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XXXVI CICLO DEL DOTTORATO DI RICERCA IN AMBIENTE E VITA

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Diatom associations on seagrass meadows: Effects of Ocean Acidification on the first colonization and plant-animal chemical relationships

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“Few objects are more beautiful than the minute siliceous cases of the diatomaceae: were these created that they might be examined and admired under the higher powers of the microscope?”

Darwin, 1886

A Tina, a Gemì

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RIASSUNTO

Le emissioni atmosferiche di CO_2, causate principalmente dall'uso dei combustibili fossili, dalla produzione di cemento e dai cambiamenti nell'uso del suolo, vengono assorbite dall'oceano in quantità sempre maggiore fin dall'inizio dell'era industriale. Questo fenomeno, noto come "acidificazione degli oceani", è considerato essere una delle principali minacce per la stabilità delle comunità marine superficiali e profonde. Tra i cambiamenti ambientali globali, l'acidificazione oceanica sta causando sia effetti diretti sia indiretti sul biota marino. Una delle principali sfide per gli scienziati, i gestori e i decisori politici è comprendere come gli ecosistemi risponderanno a questi cambiamenti, considerando sia le specie 'vincitrici' che le 'perdenti' nel loro complesso. In questo contesto, i *vent* di CO_2 producono condizioni ecologiche potenzialmente analoghe a quelle dei futuri oceani acidificati e rappresentano una grande opportunità per capire le risposte delle specie e delle comunità esposte a lungo termine a basse condizioni di pH.

I *vent* sono ambienti localmente acidificati e vengono usati in tutto il mondo come laboratori naturali per testare gli effetti dell'acidificazione non solo su singole specie, ma anche su intere comunità, valutando gli effetti indiretti sugli ecosistemi. I produttori primari, come ad esempio le praterie di fanerogame, possono essere avvantaggiati dagli elevati livelli di CO_2 presente nell'acqua marina. In particolare, la pianta marina *Posidonia oceanica* ha un'importanza fondamentale in Mediterraneo, grazie soprattutto a tutti i servizi ecosistemici che essa svolge. In condizioni naturalmente acidificate (come ad esempio nei *vent* superficiali di CO_2 di origine vulcanica), negli ecosistemi a *P. oceanica* aumenta la complessità strutturale delle comunità associate, ma diminuisce l'abbondanza delle specie epifite calcaree (ad esempio le alghe Corallinaceae). Tra gli epifiti che vivono associati alle foglie di *P. oceanica,* un ruolo ecologico rilevante è svolto dalle diatomee bentoniche. Le diatomee bentoniche rappresentano organismi chiave attraverso la formazione di biofilm primari e secondari, giocando un ruolo molto importante nel determinare la struttura e la

dinamica delle comunità bentoniche successive. Per questa ragione, le diatomee bentoniche rappresentano importanti elementi nella determinazione dei pattern di colonizzazione.

La presente ricerca di dottorato ha mirato alla valutazione degli effetti delle condizioni di alta pCO_2 / basso pH sull'organizzazione strutturale e funzionale delle comunità associate di diatomee bentoniche in un *vent* superficiale di CO_2 in Mediterraneo. Per prima cosa, analizzando la letteratura disponibile, abbiamo riassunto le conoscenze attuali in merito agli impatti dell'acidificazione sugli stadi di colonizzazione primari e la successione di comunità bentoniche nel tempo (**Capitolo 1**). Successivamente sono state raccolte le diatomee epifitiche attraverso pannelli di campionamento a bassa adesione costruiti per selezionare principalmente gli stadi di colonizzazione primari. Da questi campioni è stato possibile risalire alle associazioni di diatomee bentoniche appartenenti a diverse condizioni di pH (siti acidificati *versus* siti a pH normale), grazie all'identificazione morfologica basata sull'analisi delle immagini dell'ultrastruttura del frustulo al Microscopio Elettronico a Scansione (**Capitolo 2**). Sfruttando i campioni ottenuti, abbiamo anche isolato, selezionato e coltivato nuove diatomee bentoniche monoclonali axeniche. Un'attenzione particolare è stata posta sull'identificazione, ottenuta mediante un approccio polifasico (molecolare e morfologico) di una diatomea "modello" isolata: *Cocconeis neothumensis* var. *marina* (**Capitolo 3**). In questa visione, la mia ricerca si è focalizzata sull'ecofisiologia di ceppi di diatomee selezionati in natura a diverse condizioni di pH. In particolare, sono stati testati i possibili effetti dell'acidificazione oceanica su crescita e metabolismo dei due ceppi coltivati (**Capitolo 4**). Per concludere, abbiamo testato gli effetti dell'acidificazione sulle relazioni chimiche pianta-animale. In particolare, le diatomee bentoniche del genere *Cocconeis*, dominanti le comunità bentoniche associate a *P. oceanica*, sono note per la produzione di composti apoptogenici che influenzano l'inversione precoce del sesso del decapode *Hippolyte inermis*. A questo proposito, abbiamo esplorato le risposte di *H. inermis* a diverse fonti di cibo. In particolare, abbiamo effettuato un esperimento di *bioassay* fornendo alle post-larve due ceppi di *C. neothumensis* isolati dai siti a pH acido e normale (**Capitolo 5**).

ABSTRACT

The atmospheric CO_2, primarily caused by combustion of fossil fuels, cement production and land use changes, is being absorbed by the ocean at faster rate since the beginning of the industrial era. This phenomenon, known as ocean acidification (OA), is considered one of the main threat for the marine communities. Among global environmental changes, OA is causing both direct and indirect effects to the marine life, and one of the main challenges for scientists, managers, and policy-makers is understanding how the ecosystems will respond to these changes, considering the ‘winners’ and the ‘losers’ species in their entirety. In this context, shallow CO_2 vents provide potential analogues of forecasted acidified ocean and represent a great opportunity to understand the responses of species and communities long-term exposed to low pH conditions.

