR) on each macroalga: *Cystoseira compressa* (CC), *Dictyota dichotoma* (DD), *Ulva cf. lacinulata* (UL), *Dictyopteris polypodioides* (DP), and *Sargassum muticum* (SM).

The number of major meiobenthic taxa (S) varied significantly (PERMANOVA results: Pseudo-F = 13.23; $p = 0.0001$; Table S9) among site-macroalgae and the highest value was found on PAS-DP (8.5 ± 0.96) and the lowest on POR-UL (2 ± 0.41) (Figure 8a). In Passetto, pairwise comparisons showed a significant highest number of taxa on PAS-DP in comparisons with all the other three macroalgae, while in Porto the number of taxa did not show significant differences (Table S9). The number of taxa on the same macroalga in the two sites resulted always significantly higher in Passetto. Results of PERMANOVA on the total abundance (N) showed a significant effect of site-macroalgae (Pseudo F = 2.15; $p = 0.049$; Table S9) (Figure 8b). Post hoc comparisons indicated that the abundance on PAS-DP resulted higher than PAS-UL, while in Porto a higher abundance occurred on POR-SM in comparison with POR-DD and POR-UL (Table S9). Comparisons between the same macroalga in the two sites showed significantly higher abundance in PAS-DP than POR-DP.

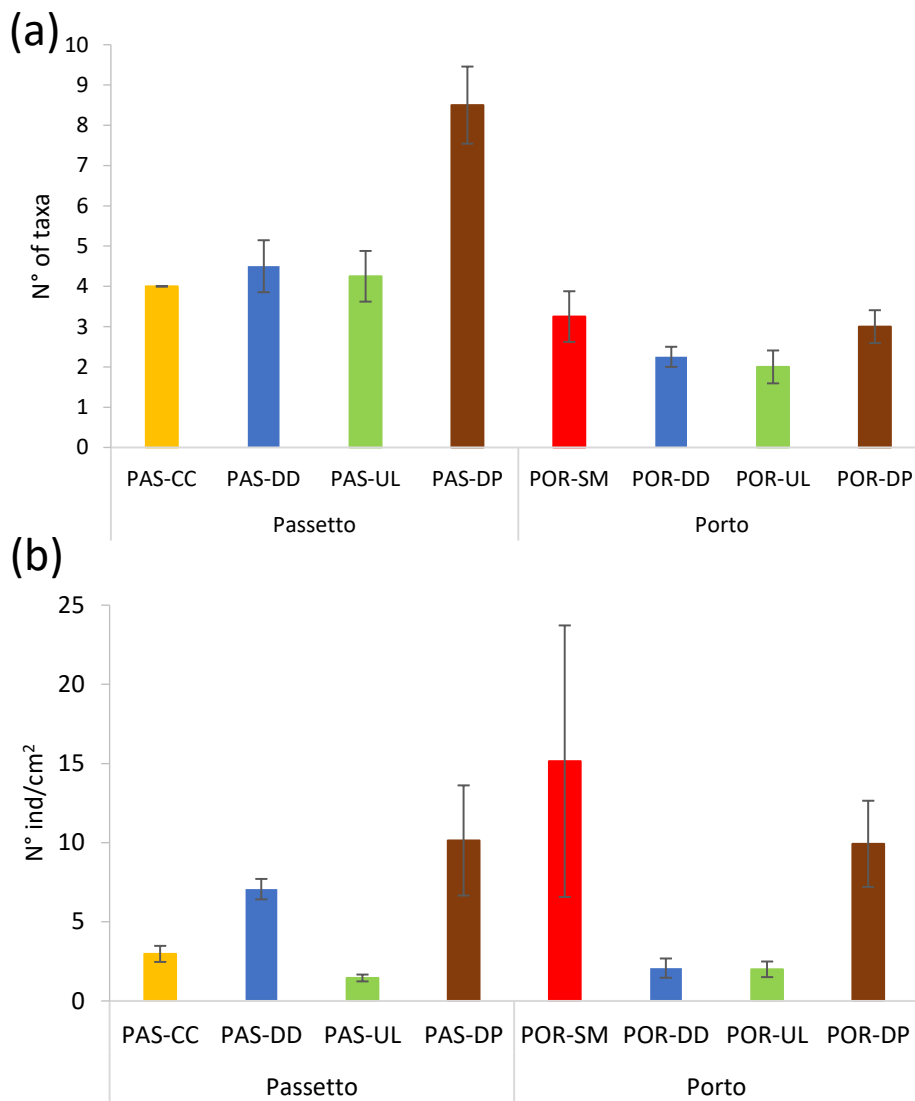


Figure 8. Mean (\pm SE; $n = 4$) of (a) number of taxa and (b) total abundance of the meiobenthic community in Passetto (PAS) and Porto (POR) on each macroalgae: *Cystoseira compressa* (CC), *Dictyota dichotoma* (DD), *Ulva cf. lacunculata* (UL), *Dictyopteris polypodioides* (DP), and *Sargassum muticum* (SM).

The nMDS plot showed the meiofauna assemblages present in the Passetto relatively separated from those in Porto but changes of meiobenthic communities depended on samples belonging to the different macroalgae (Figure 9).

PERMANOVA carried out on taxonomic structure (PseudoF = 4.58, $p = 0.0001$; Table S10) showed significant differences among site–macroalgae. In Passetto, the significantly different assemblages resulted between PAS-CC vs PAS-DP, PAS-DD vs. PAS-UL and PAS-UL vs. PAS-DP, while in Porto significantly different assemblages occurred between POR-SM vs. POR-UL, POR-DD vs. POR-DP and POR-UL vs. POR-DP. Moreover, the community structure was significantly different on corresponding macroalgae in the two sites. (Table S10).

SIMPER analysis revealed that average dissimilarities between macroalgae in Passetto ranged from 60.80% between PAS-UL and PAS-DP to 37.61% between PAS-CC and PAS-UL. In general, dissimilarities between macroalgae were largely due to the variations in abundance of almost all identified taxa, without a clear pattern. On the other hand, in Porto, average dissimilarities between macroalgae were lower, ranging from 48.98% between POR-SM and POR-UL, to 23.59% between POR-DD and POR-UL and were mainly due to abundance variation of Copepod *nauplii* and harpacticoids that, in combination contributed to about 62–84% of total dissimilarities (Table S11).

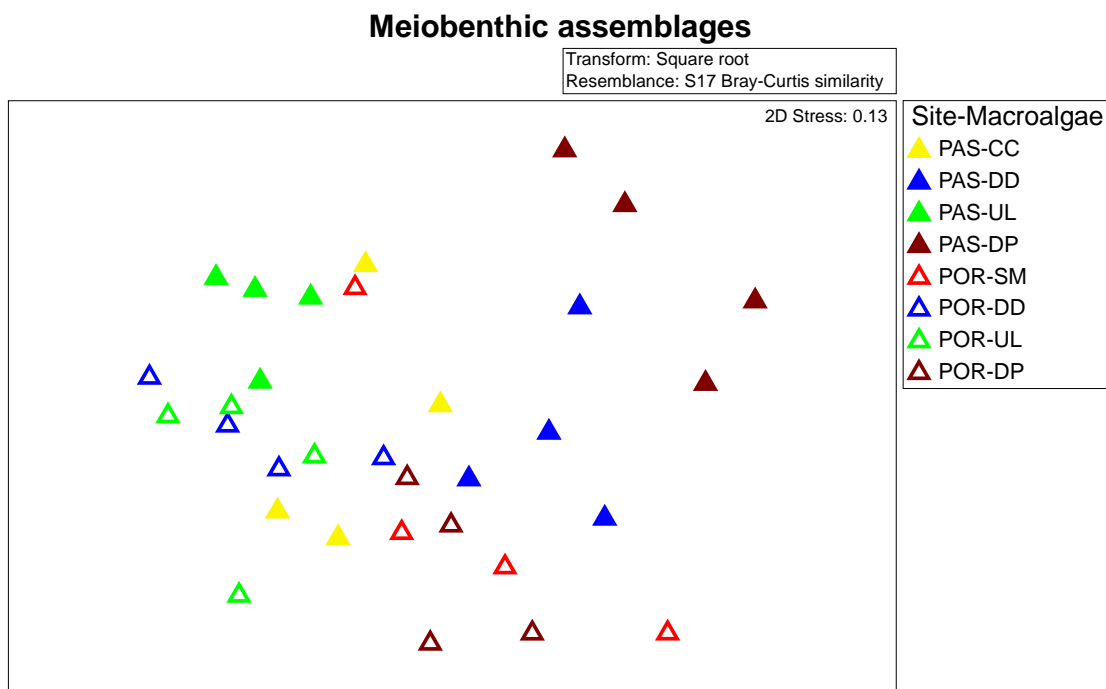


Figure 9. nMDS ordination of sample of meiobenthic assemblages based on square-root transformed abundance (N/cm²) and Bray–Curtis similarity in Passetto (PAS) and Porto (POR) associated with each macroalga: *Cystoseira compressa* (CC), *Dictyota dichotoma* (DD), *Ulva cf. lacinulata* (UL), *Dictyopterus polypodioides* (DP), and *Sargassum muticum* (SM).

Comparing meiobenthic communities on the same macroalga in the two sites, the highest dissimilarity was between PAS-DP and POR-DP (60.34%) and was due to the higher abundance of copepod *nauplii* on POR-DP and the absence of the other taxa present only on PAS-DP (Table S11). Furthermore, considering the possible effect of PUA production on copepods, the number of dead harpacticoids (copepodites and adults), dead copepod *nauplii*, and expelled egg sacs were also counted (Table 2). The highest number of dead *nauplii* and adults were found on DP in Passetto. In general, dead copepod *nauplii* were more present on all macroalgae sampled in Porto, especially on POR-SM, while dead adult harpacticoids occurred on all macroalgae in both sites. A different result was obtained for expelled egg sacs that were high on PAS-CC (8.25 egg sac/cm²) and PAS-DD (16.02 egg sacs/cm²), and to a lesser extent on POR-SM (4.03 egg sacs/cm²).

Table 2. Mean value (\pm SE; n = 4) of the abundance (Ind/cm²) of dead copepod *nauplii*, dead harpacticoids and expelled egg sacs in Passetto (PAS) and Porto (POR) on each macroalga: *Cystoseira compressa* (CC), *Dictyota dichotoma* (DD), *Ulva cf. lacinulata* (UL), *Dictyopteris polypodioides* (DP), and *Sargassum muticum* (SM).

	Dead Copepod <i>nauplii</i>	Dead Harpacticoids	Expelled Egg Sacs
PAS-CC	- \pm -	0.06 \pm 0.06	8.25 \pm 0.06
PAS-DD	- \pm -	0.10 \pm 0.06	16.02 \pm 0.06
PAS-UL	- \pm -	0.05 \pm 0.02	1.11 \pm 0.02
PAS-DP	0.31 \pm 0.17	0.37 \pm 0.12	2.84 \pm 0.12
POR-SM	0.11 \pm 0.11	0.05 \pm 0.03	4.03 \pm 0.03
POR-DD	0.03 \pm 0.03	0.03 \pm 0.02	0.08 \pm 0.02
POR-UL	0.03 \pm 0.02	0.01 \pm 0.01	0.11 \pm 0.01
POR-DP	0.08 \pm 0.04	0.09 \pm 0.03	0.91 \pm 0.03

3.3.5. Relationship between the Microphytobenthos and Meiofauna Assemblages with Macroalgae Complexity and PUAs Production

To evaluate the relationships between the community structure of both microphytobenthos and meiofauna with macroalgae complexity and PUAs production, D, weight/area, perimeter/area, and volume/area and PUAs tot (nmol/g) were entered in DistLM analysis.

The results of DistLM carried out to analyze the relationship between PUAs and macroalgal variables and the microphytobenthos were shown in Table 3a. The marginal test showed that all the analyzed variables had a significant relationship with the assemblages when considered alone and the fractal D explained nearly 15% of the variability in the data. The best selection included three variables: D, perimeter/area and PUAs tot (nmol/g) that explained 30% of variance in microphytobenthos structure.

The DistLM carried out to analyze the relationship between PUAs and macroalgal variables and the meiofauna assemblages, showed that none of the variables were significant predictors and the best selection of analyzed variables included only D (Table 3b), but explained only 5% of variance in the meiobenthic community structure. The analysis was repeated adding the microphytobenthos abundance to the other variables considered in the previous analysis. Results (Table 3c) showed that the only significant predictor resulted total abundance of microphytobenthos, which however explained only the 11% of variance in meiobenthic assemblage structure (Table 3c). The best selection of analyzed variables included PUAs tot and total abundance of microphytobenthos that,

however, explained only 18% of variance in meiobenthic community structure. Finally, the DistLM was performed between PUAs and the macroalgal variables and the meiofauna, including also the number of dead copepod *nauplii*, dead harpacticoids, and expelled egg sacs. In this analysis, PUAs concentration resulted in the only significant predictor which however explained only 7% of variance in meiobenthic assemblage structure and the best solution explaining overall variation of assemblages included only D and PUAs that all together explained a very low variance (16%) in meiofauna (Table 3d).

Table 3. Results from the DistLM analysis of the influence of measure of macroalgae complexity and PUAs concentration on (a) microphytobenthic assemblage structure; (b) meiobenthic assemblage structure; (c) meiobenthic assemblage structure considering also total abundance of microphytobenthos (abund-microphyto); and (d) meiobenthic assemblage structure considering also dead harpacticoids, dead copepod nauplii end expelled egg sacs. Statistically significant p-values are in bold.

(a)	Variable	Pseudo-F	P	Prop
Marginal test	D	5.441	0.0001	0.154
	Weight/Area	3.850	0.0033	0.114
	Perimeter/Area	4.288	0.0011	0.125
	Volume/Area	3.184	0.0072	0.096
	PUAs (nmol/g)	3.745	0.0061	0.111
	Model	AICc	R ²	RSS
Best solution		222.0300	0.30	24533
D; Perimeter/Area; PUAs				
(b)	Variable	Pseudo-F	P	Prop
Marginal test	D	1.598	0.1684	0.051
	Weight/Area	0.640	0.6566	0.021
	Perimeter/Area	0.942	0.4193	0.030
	Volume/Area	0.706	0.6045	0.023
	PUAs (nmol/g)	1.438	0.2063	0.046
	Model	AICc	R ²	RSS
Best solution		227.72	0.05	34342
D				
(c)	Variable	Pseudo-F	P	Prop
Marginal test	D	1.598	0.165	0.051
	Weight/Area	0.640	0.648	0.021
	Perimeter/Area	0.942	0.427	0.030
	Volume/Area	0.706	0.593	0.023
	PUAs (nmol/g)	1.438	0.204	0.046
	Abund Microphyto	3.610	0.010	0.107
	Model	AICc	R ²	RSS
Best solution		225.60	0.18	29777
PUAs; Abund Microphyto				
(d)	Variable	Pseudo-F	P	Prop
Marginal test	D	2.153	0.0561	0.067
	Weight/Area	1.764	0.1056	0.056
	Perimeter/Area	1.542	0.1617	0.049
	Volume/Area	1.172	0.3007	0.038
	PUAs (nmol/g)	2.226	0.0485	0.069
	Model	AICc	R ²	RSS
Best solution		233.70	0.16	38351
D; PUAs				

The marginal test reports Pseudo-F and *p* value and explained the variation for each variable, and the best solution reports the selection of variables that generate the highest explained variation at the lower AICc value.

The dbRDA ordination of microphytobenthic assemblages with superimposed explanatory variables (Figure 10a) showed that the first axis detected a variation in community structure among algae with different D and PUAs production, while the second axis highlighted the variability among samples between sites and is mainly driven by perimeter/area ratio (Figure 10a). The dbRDA ordination of meiobenthic assemblages, with also dead harpacticoids, dead nauplii, and expelled egg sacs with superimposed explanatory variables showed that D and PUAs were the variables that influence the communities, but in the opposite way (Figure 10b).

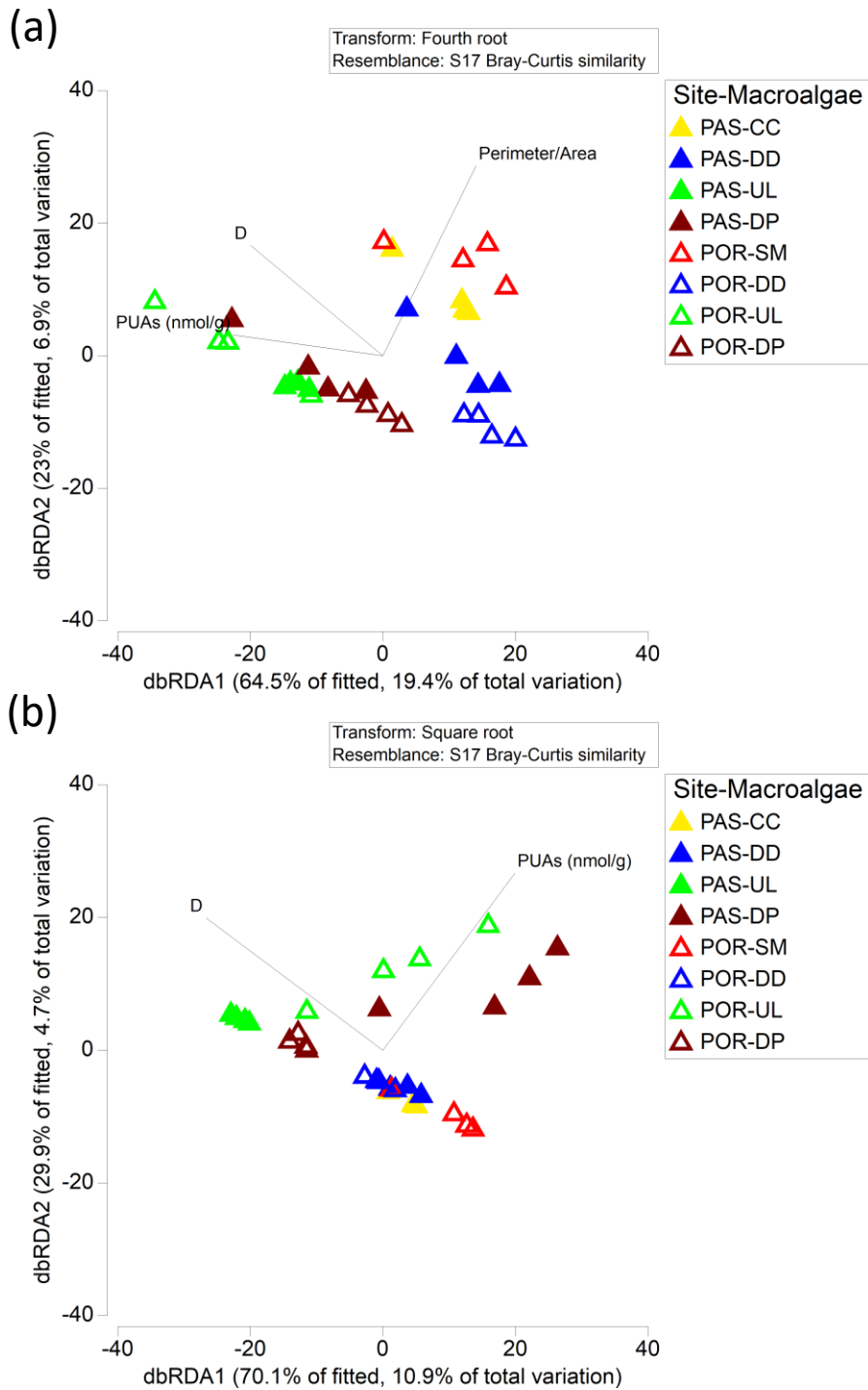


Figure 10. Distance-based redundancy analysis (dbRDA) of (a) microphytobenthos samples and (b) meiofauna samples including dead copepod nauplii, dead harpacticoids, and expelled egg sacs in Passetto (PAS) and Porto (POR) on each macroalgae: *Cystoseira compressa* (CC), *Dictyota dichotoma* (DD), *Ulva cf. lacunculata* (UL), *Dictyopteris polypodioides* (DP), and *Sargassum muticum* (SM). Normalized predictor variables (based on distLM analysis in Table 3) are overlaid.

The different response of microphytobenthic and meiobenthic assemblages to macroalgae and PUA production in the two sites was confirmed by the RELATE result that showed a not significant correlation between the two multivariate matrices ($Rho = 0.13$; $p = 0.08$).

3.4. Discussion

Differences in the morphological complexity of five macroalgal species, including the introduced species *Sargassum muticum*, were explored in this study. Different variables estimating the structural complexity of macrophytes have been considered in different studies [50]. Most studies have used variables related to the amount of habitat provided (i.e., biomass, surface area, or volume), rather than true complexity measures. On the other hand, variables such as perimeter and fractal dimensions (D) are more suitable to provide a proxy of the habitat architecture [35]. In this study we decided to use volume/area ratio (for which high values indicate a more three-dimensional morphology), weight/area ratio (which provides a measure of available habitat standardized per surficial area), and perimeter/area ratio (to compare thalli that do not present a regular geometrical shape).

Multivariate PCA results allowed to discern the main variables that described the complexity of the macroalgae in the two sites and did not seem to be redundant. Moreover, all the measures collected for the same macroalga were consistently comparable in the two sites, suggesting that at the time of sampling each species exhibits consistent morphological characteristics. The macroalgae were sampled within one week at the same time of the year, in order to guarantee that comparisons between sites and species would not be affected by seasonal variation, which strongly influences the morphological structure of the species considered [51,52].

High values of all ratios (weight, perimeter, or volume to area) were calculated for *S. muticum* and *C. compressa*, indicating that these species were more complex compared to the others, while D values resulted as high in *U. cf. lacunculata* and *D. polypodioides* in both sites. The low D values measured for *S. muticum* and *C. compressa* seem to contradict the notion that fractal of macroalgae increases with the complexity [56]. Although efforts were made to obtain two-dimensional samples by breaking up the thalli, recent studies remarked the limitation in using a two-dimensional photographs to estimate the true complexity found in naturally suspended plants and recognized that the three-dimensional architecture typical of a plant is not completely represented by a two-dimensional image [54].

To understand if the production of bioactive compounds could influence the microphyto and meiobenthic communities associated with the different macroalgae, we focused on a class of compounds well known for having allelopathic effects, i.e., polyunsaturated aldehydes (PUAs). The production of a variety of biomolecules by algae is widely recognized [55]. In particular, macroalgae are among the main producers of polyunsaturated fatty acids (PUFAs), such as linoleic acid, which

are not only essential components of membranes but can be involved in the regulation of physiological processes, serving also as precursors in the biosynthesis of structurally diverse oxylipins, including PUAs [56]. Recent studies [24,31] highlighted a great difference in the profile of aldehydes produced by different macroalgae, including species belonging to the same family, especially in relation to the length of their carbon chains and number of unsaturations. In particular, some species such as *D. polypodioides* and *U. cf. lacinulata*, produce higher amounts of PUAs compared to other macroalgae (e.g., *C. compressa*, *Padina pavonica*, *Gracilaria* sp.) [31]. The short-chain PUA C6:2 was the main compound in several species (e.g., *Ceramium ciliatum*, *Padina pavonica*, *Cystoseira compressa*), while medium-chain PUAs (i.e., C9:4 and C10:4) were dominant in *Ulva cf. rigida* (now referred to as *U. cf. lacinulata*) [31]. For long-chain aldehydes (i.e., C20:5 C14:5, C16:3 and C16:4.), a higher production is known for *Dictyota dichotoma* and *Dictyopteris polypodioides* [24,31]. These results were confirmed in the present study, where the same algal species previously analyzed [31] yielded a similar PUAs profile, regardless of the site. In fact, the macroalgae that showed the highest production of long and medium-chain PUAs were *D. polypodioides*, which produced C14:5 and C16:4, and *D. dichotoma*, which produced C10:4, together with *S. muticum*. The short-chain PUA C6:2 was produced by several species such as *C. compressa*, *U. cf. lacinulata*, *D. dichotoma*, and *S. muticum*.

From a quantitative point of view, *D. polypodioides* showed the highest concentration of PUA (1032.8 nmol/g), especially in the Passetto site, similarly to the results of a previous study [24], where concentrations of 225.5 µg/g ww (corresponding to 2127.3 nmol/g) were reported. These results confirmed that PUA profiles can be used as a fingerprint of each algal species, as the same compounds were consistently detected regardless of the sampling site. Conversely, their relative and total amount may vary depending on environmental conditions or morphotypes [31,33].

Differences between specimens from different sites were detected for *Ulva cf. lacinulata*, in which high qualitatively and quantitatively different profile PUAs were observed in samples collected in Porto and in Passetto. Specifically, *U. cf. lacinulata* in the Passetto produced only the short-chain compound C6:2 at a low concentration (11.8 nmol/g), while in the Porto it produced several compounds at high concentrations, including short-chain aldehydes, such as C6:2 (100.6 nmol/g), medium chain PUAs such as C8:2, C9:2, C10:4 (110.9 nmol/g, 137.5 nmol/g, and 128.8 nmol/g, respectively), and long chain ones such as C16:3 (94.0 nmol/g). Middle and long-chain PUAs had already been described for *Ulva* spp., specifically C7:2, C10:2, and C10:3 [33], as well as C16:3, C17:2, and C17:3 in *U. pertusa* [57,58]. The fact that PUA profiles represent a fingerprint for each species

leads to the hypothesis that the *Ulva* samples collected in the two sites may belong to different species. Species of *Ulva* (Ulvophyceae, Chlorophyta) are among the most common algae in intertidal environments, and their morphological identification at the species level is traditionally difficult due to the well-known cryptic diversity and morphological plasticity typical of this genus, which have caused major taxonomic and nomenclatural confusion [59]. Several cryptic species within *Ulva* have been detected using genetic methods, such as DNA barcoding [60,61]. Unfortunately, for the purposes of this study it was not possible to obtain DNA sequence data from the *Ulva* samples analyzed; thus, future studies should investigate the taxonomical diversity of *Ulva* spp. in this area. To our knowledge, PUAs production, specifically of short- (i.e., C6:2 and C8:1) and medium-chain (i.e., C10:4) compounds by *S. muticum* is here reported for the first time (46.8 nmol/g), attesting the potential allelopathic effect of this species, which could contribute to its strong invasive potential. The analysis of the microphytobenthic community revealed that diatoms were the dominant group, as typically reported, and as attested also in previous studies concerning the micro-epiphytic communities in this Adriatic area [24,29,62]. In these studies, Scanning Electron Microscopy (SEM) observations documented the great biodiversity of this community, while in the present study only light microscopy was used and only identifications at genera or even higher taxonomic levels could be obtained.

Microphytobenthic diatom populations are usually composed of adnate forms (strongly adhering horizontally to the substrate by means of the raphidic valve), which represent the first encrusting and more stable component in the diatom assemblage, or erect growth forms (adhering vertically to the substrate by means of mucous pillows or stalks/peduncles), which are less stable and colonize the substrate after the adnates, as well as motile forms (having high movement capability) [63]. Generally, the motile forms (e.g., *Nitzschia* and *Navicula* spp.) spread more effectively above the substrate than other forms but are less stable and can be easily removed by water movements. In this study, motile forms represented the main fraction in terms of cell abundance, probably due to the ability of these biraphid taxa to move within a mature substrate, which could make them superior competitors for nutrients and light [62]. Conversely, erect (e.g., *Grammatophora* and *Licmophora* spp.) or adnate (e.g., *Amphora* and *Cocconeis* spp.) forms reported low abundances (lower than 20%).

Notably, the microphytobenthic communities differed on thalli of the same macroalgae sampled in the two sites. In general, variations in the abundance of some diatoms (e.g., species belonging to the genera *Leptocylindrus* and *Cocconeis*, and unidentified pennate diatoms) were found at the

Passetto. Conversely, in Porto variations were due to the abundance of motile forms (i.e., *Navicula* spp., *Pseudo-nitzschia* spp., and *Nitzschia* spp.), which are able to move in response to a multitude of factors (e.g., light, hydrodynamics, tides, nutrient) and perhaps as a defence strategy against grazing or other stressors [64,65]. Similarly, a previous study [66] performed in the Adriatic Sea reported that the dominant taxa on the fronds of the invasive green alga *Caulerpa taxifolia* were characterized by high motility (e.g., *Navicula* and *Nitzschia* spp.), capable of moving on the substrate to find optimal conditions.

The results of this study highlighted differences in the taxonomic composition and abundance of epiphytic diatoms among the analyzed macroalgae and between the two sampling sites, as shown by MDS results where microphytobenthic communities clustered in two distinct groups. Differences among macroalgae could be explained, at least in part, by the different thallus architecture of the studied species, but also by other uninvestigated environmental factors reflecting site-specific conditions, which could be reflected in the structure and species composition of the algal communities. The population of the invasive alga *Sargassum muticum* in Porto may affect the community living under its canopy, especially considering its floating ability and long-branched habitat, which limits light penetration, together with other potential consequences caused by its presence (e.g., interactions with native species, competition for nutrients). In fact, benthic diatoms have a highly adaptive photosynthetic pigment apparatus, and can adapt well to low-light regimes [67]. In addition, different microalgal taxa have different light requirements and consequently light conditions can regulate colonization patterns of microalgal assemblages [68]. It should also be considered that the Porto site is within the area of a commercial harbor, and therefore represents a more stressful environment than the rocky coastal inlet of the Passetto site.

Concerning the meiobenthic community, harpacticoid copepods (including their *nauplii*) were the most abundant taxon, comprising 82.4% of the total meiofauna, followed by Gastropoda (7.8%) and juveniles of Amphipoda (2.2%). Nematodes accounted only for 1.9%, which only partially agrees with information reported in the literature [53]. Copepods have been reported as dominant in epiphytic environments with abundances ranging from 30 to 60% of the total meiofauna, followed by nematodes [69]. The ecological structure of the meiofauna community, in terms of relative abundance of major taxa, differed among macroalgae in the two sites. In Passetto, more diverse communities were found on all the four macroalgae, in particular on *D. polypodioides* where the highest number of taxa was recorded and resulted more evenly distributed. On the contrary, in Porto the communities were mainly represented by copepod *nauplii* followed by harpacticoids. The

average abundances of total meiofauna, however, did not show a clear pattern, with a general higher abundance on *D. dichotoma* and *D. polypoidioides* in Passetto and on *S. muticum* and *D. polypoidioides* in Porto. The community structure analyzed at major taxonomic levels resulted as significantly different among macroalgae and between sites on the same macroalga, except for communities associated to *Ulva* cf. *lacunculata*, which were not significantly different. Multivariate pairwise comparisons detected more significant differences among macroalgal species in the two sites than univariate post hoc. These different results between univariate and multivariate analyses suggested that differences among meiobenthic assemblages settled on macroalgae were mainly due to differences in the identity and relative abundance of meiofaunal taxa rather than to the total number of taxa and abundance [70]. These results agree with those reported by Richardson and Stephens [71], where *S. muticum* supported a lower diversity of meiofauna compared to native species, but disagree with Veiga et al. [14], who found that *S. muticum* apparently harbored more meiofaunal taxa than native macroalgae.

These contrasting results could be ascribed to differences in distribution of macroalgae between the two investigated sites. As also observed for microphytobenthic assemblages, in Passetto a more diversified macroalgal community was present, while in Porto *S. muticum* tended to dominate and grow until floating on the water surface, producing more sheltered and shaded conditions underneath. Therefore, we may speculate that different assemblages of macroalgal species may influence meiofauna communities [72]. In Passetto, a more diverse macroalgal community seems to offer a more stable habitat to meiofauna. Instead, in Porto a less diverse macroalgal community dominated by *S. muticum* may explain the very high dominance of copepod *nauplii* and harpacticoids, that are well known for their high mobility [73].

Macroalgae, in addition to being key primary producers, provide the substrate for many organisms ranging from microbes to fish. Therefore, the community structure of a particular epibenthic community depends on the relationship of the different communities associated with different macroalgae. Many invertebrates use macroalgae as a refuge from physical stress, protection from predators, and many of them are herbivores that consume epiphytic algae or the host macrophyte itself [74]. Chemical defenses also play an important role in structuring associated epiphytic communities, as allelopathic compounds can reduce the settlement rate and development of sessile organisms. Furthermore, successful colonists must have a wide tolerance range and establish themselves during phases in which the composition and concentration of biomolecules are not harmful. Recently the importance of including the potential role of macroalgae, both in terms of

structural complexity and production of allelochemical compounds, in the study of the interactions between different epibenthic associated organisms has been stressed [24].

Responses of microphytobenthic assemblages to PUAs production and macroalgal complexity, measured by fractal values (D), weight/area, volume/area, and perimeter/area ratios, showed that all the considered variables resulted significantly correlated with the differences in the microphytobenthos community structure. Thus, differences in microphytobenthos could be mainly due to variations in the morphological characteristics of the macroalgae but also to PUAs production by the different species.

On the contrary, responses of meiobenthic assemblages to macroalgal complexity measures and PUAs production showed that none of the considered variables were significant predictors of differences among the communities settled on macroalgae in the two sites. Many studies found that complexity did not correlate with invertebrate taxon richness, influencing only epifaunal abundance [75–77] or showing that complexity is not a consistent predictor of either the abundance or the diversity of the epifauna [78]. For example, Russo [70] and similarly Schreider et al. [79] showed that the complexity of algae did not explain amphipod abundance between macroalgal species with different structural complexity. Our results suggest that differences in the meiobenthic community structure may be mainly due to heterogeneity of macroalgal communities in terms of different species present in a site, while size and morphology of macroalgae have an unclear effect. The less structured meiobenthic community associated with the invasive species *S. muticum* seems to support this hypothesis.