Vents are locally acidified environments that are used worldwide as natural laboratories to test acidification effects not only on single species, but also on whole communities, evaluating indirect effects on ecosystems. Primary producers, such as seagrass meadows, may take advantage from the higher seawater CO_2 levels. In particular, the foundation species *Posidonia oceanica* has a key importance for the Mediterranean Sea, for all the ecosystem services that it supports. Under naturally acidified conditions (*i.e.*, shallow volcanic CO_2 vents), *P. oceanica* increases its habitat complexity but lower the abundance of epiphytic calcareous species (*e.g.*, coralline algae). Among the epiphytes that live associated with *P. oceanica* leaves, a relevant ecological role is covered by benthic diatoms. Benthic diatoms represent key organisms riding through primary and secondary biofilm formation, playing important roles in determining the structure and the dynamic of the overlying benthic communities. For this reason, they represent important elements in the determination of colonization patterns.

Here, the current PhD research aimed to assess the effect of high $p\mathrm{CO}_2$ / low pH conditions on the structural and functional organization of benthic diatoms assemblages in a Mediterranean shallow CO_2 vent. First, by reviewing the available literature, we summarize the present knowledge

about the impacts of OA on the early colonisation stages and the succession of benthic communities over time (**Chapter 1**). Then we collected epiphytic diatoms through *ad-hoc* developed low adhesive sampling panels capable of selecting mainly the early colonization stages. Thus, we were able to reconstruct the benthic diatoms assemblages belonging to different pH conditions (acidified *versus* control sites) thanks to the morphological identification base on the analyses of frustule ultra-structures on Scanning Electron Microscope (SEM) images (**Chapter 2**). Exploiting the collected samples, we also isolated, selected and cultured new axenic monoclonal benthic diatoms. Special attention was paid to the identification through a polyphasic approach (molecular and morphological) of a well-established model diatom isolated: *Cocconeis neothumensis* var. *marina* (**Chapter 3**). In this view, my research work focused on the ecophysiology of diatom strains selected in nature under different pH conditions. In particular, the possible effects of ocean acidification on the growth and metabolism of the two cultured strains were tested (see **Chapter 4**). Finally, we tested the effect of OA on the plant-animal chemical relationships. In particular, the benthic diatoms of the genus *Cocconeis*, dominating benthic communities associated with *P. oceanica*, are known for the production of apoptogenic compounds influencing the early sex reversal of the decapod *Hippolyte inermis*. To this purpose, we approached this issue by exploring the responses of *H. inermis* to different food source. In particular, we carried out a bioassay experiment supplying to the post-larvae two strains of *C. neothumensis* isolated both from control and low pH sites (**Chapter 5**).

GENERAL INTRODUCTION AND LINK TO THE CHAPTERS

Global environmental changes are an incontrovertible phenomenon that poses pressing questions about the future of our planet. Among all, Ocean Acidification (OA) represents a key-problem for marine environments from which a multitude of deleterious effects arises (Caldeira & Wickett, 2003; Doney et al., 2009; Gattuso et al., 2015). The increase in the concentration of atmospheric CO_2, mainly due to anthropogenic emissions, is causing the decrease of seawater pH, causing both direct and indirect effects to the marine life (Zunino et al., 2019). Direct effects, such as the erosion of carbonate shells and exoskeletons, are more evident and consequently better studied. On the other hand, only in recent years investigations have been carried out on the indirect OA effects, especially regarding changes in taxonomic and functional biodiversity, and the overall functioning of ecosystems at community level (Garrard et al., 2013).

In this view, seagrass meadows, and in particular *Posidonia oceanica*, represent important habitats because they offer shelter to a rich associated community of fish and invertebrates, for their high biodiversity, because of their effects on the ecology of coastal waters, because they may represent reproduction sites for several marine organisms and also for their resilience, as compared to other environments. The well-known time succession of the leaf stratum communities leads to stable associations that assure their biodiversity and the correct seasonal functioning. Furthermore, such features determine well-known ecosystem services, and their importance both for an ecological and an economic point of view. Indeed, *P. oceanica* produces highly complex ecosystems and their associated plant and animal communities are stabilised by specific chemical and trophic relationships.

The importance of early colonisation stages is mainly due to their influences over the well-known succession of benthic communities over time. Indeed, the modifications to the surface properties promoted by pioneer organisms, such as diatoms, ease the surfaces consortia and associations. This process might be negatively affected by climate changes. The impact of global stressors such as OA on benthic ecosystems, especially on the microphytobenthic assemblages, is still poorly known, and might produce deleterious effects for benthic successions (see Chapter 1).

Hence, my PhD project is aimed at studying the changes of key communities such as the epiphytic diatoms in the *P. oceanica* leaf stratum and, in particular, focusing on the early colonisers and plant-animal chemical relationships in the leaf stratum. Consequently, here we dealt with the assessment of the effects of high $p\mathrm{CO_2}$/low pH conditions on the structural and functional organization of diatoms assemblages in a Mediterranean shallow CO_2 vent.

In the Mediterranean Sea, one of the best-known shallow CO_2 vents is located in Ischia Island (Gulf of Naples, Italy). Here, exploiting the unique features of this area as “a window on the future” (Hall-Spencer et al., 2008), we compared the responses of epiphytic diatoms assemblage over a long-term exposed to high CO_2 emissions (Low pH site, pH = 7.7), against two assemblages living at normal pH (Controls, pH = 8.2). We hypothesized that OA might facilitate the selection of specific genera of benthic diatoms, altering the organization of the assemblages in the low pH site from that found in the two controls, with consequences for the structure and the overall trophic organization of leaf-associated communities. To test this hypothesis, we collected epiphytic diatoms through *ad-hoc* developed low adhesive sampling panel system capable of selecting mainly the early colonization stages. Thus, we were able to reconstruct the benthic diatoms assemblages belonging to different sampling sites (exposed to different pH conditions) thanks to the morphological identification carried out through analysis of frustule ultra-structures on Scanning Electron Microscope (SEM) images. The collected material also underwent an isolation process through which we succeeded in the difficult task of selecting and culturing new axenic monoclonal benthic diatoms. Special attention was paid to the identification through a polyphasic approach of a well-established model diatom isolated: *Cocconeis neothumensis* var. *marina* (see Chapter 3).

An interesting achievement deriving from the isolation process was the culture of two strains (three replicates for each strain) of the same diatom species collected in sites characterized by different pH conditions. Taking advantage of the above-mentioned identification based both on molecular and morphological analyses, it was possible to properly identify the species. In this view, my research work focused on the possible effect of OA on the selection of diatom strains with a special attention on the growth and the metabolism of the two strains cultured (see Chapter 4).

It is worth to note that epiphytic diatoms are among the early colonisers of *P. oceanica* leaves, representing important elements in the determination of the ecological succession patterns. They also play a key role in the trophic web, constituting primary energy source for herbivore grazers. Furthermore, diatoms exert several ecological roles in marine benthos, especially for what concern plant-animal chemical relationships. In this regard, there is a peculiar example involving benthic diatoms and crustaceans. Diatoms of the genus *Cocconeis*, a genus dominating benthic communities associated with *P. oceanica*, are known for the production of apoptogenic compounds influencing the early sex reversal of the caridean decapod *Hippolyte inermis*. These relationships are vital to assure stability to the shrimp populations. Nevertheless, OA may affect diatom physiology and metabolism, provoking the interruption or the disruption of such important chemical communication. This scenario might have drastic impact overall ecosystem due to the great importance of *H. inermis* in the trophic web of *P. oceanica* meadows. To this purpose, we approached this issue by exploring the responses of *H. inermis* to different food source. In particular, we carried out a bioassay experiment supplying to the post-larvae two strains of *C. neothumensis* isolated both from Control and Low pH sites (see Chapter 5).

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CHAPTER 1

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Global Changes Alter the Successions of Early Colonizers of Benthic Surfaces

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Abstract

The successions of benthic communities over time are strongly influenced by the first colonizers, because surface associations are facilitated by modifications to the adhesive properties promoted by primary colonizers, such as bacteria, protozoans, diatoms, algal propagules, spores, and invertebrate larvae. Bacteria are often the first colonizers on marine submerged surfaces, both organic (e.g., algae, seagrasses and invertebrates) and inorganic. However, they are promptly followed by diatoms and other microorganisms. Consequently, diatoms may represent key elements in the determination of the colonization patterns, although the development of epiphytic communities is a dynamic process influenced by several factors, including nutrient availability, the ability to synthesize and secrete extracellular material, the competition among species and the influence of grazers on individual colonizers. The process may be drastically impacted by global warming and ocean acidification due to the increasing atmospheric levels of CO_2. The impact of such global stressors on benthic ecosystems, especially on the primary microphytobenthic assemblages, is still poorly investigated, and may have deleterious consequences for the benthic successions. In this review, we analyze the adhesion patterns of marine microorganisms according to their surface features and the effects of global changes on critical pioneer colonizers, such as the benthic diatoms. The results are remarkable, as they highlight emergent concerns in ecosystem conservation and the prediction of benthic communities.

Keywords: biofilm, diatom, global change, global warming, ocean acidification.

Introduction

The settlement of organisms anchoring to any submerged surface, including deposit formation, encrustation, crudding, deposition, scaling, scale formation, slagging, and sludge (Characklis & Cooksey, 1983), is a well-known phenomenon which has historically been the object of basic and applied studies for its potential influence on many human activities, including the installation of structures in coastal areas and the navigation (Molino & Wetherbee, 2008). Global changes, such as ocean warming and acidification due to increasing $p\mathrm{CO}_2$, are changing the complex relationships between organic and inorganic components, leading to new paradigms and requiring new actions to control the expansion of encrusting communities (Wetherbee et al., 1998). However, encrusting organisms are also important components of benthic communities, because various micro- and macro-organisms may succeed up to the reaching of mature communities. Epiphytic communities are crucial in the food webs of important marine habitats, such as those characterizing seagrasses. The complex ecosystems of seagrasses, which provide a nursery for several species of fish and invertebrates, are important for fisheries and other human activities. They depend on the succession of organisms anchoring to the surface of leaves. Often, this succession starts with bacteria and diatoms, which grow at the base of leaves (the younger portions), followed by algae, bryozoans, polychaetes and other invertebrates (Figure 1), leading to the complete decay of the oldest portion of leaves (the upper parts), which is lost as detritus provide a nursery for several species of fish and invertebrates, are important for fisheries and other human activities.