Epiphytic communities could play a role in structuring meiobenthic associates assemblages [24]. In fact, when the DistLM analysis was performed, adding the total abundance of microphytobenthos, this variable resulted as a significant predictor of meiobenthic assemblage structure. This result agrees with Bologna and Heck [80] who showed a more important trophic role of epiphytes in structuring associated epibenthic assemblages than that of structural complexity, and with Lenzo et al. [24] who highlighted the role of microphytobenthos as an important driver in differentiating the meiofauna community among macroalgae. Indeed epiphytes could influence meiobenthic taxa by supplying food resource and adding complexity to the habitat [81]. The lack of correlation between PUAs and meiobenthic communities could be ascribed to the high taxonomic resolution used that might have failed to highlight the role of PUAs production on meiofauna, whose effects are known to be species-specific [24]. When the responses of meiofauna to PUAs and macroalgal complexity variables were analyzed including the number of dead copepods *nauplii*, dead harpacticoids, and

the expelled egg sacs, whose release was reported as a way to reduce the load of toxic compounds [82], PUAs concentration became a significant predictor, suggesting that these compounds have some roles in structuring meiobenthic communities.

3.5. Conclusions

This study showed how two different macroalgal-associated communities (i.e., microphyto and meiobenthic assemblages) respond to the habitat provided by thalli of different species. Results showed that epibenthic communities respond differently to the variability of the macroalgal species and that, to better understand the global role of macroalgae as habitat formers on coastal ecosystems, it is necessary to analyze the interactions between organisms belonging to different trophic groups (e.g., microphytobenthos and meiofauna). The dominance of the large-sized invasive macroalga *Sargassum muticum* in Porto seemed to have a strong effect on the associated microphytobenthos, mainly characterized by motile diatoms (i.e., *Navicula* spp., *Pseudo-nitzschia* spp., and *Nitzschia* spp.) and meiofauna which was composed mainly by copepod *nauplii* and harpacticoids. The effects of invasive species on associated assemblages are difficult to predict and study as they depend on many factors, such as the identity of species and the macroalgal community structure, in terms of number and type of algae. Moreover, the interactions among complexity of macroalgae, the produced allelopathic compounds and the different epibenthic communities inhabiting them resulted very complex. The results of the present study highlight that when assessing the impact of the introduction of a non-native macroalga on the associated communities of a particular habitat and, more generally, on the environment, several aspects must be considered, including the allelochemicals potential of the macroalgae, which so far has been poorly considered. Further studies aimed at investigating the effects due to the invasiveness of macroalgal species should also consider the seasonal variability and should disentangle the effects of macroalgal complexity and allelochemical production on epibenthic communities performing field experiments using artificial macroalgae, which mimic substrates with different complexity, without being able to produce allelochemicals (e.g., PUAs).

Supplementary Materials: The following supporting information can be downloaded at: www.mdpi.com/xxx/s1, Figure S1: Macroalgae sampled in Passetto (PAS) and Porto (POR): *Cystoseira compressa* (CC), *Dictyota dichotoma* (DD), *Ulva* cf. *lacinulata* (UL), *Dictyopteris polypodioides* (DP) and *Sargassum muticum* (SM); Table S1: Results of PCA carried out on the variables measured for macroalgal morphology; Table S2: Results of PERMANOVA and pairwise comparison test carried out on morphological measure taken in Passetto (PAS) and Porto (POR) for each macroalga: *Cystoseira compressa* (CC), *Dictyota dichotoma* (DD), *Ulva* cf. *lacinulata* (UL), *Dictyopteris polypodioides* (DP) and *Sargassum muticum* (SM); Table S3: Results of one-way PERMANOVA and pairwise comparisons carried out on the total concentration of PUAs taken in Passetto (PAS) and Porto (POR) for each macroalga: *Cystoseira compressa* (CC), *Dictyota dichotoma* (DD), *Ulva* cf. *lacinulata* (UL), *Dictyopteris polypodioides* (DP) and *Sargassum muticum* (SM); Table S4: Microphytobenthos assemblages (cells/cm²) associated to the macroalgae collected in Passetto (PAS) and Porto (POR): *Cystoseira compressa* (CC), *Dictyota dichotoma* (DD), *Ulva* cf. *lacinulata* (UL), *Dictyopteris polypodioides* (DP) and *Sargassum muticum* (SM); Table S5: Results of one-way PERMANOVA and pairwise comparisons carried out on the number of taxa (S) and on total density (N) of microphytobenthos taken in Passetto (PAS) and Porto (POR) for each macroalga: *Cystoseira compressa* (CC), *Dictyota dichotoma* (DD), *Ulva* cf. *lacinulata* (UL), *Dictyopteris polypodioides* (DP) and *Sargassum muticum* (SM); Table S6: Results of PERMANOVA and pairwise comparison test carried out on microphytobenthos community, taken in Passetto (PAS) and Porto (POR) for each macroalga: *Cystoseira compressa* (CC), *Dictyota dichotoma* (DD), *Ulva* cf. *lacinulata* (UL), *Dictyopteris polypodioides* (DP) and *Sargassum muticum* (SM); Table S7: Results of SIMPER analysis based on four root transformed data, used to identify organisms that mostly contribute to microphytobenthos dissimilarity among site-macroalgae (Cut-off 60%). Av.Ab, mean abundance; Diss, mean dissimilarity; Diss/SD, dissimilarity/standard deviation; Contrib%, contribution relative to single taxon; Cum%, cumulative contribution; Table S8: Meiofauna assemblages (cells/cm²) associated to the macroalgae collected in Passetto (PAS) and Porto (POR): *Cystoseira compressa* (CC), *Dictyota dichotoma* (DD), *Ulva* cf. *lacinulata* (UL), *Dictyopteris polypodioides* (DP) and *Sargassum muticum* (SM); Table S9: Results of one-way PERMANOVA and pairwise comparisons carried out on the number of taxa (S) and on total density (N) of meiobenthos taken in Passetto (PAS) and Porto (POR) for each macroalga: *Cystoseira compressa* (CC), *Dictyota dichotoma* (DD), *Ulva* cf. *lacinulata* (UL), *Dictyopteris polypodioides* (DP) and *Sargassum muticum* (SM); Table S10: Results of PERMANOVA and pairwise comparison test carried out on meiobenthic community, taken

in Passetto (PAS) and Porto (POR) for each macroalga: *Cystoseira compressa* (CC), *Dictyota dichotoma* (DD), *Ulva cf. lacinulata* (UL), *Dictyopteris polypodioides* (DP) and *Sargassum muticum* (SM); Table S11: Results of SIMPER analysis based on four root transformed data, used to identify organisms that mostly contribute to meiobenthos dissimilarity among site-macroalgae (Cut-off 60%). Av.Ab, mean abundance; Diss, mean dissimilarity; Diss/SD, dissimilarity/standard deviation; Contrib%, contribution relative to single taxon; Cum%, cumulative contribution.

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Supplementary data

Type of the Paper (Article) – SUPPLEMENTARY FILE

Understanding the role of macroalgal complexity and allelochemicals production in invasive and non-invasive macroalgae in the north-western Adriatic Sea: effect on the associated communities

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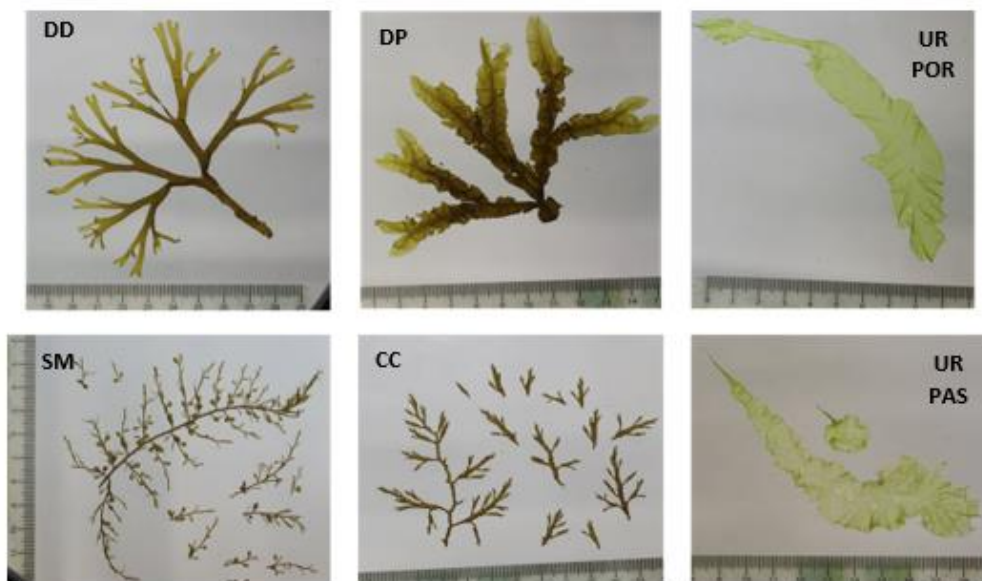


Figure S1- Macroalgae sampled in Passetto (PAS) and Porto (POR): *Cystoseira compressa* (CC), *Dictyota dichotoma* (DD), *Ulva cf. lacinulata* (UL), *Dictyopteris polypodioides* (DP) and *Sargassum muticum* (SM).

Table S1. Results of PCA carried out on the variables measured for macroalgal morphology.

	PC1	PC2	PC3	PC4
% variation	83.3	11.9	3.3	1.5
D	0.478	-0.676	0.206	-0.522
Weight/Area	-0.513	-0.283	0.783	0.206
Perimeter/Area	-0.529	0.239	-0.047	-0.813
Volume/Area	-0.478	-0.637	-0.584	0.157

Table S2. Results of PERMANOVA and pairwise comparison test carried out on morphological measure taken in Passetto (PAS) and Porto (POR) for each macroalga: *Cystoseira compressa* (CC), *Dictyota dichotoma* (DD), *Ulva cf. lacinulata* (UL), *Dictyopteris polypodioides* (DP) and *Sargassum muticum* (SM).

Source of Variation	df	MS	Pseudo-F	P(perm)
Site-Macroalgae	7	16.60	50.86	0.0001
Error	24	0.33		
Total:	31			
Comparisons			P(MC)	
PAS-CC vs PAS-DD			0.0001	
PAS-CC vs PAS-UL			0.0001	
PAS-CC vs PAS-DP			0.0002	
PAS-DD vs PAS-UL			0.0002	
PAS-DD vs PAS-DP			0.0026	
PAS-UL vs PAS-DP			0.0013	
POR-SM vs POR-DD			0.0001	
POR-SM vs POR-UL			0.0003	
POR-SM vs POR-DP			0.0001	
POR-DD vs POR-UL			0.0006	
POR-DD vs POR-DP			0.0005	
POR-UL vs POR-DP			0.1091	
PAS-DD vs POR-DD			0.0212	
PAS-UL vs POR-UL			0.1813	
PAS-DP vs POR-DP			0.1203	

Table S3. Results of one-way PERMANOVA and pairwise comparisons carried out on the total concentration of PUAs taken in Passetto (PAS) and Porto (POR) for each macroalga: *Cystoseira compressa* (CC), *Dictyota dichotoma* (DD), *Ulva cf. lacinulata* (UL), *Dictyopterus polypodioides* (DP) and *Sargassum muticum* (SM).

Source of Variation	df	MS	Pseudo-F	P(perm)
Site-Macroalgae	7	721740.00	14.39	0.0003
Error	24	50160.00		
Total:	31			

Comparisons	P(MC)
PAS-CC vs PAS-DD	0.010
PAS-CC vs PAS-UL	0.065
PAS-CC vs PAS-DP	0.003
PAS-DD vs PAS-UL	0.013
PAS-DD vs PAS-DP	0.005
PAS-UL vs PAS-DP	0.003
POR-SM vs POR-DD	0.700
POR-SM vs POR-UL	0.014
POR-SM vs POR-DP	0.985
POR-DD vs POR-UL	0.012
POR-DD vs POR-DP	0.824
POR-UL vs POR-DP	0.014
PAS-DD vs POR-DD	0.155
PAS-UL vs POR-UL	0.013
PAS-DP vs POR-DP	0.004

Table S4. Microphytobenthos assemblages (cells/cm²) associated to the macroalgae collected in Passetto (PAS) and Porto (POR): *Cystoseira compressa* (CC), *Dictyota dichotoma* (DD), *Ulva cf. lacinulata* (UL), *Dictyopteris polypodioides* (DP) and *Sargassum muticum* (SM).

	PAS-CC	PAS-DD	PAS-UL	PAS-DP	POR-SM	POR-DD	POR-UL	POR-DP
	cell/cm ²	cell/cm ²	cell/cm ²	cell/cm ²	cell/cm ²	cell/cm ²	cell/cm ²	cell/cm ²
<i>Amphora</i> spp.	345 ± 137	243 ± 60	41 ± 9	1368 ± 794	-	99 ± 69	55 ± 55	-
<i>Ardissonea</i> spp.	2 ± 1	-	1 ± 1	-	-	-	-	-
<i>Chaetoceros</i> spp.	-	-	-	-	-	-	3 ± 3	-
<i>Cocconeis</i> spp.	3697 ± 596	170 ± 96	1446 ± 390	7743 ± 2002	2712 ± 1092	1146 ± 218	68 ± 44	690 ± 132
<i>Cylindrotheca</i> spp.	409 ± 325	778 ± 287	98 ± 28	1434 ± 1022	2524 ± 1379	41 ± 29	-	100 ± 62
<i>Entomoneis</i> spp.	478 ± 110	442 ± 123	82 ± 27	670 ± 330	16 ± 16	-	3 ± 3	-
<i>Fragilariopsis</i> spp.	-	-	-	-	390 ± 204	-	-	-
<i>Grammatophora</i> spp.	-	-	-	-	12 ± 12	6 ± 2	-	-
<i>Leptocylindrus</i> spp.	16099 ± 2502	814 ± 414	-	140 ± 91	1666 ± 637	1376 ± 819	3 ± 3	143 ± 52
<i>Licmophora</i> spp.	2229 ± 928	577 ± 240	351 ± 173	783 ± 378	2888 ± 1388	1144 ± 515	5 ± 3	191 ± 103
<i>Meringosphaera</i> spp.	121 ± 59	66 ± 26	51 ± 26	4 ± 4	130 ± 87	29 ± 14	2 ± 2	-
<i>Meuniera membranacea</i>	163 ± 84	168 ± 55	5 ± 5	356 ± 153	-	-	-	-
<i>Navicula</i> spp.	5221 ± 1538	17360 ± 4267	4521 ± 1997	16644 ± 3759	5750 ± 2114	11568 ± 1720	268 ± 184	5911 ± 342
<i>Nitzschia</i> spp.	1227 ± 232	713 ± 155	140 ± 30	2551 ± 849	21321 ± 9841	112 ± 57	33 ± 5	2341 ± 1020
<i>Pleurosigma</i> spp.	3 ± 2	1 ± 1	-	2 ± 1	-	-	-	1 ± 0
<i>Pseudo-nitzschia</i> spp.	-	-	-	-	1761 ± 704	-	-	-
<i>Striatella unipunctata</i>	1 ± 1	-	2 ± 1	-	-	-	-	-
<i>Thalassionema</i> spp.	49 ± 49	-	497 ± 425	-	-	-	-	-
<i>Toxarium</i> spp.	-	-	-	-	-	-	-	-
undetermined centric diatoms	213 ± 213	305 ± 116	24 ± 14	446 ± 265	-	59 ± 35	3 ± 3	-
undetermined pennate diatoms	1642 ± 1298	13420 ± 3414	988 ± 302	4510 ± 2720	5729 ± 1842	16885 ± 3810	8 ± 8	5110 ± 1014
<i>Alexandrium</i> spp.	2 ± 1	-	3 ± 1	12 ± 6	-	-	-	-
<i>Amphidinium</i> sp.	660 ± 262	1928 ± 280	321 ± 88	1819 ± 664	-	-	-	-
cf. <i>Gyrodinium</i>	-	-	-	0 ± 0	-	-	8 ± 8	-
cf. <i>Heterocapsa</i>	74 ± 40	83 ± 83	38 ± 23	151 ± 105	-	332 ± 144	2 ± 2	-
<i>Dinoflagellata</i> sp.1	-	-	-	33 ± 33	-	17 ± 17	8 ± 8	-
<i>Dinoflagellata</i> sp.2	-	-	-	1 ± 1	-	-	155 ± 148	-
<i>Prorocentrum lima</i>	2 ± 1	-	1 ± 0	15 ± 10	-	-	19 ± 19	-
<i>Prorocentrum micans</i>	2 ± 1	1 ± 1	-	-	2 ± 1	1 ± 0	-	1 ± 1
microalgal cells < 20µm	1830 ± 664	9343 ± 2599	2996 ± 2661	411 ± 248	179 ± 86	-	1835 ± 1494	-

tot	34470	46412	11606	39093	45080	32814	2480	14488
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Table S5. Results of one-way PERMANOVA and pairwise comparisons carried out on the number of taxa (S) and on total density (N) of microphytobenthos taken in Passetto (PAS) and Porto (POR) for each macroalga: *Cystoseira compressa* (CC), *Dictyota dichotoma* (DD), *Ulva cf. lacinulata* (UL), *Dictyopteris polypodioides* (DP) and *Sargassum muticum* (SM).

		N° of Taxa			Total abundance		
Source of Variation	df	MS	Pseudo-F	P(perm)	MS	Pseudo-F	P(perm)
Site-Macroalgae	7	41.89	10.50	0.0001	1.1E+09	4.613	0.003
Error	24	3.99			2.4E+08		
Total:	31						
Comparisons		P(MC)			P(MC)		
PAS-CC vs PAS-DD		0.414			0.248		
PAS-CC vs PAS-UL		0.270			0.009		
PAS-CC vs PAS-DP		0.367			0.714		
PAS-DD vs PAS-UL		0.105			0.010		
PAS-DD vs PAS-DP		0.805			0.612		
PAS-UL vs PAS-DP		0.119			0.057		
POR-SM vs POR-DD		0.321			0.468		
POR-SM vs POR-UL		0.082			0.028		
POR-SM vs POR-DP		0.002			0.085		
POR-DD vs POR-UL		0.144			0.002		
POR-DD vs POR-DP		0.000			0.017		
POR-UL vs POR-DP		0.593			0.001		
PAS-DD vs POR-DD		0.029			0.229		
PAS-UL vs POR-UL		0.003			0.093		
PAS-DP vs POR-DP		0.128			0.617		

Table S6. Results of PERMANOVA and pairwise comparison test carried out on microphytobenthos community, taken in Passetto (PAS) and Porto (POR) for each macroalga: *Cystoseira compressa* (CC), *Dictyota dichotoma* (DD), *Ulva cf. lacinulata* (UL), *Dictyopteris polypodioides* (DP) and *Sargassum muticum* (SM).

		Multivariate structure		
Source of Variation	df	Pseudo-MS	F	P(perm)
Site-Macroalgae	7	3584.10	8.59	0.0001
Error	24	417.00		
Total:	31			
Comparisons		P(MC)		
PAS-CC vs PAS-DD		0.011		
PAS-CC vs PAS-UL		0.007		
PAS-CC vs PAS-DP		0.227		
PAS-DD vs PAS-UL		0.002		
PAS-DD vs PAS-DP		0.075		
PAS-UL vs PAS-DP		0.052		
POR-SM vs POR-DD		0.003		
POR-SM vs POR-UL		0.002		
POR-SM vs POR-DP		0.008		
POR-DD vs POR-UL		0.002		
POR-DD vs POR-DP		0.002		
POR-UL vs POR-DP		0.001		
PAS-DD vs POR-DD		0.001		
PAS-UL vs POR-UL		0.005		
PAS-DP vs POR-DP		0.011		

Table S7. Results of SIMPER analysis based on four root transformed data, used to identify organisms that mostly contribute to microphytobenthos dissimilarity among site-macroalgae (Cut-off 60%). Av.Ab, mean abundance; Diss, mean dissimilarity; Diss/SD, dissimilarity/standard deviation; Contrib%, contribution relative to single taxon; Cum%, cumulative contribution.

Groups PAS-CC & PAS-DD		Average dissimilarity = 30.19					
Species	Group PAS-CC	Group PAS-DD	Av.Diss	Diss/SD	Contrib%	Cum.%	
	Av.Abund	Av.Abund					
<i>Leptocylindrus spp.</i>	11.19	4.96	3.92	3.91	12.99	12.99	
undetermined pennate diatoms	4.52	10.57	3.80	1.74	12.60	25.58	
<i>Cocconeis spp.</i>	7.73	2.79	3.09	2.72	10.24	35.82	
microalgal cells < 20µm	5.25	9.65	2.73	1.34	9.03	44.85	
undetermined centric diatoms	1.35	3.99	2.10	2.38	6.95	51.81	
<i>Navicula spp.</i>	8.30	11.26	1.87	1.88	6.18	57.99	
<i>Licmophora spp.</i>	6.54	3.88	1.80	1.04	5.96	63.95	
Groups PAS-CC & PAS-UL		Average dissimilarity = 35.37					
Species	Group PAS-CC	Group PAS-UL	Av.Diss	Diss/SD	Contrib%	Cum.%	
	Av.Abund	Av.Abund					
<i>Leptocylindrus spp.</i>	11.19	0.00	8.14	9.84	23.03	23.03	
microalgal cells < 20µm	5.25	5.65	2.60	2.17	7.36	30.38	
<i>Thalassionema spp.</i>	0.94	3.24	2.03	1.38	5.75	36.13	
undetermined pennate diatoms	4.52	5.33	2.03	1.37	5.74	41.87	
<i>Meuniera membranacea</i>	2.80	0.52	1.87	1.52	5.27	47.14	
<i>Licmophora spp.</i>	6.54	4.10	1.84	1.74	5.20	52.35	
<i>Nitzschia spp.</i>	5.86	3.37	1.82	3.50	5.15	57.50	
<i>Amphidinium sp.</i>	4.05	4.13	1.45	1.40	4.10	61.59	
Groups PAS-DD & PAS-UL		Average dissimilarity = 36.40					
Species	Group PAS-DD	Group PAS-UL	Av.Diss	Diss/SD	Contrib%	Cum.%	
	Av.Abund	Av.Abund					
undetermined pennate diatoms	10.57	5.33	3.83	2.88	10.52	10.52	
<i>Leptocylindrus spp.</i>	4.96	0.00	3.57	4.70	9.80	20.32	
microalgal cells < 20µm	9.65	5.65	3.32	1.91	9.11	29.43	
<i>Navicula spp.</i>	11.26	7.77	2.57	1.82	7.07	36.50	
<i>Cocconeis spp.</i>	2.79	6.00	2.30	1.67	6.32	42.82	
<i>Thalassionema spp.</i>	0.00	3.24	2.26	1.49	6.21	49.03	
<i>Meuniera membranacea</i>	3.49	0.52	2.21	2.45	6.08	55.10	
undetermined centric diatoms	3.99	1.31	1.99	1.64	5.46	60.56	
Groups PAS-CC & PAS-DP		Average dissimilarity = 35.20					
Species	Group PAS-CC	Group PAS-DP	Av.Diss	Diss/SD	Contrib%	Cum.%	
	Av.Abund	Av.Abund					
<i>Leptocylindrus spp.</i>	11.19	2.60	5.82	3.29	16.54	16.54	
undetermined pennate diatoms	4.52	4.85	3.22	1.44	9.14	25.68	
microalgal cells < 20µm	5.25	2.66	2.72	1.21	7.72	33.40	
<i>Amphidinium sp.</i>	4.05	5.24	2.20	1.17	6.26	39.66	
<i>Cylindrotheca spp.</i>	3.19	4.05	2.13	1.56	6.06	45.72	
<i>Licmophora spp.</i>	6.54	4.15	2.09	0.89	5.93	51.65	
<i>Navicula spp.</i>	8.30	11.13	1.85	2.17	5.26	56.90	
undetermined centric diatoms	1.35	2.73	1.70	0.96	4.82	61.73	
Groups PAS-DD & PAS-DP		Average dissimilarity = 35.12					

Species	Group PAS-DD		Group PAS-DP			
	Av.Abund	Av.Abund	Av.Diss	Diss/SD	Contrib%	Cum.%
microalgal cells < 20µm	9.65	2.66	4.83	1.85	13.74	13.74
undetermined pennate diatoms	10.57	4.85	4.43	1.10	12.63	26.37
<i>Cocconeis</i> spp.	2.79	9.17	4.16	3.19	11.84	38.21
<i>Cylindrotheca</i> spp.	5.09	4.05	2.11	1.78	6.01	44.22
undetermined centric diatoms	3.99	2.73	1.96	1.47	5.59	49.81
<i>Licmophora</i> spp.	3.88	4.15	1.76	0.96	5.00	54.81
<i>Amphidinium</i> sp.	6.58	5.24	1.71	0.69	4.88	59.69
<i>Leptocylindrus</i> spp.	4.96	2.60	1.68	1.34	4.78	64.48
Groups PAS-UL & PAS-DP		Average dissimilarity = 40.37				
Species	Group PAS-UL		Group PAS-DP			
	Av.Abund	Av.Abund	Av.Diss	Diss/SD	Contrib%	Cum.%
undetermined pennate diatoms	5.33	4.85	3.92	2.46	9.72	9.72
microalgal cells < 20µm	5.65	2.66	3.09	1.27	7.66	17.37
<i>Navicula</i> spp.	7.77	11.13	2.62	2.13	6.50	23.87
<i>Amphidinium</i> sp.	4.13	5.24	2.61	2.07	6.46	30.33
<i>Thalassionema</i> spp.	3.24	0.00	2.47	1.40	6.11	36.45
<i>Cocconeis</i> spp.	6.00	9.17	2.39	2.12	5.93	42.37
<i>Nitzschia</i> spp.	3.37	6.46	2.35	2.09	5.82	48.19
<i>Meuniera membranacea</i>	0.52	3.45	2.29	1.56	5.66	53.86
<i>Cylindrotheca</i> spp.	3.04	4.05	2.20	2.30	5.44	59.30
<i>Leptocylindrus</i> spp.	0.00	2.60	2.01	1.55	4.97	64.26
Groups POR-SM & POR-DD		Average dissimilarity = 41.06				
Species	Group POR-SM		Group POR-DD			
	Av.Abund	Av.Abund	Av.Diss	Diss/SD	Contrib%	Cum.%
<i>Nitzschia</i> spp.	10.94	3.02	6.18	2.59	15.05	15.05
<i>Pseudo-nitzschia</i> spp.	6.03	0.00	4.79	6.08	11.66	26.71
cf. <i>Heterocapsa</i>	0.00	4.03	3.32	3.54	8.10	34.81
<i>Leptocylindrus</i> spp.	6.04	3.61	3.10	1.37	7.56	42.37
<i>Cylindrotheca</i> spp.	5.69	1.87	3.06	1.47	7.46	49.83
microalgal cells < 20µm	3.45	0.00	2.79	5.61	6.78	56.61
<i>Fragilariopsis</i> spp.	3.47	0.00	2.61	1.59	6.35	62.96
Groups POR-SM & POR-UL		Average dissimilarity = 68.84				
Species	Group POR-SM		Group POR-UL			
	Av.Abund	Av.Abund	Av.Diss	Diss/SD	Contrib%	Cum.%
<i>Nitzschia</i> spp.	10.94	2.38	8.76	3.27	12.72	12.72
undetermined pennate diatoms	8.36	0.60	8.20	6.66	11.91	24.63
<i>Pseudo-nitzschia</i> spp.	6.03	0.00	6.31	7.53	9.17	33.80
<i>Leptocylindrus</i> spp.	6.04	0.46	6.03	3.59	8.76	42.56
<i>Cylindrotheca</i> spp.	5.69	0.00	5.71	2.50	8.30	50.86
<i>Licmophora</i> spp.	6.41	0.89	5.68	2.44	8.25	59.11
<i>Navicula</i> spp.	8.24	3.54	4.79	2.74	6.96	66.07
Groups POR-DD & POR-UL		Average dissimilarity = 69.46				
Species	Group POR-DD		Group POR-UL			
	Av.Abund	Av.Abund	Av.Diss	Diss/SD	Contrib%	Cum.%

undetermined pennate diatoms	11.26	0.60	13.69	8.71	19.70	19.70
<i>Navicula</i> spp.	10.30	3.54	8.76	4.42	12.61	32.31
microalgal cells < 20µm	0.00	5.41	7.02	2.40	10.10	42.42
<i>Licmophora</i> spp.	5.03	0.89	5.23	1.94	7.53	49.94
cf. <i>Heterocapsa</i>	4.03	0.42	4.72	2.84	6.79	56.73
<i>Leptocylindrus</i> spp.	3.61	0.46	4.44	1.07	6.39	63.12

Groups POR-SM & POR-DP Average dissimilarity = 33.59

Species	Group POR-SM		Group POR-DP			
	Av.Abund	Av.Abund	Av.Diss	Diss/SD	Contrib%	Cum.%
<i>Pseudo-nitzschia</i> spp.	6.03	0.00	5.40	6.75	16.08	16.08
<i>Nitzschia</i> spp.	10.94	6.42	4.35	1.74	12.94	29.02
microalgal cells < 20µm	3.45	0.00	3.15	5.67	9.38	38.40
<i>Licmophora</i> spp.	6.41	3.31	2.99	1.64	8.90	47.30
<i>Fragilariopsis</i> spp.	3.47	0.00	2.91	1.59	8.68	55.98
<i>Cylindrotheca</i> spp.	5.69	2.84	2.79	1.42	8.31	64.28

Groups POR-DD & POR-DP Average dissimilarity = 31.50

Species	Group POR-DD		Group POR-DP			
	Av.Abund	Av.Abund	Av.Diss	Diss/SD	Contrib%	Cum.%
cf. <i>Heterocapsa</i>	4.03	0.00	4.35	4.23	13.80	13.80
<i>Leptocylindrus</i> spp.	3.61	3.29	3.87	4.71	12.28	26.08
<i>Nitzschia</i> spp.	3.02	6.42	3.69	1.84	11.72	37.80
undetermined pennate diatoms	11.26	8.37	3.04	2.60	9.67	47.47
<i>Amphora</i> spp.	2.35	0.00	2.61	1.49	8.29	55.76
<i>Licmophora</i> spp.	5.03	3.31	2.56	1.72	8.14	63.89

Groups POR-UL & POR-DP Average dissimilarity = 66.52

Species	Group POR-UL		Group POR-DP			
	Av.Abund	Av.Abund	Av.Diss	Diss/SD	Contrib%	Cum.%
undetermined pennate diatoms	0.60	8.37	12.25	5.69	18.41	18.41
microalgal cells < 20µm	5.41	0.00	8.57	2.40	12.88	31.30
<i>Navicula</i> spp.	3.54	8.76	8.25	4.23	12.40	43.70
<i>Nitzschia</i> spp.	2.38	6.42	6.34	2.36	9.53	53.22
<i>Leptocylindrus</i> spp.	0.46	3.29	4.46	2.69	6.70	59.92
<i>Cylindrotheca</i> spp.	0.00	2.84	4.44	4.12	6.68	66.60

Groups PAS-UL & POR-UL Average dissimilarity = 58.36

Species	Group PAS-UL		Group POR-UL			
	Av.Abund	Av.Abund	Av.Diss	Diss/SD	Contrib%	Cum.%
undetermined pennate diatoms	5.33	0.60	5.73	3.15	9.82	9.82
<i>Navicula</i> spp.	7.77	3.54	5.20	2.09	8.90	18.72
<i>Amphidinium</i> sp.	4.13	0.00	5.05	8.02	8.66	27.38
<i>Cocconeis</i> spp.	6.00	2.58	4.16	2.99	7.12	34.50
<i>Licmophora</i> spp.	4.10	0.89	3.85	3.06	6.61	41.10
<i>Thalassionema</i> spp.	3.24	0.00	3.76	1.57	6.44	47.54
<i>Cylindrotheca</i> spp.	3.04	0.00	3.72	5.55	6.37	53.91
<i>Entomoneis</i> spp.	2.91	0.46	2.97	2.65	5.09	59.00
microalgal cells < 20µm	5.65	5.41	2.92	1.08	5.00	64.00

Groups PAS-DP & POR-DP Average dissimilarity = 46.16

Group PAS-DP Group POR-DP

Species	Av.Abund	Av.Abund	Av.Diss	Diss/SD	Contrib%	Cum.%
undetermined pennate diatoms	4.85	8.37	5.32	1.12	11.52	11.52
<i>Amphora</i> spp.	5.25	0.00	4.47	4.77	9.68	21.20
<i>Amphidinium</i> sp.	5.24	0.00	4.12	1.67	8.93	30.13
<i>Entomoneis</i> spp.	4.50	0.00	3.85	7.18	8.35	38.48
<i>Cocconeis</i> spp.	9.17	5.07	3.63	3.90	7.86	46.34
<i>Meuniera membranacea</i>	3.45	0.00	2.76	1.55	5.97	52.31
<i>Cylindrotheca</i> spp.	4.05	2.84	2.50	1.97	5.42	57.74
<i>Licmophora</i> spp.	4.15	3.31	2.42	1.47	5.25	62.99
Groups PAS-DD & POR-DD	Average dissimilarity = 40.42					
	Group PAS-DD	Group POR-DD				
Species	Av.Abund	Av.Abund	Av.Diss	Diss/SD	Contrib%	Cum.%
microalgal cells < 20µm	9.65	0.00	7.19	9.85	17.79	17.79
<i>Amphidinium</i> sp.	6.58	0.00	4.92	10.80	12.17	29.96
<i>Entomoneis</i> spp.	4.46	0.00	3.35	5.03	8.29	38.25
<i>Leptocylindrus</i> spp.	4.96	3.61	2.74	1.96	6.77	45.02
<i>Meuniera membranacea</i>	3.49	0.00	2.62	5.61	6.47	51.49
cf. <i>Heterocapsa</i>	1.07	4.03	2.43	1.85	6.02	57.51
<i>Cylindrotheca</i> spp.	5.09	1.87	2.42	2.11	5.98	63.49

Table S8. Meiofauna assemblages (cells/cm²) associated to the macroalgae collected in Passetto (PAS) and Porto (POR): *Cystoseira compressa* (CC), *Dictyota dichotoma* (DD), *Ulva cf. lacinulata* (UL), *Dictyopterus polypodioides* (DP) and *Sargassum muticum* (SM).