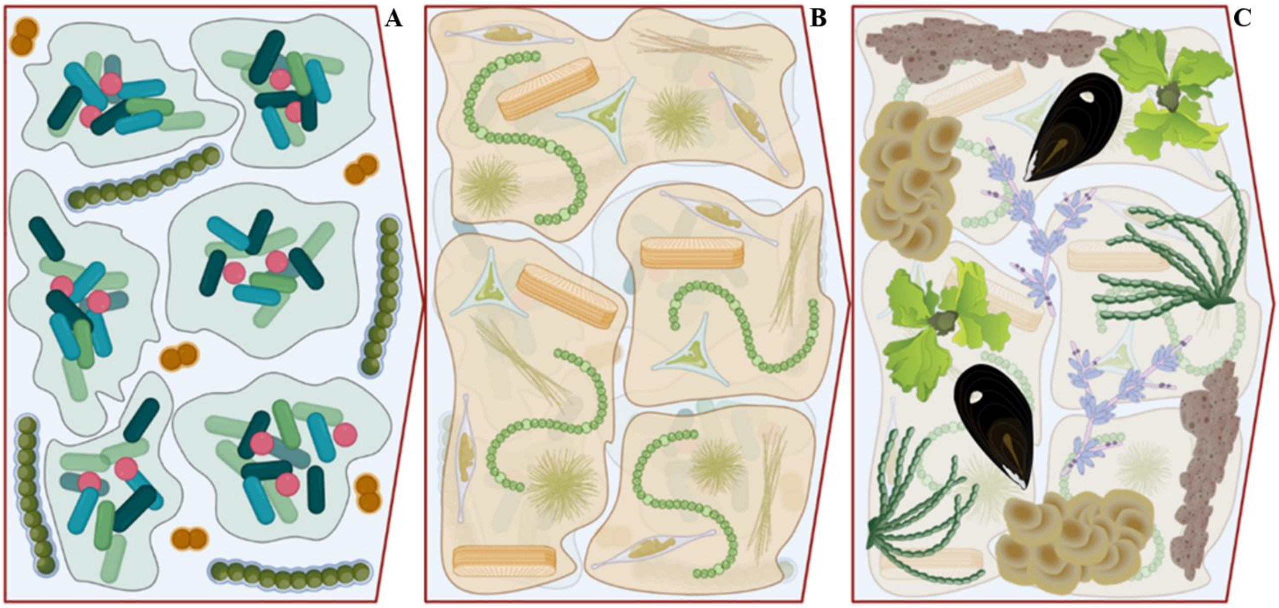

Figure 1. Bacteria are the first colonizers on almost all marine submerged surfaces (A), both live (as algae, seagrasses and invertebrates) and abiotic. They are promptly followed by diatoms and other microalgae, producing a first basal layer (B), named the "conditioning film". This film is essential for further successions, promoting the adhesion of plant and animal macro-epibionts (C).

The detritus itself is a source of nutrition for a complex food web and a means of exporting organic matter to closer ecosystems and micro-epiphytic matter to other surfaces. Possible alterations in the epiphytic communities occur due to global changes, and they may have consequences both for the control of biofouling on submerged structures and the functioning of ecologically relevant seagrass meadows. Here, we reviewed the available literature to: i. state the role and ecology of the first colonizers; ii. analyse their actual roles in the determination of further successions; iii. analyse the effects of global stressors, such as ocean acidification and warming, on the process of colonization; iv. forecast newer management strategies to control the expansion of organisms encrusting on various submerged structures, while conserving the services provided by complex environments, such as the seagrass meadows.

Epiphytes and Early Colonizers

A variety of surfaces, both biotic (e.g., seagrasses leaves, algae, sessile and even motile animals) and abiotic (e.g., rocks, mineral aggregates), are available for the adhesion of benthic organisms in the marine environment (Wetherbee et al., 1998). Although all these surfaces are characterized by specific chemical, physical and biological properties, they all share an important feature (Huggett et al., 2009): they are, sooner or later, colonized by marine microorganisms that establish a complex biofilm (Davey & O'toole, 2000). Since the pioneering paper by Zobell & Allen (1935), it has been acknowledged that the formation of epiphytic/epizoic communities leads to a predictable succession of events, although the specific interactions involved in the mechanisms of the initial surface adhesion are still largely unexplored (Wahl et al., 2012) and may rule both the benthic communities and the epiphytic associations characterizing any natural substrate in benthic environments. However, clear evidence indicates that the succession events are largely influenced by the nature and composition of the first colonizers (Salta et al., 2013). Benthic microorganisms may be selected as well, according to the molecular properties of the submerged surfaces (Dang & Lovell, 2000). Marine biofilms are composed of heterogeneous communities and also contain metabolic by-products (Allison, 2010; Salta et al., 2013) such as exopolysaccharides and algal mucilage, which will be further detailed. The development of these communities is a dynamic process influenced by several factors, such as nutrient availability, the ability to synthesize and secrete extracellular material, competition among species and the influences of grazers (Flemming et al., 2016).

Biofilms facilitate the settlement of microorganisms and their shift to a lifestyle that is different from planktonic life (Annuk & Moran, 2010). Whether the biofilm matrix confers an ecological advantage to benthic organisms is still unknown. However, a strong evolutionary benefit for polymer-producing cells has been demonstrated (Flemming & Wingender, 2010). The formation of a matrix starts with the shift from planktonic to benthic life (Pawlik, 1992). This process is likely to depend on the bacterial motility and cell-to-cell communication processes, also known as quorum sensing (QS)

(Dobretsov et al., 2009; Landini et al., 2010). The succession of marine organisms within a biofilm is a complex multi-step process triggered by the formation of a conditioning film composed of adsorbed molecules (mainly glycoproteins), which quickly form on each submersed surface (Flemming & Wingender, 2010). The development of a given community within a microbial biofilm generally starts with the adhesion of a bacterial consortium to the surface (Flemming et al., 2016). Bacterial cells produce extracellular polymeric substances (EPS), mainly composed of exopolysaccharides, proteins, nucleic acids, glycoproteins, phospholipids and humic substances (Vu et al., 2009). Bacterial colonies encased in their EPS promote the formation of a highly hydrated three-dimensional matrix on which the development and maintenance of a marine biofilm structured community depend (Flemming et al., 2016). As observed for the initial conditioning film, the EPS–bacteria complex can modify the available surfaces, forming micro-colonies that modify the physical and chemical properties of the substrate and affecting the subsequent colonization by algae, spores and larvae (Hadfield, 2011; Mieszkin et al., 2012). The recruitment of diatoms and cyanobacteria immediately follows, taking advantage of the changes in the surface of the substrates operated by bacteria (Dang & Lovell, 2016).