	PAS-CC	PAS-DD	PAS-UL	PAS-DP	POR-SM	POR-DD	POR-UL	POR-DP
Nematoda	0.04 ± 0.04	0.23 ± 0.23	0.06 ± 0.02	0.47 ± 0.07	0.17 ± 0.17			
Kinorhyncha				0.08 ± 0.08				
Ostracoda				0.12 ± 0.08			0.03 ± 0.03	0.14 ± 0.08
Harpacticoida	0.60 ± 0.16	2.11 ± 0.21	0.25 ± 0.02	3.58 ± 0.82	1.46 ± 0.38	0.40 ± 0.23	0.20 ± 0.10	1.58 ± 0.33
Copepod <i>nauplii</i>	1.84 ± 0.42	2.79 ± 0.38	0.97 ± 0.21	1.33 ± 0.66	13.17 ± 8.10	1.65 ± 0.42	1.77 ± 0.51	8.10 ± 2.59
Cirripeda				0.08 ± 0.05				
Chironomidae	0.04 ± 0.04	0.31 ± 0.19	0.02 ± 0.02	0.28 ± 0.22				
Isopoda				0.29 ± 0.15	0.06 ± 0.06			
Amphipoda	0.06 ± 0.06	0.50 ± 0.38	0.02 ± 0.02	0.40 ± 0.15	0.13 ± 0.07	0.02 ± 0.02		
Halacaridae	0.00 ± 0.00	0.23 ± 0.23		0.07 ± 0.07				
Polychaeta	0.05 ± 0.05	0.08 ± 0.08	0.05 ± 0.03	0.53 ± 0.12				
Gastropoda	0.16 ± 0.10	0.61 ± 0.47	0.08 ± 0.05	2.90 ± 2.33	0.17 ± 0.17			
Bivalvia	0.17 ± 0.11	0.20 ± 0.20						0.11 ± 0.08

Table S9. Results of one-way PERMANOVA and pairwise comparisons carried out on the number of taxa (S) and on total density (N) of meiobenthos taken in Passetto (PAS) and Porto (POR) for each macroalga: *Cystoseira compressa* (CC), *Dictyota dichotoma* (DD), *Ulva cf. lacinulata* (UL), *Dictyopterus polypodioides* (DP) and *Sargassum muticum* (SM).

		N° of Taxa			Total abundance		
Source of Variation	df	MS	Pseudo-F	P(perm)	MS	Pseudo-F	P(perm)
Site-Macroalgae	7	16.67	13.23	0.0001	101.49	2.15	0.049
Error	24	1.26			47.21		
Total:	31						
Comparisons		P(MC)			P(MC)		
PAS-CC vs PAS-DD		0.466			0.003		
PAS-CC vs PAS-UL		0.715			0.032		
PAS-CC vs PAS-DP		0.004			0.089		
PAS-DD vs PAS-UL		0.793			0.000		
PAS-DD vs PAS-DP		0.014			0.418		
PAS-UL vs PAS-DP		0.009			0.031		
POR-SM vs POR-DD		0.194			0.177		
POR-SM vs POR-UL		0.146			0.176		
POR-SM vs POR-DP		0.744			0.580		
POR-DD vs POR-UL		0.623			0.927		
POR-DD vs POR-DP		0.168			0.032		
POR-UL vs POR-DP		0.137			0.025		
PAS-DD vs POR-DD		0.017			0.001		
PAS-UL vs POR-UL		0.023			0.347		
PAS-DP vs POR-DP		0.002			0.964		

Table S10. Results of PERMANOVA and pairwise comparison test carried out on meiobenthic community, taken in Passetto (PAS) and Porto (POR) for each macroalga: *Cystoseira compressa* (CC), *Dictyota dichotoma* (DD), *Ulva cf. lacunculata* (UL), *Dictyopteris polypodioides* (DP) and *Sargassum muticum* (SM).

Source of Variation	df	MS	F	P(perm)
Site-Macroalgae	7	2954.90	4.58	0.0001
Error	24	645.32		
Total:	31			

Comparisons	P(MC)
PAS-CC vs PAS-DD	0.236
PAS-CC vs PAS-UL	0.250
PAS-CC vs PAS-DP	0.008
PAS-DD vs PAS-UL	0.007
PAS-DD vs PAS-DP	0.078
PAS-UL vs PAS-DP	0.004
POR-SM vs POR-DD	0.064
POR-SM vs POR-UL	0.046
POR-SM vs POR-DP	0.553
POR-DD vs POR-UL	0.729
POR-DD vs POR-DP	0.011
POR-UL vs POR-DP	0.010
PAS-DD vs POR-DD	0.014
PAS-UL vs POR-UL	0.028
PAS-DP vs POR-DP	0.003

Table S11. Results of SIMPER analysis based on four root transformed data, used to identify organisms that mostly contribute to meiobenthos dissimilarity among site-macroalgae (Cut-off 60%). Av.Ab, mean abundance; Diss, mean dissimilarity; Diss/SD, dissimilarity/standard deviation; Contrib%, contribution relative to single taxon; Cum%, cumulative contribution.

Groups PAS-CC & PAS-DD		Average dissimilarity = 42.39					
Species	Group PAS-CC Av.Abund	Group PAS-DD Av.Abund	Av.Diss	Diss/SD	Contrib%	Cum.%	
Harpacticoida	0.75	1.45	8.50	2.54	20.05	20.05	
Amphipoda	0.12	0.47	6.05	0.98	14.28	34.33	
Gastropoda	0.28	0.52	5.94	1.15	14.02	48.34	
Copepod <i>nauplii</i>	1.32	1.66	4.90	1.13	11.55	59.90	
Chironomidae	0.10	0.39	4.36	1.14	10.30	70.19	
Groups PAS-CC & PAS-UL		Average dissimilarity = 37.61					
Species	Group PAS-CC Av.Abund	Group PAS-UL Av.Abund	Av.Diss	Diss/SD	Contrib%	Cum.%	
Copepod <i>nauplii</i>	1.32	0.97	8.34	1.79	22.16	22.16	
Bivalvia	0.29	0.00	5.50	0.94	14.62	36.78	
Gastropoda	0.28	0.19	5.27	1.16	14.01	50.79	
Harpacticoida	0.75	0.50	5.23	1.61	13.90	64.69	
Nematoda	0.10	0.22	4.01	1.58	10.67	75.36	
Groups PAS-CC & PAS-DP		Average dissimilarity = 58.69					
Species	Group PAS-CC Av.Abund	Group PAS-DP Av.Abund	Av.Diss	Diss/SD	Contrib%	Cum.%	
Harpacticoida	0.75	1.85	10.20	3.71	17.38	17.38	
Gastropoda	0.28	1.20	8.91	1.15	15.18	32.56	
Polychaeta	0.12	0.71	6.28	1.77	10.70	43.27	
Copepod <i>nauplii</i>	1.32	1.04	6.10	1.30	10.40	53.66	
Nematoda	0.10	0.68	6.01	1.93	10.24	63.90	
Isopoda	0.00	0.49	4.60	3.79	7.83	71.74	
Groups PAS-DD & PAS-UL		Average dissimilarity = 53.72					
Species	Group PAS-DD Av.Abund	Group PAS-UL Av.Abund	Av.Diss	Diss/SD	Contrib%	Cum.%	
Harpacticoida	1.45	0.50	12.96	4.72	24.12	24.12	
Copepod <i>nauplii</i>	1.66	0.97	9.60	2.07	17.87	42.00	
Amphipoda	0.47	0.07	6.93	0.98	12.91	54.90	
Gastropoda	0.52	0.19	6.50	1.14	12.11	67.01	
Chironomidae	0.39	0.08	4.77	1.09	8.89	75.90	
Groups PAS-DD & PAS-DP		Average dissimilarity = 46.77					
Species	Group PAS-DD Av.Abund	Group PAS-DP Av.Abund	Av.Diss	Diss/SD	Contrib%	Cum.%	
Gastropoda	0.52	1.20	7.97	1.19	17.03	17.03	
Copepod <i>nauplii</i>	1.66	1.04	6.39	1.22	13.65	30.69	
Nematoda	0.24	0.68	4.89	1.88	10.45	41.14	
Polychaeta	0.14	0.71	4.87	1.76	10.41	51.54	
Amphipoda	0.47	0.54	4.08	1.14	8.73	60.28	
Isopoda	0.00	0.49	3.78	3.45	8.07	68.35	
Chironomidae	0.39	0.36	3.37	1.09	7.21	75.56	
Groups PAS-UL & PAS-DP		Average dissimilarity = 60.80					
Species	Group PAS-UL Av.Abund	Group PAS-DP Av.Abund	Av.Diss	Diss/SD	Contrib%	Cum.%	
Harpacticoida	0.5	1.9	14.0	10.2	23.0	23.0	
Gastropoda	0.2	1.2	9.5	1.1	15.6	38.7	
Polychaeta	0.2	0.7	6.6	1.8	10.8	49.5	
Nematoda	0.2	0.7	5.5	1.7	9.1	58.6	

Copepod <i>nauplii</i>	1.0	1.0	5.1	1.7	8.4	67.0
Isopoda	0.0	0.5	5.1	3.9	8.3	75.3
Groups POR-SM & POR-DD	Average dissimilarity = 45.59					
	Group POR-SM		Group POR-DD			
Species	Av.Abund	Av.Abund	Av.Diss	Diss/SD	Contrib%	Cum.%
Copepod <i>nauplii</i>	3.11	1.25	25.13	1.73	55.11	55.11
Harpacticoida	1.18	0.57	9.66	2.09	21.19	76.31
Groups POR-SM & POR-UL	Average dissimilarity = 48.98					
	Group POR-SM		Group POR-UL			
Species	Av.Abund	Av.Abund	Av.Diss	Diss/SD	Contrib%	Cum.%
Copepod <i>nauplii</i>	3.11	1.29	24.81	1.7	50.66	50.66
Harpacticoida	1.18	0.37	12.21	2.28	24.92	75.58
Groups POR-SM & POR-DP	Average dissimilarity = 33.32					
	Group POR-SM		Group POR-DP			
Species	Av.Abund	Av.Abund	Av.Diss	Diss/SD	Contrib%	Cum.%
Copepod <i>nauplii</i>	3.11	2.73	17.01	1.66	51.05	51.05
Harpacticoida	1.18	1.23	3.62	1.31	10.85	61.91
Nematoda	0.20	0.00	2.98	0.55	8.94	70.84
Groups POR-DD & POR-UL	Average dissimilarity = 23.59					
	Group POR-DD		Group POR-UL			
Species	Av.Abund	Av.Abund	Av.Diss	Diss/SD	Contrib%	Cum.%
Copepod <i>nauplii</i>	1.25	1.29	10.89	1.36	46.16	46.16
Harpacticoida	0.57	0.37	9.02	1.61	38.24	84.41
Groups POR-DD & POR-DP	Average dissimilarity = 42.35					
	Group POR-DD		Group POR-DP			
Species	Av.Abund	Av.Abund	Av.Diss	Diss/SD	Contrib%	Cum.%
Copepod <i>nauplii</i>	1.25	2.73	22.59	1.88	53.34	53.34
Harpacticoida	0.57	1.23	11.59	1.84	27.38	80.72
Groups POR-UL & POR-DP	Average dissimilarity = 43.78					
	Group POR-UL		Group POR-DP			
Species	Av.Abund	Av.Abund	Av.Diss	Diss/SD	Contrib%	Cum.%
Copepod <i>nauplii</i>	1.29	2.73	21.99	1.85	50.23	50.23
Harpacticoida	0.37	1.23	14.5	2.19	33.12	83.35
Groups PAS-DD & POR-DD	Average dissimilarity = 49.11					
	Group PAS-DD		Group POR-DD			
Species	Av.Abund	Av.Abund	Av.Diss	Diss/SD	Contrib%	Cum.%
Harpacticoida	1.45	0.57	12.92	2.25	26.31	26.31
Amphipoda	0.47	0.08	7.29	0.96	14.85	41.16
Copepod <i>nauplii</i>	1.66	1.25	6.85	1.23	13.95	55.1
Gastropoda	0.52	0	6.42	0.87	13.08	68.18
Chironomidae	0.39	0	4.84	0.95	9.86	78.05
Groups PAS-UL & POR-UL	Average dissimilarity = 35.79					
	Group PAS-UL		Group POR-UL			
Species	Av.Abund	Av.Abund	Av.Diss	Diss/SD	Contrib%	Cum.%
Copepod <i>nauplii</i>	0.97	1.29	10.24	1.29	28.62	28.62
Harpacticoida	0.5	0.37	6.15	1.32	17.17	45.8
Nematoda	0.22	0	5.49	1.63	15.34	61.14
Gastropoda	0.19	0	4.86	0.89	13.57	74.71
Groups PAS-DP & POR-DP	Average dissimilarity = 60.34					
	Group PAS-DP		Group POR-DP			
Species	Av.Abund	Av.Abund	Av.Diss	Diss/SD	Contrib%	Cum.%
Copepod <i>nauplii</i>	1.04	2.73	15.27	1.55	25.31	25.31
Gastropoda	1.20	0.00	8.40	1.03	13.91	39.23
Polychaeta	0.71	0.00	6.49	2.53	10.75	49.98

Nematoda	0.68	0.00	6.24	2.53	10.34	60.31
Harpacticoida	1.85	1.23	5.00	1.70	8.28	68.60
Amphipoda	0.54	0.00	4.19	1.62	6.95	75.55

3.5 Case study: *Rugulopterix okamurae*, Tarifa (Spain)

3.5.1 The macroalga *Rugulopterix okamurae*

Rugulopterix okamurae (E.Y.Dawson) I.K.Hwang, W.J.Lee & H.S.Kim (Dictyotaceae) (Fig.3.13) is a macroalga belonging to the class Phaeophyceae which is common in temperate areas of the Northwest Pacific (e.g., Philippines, Taiwan, China, Korea, and Japan) but it ranges from tropical areas to the Gulf of California (see also Norris, 2010). In the Mediterranean Sea, it is an exotic seaweed that has been recorded since 2002 in the northwestern zone where it was accidentally introduced in the Thau lagoon of the French coast through seed importation for Japanese oyster (*Cassostrea gigas*) culturing (Verlaque et al., 2009). In European waters, *R. okamurae* is the more recent example of an unprecedented case of bioinvasion by marine macroalgae. In 2015 and 2016, it was recorded in Ceuta and in Andalusian waters, respectively (Altamirano et al., 2016, 2017; Ocana et al., 2016). In its native area, the alga dwells on sublittoral rocky substrata from 0.5 to 35 m deep, while it can occur down to 40 m deep in the northern Bay of Ceuta (southern sector of the Strait of Gibraltar). In the Strait of Gibraltar, *R. okamurae* has expanded massively causing considerable ecological impacts on coastal communities (García-Gomez et al., 2018, 2020). For instance, after its arrival, *R. okamurae* became the most abundant species over a period of only one year covering over 90% of the bottom between 10 and 20 m depth, which implied a significant change in the structure of benthic communities (García-Gomez et al., 2020). Additionally, hundreds of tons of *R. okamurae* accumulated on beaches becoming a nuisance with implications for both the tourism and public health (Ocana et al., 2016; García-Gomez' et al., 2018, 2020, 2021). Fishermen have also reported that the species clogs fishing nets causing a substantial reduction in their ability to catch fish (Sempere Valverde et al., 2019). In 2019, *R. okamurae* was first record of north-eastern Atlantic archipelago of the Azores close to one of the islands largest harbor (Ponta Delgada, Sao-Miguel Island). As observed in the Strait of Gibraltar, the alga quickly proliferated and over a period of only two years it became the dominant species covering most of the rocky rbottom along the south coast of the island and producing substantial accumulations of algal wrack on coastal areas (Faria et al., 2021). Nevertheless, it is yet not known the potential deleterious impact of this species in the structure of shallow-water benthic communities. Until now, there are no known autochthonous herbivore species that find *R. okamurae* suitable as a food source.

This is probably due to the species anti-herbivory bioactive defenses (Paula et al., 2011). Given its high proliferation in the region, food web structure and trophic dynamics are likely to be affected with potential negative impacts on local fisheries. The chemical study of *R. okamurae* from the Strait of Gibraltar led to the isolation of six secondary metabolites, among which the compound dilkamural stands out because of its high concentration (Casal-Porras et al., 2021; Cuevas et al., 2021).

R. okamurae in the Strait of Gibraltar, is sympatric with the native species *Dictyota dichotoma*. Despite the high morphological similarity between both habitat-forming species, native and exotic macroalgae hosted different macrofaunal assemblages. *D. dichotoma* showed lower number of species, abundance of individuals, and diversity values than the introduced macroalga. Most shared species showed higher abundance on *R. okamurae*, but there was high variability in the response to macroalgal identity across higher taxa. Recent observation in the Strait of Gibraltar and in the Azoras (García-Gomez et al., 2021) demonstrated that *R. okamurae* quickly impacted the structure of marine benthic communities replacing the previously dominant assemblages. It is yet unclear whether the results persist throughout time. It appears that in the Azores, *R. okamurae* undergoes a seasonal fluctuation in size, and perhaps in coverage. Individuals of *R. okamurae* are more abundant and grow larger from mid-winter until summer, when massive amounts of the alga become detached and cast away the shore. After this (in late summer, early autumn), the species is still present but forming a much shorter canopy, with the thallus often reduced to a basal system of perennial rhizoids, as described for its native location in the Pacific (Kajimura, 1992; Hwang et al., 2009) and also for the waters of the Strait of Gibraltar (García-Gomez et al., 2018). This somehow “resting state” would putatively allow native species to recover. The extraordinary competitive and colonization capacity shown by *R. okamurae* can eventually lead to the extinction of native biota and a critical decline in biodiversity. Moreover, substantial habitat modification, community homogenization and accumulations of detached biomass can result in huge economic impacts and fundamental disruptions in the marine ecosystem.



Fig. 3.13 **A)** *Rugulopteryx okamurae*. Photo © Sandrine Ruitton **B)** A heap of dry fragments of *Rugulopteryx okamurae* on the shore

3.5.2 Aim of the study

Invasive marine species often have a disproportionate influence on ecological processes (Molnar et al., 2008; Occhipinti-Ambrogi and Galil, 2010) and could produce greater shifts in species composition, diversity, and abundance, leading to local extinction of native biota; they may cause severe ecological impacts by modifying the invaded habitat, its food webs, and community structure, as well as by displacing native species (Viard and Comtet, 2016). These invasive algae have also been shown to be a source of several bioactive compounds with biological activities, probably to compete with native algae (Máximo et al., 2018). This study aims to understand the important role of the production of some allelochemicals, in structuring the associated benthic communities.

Specifically, the aim is to analyze in a benthic environment, located in the Mediterranean Sea, Tarifa (within the Strait of Gibraltar), characterized by the presence of an invasive macroalga (*R. okamurae*) and a native macroalga (*H. scoparia*), the relationship between the qualitative-quantitative profile of the PUAs produced by the two macroalgae and the associated meio- and microphytobenthic community. This was performed considering that *R. okamurae* is known to produce the newly discovered compound dilkamural, whose production has been hypothesized as defense strategy.

3.5.3 Material and Methods

3.5.3.1 Study site

Sampling of *R. okamurae* was done in one site located in the strait of Gibraltar (Tarifa, Cadiz, Spagna). The Strait of Gibraltar is bounded by the southern end of the Iberian Peninsula and to the south by the African continent. It has a minimum width of 32 km and a length of 60 km and connects the Atlantic Ocean with the Mediterranean Sea. The Strait of Gibraltar is a biodiversity hotspot but is also a highly populated and urbanized area with an intense maritime traffic (Coll et al., 2010). Moreover, the Strait of Gibraltar is characterized by the presence of two different currents that move in opposite directions at different altitudes: one of depth (towards the Atlantic) and one of surface (towards the Mediterranean), the regime of these two currents is subject to periodic variations depending on the tide. The average depth of the Strait of Gibraltar is 300 m while the maximum depth is 900 m. The coasts on both the African and Iberian sides are very diversified, in some points there are sandy dunes in other rocky areas. The area involved in this study includes the Iberian costs, an area characterized by rocky substrate.

The study site was in Tarifa (Cadiz, southern Spain 36°00' N, 5°28'W). This coast represents a transition between Atlantic and Mediterranean waters. The upper water mass (up to 150 m depth) is the Atlantic component, which is characterized by temperature higher than 20°C and salinities lower than 36.5‰ in summer. Down to a depth of 100-150 m it is possible to distinguish the deep Mediterranean water layer, which is denser and exhibits a lower temperature and higher salinity (38.4‰ and 12.9°C) than the upper one. Thus, the photic zone and especially the littoral assemblages are basically subjected to Atlantic conditions (Rodríguez, 1982). Between 10 and 20 m depth, temperature decreases by 4°C (Flores-Moya, 1997). Tides are semidiurnal, and in Tarifa the maximum range does not exceed 2 m amplitude (Fig.3.14).

This area is characterized by the presence of different macroalgae which are distributed in the water column. *Gelidium corneum* and *Gymnogongrus patens* were dominant at the lower levels, close to the subtidal. *Valonia utricularis*, *Osmundea pinnatifida*, *Corallina elongata* and *Jania rubens* were distributed in intermediate levels while *Ulva cf. rigida*, *Chaetomorpha aerea* and *Fucus spiralis* (habitat-forming species) were distributed in upper levels. Also, gorgonian *Paramuricea clavata* is always present. In July 2016, it was detected the presence of a non-indigenous species (NIS) *Rugulopteris okamurae* and recorded the subsequent increase in coverage (García-Gómez et al 2020).



Fig.3.14. Map of study site at Tarifa and sampling sites: Tarifa 1 and Tarifa 2 (southern Spain).

3.5.3.2 Sampling and sample processing

The samplings were carried out on 7 November 2021.

Two different macroalgae were chosen: the invasive species *Rugulopteryx okamurae* (RO) and *Halopteris scoparia* (HS), in two sites, Tarifa1 and Tarifa2, far from each other of about 1 km. Three replicates for each macroalga were sampled by snorkeling in each site. The sampling design resulted as a two-way mixed model with macroalgae as fixed factor (two levels: *Rugulopteryx okamurae* (RO), *Halopteris scoparia* (HS)) and sites, as random factor (Tarifa1 and Tarifa2).

Macroalgae were treated to remove all associated benthic organisms as described in Pezzolesi et al. (2021) and Lenzo et al. (2022). Briefly, each tube containing thalli and their storage water was vigorously shaken to separate the macroalgae from the epiphytic microalgae and the meiofauna. Then the tube was rinsed with filtered seawater and vigorously washed several times until epiphytic organisms were completely removed. The seaweed thalli were dried with absorbent paper, then weighed to determine wet weight (g WW) and photographed; finally, they were stored at -80 °C in new tubes.

The total volume of washing seawater of each sample (200 mL) was measured and then divided into two aliquots, one for the microphytobenthos and the other for the meiofauna analyses. Aliquots for microphytobenthos analysis (100 mL) were fixed with Lugol and stored in 250 mL dark glass bottles. Aliquots for meiofauna analysis were sieved through a 1000- μ m mesh and a 45- μ m mesh. The meiofauna retained in latter sieve was preserved in alcohol 70% and stored in a 50 ml falcon until subsequent analyses.

3.5.3.3 Aldehydes (PUAs) produced by macroalgae

The extraction of PUAs produced by the two macroalgae was carried out as described in Pezzolesi et al., (2021).

To quantify PUA concentrations, we used the protocol detailed by Morillo-García et al. (2014). Specifically, the analysis of extracts was conducted using an Agilent 7890A GC system (Agilent Technologies Inc., Santa Clara, CA, USA) coupled to a high-resolution mass spectrometer Waters Synapt G2 Q-TOF (Milford, MA, USA) equipped with an Atmospheric Pressure Gas Chromatography (APGC) ionization source. Capillary gas chromatography separation of PUAs was performed using an HP-5MS column (30 m x 0.25 mm inner diameter (i.d.) x 0.25 mm film thickness consisting of 5% phenyl and 95% polydimethylsiloxane), keeping the helium carrier gas flow at 1 mL/min and the injection port temperature at 280 °C. The column temperature ramp was as follows: 70 °C for 1 min, increased by 35 °C/min to 180 °C, then by 4.50 °C/min to 290 °C, and held for 8 min. Time-of-flight mass spectrometry was used for the identification and quantification of analytes (Waters Synapt G2). Atmospheric Pressure Ionization (API) positive polarity mode was selected. The mass range considered was $m/z = 50\text{--}1200$. Corona voltage was 2 kV, and the source temperature was 130 °C.

Different sampling cone voltages (from 10 to 40V) were tested. Identification of analytes was based on comparing retention times and accurate mass measurements (allowing an error of less than 5 ppm) to those for commercially available pure standards, 2E,4E-heptadienal (90%, Sigma-Aldrich Chemie GmbH, Steinheim, Germany), 2E,4E-octadienal (>96%, Sigma-Aldrich Chemie GmbH), and 2E,4E-decadienal (85%, Sigma-Aldrich Chemie GmbH). Quantification of target compounds was performed using calibration curves (from 1 to 7000 nM, prepared in hexane 2-mL vials, and taking into account the signal intensities of the standards at the pseudomolecular ions. The results were obtained by plotting the peak area of each aldehyde $[M + H]^+$ relative to the internal standard (benzaldehyde, $[M + H]^+$, 302.0609). The reproducibility and repeatability of the methods were evaluated by performing three successive extractions and injections of the sample and by re-analyzing a batch of standards two weeks after the first analysis. Chromatograms were evaluated with the MassLynx software (version 4.1, Waters, Milford, MA, USA).

3.5.3.4 Microphytobenthic assemblages

The quali-quantitative analysis of the microphytobenthos associated with the two macroalgae was performed using an inverted optical microscope (Nikon Eclipse Ti) at 320× and 200× magnification.

Subsamples (3-5 mL) of epiphytic communities fixed with Lugol were settled in counting chambers after homogenization, according to the Utermöhl's sedimentation method (Utermöhl, 1958; Edler and Elbrächter, 2010). Counting was performed with 4–5 transects and the software for image analysis and counting was NiSelement from Nikon.

The microphytobenthos was identified and recognized following various manuals and identification keys (e.g. Tomas, 1997; Kraberg et al., 2010). The identification of individuals was based exclusively on observable morphological characters (such as shape, size, number of chloroplasts).

3.5.3.5 Meiobenthic assemblages

Meiobenthic organisms of each sample were counted and identified to major taxa under a stereomicroscope (Nikon SMZ 1500). Since harpacticoid copepods were recognized as the most sensitive group to aldehydes (Lenzo, et al., 2022), live adults, copepodites, and *nauplii* (referred from now on as copepod *nauplii* and defined as the larvae of the copepod, mainly harpacticoids) were categorized into 3 different groups. Furthermore, dead copepod harpacticoids (copepodites and adults together) and dead *nauplii*, recognizable by the empty exuviae, were separately counted. Finally, taking into account that PUAs may impacts also harpacticoids reproduction causing a decreased egg viability, expelled egg sacs were also counted (Lenzo, et al., 2022). Abundance of meiobenthic organism were expressed as ind/g wet weight (ww).

3.5.4 Data analysis

Data were analyzed using univariate and multivariate techniques. The differences in all univariate variables (eg. Total PUAs, total meio and phytobenthos abundance, number of taxa) and in the structure of meio and phytobenthic populations were assessed by permutational analysis of variance (PERMANOVA; Anderson, 2001; Anderson and Ter Braak, 2003). All variables were analysed using a two-way crossed model with macroalgae (fixed, two levels: *Rugulopteryx okamurae* (RO), *Halopteris scoparia* (HS)) and sites

(random, two levels: Tarifa1 and Tarifa2) as factors.

Univariate tests were performed on Euclidean distances calculated on untransformed data (Anderson and Robinson, 2001). Multivariate analyses were carried out on Bray-Curtis similarity after square root transformation of standardized data (ind/g ww). Significant results were further analyzed by pairwise “post-hoc” comparative tests. When less than 100 unique values in the permutation distribution were available, asymptotical Monte Carlo p-values (pMC) were used instead of permutational p-values (Anderson and Robinson, 2001; Anderson and Ter Braak, 2003). The analyses were performed using “Unrestricted permutation of raw data” and 9999 permutations.

Significance levels was set at $\alpha = 0.05$ for all tests. All analyses were performed by software PRIMER 6 and PERMANOVA+.

3.5.5 Results

A total of 12 meiofauna samples and 12 microphytobenthos samples were collected from Tarifa. Environmental parameters of the seawater were measured in the sites and the temperature resulted 19°C and the salinity 40. The wet weight (g) of each replicate of macroalgae collected in the two sites was reported in Table 3.4 and used to standardize the densities of individuals, belonging to the associated meio and microphytobenthic community.

Table 3.4. Wet weights of the two macroalgae *Rugulopterix Okamurae* (RO) and *Halopteris scoparia* (HS) replicates sampled in sites Tarifa1 and Tarifa2.

Tarifa	
Sample	wet-weight (g)
Tarifa1-RO1	0.64
Tarifa1-RO2	0.6
Tarifa1-RO3	2.54
Tarifa2-RO1	0.7
Tarifa2-RO2	0.33
Tarifa2-RO3	1.47
Tarifa1-HS1	3.72
Tarifa1-HS2	1.59
Tarifa1-HS3	1.23
Tarifa2-HS1	3.9
Tarifa2-HS2	0.5
Tarifa2-HS3	2.5

3.5.5.1 PUAs results

The interpretation of the chromatograms and relative mass spectra obtained by GC-MS allowed to obtain the total concentrations of the main aldehydes produced by the macroalgae sampled in Tarifa.

Quantitative PUAs analysis highlighted RO as major producer of PUAs (Fig. 3.15) whose concentration was 15.08 nmol g⁻¹, while for HS PUAs resulted 4.74 nmol g⁻¹. The concentration found for RO was high compared to HS, but this difference was not supported by PERMANOVA analysis (Table 3.5).

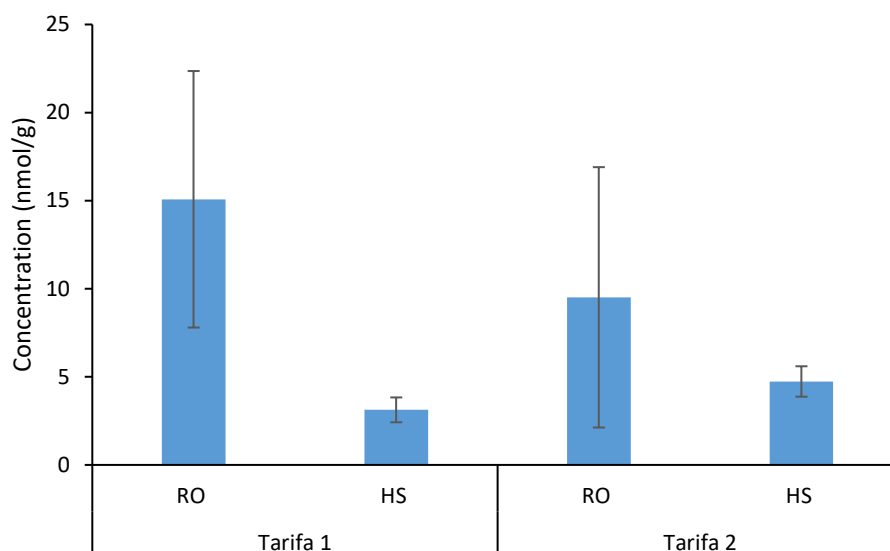


Fig.3.15- Average concentration (\pm E.S.) of PUAs produced by the two macroalgae (RO, HS) in Tarifa 1 and Tarifa 2.

Table 3.5. Results of PERMANOVA analyses performed on PUAs concentration in the two macroalgae (RO, HS). df, degrees of freedom; MS, mean square; F, F-ratio; P, probability.

PERMANOVA				
Source of variation	df	MS	F	P
ALGAE (AL)	1	200	5.44	0.27
SITE (SI)	1	14	0.18	0.69
ALxSi	1	37	0.46	0.5
RES	8	79		
TOT	11			

From a qualitative point of view, the main compounds detected were: the short-chain PUA hexadienal (C6:2) and heptadienal (C7:2), which were present in both algae, the first compound in low relative amounts, about 18% of total PUAs, while the second compound about 27% of total PUAs; middle chain PUAs decadienal (C10:2), which reported a relative abundance about 21% of total PUAs, and long chain PUAs, namely hexadecaheptaenal

(C16:7) and pentadecanal (C15:0). C16:7 showed a higher concentration in RO (relative abundance of 48%), while C15:0 showed a higher concentration in HS (relative abundance of 71%) (Fig. 3.16).