Moreover, even organisms such as benthic dinoflagellates, fungi and protozoa can attach themselves to these surfaces by exploiting the mucilage secretions produced by previous colonizers (Molino & Wetherbee, 2008). These communities generally play important roles in determining the structure and dynamics of the overlying benthic communities by enhancing and/or inhibiting the subsequent colonization stages (Terlizzi et al., 2000). The settlement of further stages, including spores, macroalgal propagules and larvae of sessile invertebrates (Freckelton et al., 2017; Hadfield et al., 2014), may be influenced by marine biofilms, which, in several cases, represent a preferred substrate for their attachment (Jenkins et al., 2001; Lau et al., 2005; Patel et al., 2003; Salta et al., 2013; Wang et al., 2012). However, some studies highlighted no effect or inhibitory effects on the settlement of macro-fouler larvae (Hadfield, 2011; Hadfield & Paul, 2001). In this context, peculiar examples are provided by the polychaetes *Spirorbis* spp. (Krug, 2006) and the bryozoan *Bugula* *neritina* (Dahms et al., 2004; Dobretsov & Qian, 2006), for which the settlement of larvae is strongly influenced by the biofilm composition. An extreme example of the selection by the attachment surface was also reported by Wieczorek et al. (1995) for cyprids of *Amphibalanus* sp. (*Balanus*) and for *Amphitrite* spp. Their larvae were induced to settle on older biofilms, which were characterized by a diverse microbial composition and abundance as compared to immature biofilms. Although some examples were provided for all kinds of effects, different experimental procedures may lead to contradictory responses regarding the relationships between macro-foulers and biofilms (de Carvalho, 2018). Although there is a noteworthy amount of literature about biofouling on benthic surfaces, the effects of primary biofilms remain partially unclear (Cacabelos et al., 2020; Sweat et al., 2017).

In addition, a wide range of biotic and abiotic factors may influence the colonization of marine surfaces (Dang & Lovell, 2000; Stegen et al., 2012). Many surface properties influence the colonization patterns, especially at the early stages of biofilm formation (Dang & Lovell, 2016; Scheuerman et al., 1998). The roughness of the surfaces represents one of the most relevant factors supporting the adhesion of marine microbes and promoting biofilm formation (Crawford et al., 2012). To this end, several marine microorganisms exploit the irregularities of the substrates, finding shelter from unfavourable environmental conditions and better adhesion properties (Kardar & Amini, 2022). Additional factors related to the physical and chemical properties of the surface and affecting the colonization are wettability (Dang & Lovell, 2000), surface polarization and tension (Jones et al., 2006), hydrophobicity (Fletcher & Loeb, 1979) and molecular topography (Lee et al., 2008). As suggested by Dobretsov et al. (2009), the succession patterns of marine biofilm communities are also influenced by a variety of environmental factors. Regarding the physical and chemical variables, the ones with the greatest influence on substrate colonization are temperature (Lau et al., 2005), pH (Baragi & Anil, 2016) and dissolved CO_2 concentration (Brown et al., 2018; V. R. Johnson et al., 2013).

Overall, it is likely that the marine environment will be subject to profound changes in the coming decades, calling for a change in the paradigms of the investigation of epiphytic communities. The general increase in water temperature and ultraviolet radiation, along with plastic pollution and changes in salinity and OA will affect the survival of dispersal stages and their development and recruitment on various submersed surfaces, both natural (such as seagrass leaves) and man-made (such as ship hulls, pipework, grates and ponds) (Sorte et al., 2010). Consequently, our aim is to analyse the mechanisms of adhesion and the patterns of colonization that characterize both the epiphytic colonization and the benthic communities in view of global changes. In fact, climate changes impact the features of the pioneer microbial communities and their production of metabolites, which, in turn, influence the settlement of dispersal stages and the fate and development of further communities (Nasrolahi et al., 2012). For this reason, it is important to analyse the mechanisms of action of the first colonizers that follow the adhesion of bacterial films, which are mainly represented by diatoms and other micro-algae (Khandeparker et al., 2014). This topic will be analysed in the following paragraphs, as well as the influence of global changes, to detect various important aspects for the ecosystem conservation.

In recent research, Khalaman et al. (2016) investigated the communities while they were allowed to develop on the top and bottom sides of horizontal plates. After a given period of exposure, all the plates were inverted. When the substrate orientation was reversed, the community adapted to the new lighting conditions. Consequently, an increased light intensity resulted in the degradation of zoocoenosis and changed its structure. In contrast, shading did not lead to degradation or changes in the algocoenoses, but stunted algal growth. These results indicate that algocoenoses are more stable and more competitive than zoocoenoses in early communities (Levich, 2000). In parallel, detailed examples of chemically mediated interactions over seagrass and seaweed surfaces were quantified in situ and a biological effect was determined. Steinberg & De Nys (2002) investigated both the deterrents and inducers of colonization on various surfaces. For example, seaweed secondary metabolites, such as brown algal phlorotannins, play an ecologically important function as a chemical defense against epiphytes and they retard the colonization of epiphytic organisms. Similarly, *Fucus* sp. phlorotannins have the potential to inhibit the settlement of invertebrate larvae, but that settlement inhibition cannot explain the lower abundance of the barnacle *Balanus improvisus* on *Fucus evanescens* compared to *F. vesiculosus,* because the mechanism of *F. evanescens* acts on post-settlement stages of *B. improvisus* (Wikström & Pavia, 2004). Consequently, Steinberg & De Nys (2002) described the interactions with the associated bacterial biofilms at the surface of seaweeds and seagrasses. There is evidently a strong need to integrate ecology, cell biology, and chemistry to understand the distribution of surface-active compounds in situ and their ecological and physiological effects. This multidisciplinary approach, merging ecological knowledge to produce biotechnologies, is indispensable to research on "biofilms grown on other organisms or surfaces". Recently, molecular tools were developed to characterize bacterial communities. These tools opened an entirely new area of research in marine chemical ecology. An integrated approach to the topic is evidently needed, because many aspects of disparate fields (e.g., larval settlement, algal pathogenesis, the molecular biology of bacterial signaling and biotechnologies linked to the control of adhesion) may be investigated within the framework of chemical mediations, facilitating the colonization of submerged surfaces.

The Role of Diatoms and the Mechanisms of Adhesion

Diatoms are a widespread and ecologically important group of microalgae (phylum Chrysophycophyta, class Bacillariophyceae). According to cell symmetry, they are divided into two groups: centric and pennate. Centric diatoms are characterized by radial symmetry and they are generally planktonic. Pennate diatoms are bilaterally symmetrical and mostly benthic (Round et al., 1990). Many are characterized by the raphe, an elongate fissure that longitudinally crosses each valve. In terms of algal diversity, they are only surpassed by green algae, representing the most diverse group of primary producers, with approximately 200,000 known species. Diatoms are major contributors to the primary production of marine and freshwater environments (Geider et al., 2001), accounting for 40% of the global primary production of oceans (Falciatore & Bowler, 2002; Medlin, 2002).

Diatoms occupy a wide range of aquatic environments. In particular, benthic diatoms adhere to any submerged surface, contributing to primary and secondary biofilm formation (Molino & Wetherbee, 2008). Benthic diatoms are among the most frequent algal early colonizers of natural and artificial substrates, of which they are the main colonizers, strongly modifying the surface properties and the ecological functions. They can also settle on sand and rocks as epiphytes of macroalgae and seagrass leaves, or even form colonial "mats" according to the species and the local ecological conditions (Underwood & Paterson, 2003), producing conspicuous amounts of adhesive mucilage during the formation of primary biofilms (Hoagland et al., 1993). Several microorganisms may form a "biofilm" (Figure 1), an assembly of adherent cells and their products on a given surface (Characklis & Cooksey, 1983). The initial event of biofilm formation on every submerged surface is represented by the adsorption of an organic layer (K. E. Cooksey & Wigglesworth-Cooksey, 1995). The adsorption of the organic layer is often referred to as a conditioning film, and is quite a fast process, lasts a few seconds, and follows a predictable pattern. The so-called "molecular marine conditioning film" (MMCF) is adsorbed by a clean surface just a few seconds after its immersion in the sea.

During this process, the surface chemistry of the substratum changes due to the adsorbed film and the MMCF makes the adhesion of microorganisms possible (Characklis & Cooksey, 1983). Normally, the adsorption is followed by the attachment of microorganisms (bacteria are considered to be the initial colonizers) and, further, by more complex and larger organisms (such as diatoms, other algae, and invertebrate larvae, in this order) (Allison, 2010; Huggett et al., 2009; Salta et al., 2013). However, there is no obligatory sequence in any succession, because various factors, both biotic and abiotic, may influence the process and, as indicated above, OA and other changes in the marine environment induce changes in the initial colonizers that are further reflected in the speed and direction of the colonization process. According to the hypothesis by Baier (1980), the film is glycoproteinaceous, and the receptors that are present on the diatom surface could be similar to the sugar receptors. Such receptors should be able to bind to the terminal sugars of the carbohydrate side chains of the adsorbed layer and set in motion the cascade of events that leads to the secretion of an adhesive polymer by the cell.

It is known (Cooksey, 1981) that diatoms can attach to clean surfaces in the laboratory, even in the absence of bacteria. The cells of diatoms may contact an available substrate in many ways, primarily by chance or due to random events, such as currents, random movements of the medium or gravity (Molino & Wetherbee, 2008). Adhesive events referred to diatoms have been reviewed by Cooksey & Wigglesworth-Cooksey (1995), indicating that a combination of physical and chemical signals synergistically influence the process. The first contact triggers the initial adhesion process, which is reversible, during which the cells consume energy to control the attachment, depending on the inhabitability of the surface (Wetherbee et al., 1998). When diatoms are suspended in the water, some polymers diffuse from the cells. To start the process of diatom adhesive synthesis, when a diatom contacts a surface, the diffusion of adhesive compounds is reduced by the proximity of the surface. Consequently, local extracellular polymer concentration will increase. If such a local increase in polymer concentration is sensed by cell surface-bound receptors, the same signal might initiate an increased polymer synthesis (Characklis & Cooksey, 1983). Chemotaxis in diatoms appears to be receptor-controlled and, as mentioned above, is mediated by Ca^{2+}-channel. At least three types of receptors for simple sugars are described but how such receptors may sense a given surface is still ignored (Dugdale et al., 2005). However, in this starting phase, the cells begin to secrete extracellular polymeric substances (EPS) that are responsible for the adhesion, the motility, the sessile attachment and the habitat stabilization. Almost simultaneously, the formation of an adhesion complex (AC) is triggered. The AC formation and its important role in cell adhesion and motility has been investigated and defined by Wetherbee et al. (1998) and may be visualized as a continuous process acting as a bridge between the actin filaments of the cytoskeleton and the substrate, made by polarized molecules and associated intracellular proteins. It has been demonstrated that adhesion, as well as motility, is

Ca^{2+}-dependent but not light-dependent (McLachlan et al., 2012). This process requires metabolic energy to sustain the synthesis of proteins and, in particular, glycoproteins. Ca^{2+}-channel blockers act at the cell membrane and prevent the adhesion of diatoms, confirming that intracellular Ca^{2+} is required (Dugdale et al., 2005). The key role played by Ca^{2+} in the adhesion process by diatoms may explain why OA so drastically influences their assemblages, taking into account its buffering influence and the mutual relationships with the $p\mathrm{CO}_2$ and the pH. In addition, such an extensive set of biochemically and energetically expensive events is evidently under the metabolic control of the diatoms (e.g., through modulation of the transmembrane signalling processes) and it is known that pH changes may dramatically impact the physiology of cells (Heath et al., 1995).

Diatoms produce EPS, which is also used for the adhesion to any substratum and secreted through the pores and the raphe (Gutiérrez-Medina et al., 2022). Consequently, EPS play a critical role in the establishment of mature epiphytic communities and the development of communities. For centric and araphid pennate diatoms, little attention has been paid regarding the primary adhesion processes, since they are mostly planktonic (Molino & Wetherbee, 2008; Wetherbee et al., 1998). The cells are likely to adhere almost instantly to the surfaces upon first contact, facilitated by the EPS layer that envelops the entire frustule, as observed in *Toxarium undulatum* by Dugdale et al. (2005). Moreover, within a few minutes from the first contact, the AC is triggered and other adhesive mucilaginous substances are secreted, which confer stronger adhesion and stability to the cells. That mucilage, mainly composed of exopolysaccharides, glycoproteins and nucleic acids encapsulating the cells, contributes to the constitution of a kind of pad that is strongly anchored to the substratum (Molino & Wetherbee, 2008).