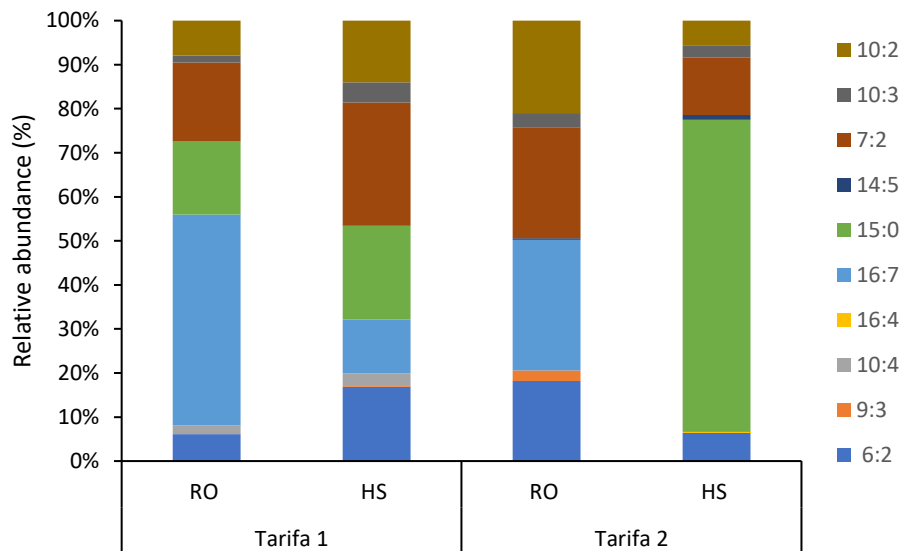


Fig. 3.16- Relative abundance of polyunsaturated aldehydes in Tarifa 1 and Tarifa 2 for each macroalga: *Rugulopterix okamurae* (RO) and *Halopteris scoparia* (HS)

3.5.5.2 Microphytobenthic community

In total 18 genera were identified, belonging mostly to diatoms and dinoflagellates.

Generally, diatoms represented the most abundant component of the microphytobenthic community in both algae, specifically, undetermined pennate diatoms (44%) and *Navicula* spp. (46%), followed by the dinoflagellate *Ostreopsis* cf. *ovata* (15% of the total organisms). The other genera were present at low concentrations (Fig.3.17).

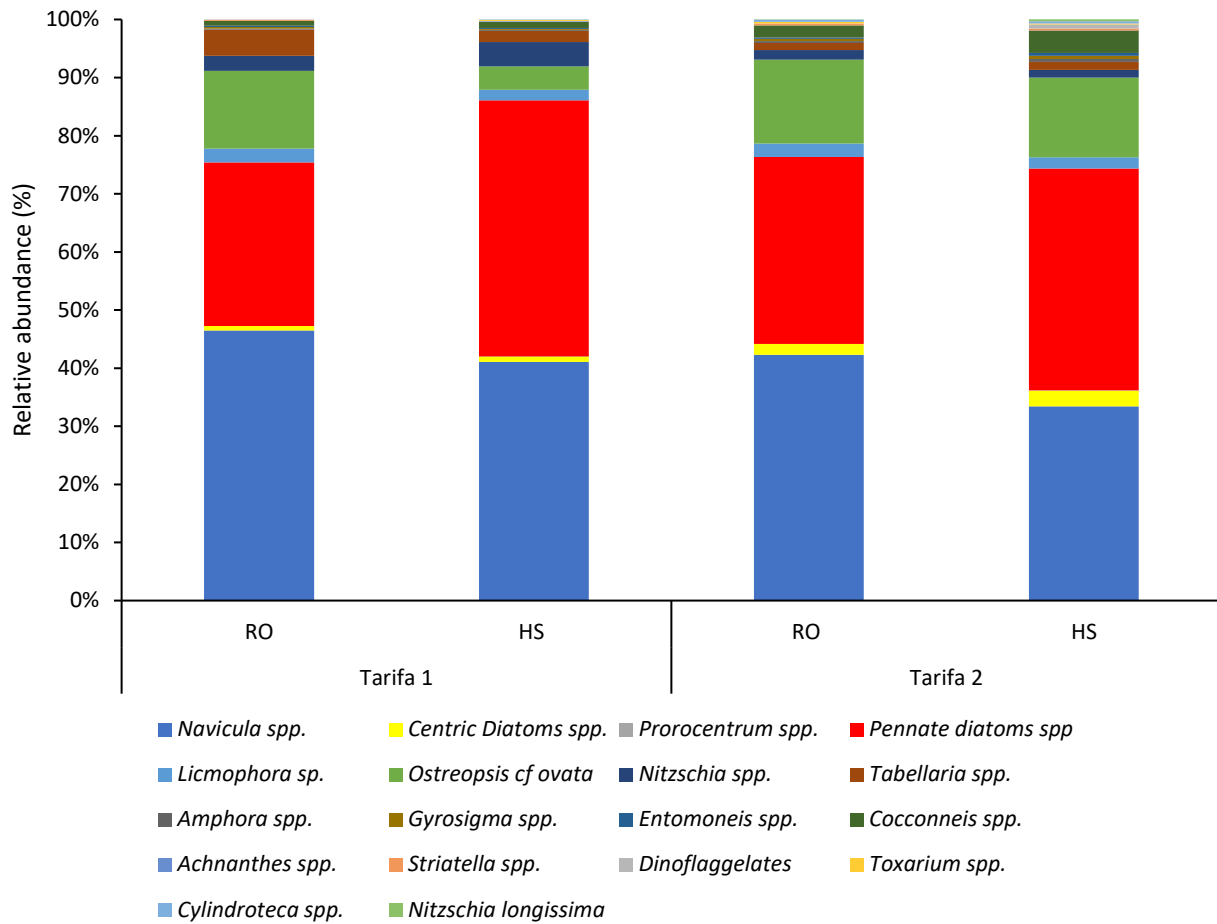


Fig. 3.17- Relative abundance of microphytobenthos in Tarifa1 and Tarifa 2, on each macroalga: *Rugulopterix Okamurae* (RO) and *Halopteris scoparia* (HS)

Microphytobenthos total abundance on the 2 macroalgae, resulted higher on HS (1,545,370± 885,533 cells/g ww) than in RO (316,874±61,288 cells/ g ww) (Fig. 3.18 A).

These differences between the two macroalgae were significant, as demonstrated by the results obtained through PERMANOVA analysis ($P < 0.05$) (Table 3.6). As for the number of taxa, high values in HS rather than RO were reached (Fig. 6 B), and this result was confirmed by the PERMANOVA analysis (Table 3.6) which highlighted a significant difference between the two macroalgae ($P < 0.05$).

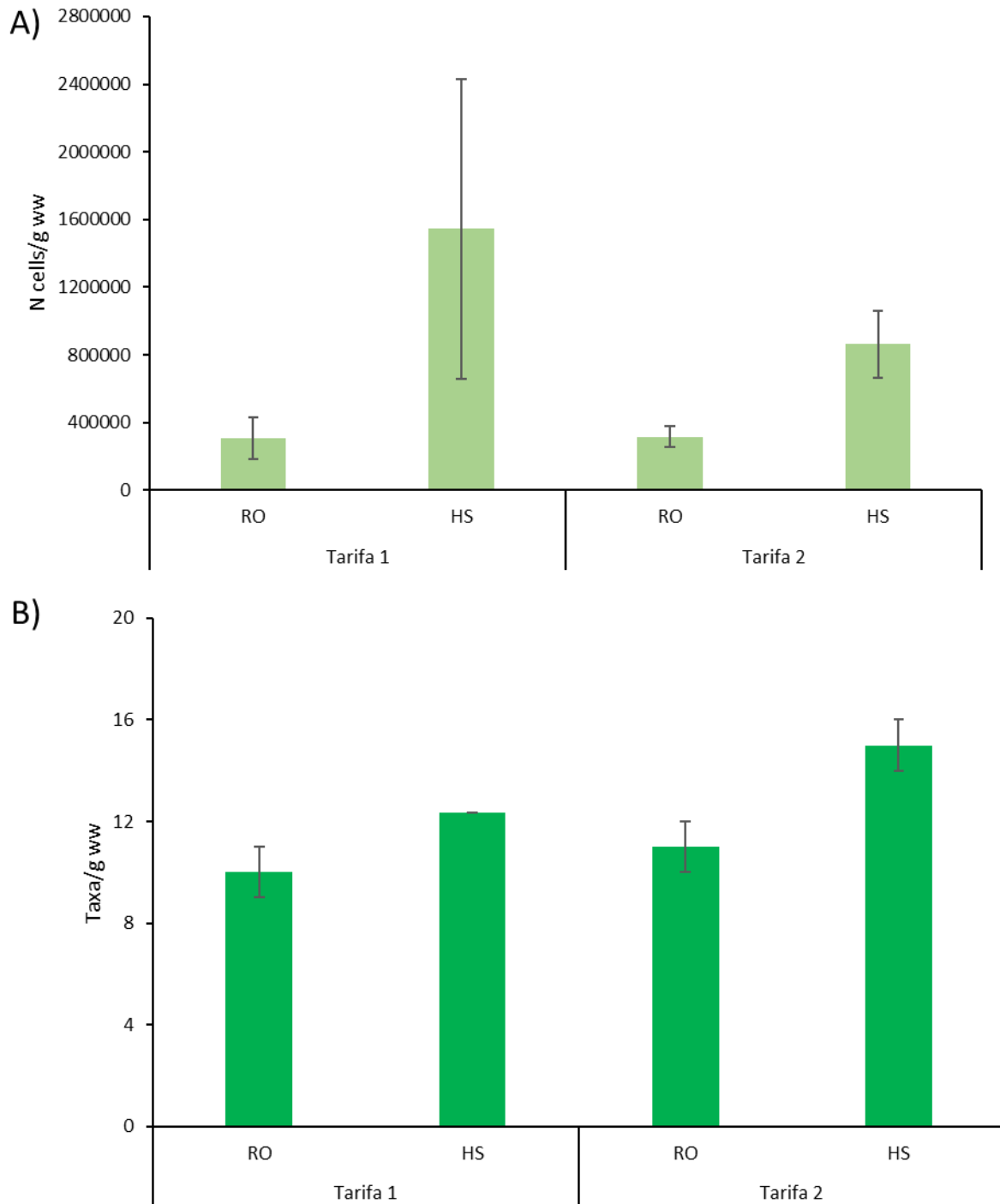


Fig.3.18-Mean values (\pm ES) of A) Total abundance of microphytobenthos (N cells/g ww), B) Number of Taxa (N° Taxa/g ww), on *Rugulopteryx okamurae* (RO) and *Halopterus scoparia* (HS).

Table 3.6 Results of PERMANOVA analysis conduct on: Total abundance and number of Taxa. df, degrees of freedom; MS, mean square; F, F-ratio; P, probability

Source of variation	Total abundance (N cells/g)				N° Taxa /g ww		
	df	MS	F	P	MS	F	P
ALGAE (AL)	1	2.39E+12	3.7847	0.0085	24.083	9.6333	0.0138
SITE (SI)	1	3.39E+11	0.53638	0.6902	10.083	4.0333	0.0913
ALxSi	1	3.58E+11	0.56679	0.6669	0.75	0.3	0.6229
RES	8	6.31E+11			2.5		
TOT	11						

The PCO run on Bray-Curtis similarity matrix on square root transformed abundance data, showed that the first two principal components accounted for 78 % of the variation on the dataset (Fig. 3.19). The arrangement of the sample points suggested significant differences between microphytobenthic assemblages on the two macroalgae, as showed by the PERMANOVA results ($P < 0.05$) (Table 3.7).

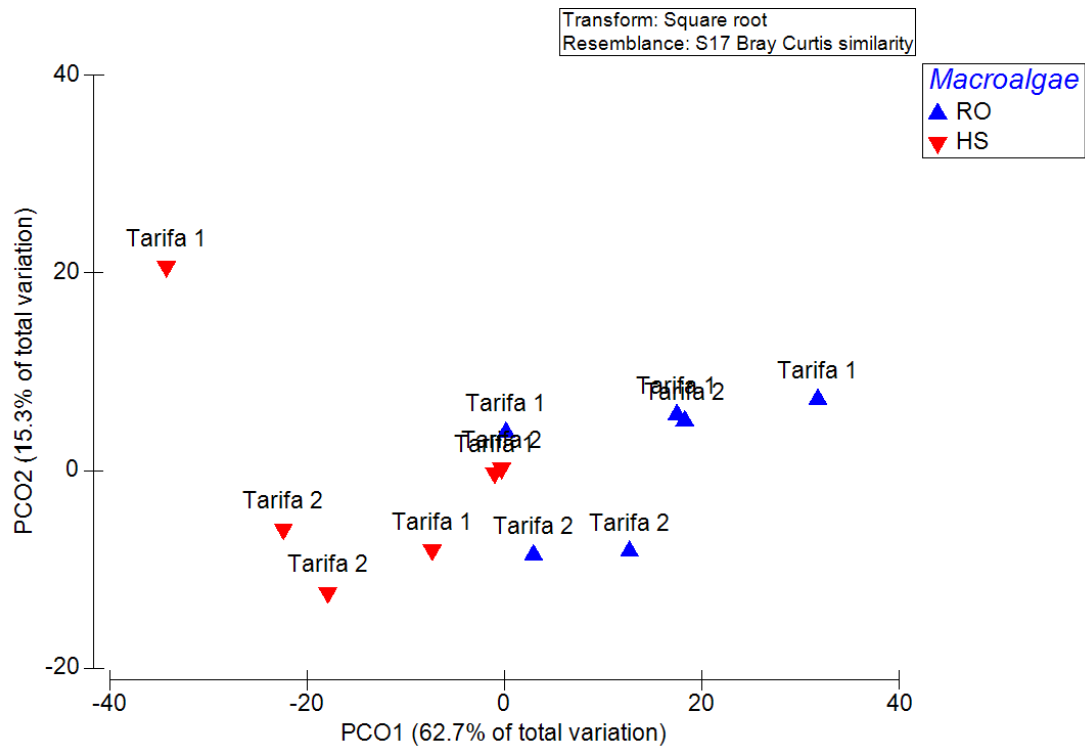


Fig.3.19- Principal coordinates analysis (PCO) plot of component microphytobenthic genera in *Rugulopteryx okamurae* (blue triangles) and *Halopteris scoparia* (red inverted triangles) based on Bray-Curtis similarity matrix, samples are indicated by Tarifa1 and Tarifa2.

Table 3.7- Results of PERMANOVA testing for differences in microphytobenthos community structure between macroalgae *Rugulopteryx okamurae* (RO) and *Halopteris scoparia* (HS). df, degrees of freedom; MS, mean square; P, probability.

PERMANOVA				
Source of variation	df	MS	F	P
ALGAE (AL)	1	2395.4	6.0069	0.0065
SITE (SI)	1	411.78	1.0326	0.3423
ALxSi	1	162.48	0.40745	0.8466
RES	8	398.77		
TOT	11			

SIMPER results (Table 3.8) revealed that communities of RO and HS showed an average dissimilarity of 53.09%. The main genera responsible for this dissimilarity were the undetermined pennate diatoms, *Navicula* spp., *Ostreopsis* cf. *ovata*, all more abundant on HS.

Table 3.8- Results of SIMPER analysis based on square root transformed data, used to identify microphytobenthic organisms that mostly contribute to dissimilarity between macroalgae. Av.Ab, mean abundance; Diss, mean dissimilarity; Diss/SD, dissimilarity/standard deviation; Contrib%, contribution relative to single taxon; Cum%, cumulative contribution.

Groups	Species	Av.Ab	Av.Ab	Av.Diss	Diss/SD	Contrib%	Cum.%
Diss		Ro	Hs				
RO & HS	Undet. pennate diatoms	94284.52	505369.7	24.6	2.66	46.4	46.4
53.03	<i>Navicula</i> spp.	138467.8	462044.7	16.62	1.33	31.34	77.74
	<i>Ostreopsis</i> cf. <i>ovata</i>	43412.92	90642.8	4.39	1.14	8.28	86.03
	<i>Cocconneis</i> spp.	4698.87	24394.22	1.46	1.26	2.75	88.78
	<i>Nitzschia</i> spp.	6580.04	37756.36	1.33	0.85	2.52	91.29

3.5.5.3 Meiobenthic community

Meiobenthic organisms were identified at the major taxa level. Copepods were counted also as *nauplii*, and copepodites and adults. Copepods and their *nauplii* were counted separately in view of their different ecology (Hicks and Coull, 1983). Moreover, dead *nauplii* and adults were counted.

A total of 13 taxa belonging to the meiobenthos were identified on the two macroalgae, two of these taxa represented by larval stages of Copepoda *nauplii* and copepodites. Nematoda (50.4%) resulted the dominant taxon followed by Harpacticoida (10.8%) and copepod *nauplii* (9.6%). While the other taxa were Polychaeta (12.8%), Gastropoda (6.9%), Bivalvia (5.0%), Isopoda (0.5%) Amphipoda (0.3%), Halacaroidea (0.5%), Kinorhyncha (0.2%), Ostracoda (0.4%) and Chironomidae (0.08%).

The percentage composition on the two macroalgae was represented in Fig. 3.20. Nematoda represented more than 50% followed by Polychaeta (about 10-17%) and

Harpacticoida (about 10-15%). Ostracoda, Halacaridae and Isopoda were present at low concentrations.

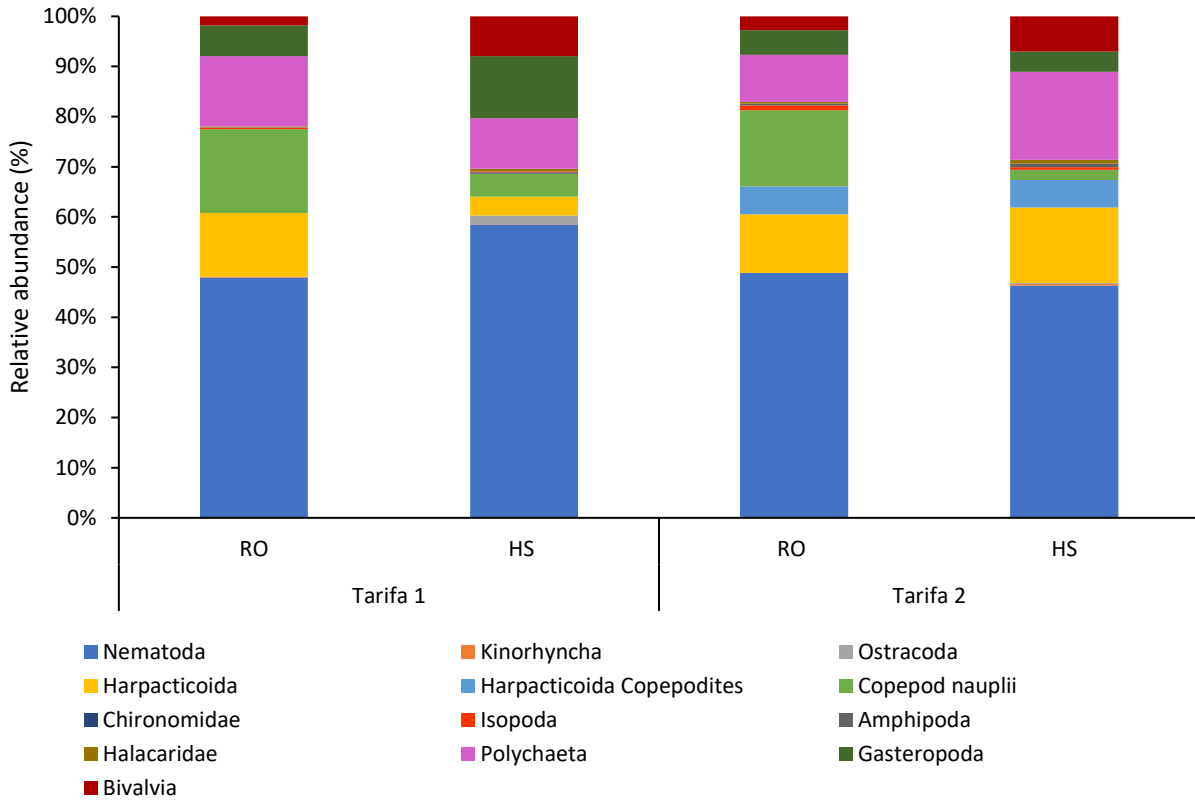


Fig. 3.20- Relative abundance of meiobenthic taxa in sites Tarifa 1 and Tarifa 2, on each macroalga: *Rugulopterix Okamurae* (RO) and *Halopteris scoparia* (HS)

Meiofauna total abundance on the 2 macroalgae resulted higher on RO than HS (Fig. 3.21 A) but only in Tarifa 2. This difference resulted not significant, as demonstrated by the results obtained through PERMANOVA analysis (Table 6). As regards the number of taxa, high values were reached in both RO and HS macroalgae (Fig. 3.21B), in particular in Tarifa2. This result was confirmed by the PERMANOVA analysis (Table 3.9), which highlighted significant differences between the two sites.

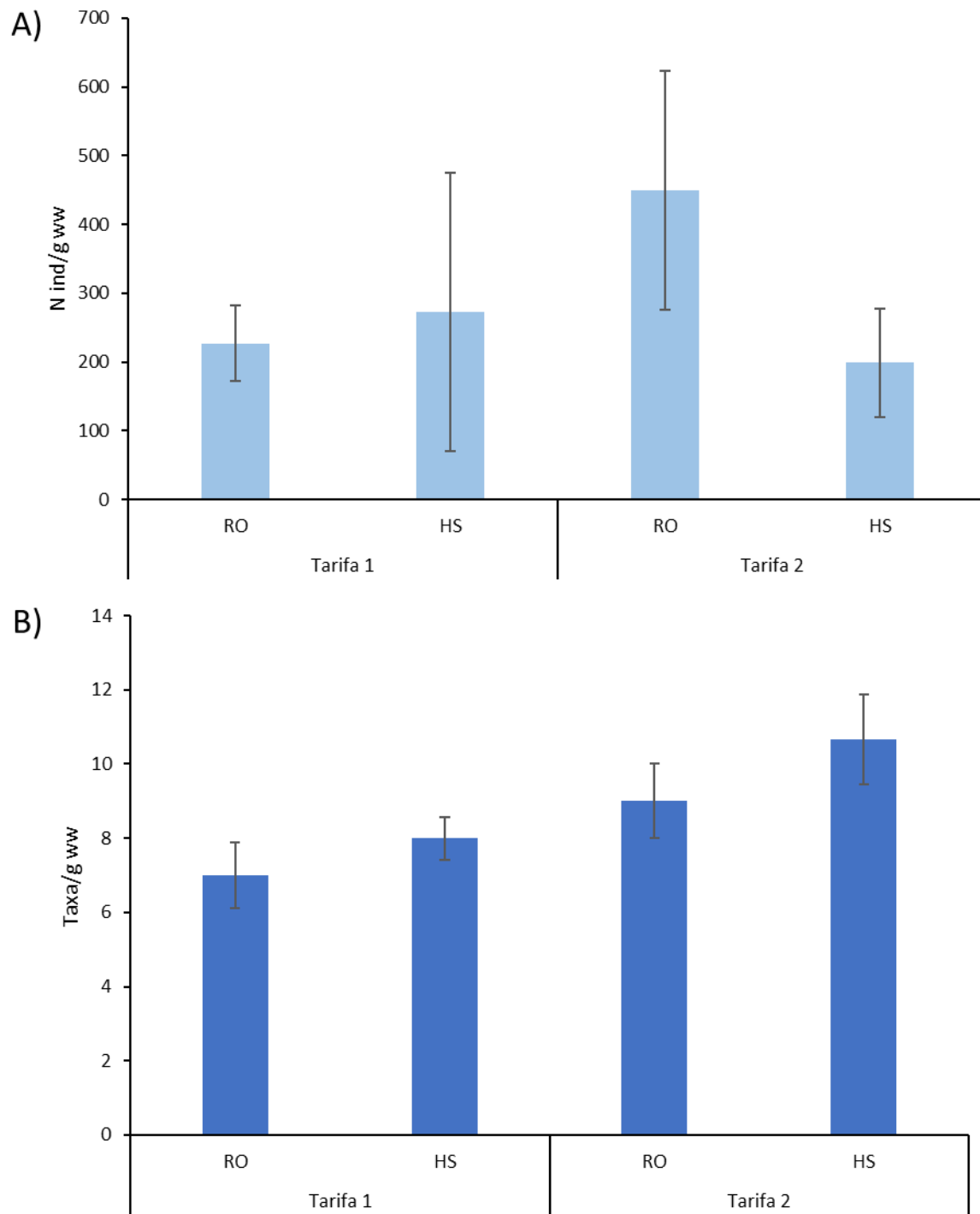


Fig.3.21- Mean values (\pm ES) of A) Total abundance of meiofauna (N ind/g ww), B) Number of Taxa (Taxa/g ww) in sites Tarifa 1 and Tarifa 2, on *Rugulopteryx okamurae* (RO) and *Halopterus scoparia*.

Table 3.9 Results of PERMANOVA analysis conduct on: meiobenthic total abundance and number of Taxa. df, degrees of freedom; MS, mean square; F, F-ratio; P, probability.

Source of variation	Total abundance (N ind/g)				Taxa /g ww		
	df	MS	F	P	MS	F	P
ALGAE (AL)	1	31485	0.54	0.47	5.33	2.91	0.13
SITE (SI)	1	16559	0.29	0.58	16.33	8.91	0.02
ALxSi	1	65725	1.14	0.32	0.33	0.18	0.65
RES	8	57794			1.83		
TOT	11						

The PCO run on Bray-Curtis similarity matrix on square root transformed abundance data, showed that the first two principal components accounted for 77.6 % of the variation on the dataset (Fig. 3.22). The arrangement of the sample points suggested differences between meiobenthic assemblages on the two macroalgae, but a very large variability between sites and replicates. PERMANOVA results did not show significant effects due to macroalgae (Tab. 3.10).

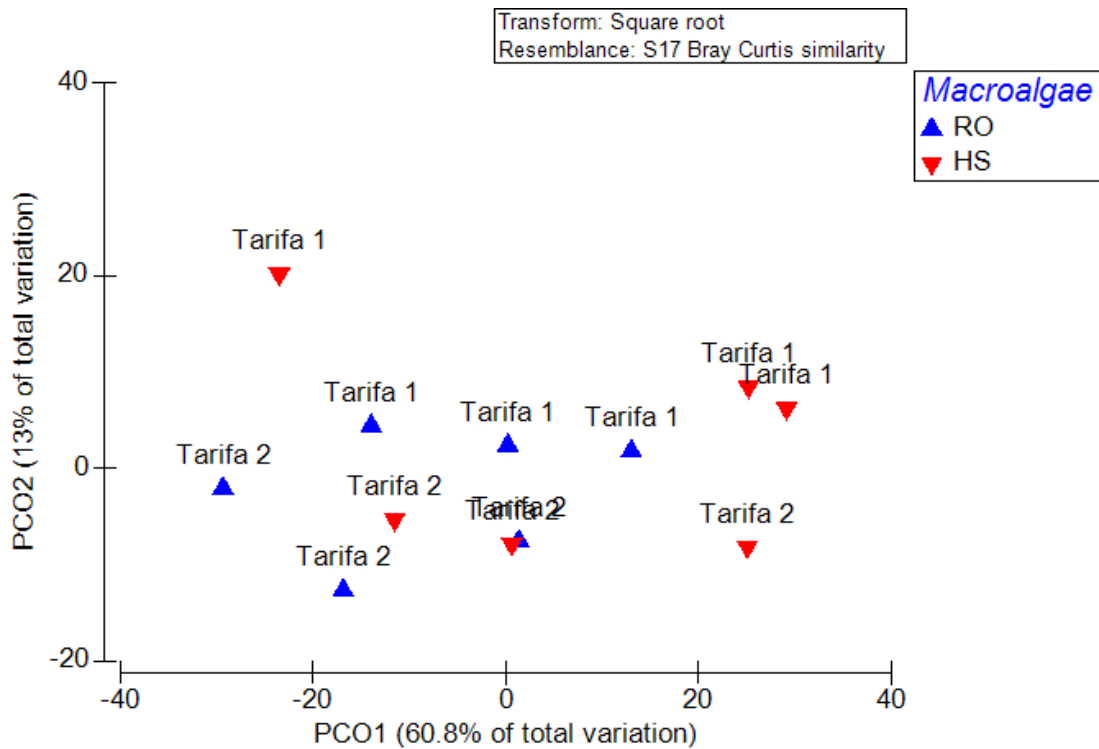


Fig.3.22 Principal coordinates analysis (PCO) plot of component meiobenthic taxa in *Rugulopteryx okamurae* (blue triangles) and *Halopteris scoparia* (red inverted triangles) based on Bray-Curtis similarity matrix, samples are indicated by Tarifa1 and Tarifa2.

Table 3.10- Results of PERMANOVA testing for differences in meiobenthic community structure between macroalgae *Rugulopteryx okamurae* (RO) and *Halopteris scoparia* (HS). df, degrees of freedom; MS, mean square; P, probability.

PERMANOVA				
Source of variation	df	MS	F	P
ALGAE (AL)	1	1112.3	1.95	0.13
SITE (SI)	1	1145	2.01	0.12
ALxSi	1	268.89	0.47	0.75
RES	8	569.21		
TOT	11			

SIMPER results (Table 3.11) revealed that communities of RO and HS showed a very low average dissimilarity of 35.18%. Taxa main responsible for this dissimilarity were Nematoda, *nauplii* and adult Copepoda, both more abundant on RO.

Table 3.11- Results of SIMPER analysis based on square root transformed data, used to identify organisms that mostly contribute to meiofaunal dissimilarity between macroalgae. Av.Ab, mean abundance; Diss, mean dissimilarity; Diss/SD, dissimilarity/standard deviation; Contrib%, contribution relative to single taxon; Cum%, cumulative contribution.

Group Diss	Species	Av.Ab RO	Av. Ab HS	Av.Diss	Diss/SD	Contrib%	Cum.%
RO vs HS 35.18	Nematoda	12.27	9.98	7.22	1.65	20.52	20.52
	<i>Nauplii</i>	6.92	2.24	6.65	1.87	18.91	39.43
	Harpacticoida	6.1	4.2	3.59	1.42	10.19	59.94
	Copepodites	2.42	1.49	3.19	1.25	9.07	69.01
	Polychaeta	5.61	5.03	3.63	1.49	10.32	49.75
	Bivalvia	2.16	3.83	3.02	1.56	8.58	77.58

Development stage abundance of harpacticoid copepods analysis showed (Fig. 3.23A) that copepodites were found only on RO and HS in Tarifa 2, instead copepod *nauplii* and adult copepods were found on both macroalgae in the two sites. In Tarifa 2 a greater amount of *nauplii* than that found on Tarifa 1 occurred on both RO and HS. Anyway, the highest abundance of *nauplii* was on RO in Tarifa 2 (about 70 N ind/g ww), while on HS the presence of *nauplii* was low in both sites.

As for the dead *nauplii*, these were found in both sites of Tarifa, while dead adults were found only in HS but at extremely low abundance (Fig. 3.23 B).

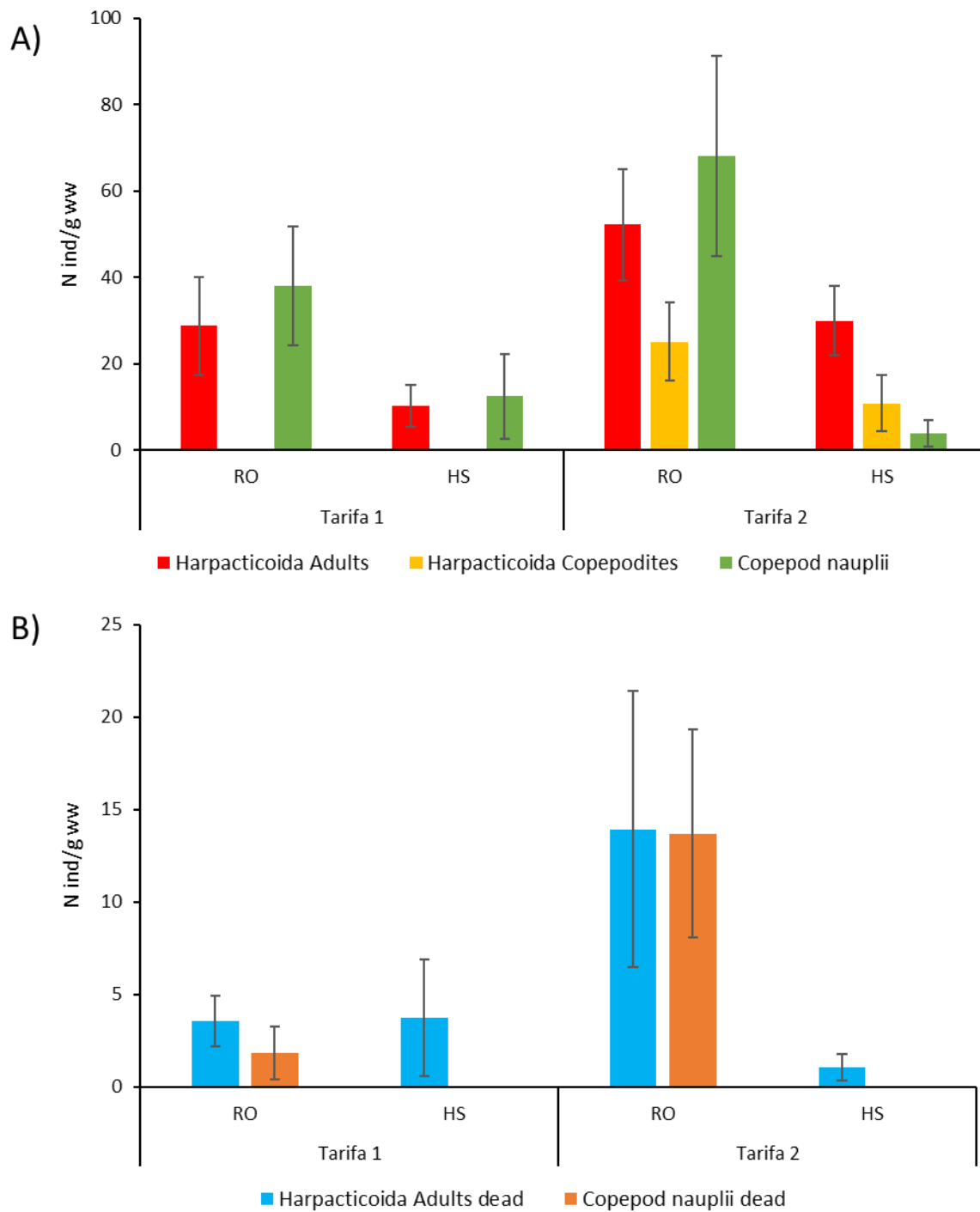


Fig.3.23- Mean values (\pm E.S) of A) Densities (N ind/g ww) of *nauplii*, copepodites and adults B) of dead *nauplii* and dead adults (N ind/g ww) on *Rugulopteryx okamurae* and *Halopteris scoparia* .

3.5.6 Discussion

The relation between the fauna and its habitat has been studied for a long time (Gunnill, 1982; Taylor and Cole, 1994; Parker et al., 2001; Christie et al., 2009) and several studies have shown that habitat type influences the associated fauna (Attrill et al., 2000; Chemello and Milazzo, 2002; Tuya et al., 2011).

To my knowledge no studies have concerned the benthic environment and included the potential involvement of macroalgae, considering invasive and non-invasive species in terms of interactions between various organisms and the production of allelochemicals.

Moreover, some recent studies (e.g., Pezzolesi et al., 2021) reported as some macroalgal species (i.e. *Dictyopteris. polypodioides* and *Ulva. cf. rigida*) could produce higher PUAs amounts than others, and even a wider variety of aldehydes (e.g. with a different length of the carbon chain and number of unsaturations).

In the present study, it was analyzed the PUAs production by one non-invasive and one invasive macroalgal species.

The invasive species (i.e., *Rugulopterix okamurae*) PUAs production was higher than that of *H. scoparia*.

Rugulopterix okamurae is known also to produce a high content of the compound dilkamural (Casal-Porras, et al., 2021), a natural product that had been found in a Japanese sample of *Rugulopterix okamurae* (Ninomiya et al., 1999). Moreover, in spite of the wide chemical research performed on the natural products from algae around the world (Carroll et al., 2019), a survey of the literature showed that so far dilkamural has only been described from the brown alga *Rugulopterix okamurae* (Ninomiya et al., 1999).

The microphytobenthic community consists of microalgae associated with benthic substrates, although it often also includes cells or colonies of phytoplankton species. Generally, in temperate zones the main component of the microphytobenthos is represented by diatoms (Totti et al., 2019), as observed also in the present study, in which diatoms are the taxon that prevails over the whole analysed community.

Coversely, benthic dinoflagellates are the major component in tropical areas, where they are known for the production of toxins that can be harmful to human health and animal organisms (Turner et al., 2021). However, benthic dinoflagellate blooms have also become common in temperate regions, and in the last decade, in the Mediterranean Sea blooms of the toxic dinoflagellate *Ostreopsis cf. ovata* have been widely reported (Accoroni et al., 2016a).

In the present study, dinoflagellates were present in low concentrations, although a bloom of *Ostreopsis cf. ovata* (representing about 15% of the community in terms of abundance) was detected. During the bloom of *Ostreopsis*, the characteristic brownish filamentous layer that completely covered the entire benthic substrate was also observed. These results are in agreement with what was previously observed in recent studies (Accoroni et al. 2016a, Gémin et al., 2020, Lenzo et al., 2021), which report maximum concentrations on macroalgae comparable to those found in the present study, while differences in the blooming period (season) are documented, starting from the end of July, even if in low concentrations, and having its maximum peak between the end of September and the beginning of October.

In the present study, the bloom of *Ostreopsis cf. ovata*, was found in early November, probably due to the environmental variables of the studied area.

In general, a greater abundance of epiphytes was reported on *Halopteris scoparia*, compared to *Rugulopterix okamurae*, regardless of the site. Furthermore, microscopic analyses also showed an unhealthy and dying community (empty cells) on *Rugulopterix okamurae*, compared to the community present on *Halopteris scoparia*, in which cells appeared healthy (Fig. 3.24).

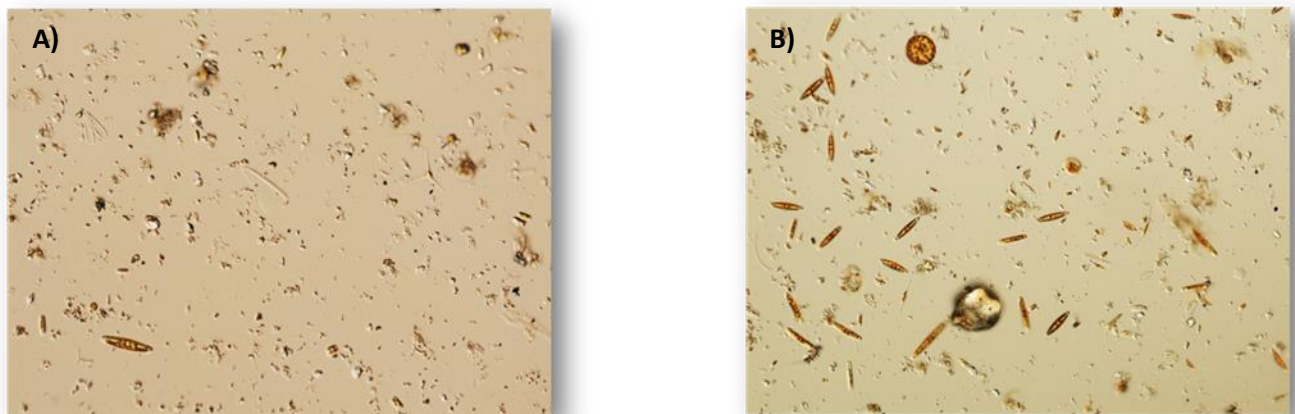


Fig 3.24- Microphytobenthic community analyzed under the microscope, in which in A) cells appear unhealthy and empty on *R. okamurae*, in B) cells are abundant and healthy on *H. scoparia*.

These results can be explained by the fact that the invasive macroalga *Rugulopterix okamurae*, in addition to the production of PUAs, produces a series of secondary metabolites in high concentrations, including the toxic compound dilkamural (Casal-Porras et al. 2021), which could impact the viability of the epiphytic community.

Instead, *Ostreopsis* cf. *ovata* did not seem to be negatively affected, in terms of abundance, by this compound, in fact this toxic dinoflagellate resulted abundant (cell/g ww) in both algae. In a previous study, Pichierri et al., (2016) had already noted that *O. cf. ovata* could be less affected by allelochemicals, such as PUAs, than other microalgae. This difference in the response of *Ostreopsis* can be explained by the toxic potential of this dinoflagellate, that in turns is able to produce several potent toxins (namely ovatoxins). Furthermore, a variability due to the cytological and/or metabolic characteristics of the algal cells has also been reported, in fact the sensitivity towards PUAs or other allelochemicals may depend on various factors, such as cell size, wall properties and lipid content. In particular, it has been reported that species with a well-structured and mineralized wall, a low surface-to-volume ratio and a certain lipid content can limit the ability of PUAs to penetrate the cell (Ribalet et al., 2007). Finally, an important role is also played by the production of mucilage by the cell, which provides an additional barrier against the substances present in the water column (Allen et al., 2016). Therefore, the presence of a rigid cover and a high volume may explain the apparent lower sensitivity of *Ostreopsis* to PUAs, but also to other metabolites produced by macroalgae (for example dilkamural) compared to other species (Fig. 3.25).

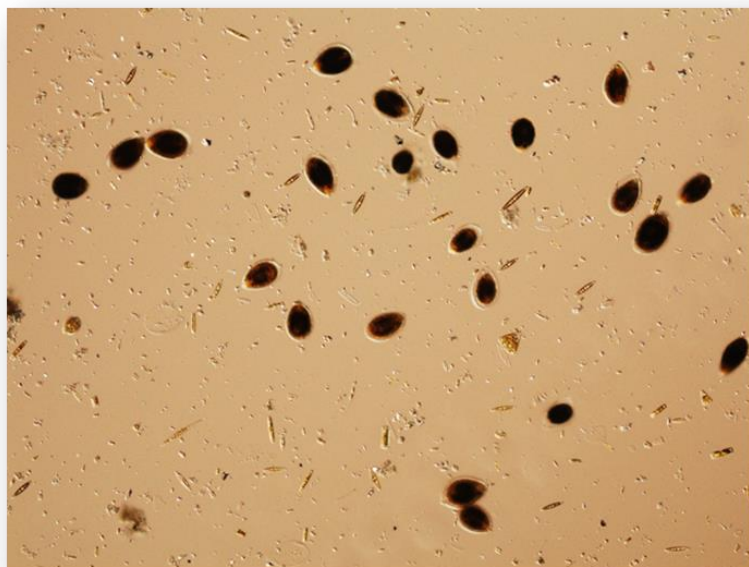


Fig. 3.25- Bloom of *Ostreopsis* cf. *ovata*

Regarding meiobenthic community, in the present work an analysis was made on the entire meiobenthic assemblages associated with the macroalgae and identification was made at the level

of major taxa to evaluate the effect of the main PUAs produced. In rocky intertidal environments, macroalgae constitute a habitat for many mobile organisms associated with them, and small invertebrates are present in these habitats at high densities (Gibbons & Griffiths, 1986). In the present study, the analysis of meiobenthic samples revealed a rich animal community associated with the different macroalgae. The most abundant taxon was Nematoda (50%), followed by Polychaeta and adult copepods. By comparing the results occurred for the two macroalgae of Tarifa, results did not highlight differences between the two communities associated with *R. okamurae* and *H. scoparia*: both were complex and with a variety of taxa; however, the lack of difference between the two community structures could be explained by the phenomenon of confounding between the studied macroalgae, in fact the invasive species *R. okamurae* at the time of sampling was strongly mixed with *H. scoparia*, to the point of making separation very difficult.

In Tarifa only the invasive species *R. okamurae* hosted dead copepod *nauplii* and the greatest abundance of dead adult copepods. Studies carried out on planktonic ecosystems have shown deleterious effects of PUAs produced by diatoms on the reproduction of copepods, which feed on them (Ianora et al., 2003, 2012; Miralto et al., 1999), as well as apoptosis in maturing oocytes (Poulet et al., 2007a) during embryo development (Romano et al., 2003) and in newly hatched *nauplii* (Ianora et al., 2004b). It has to be considered that the present study was carried out in field, so the link between PUAs effects on the community structure was more difficult to evaluate, due to the variability of PUAs production both by diatoms (Wichard et al., 2005a, 2005b) and macroalgae (Pezzolesi et al., 2021), but also to the production of the toxic compound dilkamural by *R. okamurae* (Cuevas et al., 2021), to the copepod sensitivity (Ianora et al., 2003; Sommer, 2009) and to the detoxification ability developed by certain species of copepods (e.g., Taylor et al., 2007; Wichard et al., 2008).

The results of the present study, which analyzed the allelopathic interactions within the phyto and meiobenthic community in a highly complex environment (characterized by the presence of the invasive alga *R. okamurae*), constitute a valid starting point for subsequent research aimed at study the possible implications of these compounds (i.e., PUAs and dilkamural) at the level of the trophic chain, thus evaluating the possible effects on invertebrates, with the targeted exposure of these compounds in laboratory studies.

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Chapter 4 - Effects of microalgal allelochemicals on meiobenthic community: a microcosm study

4.1 Introduction

Marine diatoms species produce an overabundance of bioactive compounds. A group of these chemicals are the polyunsaturated aldehydes (PUAs), an oxylipin-type compound, that have been found to be highly reactive (Caldwell, 2009).

PUAs are produced after lipoxidation of intracellular polyunsaturated fatty acids (PUFAs), after the cell membrane has been damaged (Bartual et al., 2020). In nature, processes producing the disruption of phytoplankton cells are viral infection, grazing or/and cell lysis during senescence (Bartual et al., 2014). As cells die, its content and derived compounds, such as these PUAs, are released into the surrounding medium. In the vicinity of the broken cell, the released PUAs create microzones, where they react with other dissolved chemicals and interact with the neighboring organisms.

To date, different types of interactions involving PUAs have been described, and different ecological roles of these compounds have been proposed. Released PUAs have been suggested as a chemical defence in diatoms against grazers. Likewise, deleterious effects on different plankton species have also been reported (Ianora et al., 2003; Romano et al., 2010).

In situ studies in planktonic systems have indicated that PUAs may exert an important influence on phytoplankton– zooplankton interactions, potentially limiting the population growth of pelagic grazers (Ianora et al., 2004; Poulet et al., 2006). This is an example of bottom-up control of herbivore populations, although several field surveys and mesocosm experiments report contradictory findings (Poulet et al., 2006; Wichard et al., 2008). There is, in fact, ongoing discussion as to the significance of PUAs impacts in the natural environment (Flynn and Irigoien, 2009). PUAs are cytotoxic (Caldwell et al., 2011) and, depending on the degree of exposure, adverse effects may be manifested, including parental sterility/infertility, abortive copepods embryogenesis, impaired hatching success, reduced larval fitness and mortality of *nauplii* (Caldwell, 2009; Ianora et al., 2004; Poulet et al., 2007).

Few studies have been directed to the benthic aspects of PUA-diatom-grazer chemical ecology, as many have focused on planktonic pathways.

In the benthic environment, PUA production has been reported in marine and freshwater diatoms; (Wichard et al., 2005).

Among the most studied PUAs-producing diatoms is *Skeletonema marinoi*, a cosmopolitan species that forms dense coastal blooms (Gallina et al., 2014; Ianora et al., 2004b), and produces several different PUAs, such as: heptadienal, octadienal and octatrienal (Ribalet et al., 2007).

Among the major damages to the microphytobenthos cell walls that trigger the release of PUAs, is the feeding by meiobenthic organisms, including harpacticoids, which can exert high grazing pressures (Taylor et al., 2012) and can be exposed to PUA for an entire or even several generations. Some studies have reported a species-specific effect of PUAs on benthic communities (Lenzo et al., 2022; Taylor et al., 2007), while other authors have found no effects of these compounds on harpacticoid copepods (Taylor et al., 2012).

It is difficult to understand the interactions between diatoms and meiobenthic assemblages, as these organisms are found in a wide range of habitats from tidal pools to sediments deposited on and macroalgae (Taylor et al., 2012). Compared to pelagic compartment, benthos is a relatively harsh environment with rapid changes in physical conditions associated with tidal regimes and the possibility of accumulations of both organic and inorganic toxicants within sediments (Raisuddin et al., 2007).

Thus, these organisms must be highly adapted to survive in such an environment, and it is hypothesized that they may have a more developed detoxification system than that of planktonic organisms and therefore be more resistant to chemical compounds (Young Mi Lee et al., 2005).

In this context, to better understand the relationship diatoms-PUAs-meiobenthos, the purpose of this study is to evaluate, through laboratory experiments with microcosm: i) the effect of *Skeletonema marinoi*, which is a diatom known to produce PUA, compared to *Phaeodactylum tricornutum*, a diatom that does not produce PUA, on the meiobenthic assemblages associated with the macroalga *Dictyopterus polypodioides*, over several weeks; ii) the effects of a decadienal (PUA C10:2) in the form of an analytical standard, on meiofauna associated with *D. polypodioides*, in a short-term (96 hours) test.

4.2 Materials and methods

4.2.1 Microcosms experiment with *Phaeodactylum tricornutum* and *Skeletonema marinoi*

The meiofauna used to populate the mesocosms was collected at a site locally known as Piscinetta del Passetto (Conero Riviera, Italy, northern Adriatic Sea: 43°37'09" N, 13°31'54" E), a semi-enclosed and shallow inlet (mean depth 1.5 m), sheltered by a natural reef, and characterized by a rocky bottom partially covered by cobbles. The sampling was carried out on 3 June 2020.

Apical parts of the thalli (first 5-8 cm) of the macroalga *Dictyopterus polypodioides* (Phaeophyceae), with their associated meiofauna, were collected by snorkeling in 90 polyethylene tubes (50 mL).

About 15 liters of sea water were taken for washing the samples and it was subsequently filtered using GF/F Whatman filters (0.7 µm porosity, 47 mm diameter).

For the experiment comparing the effect of different microalgae species on the meiofauna, two diatoms were grown as monosporic cultures: *Skeletonema marinoi* (which produces PUAs) and *Phaeodactylum tricornutum* (which does not produce PUAs).

Fiftysix microcosms were set up, each made of a sterile 250 mL Erlenmeyer flask to which 10 ml of either *S. marinoi* or *P. tricornutum* culture were added, with a density of 633966 cells/ml and 588466 cells/ml, respectively.

The meiofauna associated with the macroalgae sampled in the field was separated by shaking the thalli directly in the tubes used to collect them and rinsing several times with filtered sea water. The rinsing water was then sieved at 63 µm. Retained organisms were rinsed from the sieve with filtered seawater using a wash bottle and all the aliquots pooled in one container. Using a wide orifice pipette, 40 mL of seawater containing the meiofauna were taken from the container and added to each 250 mL flask previously prepared with microalgae. Finally, the volume in each flask was brought up to 150 mL with filtered seawater.

The flasks were placed in an incubator at 18°C, light intensity 130 µmol m⁻² s⁻¹, 16 h light and 8 h dark. Four flasks for each treatment (microalgae species) were destructively sampled immediately after they were set up (time 0) and then at one week intervals (times 1 to 6). At any time, the meiofauna samples were sieved through a 45 µm mesh and the meiobenthic organisms retained were killed and preserved with 70% alcohol, and stored in a 50 ml tube for the analysis, while the water and the residual microalgae were separated by centrifugation and stored at -80°C for subsequent PUAs analysis.

4.2.2 Microcosms experiment with decadienal standard

For the experiment testing the effect of decadienal, 4,8 mg/ml stock solution was prepared diluting a decadienal analytical standard in methanol (MeOH). All reagents were purchased from Sigma-Aldrich (Milan, Italy) and used without any further purification.

Thirty two microcosms were set up, each made of a sterile 250 mL Erlenmeyer flask to which 10 mL of *Phaeodactylum tricornutum* culture were added. Meiofauna to populate the microcosms was obtained and added to the flasks as already described. Aliquots of MeOH or decadienal stock solution were added as required to obtain eight treatments: a control, a solvent control (1.5 mg/ml MeOH) and the six decadienal concentrations (C1: 1.6 mg/L, C2: 3.1 mg/L, C3: 6.3 mg/L, C4: 12.5 mg/L, C5: 25 mg/L, C6: 50 mg/L). Finally, the volume was brought up to 150 mL with filtered seawater. Four replicate flasks were set up for each treatment and kept in an incubator at the same conditions described above. After 96 h the experiment was terminated and the meiofauna of each flask collected and preserved as described above.

4.2.3 Meiofauna and harpacticoid copepods analysis

Meiofaunal organisms of each sample were counted and identified at higher taxa level. All harpacticoids were picked out under a stereomicroscope (Nikon SMZ 1500) and stored in 70% alcohol inside 1.5 mL-Eppendorf tubes labeled for subsequent identification. Harpacticoids were identified to species level using Lang (1948, 1965), and Boxshall & Halsey (2004).

The meiofauna community structure of the microcosms was analyzed by non-metric multidimensional scaling (nMDS) based on Bray-Curtis similarity of square root-transformed data. Effect of time or treatments on the community structures due to time or treatments were assessed by permutational non-parametric multivariate analysis of variance (PERMANOVA) (Anderson, 2001, Anderson et al., 2005) and pairwise comparisons were done after significant overall effects was detected.

The toxicity of PUAs on copepods was expressed as EC50, which is the concentration of PUA inducing 50% effect relative to the controls, after a 96 h exposure.

All multivariate analyses were carried out with PRIMER v7 (Clarke and Gorley, 2015) with the PERMANOVA + add on (Anderson et al., 2008). EC50 were estimated with a three-parameter log-logistic function using the drc package in R (Ritz et al 2015).

4.3 Results

4.3.1 Comparison between *Phaeodactylum tricornutum* and *Skeletonema marinoi*

A total of 12 major taxa belonging to the meiobenthos were identified, one represented by *nauplii*, the larvae of harpacticoid copepods. Copepods (adults plus their copepodites) and their *nauplii* were counted separately due to their different ecology (Hicks and Coull 1983).

ANOVA did not detect significant differences in total abundance and number of taxa between microcosms with *P. tricornutum* and microcosms with *S. marinoi* (Table 4.1). The nMDS plot of meiofauna communities (Fig. 4.1) did not show a clear separation between the two microalgae but, for each microalga, the composition of the meiofaunal assemblage changed over time. PERMANOVA supported this result, with a significant difference among times ($P \leq 0.05$) (Table 4.2). In particular, the assemblages at time T4, T5 and T6 were all significantly different from T0, as shown by the post hoc comparisons.

SIMPER analysis revealed that average similarities among microcosms with the same microalga were 69.1% for Pt and 67.8% for Sm. Moreover, the dissimilarities between times ranged from 27% to 38%. Both similarities and dissimilarities were largely due to the variations in abundance of the following dominant taxa: bivalvia, copepoda, copepod *nauplii*, gastropods, and cirripeds.

In Pt microcosms (Fig. 4.2), the number of Bivalvia increased from T0 ($2,25 \pm 1,93$) to a maximum in T2 ($14,75 \pm 6,1$), while the average number of Copepoda increased from T0 ($49,75 \pm 9,7$) to T6 ($60,75 \pm 16,2$). *Nauplii* showed a peak at T2 ($40,25 \pm 15,4$) and at the following time decreased. Gastropoda had a constant temporal trend, with a maximum at T2 ($21,75 \pm 12,5$); Cirripeda, showed two peaks at times T5 ($6,75 \pm 3,3$) and at T6 ($4,25 \pm 2,1$), at the other times they were absent or very scarce.

In Sm microcosms (Fig. 4.3), Bivalvia, Copepoda, Gastropoda, and Cirripeda had higher mean abundances at each time when compared with Pt microcosms. In particular the density of Copepoda showed a peak at T4 ($95,75 \pm 32,8$). Conversely, *nauplii* were less abundant in Sm microcosms, with a maximum at T4 ($16 \pm 7,5$).

Table-4.1. Results of ANOVA conducted on the taxa diversity and taxa abundance in laboratory microcosms in the presence of either *Phaeodactylum tricornutum* or *Skeletonema marinoi*

Source	df	ANOVA Taxa diversity			ANOVA Taxa abundance		
		MS	F	P	MS	F	P
time	6	0.90	0.49	0.81	1436.10	0.63	0.70
microalga	1	3.02	1.64	0.21	92.57	0.04	0.84
Time x microalga	6	3.48	1.89	0.11	2141.80	0.95	0.48
residual	42	1.84			2265.80		
total	55						

Table-4.2. Results of PERMANOVA conducted on the abundances of meiobenthic communities in laboratory microcosms in the presence of either *Phaeodactylum tricornutum* or *Skeletonema marinoi*

Source	df	MS	F	P
time	6	861.21	1.76	0.014
microalga	1	347.46	0.71	0.62
Time x microalga	6	572.44	1.17	0.25
residual	42	489.2		
total	55			

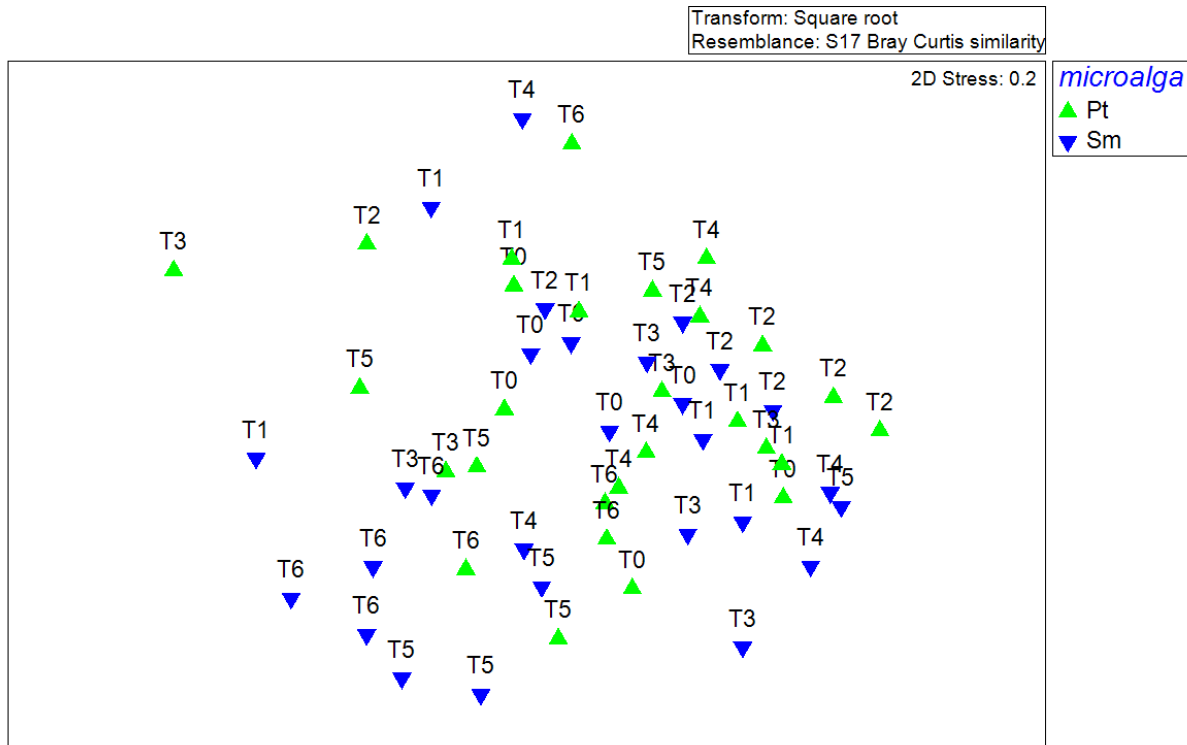


Fig. 4.1.- Two-dimensional nMDS plot based on abundances of major meiobenthic taxa in laboratory microcosms in presence of either *Phaeodactylum tricornutum* (Pt) or *Skeletonema marinoi* (Sm) at seven sampling times (T0–T6, where the number indicates weeks from the beginning of the experiment).

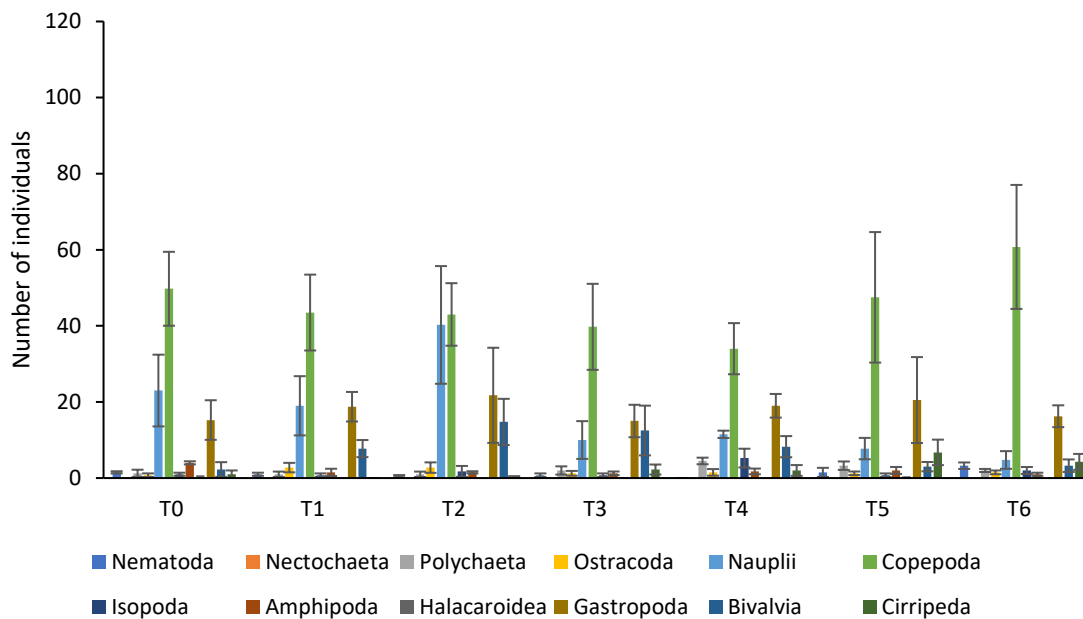


Fig. 4.2- Abundances of major meiobenthic taxa in laboratory microcosms in presence of the diatom *Phaeodactylum tricornutum* at seven sampling times (T0–T6, where the number indicates weeks from the beginning of the experiment). Mean \pm standard error ($n = 4$).

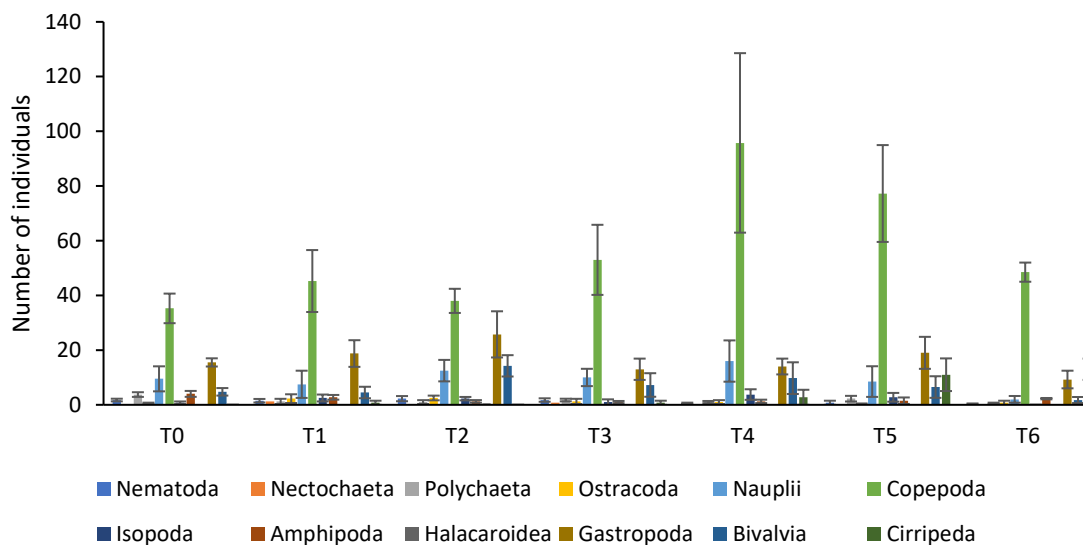


Fig. 4.3- Abundances of major meiobenthic taxa in laboratory microcosms in presence of the diatom *Skeletonema marinoi* at seven sampling times (T0–T6, where the number indicates weeks from the beginning of the experiment). Mean \pm SE, $n = 4$.

To evaluate the effect of PUAs produced by *S. marinoi*, dead *nauplii* and copepods, which appeared completely transparent under the microscopy were counted. Mortality was expressed as percentage of dead individuals with respect to the sum of dead and alive individuals.

The results showed a high mortality of *nauplii* at times T2 (64%) and T3 (66%) in microcosms with *S. marinoi*, while in microcosms with *P. tricornutum* a lower mortality was recorded, with a peak at T6 (14 %) (Fig. 4).

Also as regards the mortality of copepods, a higher mortality was recorded in microcosms with *S. marinoi*, with a peak at T3 (45%), while in microcosms with *P. tricornutum*, mortality is almost constant over time from T1 (12%) to T6 (14%) (Fig 4.5).

These results were confirmed by PERMANOVA, that showed a significant interaction between microalga and time ($P < 0.05$). The number of dead *nauplii* and copepods was significantly different between the two types of microcosms at corresponding sampling times.

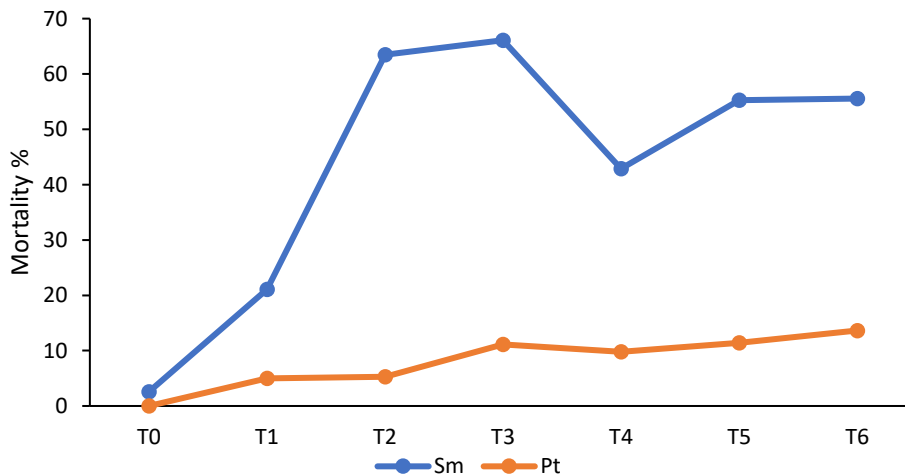


Fig. 4.5- Mortality of copepd *nauplii* in laboratory microcosms in presence of either *Phaeodactylum tricornutum* (Pt) or *Skeletonema marinoi* (Sm) at seven sampling times (T0–T6, where the number indicates weeks from the beginning of the experiment).