Remarkably, the settlement on a given substrate of the raphid pennate diatoms is quite different. It is worth noting that diatoms move by gliding (Bedoshvili et al., 2018). They cannot move when they are not adherent, and the signals that induce motility (chemotaxis) must also induce adhesive mechanisms. Consequently, after the first contact the cells may come into contact directly due to the raphe, leading to the immediate formation of the AC and producing EPS that provide strong attachment to the substrate (Lind et al., 1997; Murray et al., 1987). More often, diatom cells land on their side and only the girdle band of the cell remains in contact with the surface (Lind et al., 1997). In this case, the diatom starts to produce EPS, which protrudes through the raphe. Further, the cells move to a set with one raphe in contact with the surface, using those extensions of mucilage and the motility apparatus to pull themselves up (Wetherbee et al., 1998). This process was observed and described for the first time by Lind et al. (1997) regarding the marine diatoms *Stauroneis decipiens*. In conclusion, it is possible to summarize that benthic diatoms represent key organisms that ride through primary and secondary biofilm formation.

It should also be noted that a natural biofilm growing on any shallow marine surface will probably contain many types of unicellular organisms, including bacteria and diatoms. In contrast, as a rule, experimental studies on the adhesion mechanisms currently consider cells that are attached to various substrates in axenic cultures. This could easily be explained by the need to model single processes because, given the difficulties concerning the study of cultures of even a single bacterial species, it is reasonable to assume that a mixed microbial population including bacteria and diatoms would be quite difficult to experimentally afford (Okabe et al., 1997). However, a few investigations (e.g., Murray et al., 1986, 1987) considered mixed populations of one species of diatoms and some bacteria. For example, diatoms of the genus *Amphora* were experimentally attached in a known ratio, along with the marine bacterium *Vibrio proteolytica*, to the surfaces of synthetic Petri dishes, and surprisingly demonstrated that bacterial growth (as measured by 3H-thymidine incorporation) was dependent on the metabolism of diatoms (Murray et al., 1986). This result might be due to the cell damage that occurred during the harvesting process, which provided the bacteria with assimilable carbon, which is not available in the intact film. In addition, natural substrates could represent themselves as an important source of nutrients, and this element may be crucial to understand the differences in the observed colonization patterns, for example, on natural substrates (as *Posidonia oceanica* leaves) as compared to abiotic substrates such as rocks and vessel hulls (Källén et al., 2012). For example, marine sediments in near-shore areas are generally covered with diatoms, although the wave action and the character of the sediment may produce water turbidity and drastically reduce the light penetration (Chen et al., 2020). Interestingly, these sediments may contain much more dissolved organic carbon in their pore water than the overlying water column, thus triggering the bloom of various microorganisms, including diatoms. Under these conditions, diatoms may be attracted by chemotactic processes. In fact, positive chemotaxis to a small number of organic compounds was demonstrated at least in two diatoms of the genus *Amphora* by Cooksey & Cooksey (1988). The chemotactic ability is common in motile, pennate diatoms. Consequently, it is likely that they use this behaviour to move towards higher concentrations of nutrients, and thus may be largely involved in the biogeochemistry of sediments and of some natural substrates, such as seagrass leaves.

In contrast, phototaxis has been a well-known process since the last century, justifying the abundance of diatoms on some substrates (Lewin, 1962). Consequently, a microorganism capable of mixotrophy or heterotrophy in a gradient of a metabolizable compound and in reduced light is potentially offered a choice, to which it should respond by tuning the possible movements according to the signals collected through its receptors. In fact, as noted above, the operation of chemotaxis requires the synthesis of specific proteins, the differential occupancy of these receptors by chemotactically active compounds (Cooksey & Chansang, 1976), the transduction of the receptor signal (Dugdale et al., 2005) to the motility apparatus and, at least in some diatoms, the differential interpretation of that signal at the two raphes; otherwise, directional response (turning) would not be possible (Cooksey & Cooksey, 1988). The distinction between cell responses during chemotaxis and the products of transmembrane transport is significant because it is more important to have a specific uptake mechanism than a highly specific chemotactic mechanism.

Of course, one of the key reasons for much of our interest in the ecology of marine biofilms, and of diatoms in particular, derives from their nuisance value to man-made structures. It is well known (Loeb et al., 1984) that the deterioration of the immersed surface of a ship, which leads to increased drag, loss of performance, increased fuel consumption and corrosion, is caused by macroinvertebrates that cause calcareous deposits (e.g., barnacles and oysters). These bear much of the responsibility for these problems, but it is also believed that the final result is due to the initial biofilms that settled on those surfaces, consisting only of microorganisms such as bacteria and diatoms. At present, we know that the consequences of the adhesion and growth of microorganisms on the hull of a ship are very significant, despite their thin and innocuous aspect. In the past, these phenomena were controlled by painting the hulls with toxic compounds such as tributyltin (TBT). At present, in most parts of the world, TBT and its derivates can no longer be used due to their higher pollutant power and strong and documented impacts on benthic and planktonic organisms. Based on the previous considerations, it is evident that the materials used to replace them must control microbial adhesion as well as the adhesion of higher forms of life, and that the physiologic modifications due to global changes must be taken into account to forecast their effects on target foulers, as well as on the marine biota (Terlizzi et al., 2001). Consequently, we should improve our knowledge of the mechanisms of adhesion of diatoms, because they are essential to conserving diverse epiphytic communities in complex ecosystems and facilitating the adhesion of foulers on man-made structures.