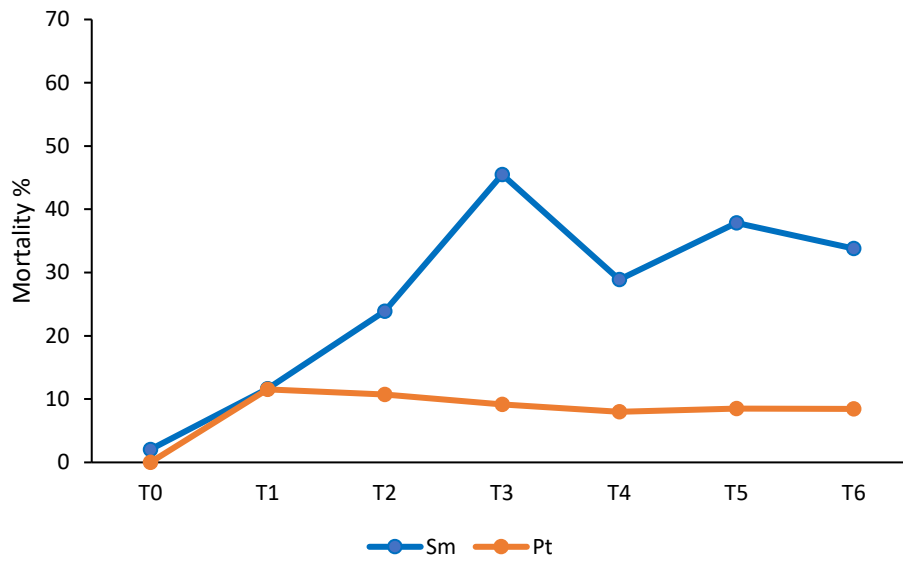


Fig. 4.5- Mortality of copepds in laboratory microcosms in presence of either *Phaeodactylum tricornutum* (Pt) or *Skeletonema marinoi* (Sm) at seven sampling times (T0–T6, where the number indicates weeks from the beginning of the experiment).

4.3.2 Analysis of harpacticoid species

In the two types of microcosms, a total of 14 species of adult harpacticoids have been identified, belonging to 11 genera and 11 families (Tables. 4.3 and 3.4).

Table 3.3- Mean abundance (\pm SE; n=4) of harpacticoid species (No ind) identified in laboratory microcosms in presence of the diatom *Phaeodactylum tricornutum*.

TIME	T0	T1	T2	T3	T4	T5	T6
Porcellidiidae							
<i>Porcellidium</i>							
<i>Porcellidium viride</i>	0	0	1.3 \pm 0.3	0.5 \pm 0.3	0	0	0.5 \pm 0.5
Ectinosomatidae							
<i>Ectinosoma</i>							
<i>Ectinosoma melaniceps</i>	0.5 \pm 0.5	1.5 \pm 0.6	1.8 \pm 0.8	0.3 \pm 0.3	3.3 \pm 1.4	3.5 \pm 1.8	6.0 \pm 2.8
Ameiridae							
<i>Ameira</i>							
<i>Ameira parvula</i>	14.8 \pm 4.6	12.7 \pm 4.5	8.0 \pm 1.9	7.5 \pm 1.4	3.0 \pm 0.7	4.5 \pm 2.0	20.0 \pm 8.4
Miraciidae							
<i>Amphiascopsis</i>							
<i>Amphiascopsis cinctus</i>	0	0.3 \pm 0.3	3 \pm 1.1	0.8 \pm 0.8	0.5 \pm 0.5	0	0
Laophontidae							
<i>Heterolaophonte</i>							
<i>Heterolaophonte sp.</i>	0	4.3 \pm 2.5	2.5 \pm 1.0	2.0 \pm 0.8	2.0 \pm 0.9	1.3 \pm 1.3	3.0 \pm 1.3
<i>Heterolaophonte minuta</i>	9.8 \pm 2.2	9.5 \pm 1.3	8.3 \pm 2.2	10.5 \pm 3.1	12.3 \pm 2.8	14.0 \pm 8.2	8.8 \pm 4.0
Parastenheliidae							
<i>Parastenhelia</i>							
<i>Parastenhelia spinosa</i>	2.0 \pm 1.2	0	2.8 \pm 1.1	2 \pm 1.4	3.8 \pm 1.3	4.5 \pm 2.0	3.8 \pm 1.9
Harpacticidae							
<i>Harpacticus</i>							
<i>Harpacticus gracilis</i>	1.0 \pm 1.0	1.8 \pm 0.6	0	0	0	0	0
<i>Harpacticus nicaeensis</i>	2.5 \pm 2.5	2.8 \pm 1.6	2.5 \pm 1.0	2.5 \pm 1.6	1.3 \pm 0.8	2.0 \pm 1.0	4.75 \pm 2.6
Dactylopusiidae							
<i>Paradactylopodia</i>							
<i>Paradactylopodia brevicornis</i>	3.3 \pm 0.3	2.0 \pm 0.7	0.0	0.8 \pm 0.8	0.0	0.5 \pm 0.5	1.0 \pm 1.0
Thalestridae							
<i>Parathalestris</i>							
<i>Parathalestris harpacticoides</i>	0.3 \pm 0.3	0	0	0	0.8 \pm 0.5	1.0 \pm 1.0	3.3 \pm 1.7
Dactylopusiidae							
<i>Diarthrodes</i>							
<i>Diarthrodes ponticus</i>	2.5 \pm 1.0	0.3 \pm 0.3	1.8 \pm 1.2	2.5 \pm 1.7	0.8 \pm 0.5	3.5 \pm 1.4	3.0 \pm 1.6
Tisbidae							
<i>Scutellidium</i>							
<i>Scutellidium ligusticum</i>	0.5 \pm 0.5	0	0	0	0	0	0
<i>Scutellidium longicaudum</i>	0	0.5 \pm 0.5	0	0	0	0	0

Table 4.4- Mean abundance (\pm SE; n=4) of harpacticoid species (No ind) identified in laboratory microcosms in presence of the diatom *Skeletonema marinoi*

TIME	T0	T1	T2	T3	T4	T5	T6
Porcellidiidae							
<i>Porcellidium</i>							
<i>Porcellidium viride</i>	0	0	0	0	0.3 \pm 0.3	0	0
Ectinosomatidae							
<i>Ectinosoma</i>							
<i>Ectinosoma melaniceps</i>	2.0 \pm 1.2	3.3 \pm 1.3	2.5 \pm 0.9	1.0 \pm 1.0	3.8 \pm 1.7	1.3 \pm 1.3	4.5 \pm 0.9
Ameiridae							
<i>Ameira</i>							
<i>Ameira parvula</i>	7.8 \pm 1.0	10.5 \pm 3.4	13.3 \pm 2.4	17.3 \pm 2.8	21.8 \pm 6.5	20 \pm 3.3	11.8 \pm 3.8
Miraciidae							
<i>Amphiascopsis</i>							
<i>Amphiascopsis cinctus</i>	0	1.0 \pm 1.0	0	0	0	0	0
Laophontidae							
<i>Heterolaophonte</i>							
<i>Heterolaophonte sp.</i>	0	0	0.8 \pm 0.8	1.3 \pm 0.8	1.8 \pm 1.8	0	0
<i>Heterolaophonte minuta</i>	11.8 \pm 1.4	15.8 \pm 2.5	5.5 \pm 0.3	4.5 \pm 2.5	9.5 \pm 4.1	8.8 \pm 4.8	1.3 \pm 0.9
Parastenheliidae							
<i>Parastenhelia</i>							
<i>Parastenhelia spinosa</i>	0.3 \pm 0.3	2.5 \pm 1.7	3.0 \pm 0.8	7.5 \pm 2.5	1.8 \pm 1.0	5.5 \pm 1.5	3.8 \pm 1.4
Harpacticidae							
<i>Harpacticus</i>							
<i>Harpacticus gracilis</i>	0.8 \pm 0.5	1.3 \pm 0.9	0	0.5 \pm 0.5	0.5 \pm 0.5	0	0
<i>Harpacticus nicaeensis</i>	1.8 \pm 1.0	1.5 \pm 1.0	1.5 \pm 1.0	2.0 \pm 0.9	8.3 \pm 5.1	2.8 \pm 0.6	10.8 \pm 2.4
Dactylopusiidae							
<i>Paradactylopodia</i>							
<i>Paradactylopodia brevicornis</i>	0	0	0.5 \pm 0.3	0.75 \pm 0.75	1.3 \pm 1.3	1.5 \pm 1	0.3 \pm 0.3
Thalestridae							
<i>Parathalestris</i>							
<i>Parathalestris harpacticoides</i>	0	0	2.8 \pm 2.4	0	3.3 \pm 1.9	1.5 \pm 1.5	0.5 \pm 0.5
Dactylopusiidae							
<i>Diarthrodes</i>							
<i>Diarthrodes ponticus</i>	0.75 \pm 0.5	1.5 \pm 1.5	2.3 \pm 1.3	5.8 \pm 1.7	5.3 \pm 3.1	17.0 \pm 4.4	4.8 \pm 1.1
Tisbidae							
<i>Scutellidium</i>							
<i>Scutellidium ligusticum</i>	1.0 \pm 0.6	0	1.0 \pm 0.7	0	0	0	0
<i>Scutellidium longicaudum</i>	0	0	0	0	0	0	0

The nMDS plot on the abundance of harpacticoid species showed a clear separation between the two types of microcosms and a different temporal pattern (Fig.4. 6).

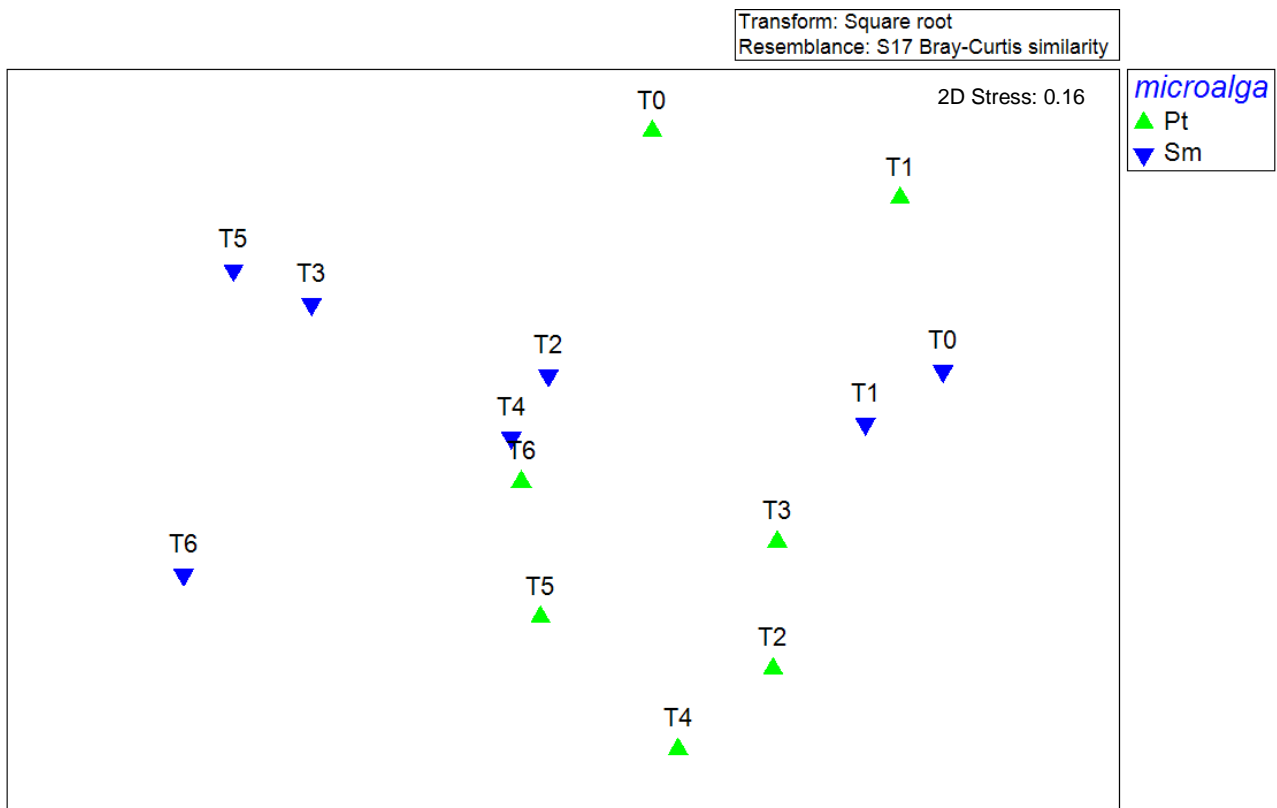


Fig. 4.6- Two-dimensional nMDS plot of centroids, based on abundances of harpacticoid copepods species in laboratory microcosms, in presence of either *Phaeodactylum tricornutum* (Pt) or *Skeletonema marinoi* (Sm) at seven sampling times (T0–T6, where the number indicates weeks from the beginning of the experiment).

These results were supported by the significant interaction between the factors, microalga and time (PERMANOVA $P < 0.01$, Tab. 4.5).

In particular, harpacticoid assemblages in Pt microcosms resulted significantly different than Sm microcosms after six weeks from the beginning of the experiment (T6), as indicated by the pairwise *post hoc* comparisons.

Table 4.5- Results of PERMANOVA conducted on the abundances of harpacticoid species in laboratory microcosms in the presence of either *Phaeodactylum tricornutum* or *Skeletonema marinoi*.

Source	df	MS	F	P
time	6	2047.6	2.5806	2.00E-05
microalga	1	3502.2	4.414	0.0005
Time x microalga	6	1332.5	1.6794	0.0152
residual	42	793.44		
total	55			

This difference at time T6 between the two microalgae is mainly due to the behavior of some harpacticoid species in the microcosms with *S. marinoi*, specifically *Heterolaophonte minuta* decreases significantly at T6, while *Heterolaophonte* sp., at the same time (T6) disappears (Fig. 4.7).

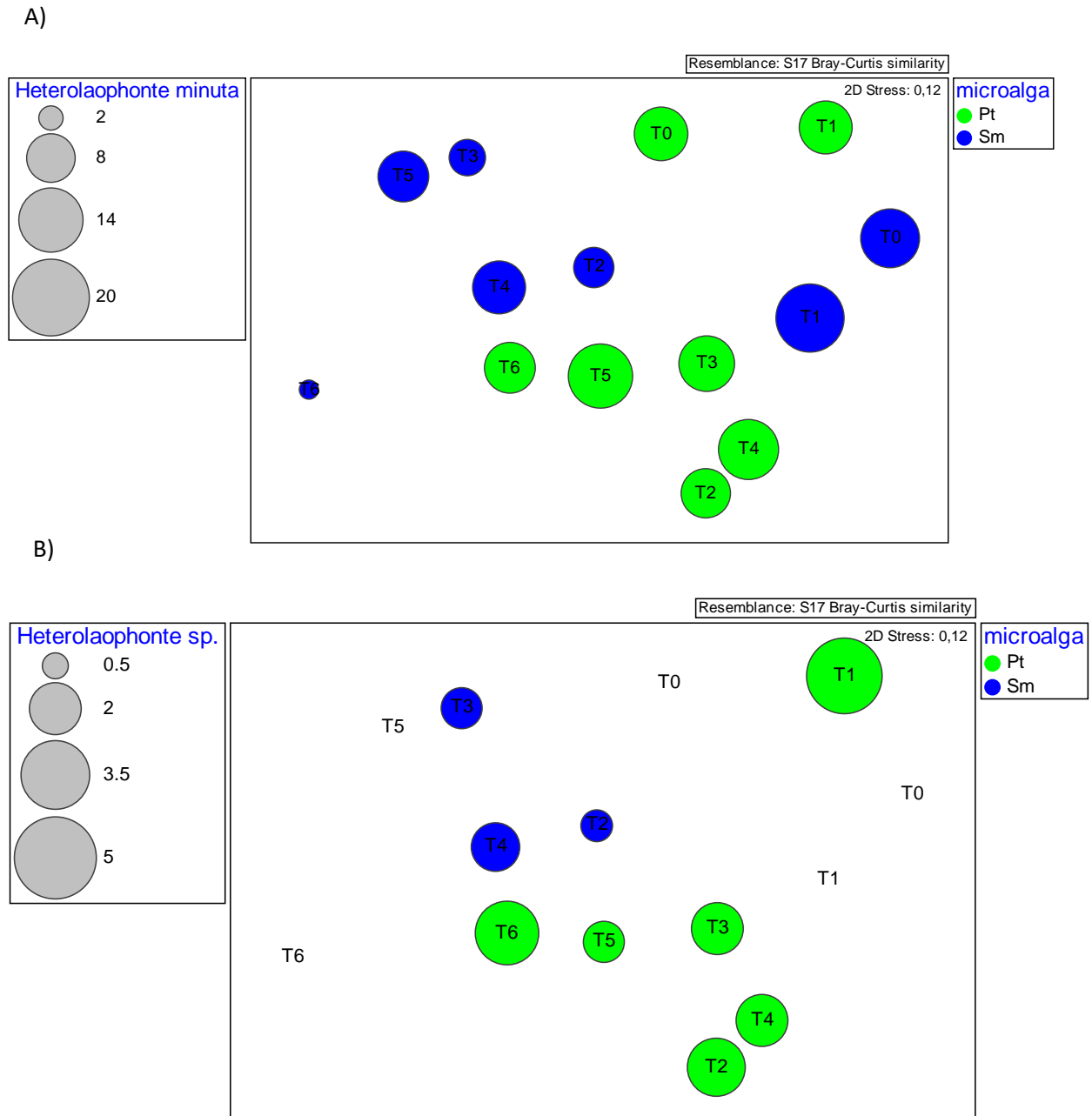


Fig. 4.7-Two-dimensional nMDS plot of centroids, based on abundances of harpacticoid copepods species in laboratory microcosms, in presence of either *Phaeodactylum tricornutum* (Pt) or *Skeletonema marinoi* (Sm) at seven sampling times (T0–T6), with superimposed circles representing abundance of: A) *Heterolaophonte minuta*, and B) *Heterolaophonte* sp.

Ameira parvula, *Diarthrodes ponticus* and *Harpacticus nicaeensis nicaeensis* on the other hand, had higher abundances, that increased over time in microcosms with *S. marinoi* (Fig. 4.8).

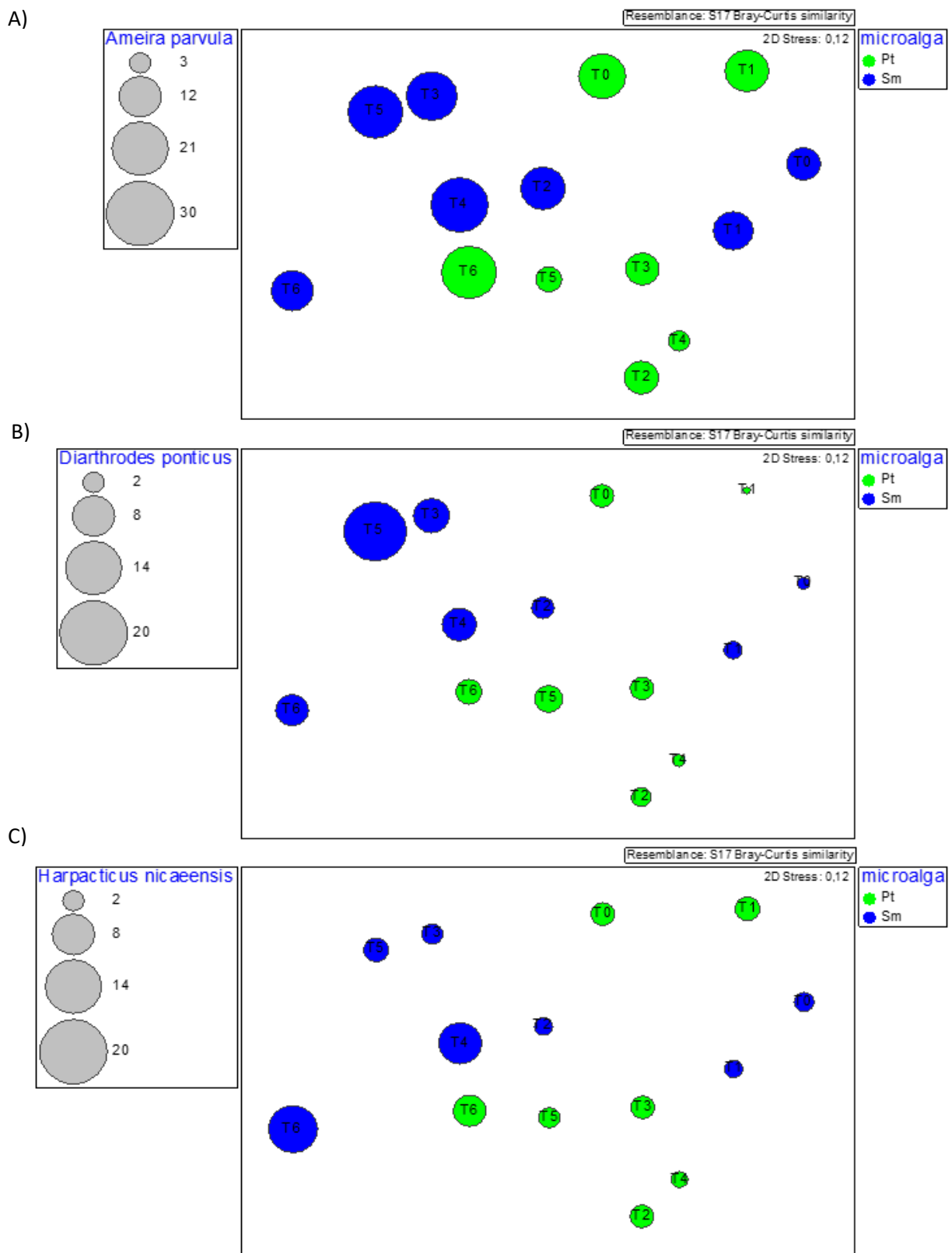


Fig. 4.8—Two-dimensional nMDS plot of centroids, based on abundances of harpacticoid copepods species in laboratory microcosms, in presence of either *Phaeodactylum tricornerum* (Pt) or *Skeletonema marinoi* (Sm) at seven sampling times (T0–T6), with superimposed circles representing abundance of: A) *Ameira parvula*, B) *Diarthrodos ponticus* and C) *Harpacticus nicaeensis*.

4.3.3 Effect of decadienal

A total of 11 major taxa belonging to the meiobenthos were identified, with copepods, *nauplii* and gastropods as dominant groups.

Regarding the total density of meiobenthos, ANOVA results showed significant differences among the eight treatments ($P < 0.05$), after the 96-h exposure and no significant difference for the number of taxa ($P > 0.05$).

The nMDS analysis of meiofauna communities showed a clear separation of the microcosms treated with different decadienal concentrations (Fig.4.9). PERMANOVA supported this result, with a significant difference among the concentrations ($P \leq 0.05$). In particular the meiobenthic assemblages resulted significantly different from the control at concentrations C4, and C5, as shown by the post hoc comparisons.

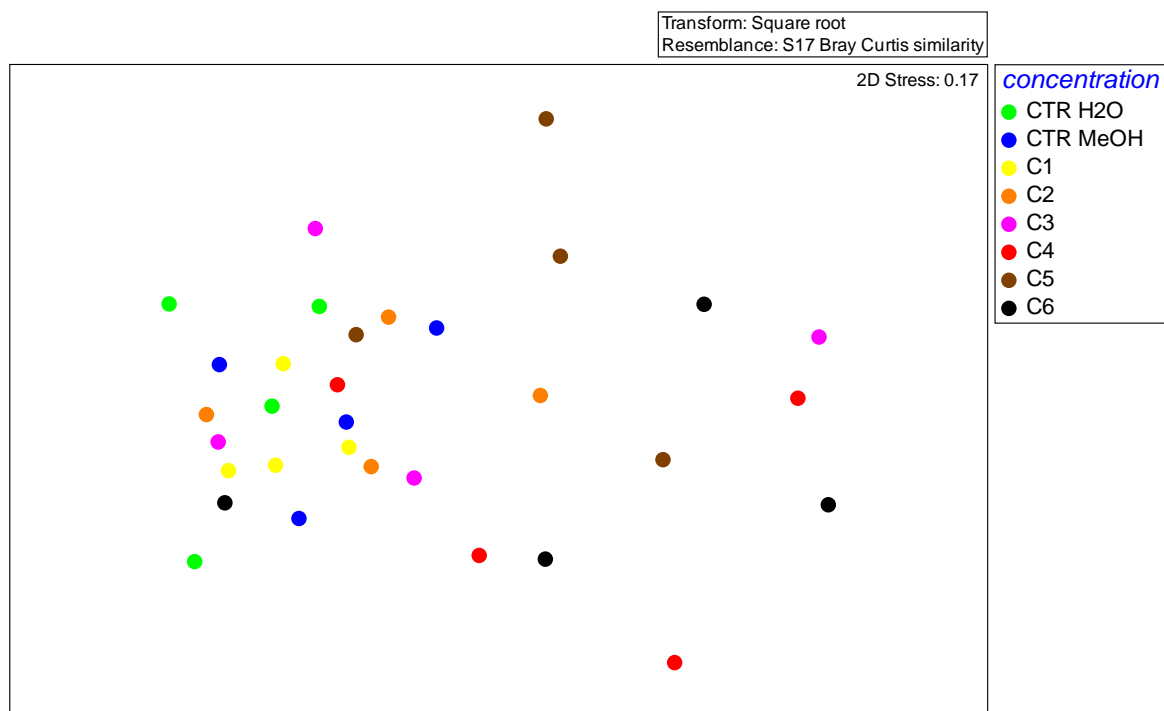


Fig. 4.9- Two-dimensional nMDS plot based on abundances of major meiobenthic taxa in laboratory microcosms treated with decadienal concentrations ranging from 1.6 mg/L (C1) to 50 mg/L (C6). Treatments include a control (CTR) and a solvent control (MeOH).

Specifically, the average density of copepods, copepods *nauplii* and gastropods decrease from C2 to C6, respectively, from 33.5 ± 1.55 to 19 ± 6.57 for copepods, from 17 ± 2.53 to 3.5 ± 2.22 for copepods *nauplii* and from 16.75 ± 7.44 to 8.75 ± 3.07 for gastropods (Fig. 4.10).

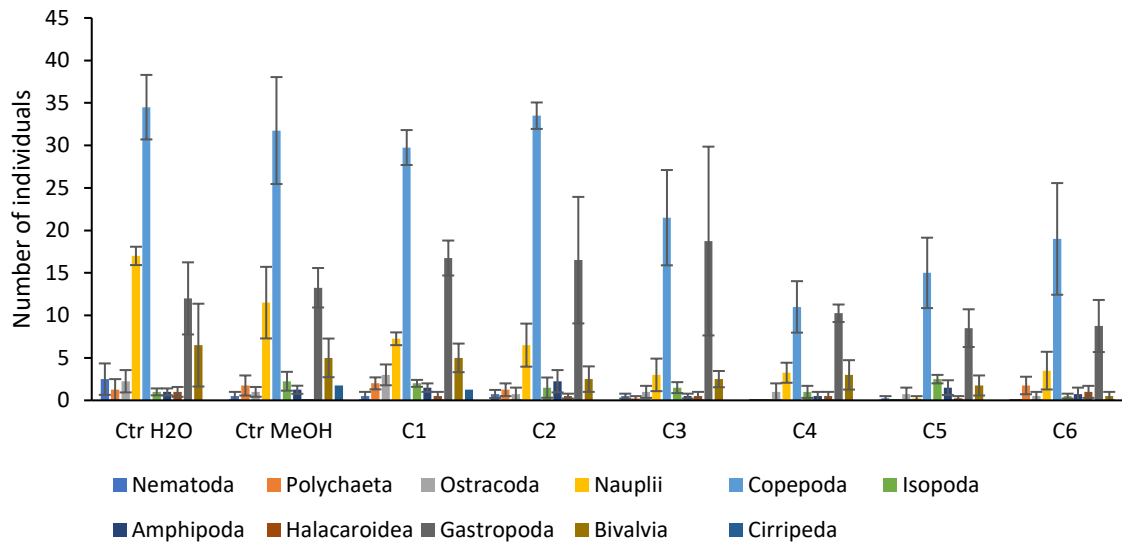


Fig. 4.10- Abundances of major meiobenthic taxa in laboratory microcosms treated with decadienal concentrations ranging from 1.6 mg/L (C1) to 50 mg/L (C6). Treatments include a control (CTR) and a solvent control (MeOH). Mean \pm standard error ($n = 4$).

The EC50 for the percentage of dead adult copepods was estimated at 53 mg/L (95% confidence interval: 20–141 mg/L). The EC50 for the percentage of dead *nauplii* was estimated at 14 mg/L (95% confidence interval: 6–30 mg/L), while the EC50 for the number of live *nauplii* was estimated at 1.7 mg/L (95% confidence interval: 0.3–8.1 mg/L). These values indicate a higher toxicity of decadienal to *nauplii* than to adult copepods.

4.3.4 Analysis of harpacticoid species

A total of 12 harpacticoid species were identified, belonging to 10 genera and 10 families (Table 4.6). The nMDS plot of harpacticoid species showed a clear separation between controls (Ctr, MeOH), lower concentrations (C1, C2) compared to higher concentrations (C4, C5, C6) (Fig. 4.11). These results were supported by the significant value of factor “concentration” (PERMANOVA $P < 0.01$) (Table 4.7).

Table 4.6. Mean abundance (\pm SE; n=4) of harpacticoid species (No ind) identified in laboratory microcosms treated with decadienal concentrations ranging from 1.6 mg/L (C1) to 50 mg/L (C6). Treatments include a control (CTR) and a solvent control (MeOH).

CONCENTRANTION	Ctr	MeOH	C1	C2	C3	C4	C5	C6
Porcellidiidae								
<i>Porcellidium</i>								
<i>Porcellidium viride</i>	0.3 \pm 0.3	0.3 \pm 0.3	0	0	0.3 \pm 0.3	0.5 \pm 0.3	1.5 \pm 1.2	0.3 \pm 0.3
Ectinosomatidae								
<i>Ectinosoma</i>								
<i>Ectinosoma melaniceps</i>	2.3 \pm 0.5	2.3 \pm 0.9	2.3 \pm 2.1	2.5 \pm 0.9	0.5 \pm 0.5	0.8 \pm 0.8	1.3 \pm 0.8	2.5 \pm 0.9
Ameiridae								
<i>Ameira</i>								
<i>Ameira parvula</i>	9.0 \pm 1.5	8.0 \pm 0.9	8.3 \pm 1.9	8.0 \pm 1.5	5.8 \pm 1.7	1.3 \pm 0.8	2.3 \pm 1.7	0
Miraciidae								
<i>Amphiascopsis</i>								
<i>Amphiascopsis cinctus</i>	2.3 \pm 1.4	0	0	0.8 \pm 0.8	0	0	0	0
Laophontidae								
<i>Heterolaophonte</i>								
<i>Heterolaophonte</i> sp.	0.8 \pm 0.8	1.8 \pm 1.0	0	0	0.8 \pm 0.5	0.3 \pm 0.3	0	0
<i>Heterolaophonte minuta</i>	10.5 \pm 1.7	6.0 \pm 2.0	8.0 \pm 1.6	5.8 \pm 1.1	3.5 \pm 0.6	2.0 \pm 0.7	1.8 \pm 0.8	3.5 \pm 1.3
Parastenheliidae								
<i>Parastenhelia</i>								
<i>Parastenhelia spinosa</i>	2.3 \pm 0.9	2.5 \pm 1.5	4.0 \pm 1.0	3.0 \pm 1.2	4.8 \pm 1.1	3.8 \pm 1.1	4.3 \pm 1.0	1.5 \pm 1.0
Harpacticidae								
<i>Harpacticus</i>								
<i>Harpacticus gracilis</i>	0.3 \pm 0.3	0.3 \pm 0.3	0	0	0	0	0	0.3 \pm 0.3
<i>Harpacticus nicaeensis</i>	0.3 \pm 0.3	0.0	0.8 \pm 0.3	0	0	0	0.3 \pm 0.3	0
Dactylopusiidae								
<i>Paradactylopodia</i>								
<i>Paradactylopodia brevicornis</i>	0.3 \pm 0.3	0	0	0	0	0	0	0
Dactylopusiidae								
<i>Diarthrodes</i>								
<i>Diarthrodes ponticus</i>	4.0 \pm 0.9	2.0 \pm 0.9	1.3 \pm 1.2	5.3 \pm 1.5	3.3 \pm 1.9	1.3 \pm 1.0	0.8 \pm 0.5	8.5 \pm 3.8
Tisbidae								
<i>Scutellidium</i>								
<i>Scutellidium longicaudum</i>	0.3 \pm 0.3	0.5 \pm 0.5	0	0	0.3 \pm 0.3	0	1.0 \pm 1.0	0.5 \pm 0.5

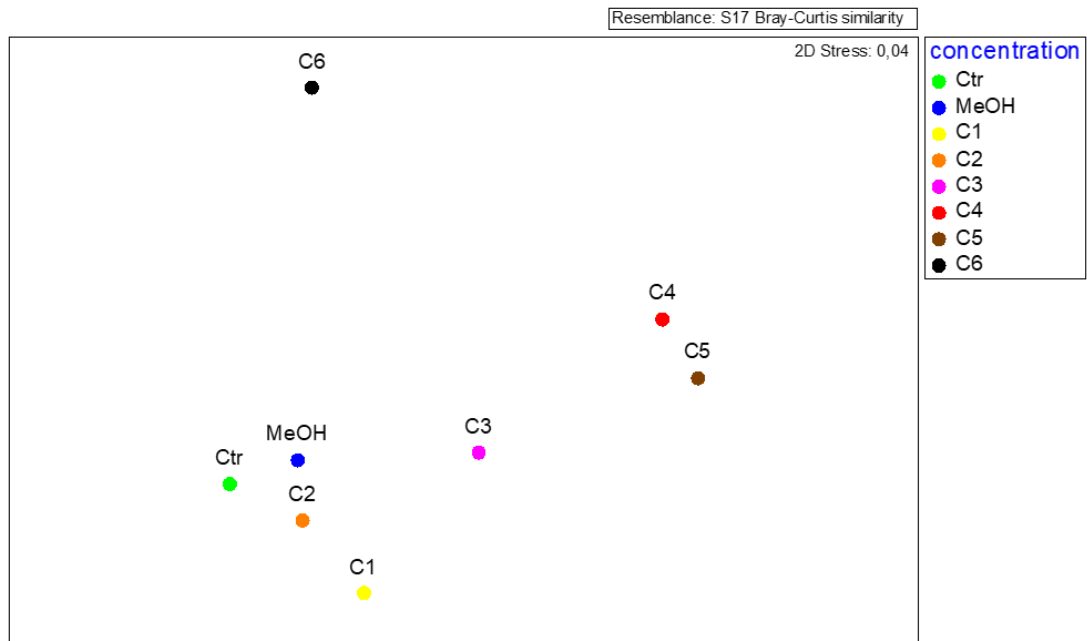


Fig. 4.11- Two-dimensional nMDS plot of centroids, based on abundances of harpacticoid copepod species in laboratory microcosms treated with decadienal concentrations ranging from 1.6 mg/L (C1) to 50 mg/L (C6). Treatments include a control (CTR) and a solvent control (MeOH).

Table 4.7- Results of PERMANOVA conducted on the abundances of harpacticoid species in laboratory microcosms treated with decadienal concentrations ranging from 1.6 mg/L (C1) to 50 mg/L (C6). Treatments include a control and a solvent control.

Source	df	MS	F	P
Concentration	7	2186	2.6066	0.0005
Residual	24	838.66		
Total	31			

In particular, differences in harpacticoid assemblages between the controls and the lower concentrations (C1 and C2) were not significant, while the differences between controls and the higher concentrations (C3, C4, C5 and C6) were significant, as evidenced by pairwise *post hoc* comparisons.

These significant differences were mainly explained by two species, *Ameira parvula* and *Heterolaophonte minuta*, whose mean abundances decreased at the higher concentrations (Fig. 4.12).

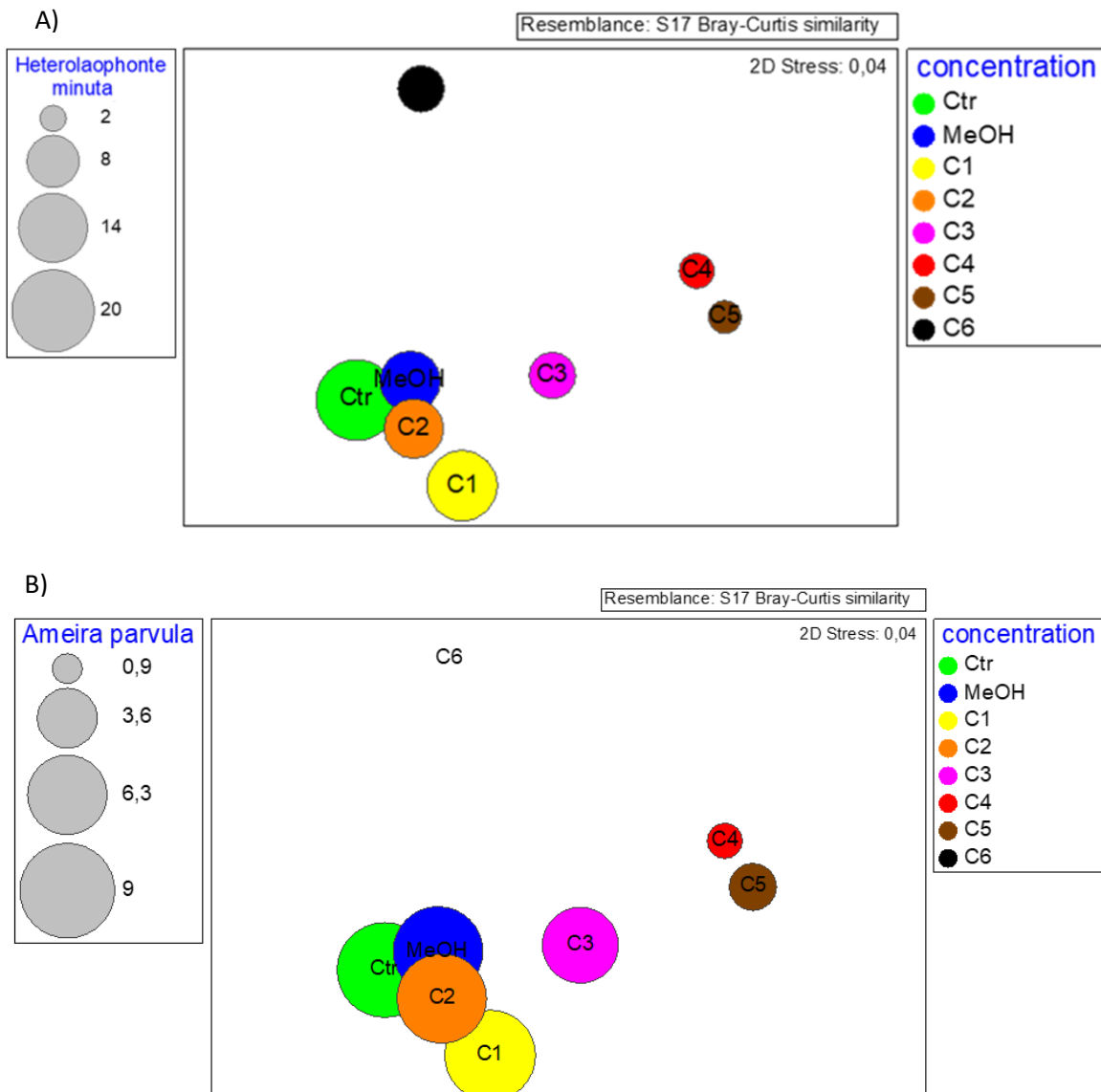


Fig. 4.12- Two-dimensional nMDS plot of centroids, based on abundances of harpacticoid copepods species in laboratory microcosms treated with decadienal concentrations ranging from 1.6 mg/L (C1) to 50 mg/L (C6), with superimposed circles representing abundance of: A) *Heterolaophonte minuta*, B) *Ameira parvula*.

The EC50 for *Heterolaophonte minuta* was estimated at 6.7 mg/L (95% confidence interval: 2.5–18.2 mg/L), while the EC50 for *Ameira parvula* was estimated at 8.0 mg/L (95% confidence interval: 5.1–12.5 mg/L).

4.4 Discussion

Several studies have considered the adverse effect of PUAs produced by diatoms on the reproduction of calanoid copepods which feed on them, e.g., *Temora stylifera* and *Calanus helgolandicus* (Miralto et al., 1999; Ianora et al., 2003, 2012;). However, few studies have examined the effect of these substances on the entire meiobenthic community (Taylor et al., 2007; Lenzo et al., 2022,).

For this reason, in the present work, a laboratory analysis was initially made of the entire meiobenthic community present within microcosms in the presence of either *Skeletonema marinoi* (a PUAs producer diatom) or *Phaeodactylum tricornutum* (a diatom not producing PUAs), and subsequently another experiment was carried out in which the effect of decadienal (PUA C10:2) was analyzed again on the whole community.

As regards the first experiment, the community present in microcosms with *S. marinoi* showed a greater abundance of organisms, although this microalga produces PUAs, compared to the benthic community present in *Phaeodactylum tricornutum*, which does not produce these compounds.

Although, studies have shown that different diatom species possess a complex infochemical system that plays an important role in allelopathy (Ribalet et al., 2009), and several observations have also demonstrated inhibitory effects of diatoms, in particular *S. marinoi*, on the reproduction of planktonic organisms during blooms (Carotenuto et al., 2014).

Our results can be explained by the fact that benthos tends to be a more stressful environment than pelagos, leading organisms to adapt to these environments, where rapid physical fluctuations occur causing the accumulation of both natural and anthropogenic toxins, at high levels within the sediments deposited on the algal fronds.

Conversely, the mean density of copepods *nauplii* and adult copepods is lower in the presence of *S. marinoi*, in fact the results show a high mortality of both in microcosms with *S. marinoi*, while in microcosms with *P. tricornutum* there is a lower mortality.

These results are supported in the literature, as studies have demonstrated detrimental effects of diatoms on copepod gametogenesis, hatching success and naupliar fitness, partly due also to the production of "oxylipins", including PUAs, in particular, following cellular wounding, e.g. after grazing of copepods.

PUAs, in fact, may act as defensive metabolites by inducing congenital malformations in copepod offspring and apoptosis in embryos, *nauplii* and adult females (Ianora et al., 2004). Reduced viability and apoptosis in copepod offspring have been reported either following feeding on oxylipin-

producing diatoms (Barriero et al., 2011) or after indirect exposure of females to known concentrations of oxylipins (Ceballos et al., 2003; Ianora et al., 2011).

The experiment carried out with decadienal, also showed an effect on copepod *nauplii* and copepods, leading to a decrease in their abundances at high concentrations of C10:2.

These results are in agreement with previous studies reporting *nauplii* mortality in the presence of diatoms and in treatments with decadienal standard (Ianora et al., 2004), and this could explain the decrease in copepod recruitment (Ianora et al., 2004).

Subsequently, the analysis performed on the species of harpacticoid copepods showed that for both experiments, there was a species-specific effect.

In fact, Sopanen et al. (2006), in her study showed that diets with higher toxin concentrations should cause greater effects. However, grazers probably have species specific responses or adaptations to different toxins.

Specifically, in my study, the mean abundances of the two species belonging to the Laophontidae family, *Heterolaophonte minuta* and *Heterolaophonte* sp., decreased in microcosms with *S. marinoi*. The first species *H. minuta*, also was affected in the decadienal experiment, along with *Ameira parvula*.

Hicks et al. (1980), report, in fact, that these species are endemic to the Mediterranean Sea, and have certain morphological characteristics which allow them to live by adhering to macroalgal surfaces; therefore, could be affected by the effect of the various chemicals compound products from both macroalgae and epiphytic microalgae.

Instead, the other identified harpacticoid species are not affected by PUAs.

Referring to the ecology of meiobenthic organisms, their persistence in their environment, depends on adaptations that allow them not only to survive but also to reproduce successfully. It is widely recognized that most benthic organisms have a relatively high tolerance to varying environmental conditions (Hicks and Coull, 1983; Riedel et al., 1997). Harpacticoids, in particular, are highly tolerant of a range of environmental stressors (examined in Raisuddin et al., 2007) including exposure to chemical agents.

For instance, also, Colin and Dam (2002) found that copepods, from areas where they are constantly exposed to blooms of toxic dinoflagellate (*Alexandrium* spp.) were less affected by ingesting toxic cells than copepods, of the same species from areas where they were not naturally exposed to these toxins. This was explained by the evolutionary adaptations and selection of grazers for resistance to the toxic effects of these algae.

Thus, harpacticoid copepods (as seen in studies by Taylor et al., 2007 for *Thisbe holothuriae* and *Tigriopus japonicus* in Lee et al., 2005, 2008) may have a more developed detoxification system than planktonic calanoid copepod, and therefore are better equipped to withstand the toxic impact of oxylipins.

Therefore, aspects such as genetic selection and physiological adaptations should be taken account when examining interactions between grazers and toxic algae.

This work showed a clear species-specific effect of PUAs on some harpacticoid copepod species, rather than on the entire benthic community. Thus, these results underlined the importance of allelopathy in structuring benthic communities, through the interactions between PUA-producing diatoms and grazers.

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Chapter 5 - Allelopathic effect of the invasive macroalga *Rugulopterix okamurae* (Phaeophyceae): potential role of the newly discovered compound Dilkamural

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Abstract

In the last decade, the brown macroalga *Rugulopterix okamurae* has shown an intensive proliferation in different coastal areas, such as the southwestern coasts of Europe. Recently, the production of defense metabolites has been suggested as one of the reasons for such huge invasive potential. A chemical investigation on *R. okamurae* from the Strait of Gibraltar led in fact to the isolation of different specialized metabolites, among which the compound dilkamural stands out for its high concentration. Whether and how allelopathic compounds produced by invasive algae could potentially change the population dynamics of native species is of great interest and is currently understudied.

In this context, this work aimed to investigate the effects of dilkamural on unicellular phototrophs to better understand its potential role as allelochemical. Growth inhibition assays were carried out testing different concentrations of dilkamural on two species of microalgae (the diatoms *Phaeodactylum tricornutum* and *Cyclotella cryptica*) and a cyanobacterium (*Synechococcus* sp.). The effects on cells were evaluated after 3, 24, 48, and 72 h from dilkamural addition by imaging flow

cytometry (IFC). Results showed an acute effect of diikamural after only three hours on all the analyzed species; particularly, effects on the viability and cell morphology of *P. tricornutum*, as well as cell integrity and division of *Synechococcus* sp. and *C. cryptica* were reported, especially after 48 or 72h. The observed results highlighted as the production of diikamural by the invasive *R. okamurae* could affect the viability of unicellular photosynthetic organisms and algal population dynamics, reporting allelopathic effects on target organisms, thus potentially reducing the biodiversity of the coastal ecosystems where this invasive algal species has been introduced and extensively proliferates.

Keywords

Non-indigenous species; diterpenoids; growth inhibition assay; *Synechococcus* sp.; *Phaeodactylum tricornutum*; *Cyclotella cryptica*

5.1 Introduction

Climate change is altering the distribution of organisms across the global oceans (Poloczanska et al., 2016) and is likely to change co-occurrence patterns and interspecific interactions of native, and non-indigenous species, whose negative impact on marine ecosystems could affect the biodiversity and economy of coastal areas. Introductions of allochthonous species to new ecosystems are one of the major threats to biodiversity, ecosystem functions, and services (Bailey et al., 2020); moreover, in synergy with other anthropogenic disturbances, such as climate change and coastal pollution, they may lead to biotic homogenization, community biodiversity reduction, and changes in species abundance (Pereira et al., 2021). Among marine invasive species declared in Europe, around 20–40% are macroalgae (Schaffelke et al., 2006). Non-native macroalgae can become invasive due to their high reproductive rates due to a greater sexual reproduction (Burns et al., 2013)

and the perennial status which makes them more competitive than native species (Pereira et al., 2021)

In aquatic environments, macroalgae provide habitat for fauna and are also able to produce different allelopathic compounds with high structural variability, that play an important role in species' successions (Saha et al., 2018). The allelochemicals released by these organisms into the environment could have beneficial or detrimental effects (e.g., phytotoxicity) on neighboring organisms (Chou, 1999). Moreover, the allelopathic potential of a native species could induce a biotic resistance against invasive organisms, whereas allelochemicals released by exotic species could favor the establishment of invasive species through the so-called "invasional meltdown", which could intensify impacts and promote secondary invasions (Green et al., 2011). Invasive species often establish monospecific patches in their introduced ranges but coexist with neighboring species in their native habitat (Ridenour and Callaway, 2001). Several studies have suggested that allelopathy may contribute to the ability of exotic species to form dense stands in invaded ecosystems (Hierro and Callaway, 2003; Garkoti et al., 2019), but the role of allelopathy on the structure and composition of marine biological communities affected by the introduction of invasive macroalgae is still relatively unexplored.

The brown algae *Rugulopteryx okamurae* (E.Y. Dawson) I.K. Hwang, WJ Lee & H. S. Kim (Dictyotaceae), is a native brown alga of the North-West Pacific. Since 2002, it has been known to be present in the French Mediterranean coast near Montpellier (Verlaque and Boudouresque, 2002). In 2018, El Aamri et al. (2018) (El Aamri et al., 2018) recorded populations of *R. okamurae* on the Moroccan Atlantic coast, increasing its distribution through the Strait of Gibraltar (Altamirano et al., 2016) and appearing for the first time in Tarifa (Cádiz, Andalusia, Southwestern of Spain) in 2017 (Altamirano et al., 2016). Nowadays, *R. okamurae* is threatening the rocky bottoms of the Andalusian coast, where it was accidentally introduced through seed importation for Japanese

oyster (*Cassostrea gigas*) culturing (Verlaque et al., 2009). *R. okamurae* is a brown seaweed similar to other native species of the genus *Dictyota* and has become the most abundant species over a period of only one year, covering over 90% of the bottom between 10 and 20 m depth, which implied a significant change in the structure of benthic communities (García-Gómez et al., 2020). Additionally, hundreds of tons of *R. okamurae* accumulated on beaches becoming a nuisance with implications for both tourism and public health (Ocana et al., 2016; García-Gómez et al., 2021; Altamirano et al., 2016). Fishermen have also reported that the species can clog fishing nets causing a substantial reduction in their ability to catch fish (Sempere-Valverde et al., 2021). The invasive success of *R. okamurae* is still unclear, but recently chemical ecologists have highlighted how research on natural products might be useful in understanding marine biological invasions (Occhipinti-Ambrogi, 2021), assessing their impact in the invaded areas, and effects on species interactions. In this regard, chemical studies of native specimens of *R. okamurae* collected at several locations on the Japanese coasts showed the presence of an array of specialized metabolites belonging to the terpenoid class (Cuevas et al., 2021). These compounds were reported to inhibit the settlement and metamorphosis of the larvae of the abalone *Haliotis discus hannai Ino* (Kurata et al., 1988b) and to possess feeding deterrent activity against the young abalone (Kurata et al., 1989; Suzuki et al., 2002) and the young sea urchin *Strongylocentrotus nudus* (Kurata et al., 1990), thus suggesting that the metabolites produced by *R. okamurae* could play a chemical defence function. A very recent publication (Casal-Porrás et al., 2021) has proposed that the large invasive capacity of *R. okamurae* in the Strait of Gibraltar (García-Gómez et al., 2020) could be favoured by the production of a newly discovered compound belonging to the class of diterpenoids, namely dilkamural, which could make *R. okamurae* less palatable to herbivores, such as sea urchins, and could cause concentration-dependent toxic effects on them in a short time.

No studies have yet been carried out on the effect of the released and dissolved dillkamural on microorganisms, such as microalgae, which are at the base of the food web. Chemical interactions are considered among the factors able to control the growth and specific biomass composition of these microorganisms since the field of aquatic chemical ecology emerged.

Bioassays using plant or algal extracts (i.e., leachates, exudates) or, when possible, isolated metabolites are one of the most useful methods to assess the allelopathic effects of the producer organism. For this reason, this study was aimed at investigating the effects of freshly extracted dillkamural from *R. okamurae* sampled in the locality of Cadiz (Spain), on unicellular phototrophs (i.e., the diatoms *Phaeodactylum tricornutum* and *Cyclotella cryptica*, and the cyanobacterium *Synechococcus* sp.) throughout growth inhibition assays, to test for the first time the potential role of this compound as allelochemical.

5.2 Material and methods

5.2.1 Species and culture conditions

The strains used for the toxicity tests were the cyanobacteria *Synechococcus* sp. PCC 7002 and the diatoms *Cyclotella cryptica* CCMM 07/0201 and *Phaeodactylum tricornutum* CCMM 07/0451. All strains were obtained from the Marine Microalgae Strain Collection at the Marine Sciences Institute of Andalusia (CSIC). The cultures were grown using natural autoclaved and filtered (0.22 μm) natural seawater enriched with f/2 medium (Guillard, 1975), with the addition of silicate in the case of diatom cultures. Cultures were prepared in 100 ml vessels at a constant temperature of 20°C, under a light intensity of 100 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ with a 14:10 h light:dark cycle and orbital shaking of 60 r.p.m using an Innova® S44i culture chamber (Eppendorf).

5.2.2 Isolation of dilkamural (DK) from *Rugulopterix okamurae*

Specimens of the brown alga *Rugulopterix okamurae* (30 g fresh weight) were collected at Punta Carnero (36°04'38.6"N; 5°25'31.1" W, Cádiz, Spain) and immediately transported to the laboratory, where the algae were washed with fresh water to remove epiphytes and organic and inorganic debris. Then, the algae were submerged in acetone/methanol (MeOH) (40 mL, 1:1, v/v), mashed for 5 min, and subjected to sonication for another 5 min. The solution was filtered, and the residual algal material was extracted five more times following the same procedure. The obtained solutions were combined, and the solvent evaporated in a rotary evaporator, to yield an aqueous residue that was extracted with diethyl ether (Et₂O) (3 x 25 mL). The organic layers were combined, dried over anhydrous MgSO₄, filtered, and the solvent evaporated in a rotary evaporator to yield a dark green oily extract (450 mg). This extract was separated by column chromatography on Merck silica gel 60 (70-230 mesh) (Merck, Darmstadt, Germany), using as eluents n-hexane (Hex)/Et₂O 50:50 v/v (100 mL), Hex/Et₂O 30:70 v/v (150 mL) and Et₂O (100 mL). Ten fractions were collected and analyzed by thin layer chromatography on silica gel (Merck) using Hex/ethyl acetate (EtOAc) (7:3, v/v) as eluent and a solution of Ce(SO₄)₂ (Sigma, St Louis, MO, USA) in 2M sulfuric acid for visualization of the spots. The fractions containing DK (R_f = 0.47) were combined and the solvent evaporated to dryness under reduced pressure. The obtained mixture (225 mg) was suspended in 2 mL of MeOH/H₂O 90:10, v/v, and transferred onto a Supelco DSC18 cartridge (1 g / 6 mL) (Supelco, Bellefonte, PA, USA) preconditioned with 3 mL of MeOH/H₂O 90:10, v/v. The cartridge was eluted with 15 mL of MeOH/H₂O 90:10, v/v, and the obtained solution was evaporated under reduced pressure. The resulting mixture was subjected to normal phase HPLC on a Lachrom-Hitachi apparatus (Merck) with a differential refractometer RI-71 (Merck), using a Luna Si (2) column (250 x 10 mm, 5 μm) (Phenomenex, Torrance, CA, USA) and Hex/EtOAc 70:30, v/v, as eluent (3 mL /min), to obtain pure

DK (95 mg). The identity and purity of DK was confirmed by ^1H NMR analysis on a Bruker 500 spectrometer (Bruker, Billerica, MA, US) using CD_3OD (Sigma) as solvent (Casal-Porras et al. 2021).

5.2.3 Growth inhibition assays

For the toxicity test, batch cultures were carried out in sterilized 25 mL Erlenmeyer flask (autoclaved at 121 °C, 1.1 atm., 20 min). Strains were acclimated to experimental conditions before the experiments. Phytoplanktonic cells in early exponential growth phase were used as inoculum for the different assays and initial cell density for each experiment was adjusted to 10^5 cells mL^{-1} . 50 μL of DK stock solutions in DMSO were added to cultures (20 mL) to reach final DK concentrations (namely C1-C4) of 8, 40, 100 and 200 μM . DMSO allowed to improve DK solubility in the culture and was selected after preliminary assays in which no toxic effects were observed on *Synechococcus* sp., *Cyclotella cryptica*, and *Phaeodactylum tricornutum* cells after the addition of 0.25% DMSO (v/v) to the cultures. Blank controls were prepared by adding 50 μL of water or DMSO to 20 mL of culture. All treated and control cultures were prepared in triplicates. Samples (1 mL) were collected at 0 h and after 3, 24, 48 and 72 hours of dikamural addition and were analysed by IFC, except for *Synechococcus* sp. where the assay ended after 48h.

5.2.4 Cell counting and cell viability analysis by IFC

Cell density was counted by IFC. Collected samples for all species and concentrations were stained using the fluorescent dye SYTOX Green dead cell stain (Invitrogen, Molecular Probes) at a final concentration of 30 nM and incubated in the dark for 20 min at room temperature. This stain was used to determine cell viability (Roth et al. 1997) and to estimate cellular membrane integrity as it does not penetrate live cells but only those with compromised membranes (Lebaron et al. 1998, Verdhuis and Kraay 2000, Verdhuis et al. 2001). Following incubation, samples were run on an ISX ImageStream[®] X Mark II (Amnis part of Luminex Corporation, Seattle, USA) multispectral image flow cytometry equipped with a CCD camera system. This is equipped with three interchangeable microscope objectives with 20x (0.5 NA), 40x (0.75 NA) and 60x (0.9 NA) magnification, and 405,

488, 645 and 785 nm excitation laser. Samples were analyzed at 60x magnification and at low flow rate, to increase imaging quality, and excited by 488 and 785 nm laser using the INSPIRE® software (Amnis Corp.). The side scatter images were collected in Channel 6 (Ex 785 nm, Em 745-800 nm) for SSC, chlorophyll autofluorescence in Channel 5 (Ex 488 nm, Em 642-745 nm), green fluorescence of stained cells in Channel 2 (Ex 488 nm, Em 505-560 nm) and brightfield image in Channel 1. Post-acquisition spectral compensation and data analysis were performed using the IDEAS® 6.2 image analysis software package (Amnis Corp.). Cell densities of live, damaged and dead cells were gating in the dot plots of green fluorescence of channel 2 vs red autofluorescence of channel 5. Morphometry analysis was also developed to evaluate cells aggregation and morphotypes; in order to differentiate them, we used Area versus Length scatter plot. Specific growth rates (μ , day⁻¹) were calculated using the following equation:

$$\mu = \frac{\ln N_1 - \ln N_0}{t_1 - t_0}$$

where N_0 and N_1 were cell density values at time t_0 and t_1 .

5.2.5 Data analysis

Statistical analysis was performed using PRIMER v6 (Clarke and Gorley, 2015) with the PERMANOVA + add -on (Anderson et al., 2008). The data were assessed by permutational non-parametric multivariate analysis of variance (PERMANOVA) following the same experimental design adopted for ANOVA (Anderson, 2001; Anderson et al., 2005). When significant main effects were detected, the specific procedure provided within PERMANOVA was used for pairwise a posteriori comparisons. The analyses were performed using unrestricted permutation of the raw data and 9999 permutation. Significance level was set at 0.05 (5%) for all tests. EC50 values were calculated with a three-parameter log-logistic function (LL.3) using the drc package in R.

5.3 Results

For all the three species analyzed, such as *Synechococcus* sp., *C. cryptica* and *P. tricornutum*, no significant differences (PERMANOVA; $p < 0.05$) were reported between the control and DMSO (Fig. 1-3), as attested also by the similar growth rates calculated for all the species cultured at these conditions during the experiments (Table 5.1).

Table 5.1 – Growth rates (μ , day⁻¹) measured in control cultures (CTR with water or DMSO) during the toxicological assays.

	μ_{CTR}	μ_{DMSO}
<i>Synechococcus</i> sp.	0.43 ± 0.19	0.38 ± 0.24
<i>Cyclotella cryptica</i>	0.25 ± 0.09	0.21 ± 0.14
<i>Phaeodactylum tricornutum</i>	0.61 ± 0.11	0.54 ± 0.09

Results on the effect of dilkamural on *Synechococcus* sp. showed that after 3h of DK addition (Fig. 5.1A) the abundance of damaged cells increased significantly (PERMANOVA; $p < 0.05$) when increasing DK concentrations, ranging from $3.29 \pm 1.05 \cdot 10^7$ cells/mL in C1 to $4.22 \pm 0.90 \cdot 10^7$ cell.mL⁻¹ in C4 (corresponding to the 67% and 91% of total cells, respectively).

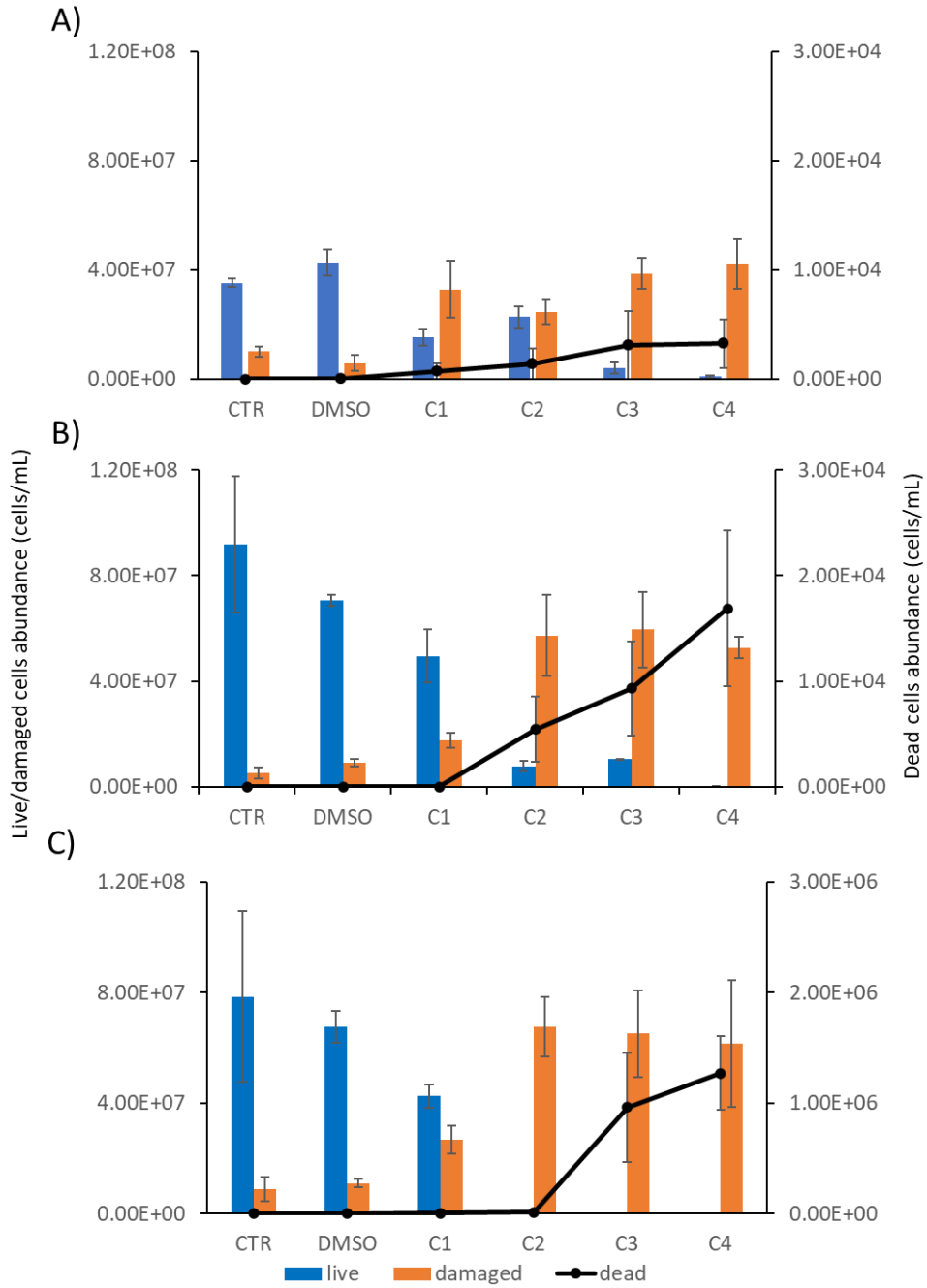


Figure 5.1 – Effect of dilkamural (DK) at different concentrations (C1-C4, range 8–200 μ M) on the abundance of live, damaged (left axis) and dead (right axis) cells of *Synechococcus* sp. after A) 3h, B) 24h, and C) 48h compared to the control conditions (i.e., CTR with water or DMSO, concentration = 0). Data are means of independent replicates (n = 3) and the error bars represent standard errors.

After 24h (Fig. 5.1B), the percentage of alive cells decreased at C2 ($7.89 \pm 1.82 \cdot 10^6$ cells mL⁻¹) and C3 ($1.05 \pm 0.01 \cdot 10^7$ cells mL⁻¹) to 12 and 15% of the total, respectively, concomitantly to a significant increase (PERMANOVA; $p < 0.05$) of damaged cells found at these concentrations (up to 89% of the total cells) and of dead cells ($1.69 \pm 0.74 \cdot 10^4$ cells mL⁻¹) at C4.

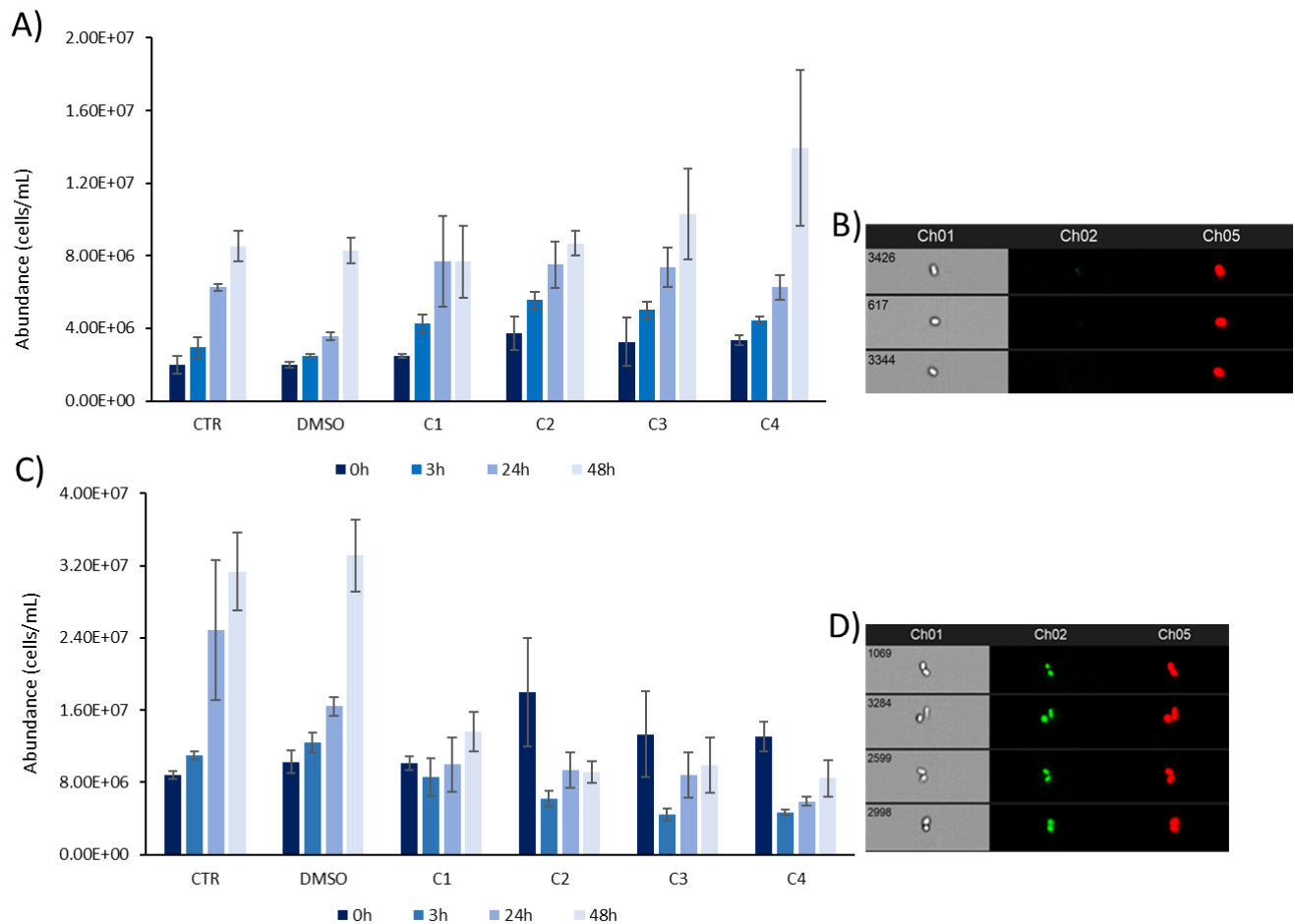


Figure 5.2 - Effect of different concentrations of dilkamural (DK) on the abundance of *Synechococcus* A) single cells, and C) double cells at all tested times (0-48h) compared to the control conditions (i.e., CTR with water or DMSO, concentration = 0). Data are means of independent replicates ($n = 3$) and the error bars represent standard errors. B) and D) images associated to Brightfield (Ch01), Chlorophyll autofluorescence (Ch05) and SYTOX green fluorescence (Ch02) of single and doubled cells of *Synechococcus* sp.

After 48h (Fig. 5.1C) the abundance of dead cells of *Synechococcus* sp. significantly increased (PERMANOVA; $p < 0.05$) at C3 ($9.62 \pm 4.93 \cdot 10^5$ cells/mL) and at C4 ($1.27 \pm 0.33 \cdot 10^6$ cells/mL), and resulted in up to 2% of total cells, while all other cells were damaged. Furthermore, results showed

that at high concentrations, particularly at C4, there was a significant difference (PERMANOVA; $p < 0.05$) between the abundance of double cells ($8.43 \pm 2.05 \cdot 10^6$ cells/mL), i.e. cells that were dividing, and the one of single cells ($1.39 \pm 0.43 \cdot 10^7$ cells/mL) (Fig. 5.2). Double cells concentration resulted significantly lower (PERMANOVA; $p < 0.05$) at high concentrations (i.e., C2, C3, C4) at all tested times, and also at C1 after 48h respect to control conditions.

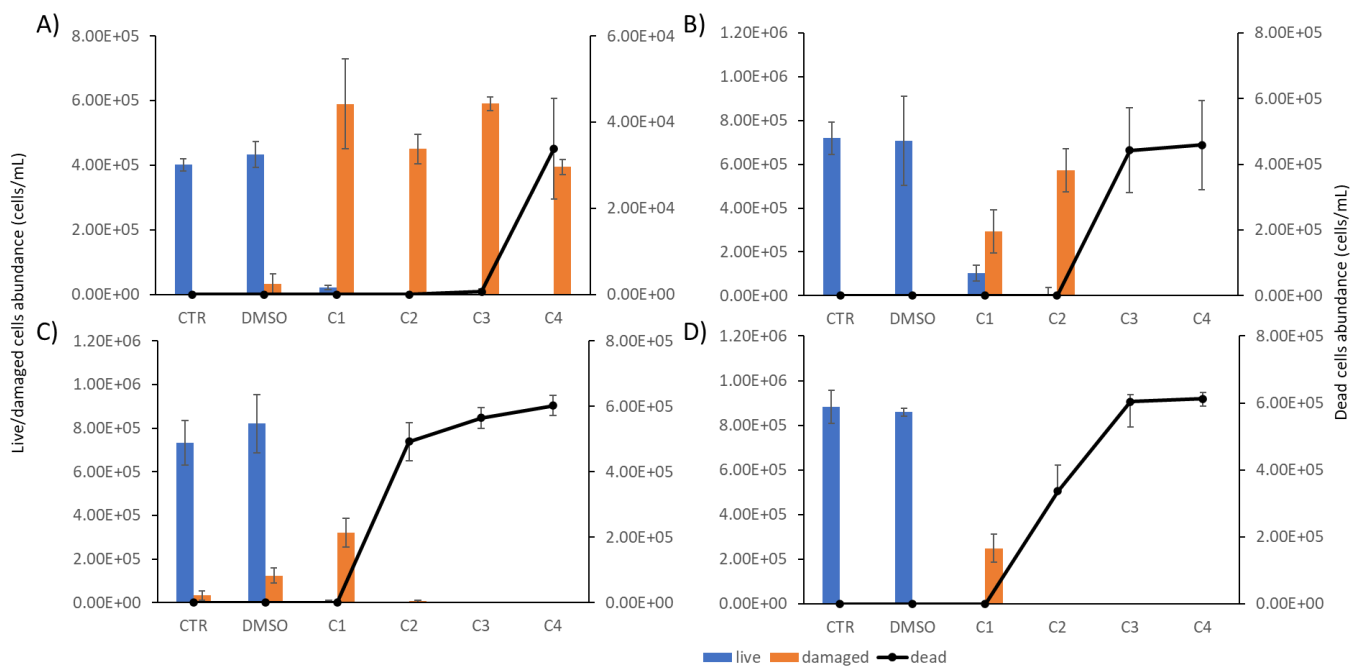


Figure 3 - Effect of dilkamural (DK) at different concentrations (C1-C4, range 8–200 μ M) on the abundance of live, damaged (left axis) and dead (right axis) cells of *Cyclotella cryptica* after A) 3h, B) 24h, C) 48h, and D) 72h compared to the control conditions (i.e., CTR with water or DMSO, concentration = 0). Data are means of independent replicates ($n = 3$) and the error bars represent standard errors.

Results on the effect of DK on *C. cryptica* showed that 3h after DK addition (Fig. 5.3A) only at C1 live cells were present ($2.21 \pm 0.67 \cdot 10^4$ cell/mL), although representing only 3.6% of the total cells, and cells were damaged at all concentrations. At the highest concentration (C4) about 8% of the total cells were already dead ($3.39 \pm 1.17 \cdot 10^4$ cells/mL). After 24h (Fig. 5.3B) the abundance of damaged cells significantly increased at C1 (PERMANOVA; $p < 0.05$) ($2.94 \pm 0.98 \cdot 10^5$ cells/mL), corresponding

to 74.2% of total cells, and at C2 ($5.73 \pm 0.98 \cdot 10^5$ cells/mL) were all cells resulted damaged (100%). After 48h and 72h (Fig. 5.3C and D) at concentrations C2, C3, and C4 cells were all dead, except for C2 where after 48h some cells resulted damaged (about 10%).

After 72h at the concentration C1 damaged cells survived but resulted at a low density ($2.49 \pm 0.63 \cdot 10^5$ cells/mL) and mostly of them (75% of total cells) tended to form aggregates or chains (Fig. 4.4).

Damaged cells were also observed in the treatment with DMSO after 3h and 48h, but their concentrations resulted low and not significant.

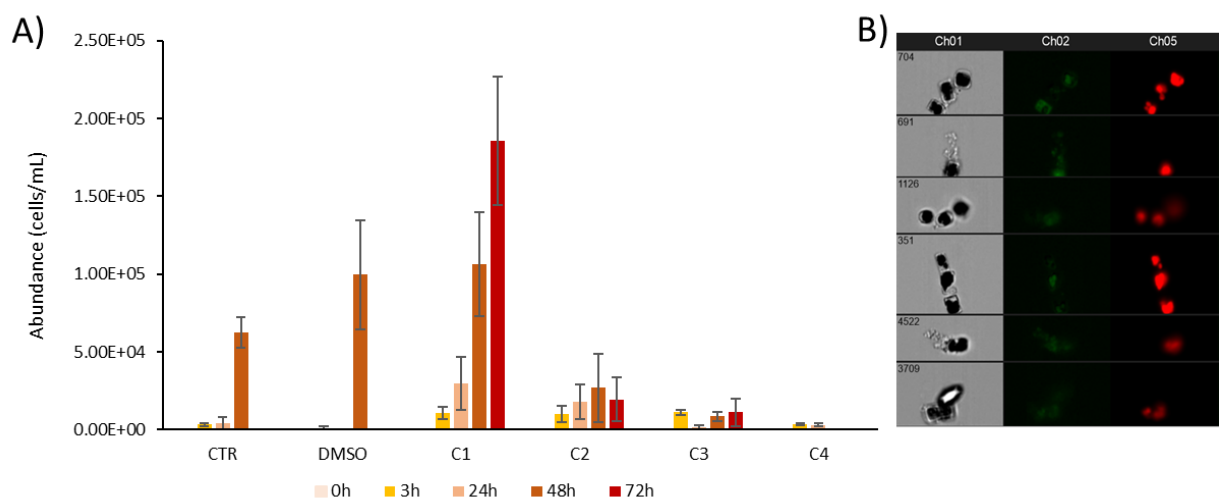


Figure 5. 4 – A) Effect of dilkamural (DK) at different concentrations (C1-C4, range 8–200 μ M) on the abundance of chains or aggregates of *Cyclotella cryptica* at all tested times (0-72h) compared to the control conditions (i.e., CTR with water or DMSO, concentration = 0). Data are means of independent replicates ($n = 3$) and the error bars represent standard errors. B) and D) images associated to Brightfield (Ch01), Chlorophyll autofluorescence (Ch05) and green fluorescence (Ch02) of *Cyclotella cryptica* stained using the fluorescent dye SYTOX Green and analysed using multispectral image flow cytometry.

As for the effect of DK on *P. tricornutum*, results showed that after 3h (Fig. 5.5A) the abundance of live cells significantly decreased (PERMANOVA; $p < 0.05$) from C1 ($8.31 \pm 0.94 \cdot 10^5$ cells/mL) to C4 ($6.26 \pm 3.51 \cdot 10^4$ cells/mL), corresponding to about 95 and 7% of the total cells, respectively. Conversely, the number of damaged cells significantly increased (PERMANOVA; $p < 0.05$) from C1 ($4.34 \pm 3.20 \cdot 10^4$ cells/mL) to C4 ($8.51 \pm 0.87 \cdot 10^5$ cells/mL) up to 93%, but no dead cells were reported (Fig. 5A). After 24h (Fig. 5B) dead cells were found at C3 ($2.82 \pm 0.52 \cdot 10^5$ cells/mL) and C4 ($3.55 \pm$

1.08 10^5 cells/mL), with an increase after 48h and 72h (Fig. 5C and D) up to 64 and 81% of the total cells, respectively, although live cells were still present at a relative abundance of about 34% ($1.14 \pm 0.40 \cdot 10^5$ and $1.63 \pm 1.30 \cdot 10^5$ cells/mL after 48h; $1.01 \pm 0.42 \cdot 10^5$ and $2.33 \pm 1.79 \cdot 10^5$ cells/mL after 72h).

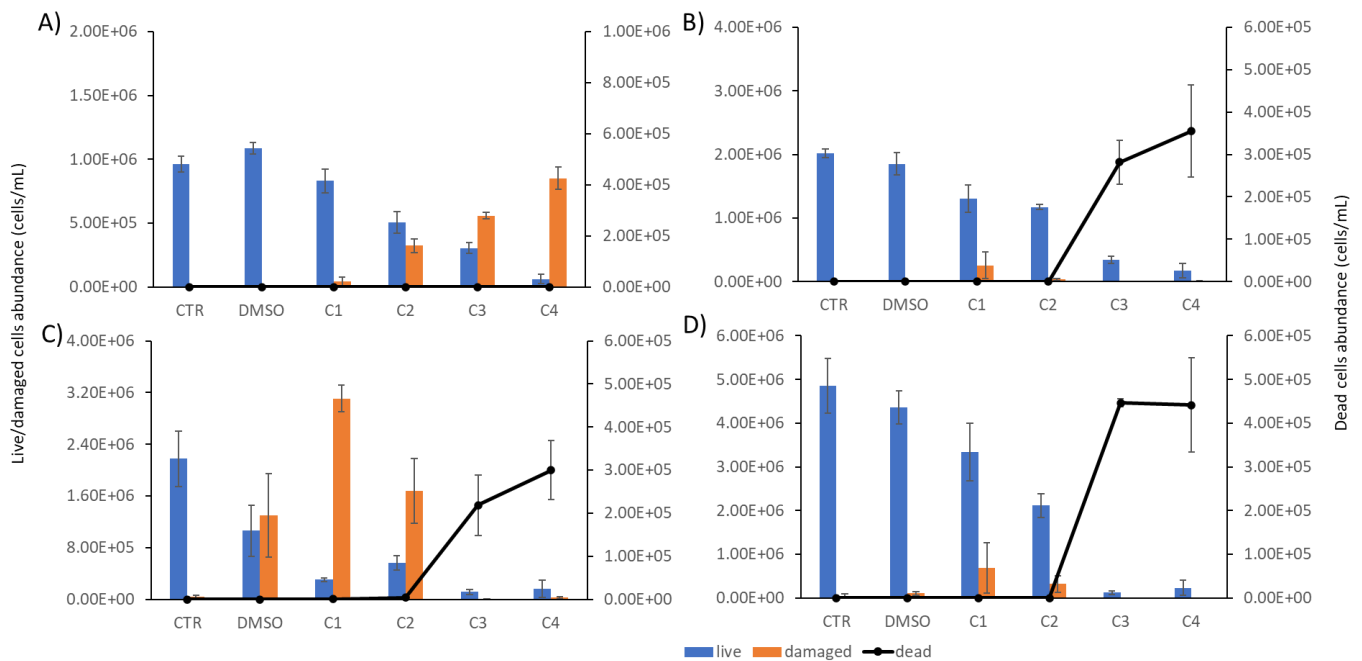


Figure 5 - Effect of dilkamural (DK) at different concentrations (C1-C4, range 8–200 μ M) on the abundance of live, damaged (left axis) and dead (right axis) cells of *Phaeodactylum tricornutum* after A) 3h, B) 24h, C) 48h, and D) 72h compared to the control conditions (i.e., CTR with water or DMSO, concentration = 0). Data are means of independent replicates (n = 3) and the error bars represent standard errors.

P. tricornutum showed the presence of three different morphotypes (Fig. 5. 6): triradiate, fusiform and oval, with the triradiate as the most abundant (50%), followed by the oval (35%) and the fusiform (15%) at control conditions. The results of the toxicity assays performed with DK demonstrated that the evolution of the three morphotypes abundance after DK addition was different. The relative abundance of oval morphotype significantly decreased (PERMANOVA; $p < 0.05$) from 3h to 72h at the highest concentrations (C3 and C4), reporting relative abundances of 39% (C3) and 22% (C4) after 3h and 12% (C3) and 8% (C4) after 72h. Contrarily, the relative

abundance of the triradiate morphotype increased from 3h to 72h at the highest concentrations, with values of 47% in C3 and 33% in C4 after 3h, and 62% (C3) and 60% (C4) after 72h. The relative abundance of the fusiform morphotype after DK addition maintained a constant trend (mean value 16.0 ± 1.3 %).

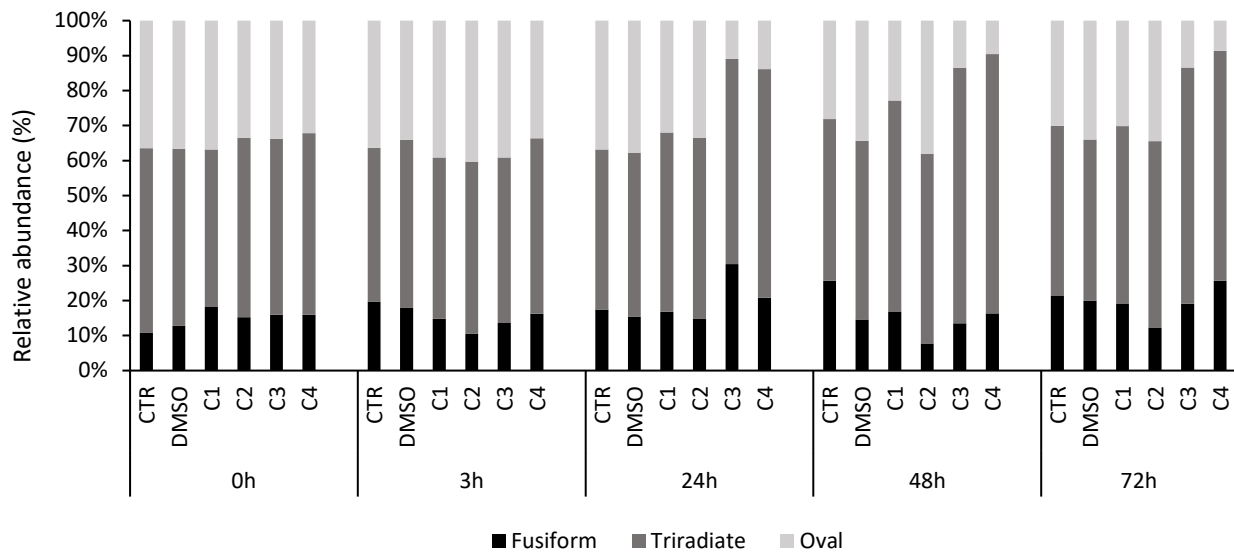


Figure 5.6 - Effect of dilkamural (DK) at different concentrations (C1-C4, range 8–200 μ M) on the relative abundance (%) of the *Phaeodactylum tricornutum* morphotypes (i.e., fusiform, triradiate and oval) at all tested times (0-72h) compared to the control conditions (i.e., CTR with water or DMSO, concentration = 0).

Table 5.2 shows the EC50 values calculated for *Synechococcus* sp. and *P. tricornutum*. These results confirmed the lower toxicity of DK towards the diatom (EC50 values of 8.86 and 29.86 μ M after 24h for *Synechococcus* sp. and *P. tricornutum*, respectively) and a time-dependent toxicity, as attested by the slight decrease in the EC50 value obtained for *P. tricornutum* after 72h (EC50 value of 21.27 μ M). No EC50 values were possible to be calculated for *C. cryptica* due to the rapid toxic effect of DK on these cells, observed even only after 3h.

Table 5.2 – EC50 values (μM) and 95% confidence limits calculated for *Synechococcus* sp. after 24h, and *P. tricornutum* after 24 and 72h of exposition to dilkamural.

	EC50	Lower limit	Upper limit
<i>Synechococcus</i> sp. (24h)	8.86 ± 2.00	4.5	13.22
<i>P. tricornutum</i> (24h)	29.36 ± 11.88	3.49	55.24
<i>P. tricornutum</i> (72h)	21.27 ± 7.27	5.44	37.11

5.4 Discussion

Our study has shown significant effects of the recently characterized metabolite dilkamural (DK) (García-Gómez et al., 2020), which was freshly extracted from the Phaeophyta *Rugulopteryx okamurae*, on the growth of three phytoplanktonic species. It has been recently reported that DK made *R. okamurae* less palatable for a generalist native herbivore as the sea urchin *Paracentrotus lividus* and had also harmful and even lethal effects on it (Casal-Porras et al., 2021). In this work allelopathic effects of dissolved DK on other phototrophic organisms, as diatoms and cyanobacteria, were demonstrated. In fact, as regards the cyanobacteria *Synechococcus* sp., this species resulted sensible to dilkamural either by decreasing cell division rates or by cellular damage as most cells were damaged or dead after being exposed for 48 h to concentrations up to 100 and 200 μM of DK. As reported in the literature, some allelochemical compounds can inhibit the growth of cyanobacteria (Zhu et al., 2021; Nakai et al., 2012; Hu and Hong, 2008; Ni et al., 2015b; Huang et al., 2016). For instance, Wu et al. (2011) documented that the disappearance of cyanobacterial blooms could be due to allelopathic phenomena between the cyanobacteria and periphyton biofilms dominated by diatoms (e.g., *Synedra*, *Fragilaria*, *Melosira*, and *Nitzschia* spp.) and bacilli and cocci bacteria. In particular, it was reported that periphyton biofilm could produce allelochemicals that significantly inhibit the growth of cyanobacteria through damage to the thylakoid membranes, electron transport disruption in photosystem II, decreasing in the effective quantum yields, and ultimately failure of photosynthesis (Wu et al., 2011). Comparing the species tested in our study, the diatom *Cyclotella criptica*, appeared to be the most sensitive to DK compared to the other ones tested in this study. Diatoms are a major constituent of the phytoplankton in oceanic and coastal

waters, and *Cyclotella* spp. are very representative of coastal phytoplankton. We found as rapid toxicity was reported and after 72h all most cells were death even at a low concentration (C2 i.e., 40 μ M). As for *Synechococcus*, this cyanobacterium is one of the most important components of photosynthetic picoplankton in temperate and tropical oceans and its presence is widespread throughout the global oceanic surface and freshwater environment, being able to adapt to different temperature, light, and nutrients conditions (Kim et al., 2018). Testing the DK effect in various genera and species resulted interesting in view of evaluating the global effect of this metabolite in coastal phytoplankton community succession in invaded areas. We observed that DK induced cell aggregation in *C. criptica* cultures. In the literature, the formation of aggregates was reported in a previous study in which *C. criptica* cells were treated with the fungicide Polyoxin D (Morin et al., 1986). These authors observed that the metabolite affected chitin fiber formation, causing a significant decrease in cell density, increase in sedimentation rates, and a strong cell aggregation tendency compared to the control. Other metabolites, as polyunsaturated aldehydes, have also shown to increase diatom size-aggregation (Bartual et al., 2017) by increasing exopolymeric production of diatom cells as a response. As a result, in both cases diatom cells aggregation was related with growth inhibition and stress. Here we also found an increase in damaged and dead cell, so, regardless of the factor that induces such observed aggregation, it resulted clear that it is an indirect effect of DK on *C. criptica*. Such aggregation phenomena could have important consequences in the natural environment, if hypothesised as a general response of coastal diatoms, since this aggregation could alter the position of these organisms in the water column, limits their nutrient availability and photosynthetic rates, inducing cell collapse. Additionally, considering that diatoms are the most abundant phytoplanktonic group in coastal areas (Carstensen et al., 2015), periphyton (Hoagland et al., 1981) and coastal macroalgal epiphytes (Totti et al., 2009), the effect of DK on invaded coastal areas could have an important ecological effect in coastal C exportation.

Regarding the effects on the diatom *Phaeodactylum tricornutum*, it appears to be the most resistant species, as after 72h at the highest concentration (200 μM) live cells were still present, although a distinct effect of dikamural on the three morphotypes (i.e., fusiform, triradiate and oval) was found. In fact, at the highest concentrations (100 and 200 μM) after 24h the oval morphotype tended to decrease. This species is not an abundant diatom in global coastal areas, however, it is a model to study diatom biology at the molecular level. *P. tricornutum* is known to have the unusual property of being pleiomorphic, and this plasticity is related to the atypical nature of the cell wall, which is only poorly silicified compared with other diatoms (Lewin et al. 1958, Borowitzka and Volcani, 1978). This diatom has the unique capability of morphotype conversion, which is relevant in acclimation to changing environmental conditions (De Martino et al., 2011). The oval cell is the only morphotype that forms a single silica frustule, embedded in an organic casing, which contains a raphe and allows the gliding motility typical of raphid diatoms (Tesson et al., 2009). Whether and how DK could affect *P. tricornutum* morphotype abundance has to be further investigated.

5.6 Conclusions

Various abiotic and biotic factors are involved in the successful settlement of invasive macroalgae (Cardeccia et al., 2018), and among these, there is a growing consensus on the crucial role that allelochemical defences produced by invasive species can have on native biota. Based on the results of this first study, where dissolved DK resulted to affect the viability of unicellular photosynthetic organisms, effects on algal population dynamics could be expected due to the production of this metabolite by *Rugulopterix okamurae*. This macroalga could be responsible of allelopathic effects towards other photosynthetic organisms or native herbivores and competitors (Casal-Porras et al., 2021), supporting the so-called “novel weapons hypothesis” proposed by Callaway and Aschehoug (2000), that invaders may use chemical weapons, which act as unusually powerful allelopathic agents and are not present in the invaded natural communities, as competitive mechanisms. If our

results are extended to more phytoplanktonic species, the main consequence of DK presence in invaded areas, could be the potential reduction of the biodiversity of the coastal ecosystems where *R. okamurai* have been introduced and extensively proliferates. Further studies aimed at investigating the effect of DK on other organisms (e.g., microphytobenthos, copepods) belonging to *R. okamurai* associated community could help in understanding this competitive ability.

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Supplementary data

Figure S5.1 – Dose-response curve obtained for *Synechococcus* sp. after 24h of exposition to the different concentrations of dilkamural. Data are means of independent replicates (n = 3).

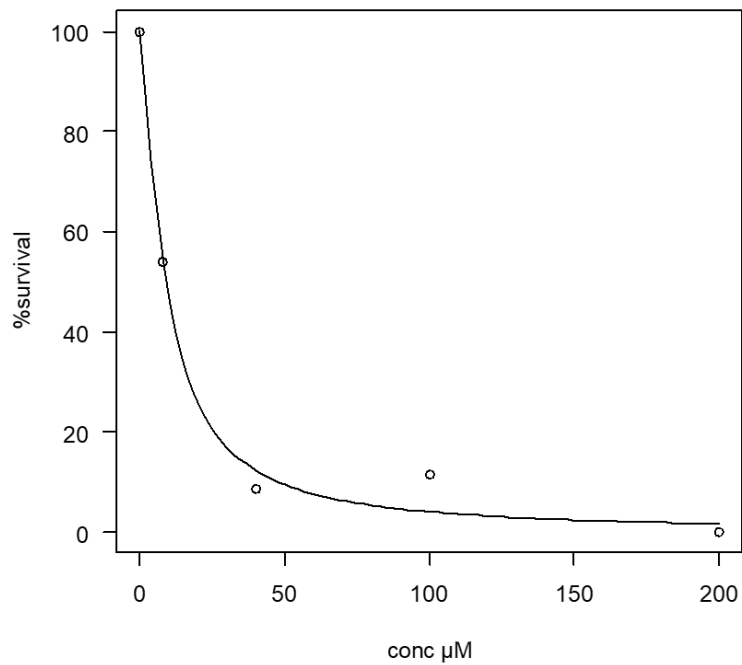


Figure S5.2 - Dose-response curve obtained for *P. tricornutum* after 24h of exposition to the different concentrations of dilkamural. Data are means of independent replicates (n = 3).

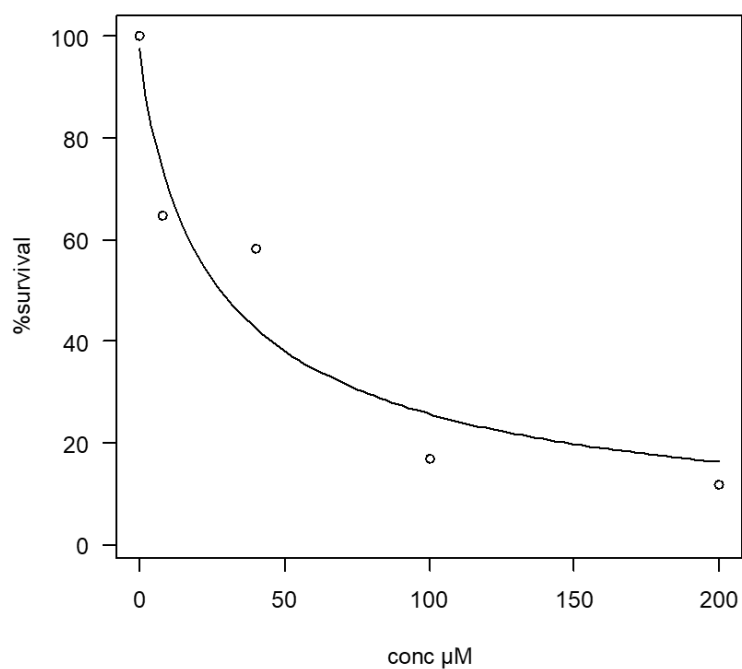
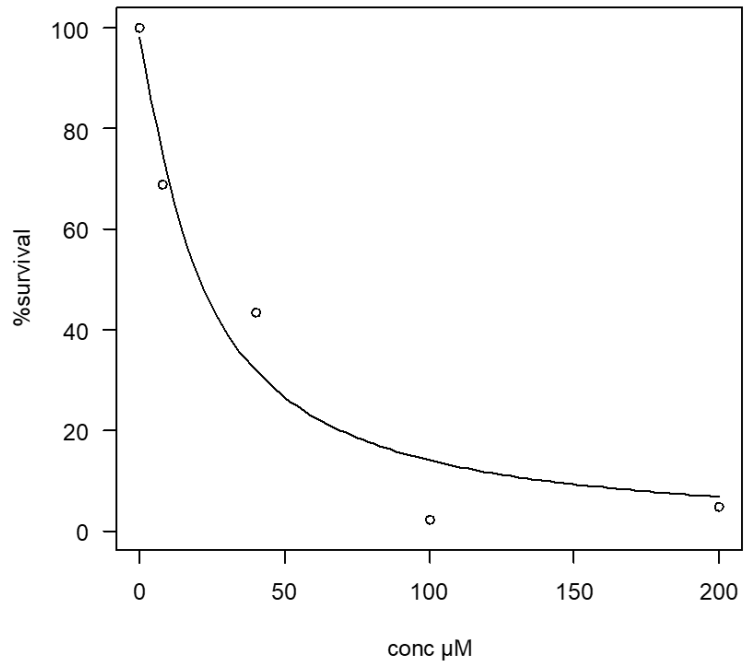


Figure S3 - Dose-response curve obtained for *P. tricornutum* after 72h of exposition to the different concentrations of diltiazem. Data are means of independent replicates (n = 3).



Chapter 6 – Conclusions

In chapter 2, it has been seen how two different species of Mediterranean macroalgae, *Dictyopteris polypodioides* and *Cystoseira compressa*, show: i) a clear separation of the associated epiphytic communities; ii) a different PUA profile (*D. polypodioides* produces long-chain compounds, mainly tetradecapentaenal (14:5) and hexadecatetraenal (16:4), while *C. compressa* produces only short-chain compound, hexadienal (6:2)); iii) different morphology and structural complexity.

The results of this study have suggested that there is a relationship between the production of PUAs and the morphological structure of macroalgae and the structure of the associated communities. They also have suggested that the effect of PUAs is species-specific. It is nevertheless essential to highlight, that this study was carried out in the field where other environmental factors play a fundamental role and it is thus difficult to demonstrate and quantify the effect of any single factor. Consequently, in chapter 4 of this thesis, I have performed laboratory experiments with microcosms to better understand the relationship between PUAs and the meiobenthic community.

These studies showed a clear effect of the presence of the PUAs producing diatom *Skeletonema marinoi* on copepods *nauplii*, as already reported in the literature (Caldwell, 2009; Ceballos and Ianora, 2003; Ianora et al., 2004; Poulet et al., 2007b). In addition, the effects of both *S. marinoi* and decadienal standard were distinctly species-specific. In fact, the most affected species (*Heterolaophonte minuta* and *Ameira parvula*) were phytal species, and therefore more sensitive by various chemical compounds produced by both macroalgae and microalgae.

In chapter 3, I investigated the effect, on epibenthic community, of PUAs production of two invasive macroalgae (*Sargassum muticum* and *R. Rugulopterix okamurae*), with other native macroalgae in different environments.

I also took into consideration the morphological structure of the analyzed macroalgae, to evaluate if this too could influence the structure of the meio and phytobenthic communities.

The study involving *S. muticum* (chapter 3), conducted in the two sites of the Adriatic Sea, has highlighted that both the morphological characteristics of macroalgae and their PUAs production can influence the phytobenthic and meiobenthic community, and above all how the presence of an invasive alga can change the composition of a habitat.

The study involving *R. okamurae*, carried out in Tarifa, Spain, also showed a clear effect on the associated phytobenthic community, especially in terms of the abundance of the taxa, which appeared unhealthy and in a state of senescence. These results can be attributed to the production,

by *R. okamurae*, of the toxic compound dilkamural. An exception was the toxic dinoflagellate *Ostreopsis. cf. ovata*, which was not affected.

Finally, I analyzed the effect of dilkamural on unicellular primary producers (such as microalgae and cyanobacteria), carrying out toxicity tests (chapter 5). The results showed that dilkamural clearly affected the growth and survival of all tested phytoplankton species responding differently: effects on cell viability and morphology of *Phaeodactylum tricornutum* as well as the cell integrity and division of *Synechococcus* sp. and *Cyclotella cryptica*. The results highlight how the production of dilkamural by *R. okamurae* could influence the viability of unicellular photosynthetic organisms and the dynamics of the algal population.

Allelopathy is a very important and complex process among benthic organisms, as there is a coevolution between emitters and targets, therefore, the allelopathy may not be visible, even if, it is responsible for the observed community structure.

More studies are still necessary and should be supported in order to know the complex relations among organisms in marine environments, to preserve biodiversity and to increase our basic knowledge about allelopathy and natural adaptation to changing physical, chemical and biological factors in marine environment.