Molecular and Genic Aspects of the Anchoring Mechanisms

Diatoms are well known for the strength of their adhesion to natural and man-made surfaces and most research on diatom adhesion aimed to develop anti-fouling coatings. Although there is little information concerning the attachment of microalgae, several extracellular proteins have been identified, which are required for the adhesion of bacterial biofilms, including amyloid-fibers for biofilm matrix cohesiveness, and flagella for bacterial attachment to abiotic surfaces (Buhmann et al., 2014). The molecular mechanisms enabling the production of these substances are essential to the ecology of first colonizers in a changing climate. Diatoms adhere through a material composed of both proteins and carbohydrates, secreted by the raphe (Poulsen et al., 2014). It is noteworthy that the extreme events of global changes, such as global warming and ocean acidification, affect the molecular pathways involved in the anchoring mechanisms to modify them, with negative consequences for the control of encrusting organisms. A bioinformatic-based approach was applied to the diatom *Halamphora coffeaeformis* (C. Agarth) Lewkov, 2009 (formerly *Amphora coffeaeformis*), one of the most common pioneer colonizers, which is used in many studies as a model organism for underwater bio-adhesion (Finlay et al., 2002; Hodson et al., 2012; Hudon & Bourget, 1981), shedding light on the molecular mechanisms of underwater adhesion and biological silica formation in diatoms. A normalized transcriptome database was generated from this diatom, which permitted the identification of five proteins exhibiting unique amino acid sequences with high similarity to the amino acid composition of the tyrosine-rich adhesion proteins from mussel footpads (Finlay et al., 2002). In addition, the molecular genetic transformation of *H. coffeaeformis* enabled an investigation into the functional characterization of one of these proteins, AC3362, revealing that it is not involved in adhesion, but in the biosynthesis and/or structural stability of the cell wall. The analysis of the amino acid sequence of AC3362 revealed a high content of lysine and tyrosine residues, resembling the cingulins of the diatom *Thalassiosira pseudonana* Hasle & Heimdal, 1970 and the silaffin-1 peptides from *Cylindrotheca fusiformis* Reimann & J. C. Lewin, 1969 (Buhmann et al., 2014), which are known to be tightly associated with the biosilica and thus cannot be extracted from the cell walls. Global stressors, such as seawater warming and acidification, could affect the expression of these genes, affecting the cellular machinery used for silica morphogenesis and mechanical performance in diatoms (Görlich et al., 2019). It is important to consider that these genes are phylogenetically well-conserved throughout the diatom realm, and essential for the biogenesis of mechanically robust diatom cell walls.

A further proteomic analysis of *H. coffeaeformis* adhesive compounds led to the identification of 21 proteins, of which 13 are diatom-specific (Lachnit et al., 2019). Ten of those proteins shared a conserved C-terminal domain, termed the GDPH domain, which is not ubiquitously present but quite widespread in all diatom classes. Some of these proteins (Ac629, Ac1442 and Ac9617) are components of the adhesive trails secreted by cells on surfaces, as revealed by the immunofluorescence localization of a GDPH domain. Raphid diatoms secrete carbohydrate-rich extracellular polymeric substances (EPS) related to their motility and underwater adhesion (Lachnit et al., 2019). Stress conditions may induce the up- or down-regulation of the genes that encode for these adhesive proteins, leading to adhesion problems for benthic diatoms, which may be seasonally abundant on the leaves of *P. oceanica*, a preferred food item for marine benthic invertebrates (De Stefano et al., 2000; Kanjer et al., 2019; Mabrouk et al., 2014; Mazzella & Spinoccia, 1992). In parallel, similar constraints play a critical role in the adhesion of diatoms to man-made structures, influencing the development of the encrusting communities.

Several proteins related to carbohydrate metabolism were also identified, comprising one glycoside hydrolase and seven exostosin-like proteins, which are known in mammalian cells to encode for glycosyltransferases involved in the synthesis of sulphated proteoglycans present in the extracellular matrix. Interestigly, these sulphated polysaccharides/proteoglycans were also identified in the EPS and cell-wall-associated glycoproteins of other two benthic diatoms, *Staurophora amphioxys* (Gregory) D. G. Mann, 1990 (formerly *Stauroneis amphioxys*) and *Craspedostauros australis* E. J. Cox, 1999, suggesting their key role in EPS synthesis in diatoms (Annuk & Moran, 2010).

In the oceans, microalgae–bacteria interactions play key roles in nutrient exchange processes and marine aggregate formation, mainly leading to the cycling, mineralization, or sedimentation of organic carbon. Aiming to study these interactions at the cellular scale, an in vitro interaction model system, consisting of the diatom *Thalassiosira weissflogii* (Grunow) G. Fryxell & Hasle, 1977 and the gamma-proteobacterium *Marinobacter adhaerens* HP15, was established (Torres-Monroy & Ullrich, 2018). This bacterium is able to attach to *T. weissflogii* cells, inducing transparent exopolymeric particle formation, which can increase the formation of marine aggregates. Its investigation facilitated the detection of 30 bacterial promoters that were specifically expressed during interactions with *T. weissflogii*, whose corresponding genes could be involved in the biochemical detection of diatom cells, bacterial attachment, metabolic exchange of nitrogen compounds and resistance toward heavy metals (Torres-Monroy & Ullrich, 2018). The interaction between *M. adhaerens* HP15 and *T. weissflogii* is multifactorial, involving a complex network of physiologic processes, which could be affected by multiple environmental stressors. In fact, temperature increases can induce shifts from the co-existence to competition in the use of inorganic and organic carbon by microalgae and bacterial communities (Sörenson et al., 2021).

Fu et al. (2020) provided molecular evidence of the mechanisms of the morphological shifts that occur during surface colonization, identifying candidate target proteins for the screening of eco-friendly anti-biofouling compounds. G-protein-coupled receptor (GPCR) genes, activators of the surface colonization pathway, were found to be differentially expressed in the model marine diatom *Phaeodactylum tricornutum* Bohlin, 1897 (Fu et al., 2020). Moreover, the transformation of *P. tricornutum* with constructs expressing individual G protein-coupled receptor (GPCR) genes were able to induce cells to adopt a morphology that is adequate for surface colonization. These transformed cells resulted in a 30% increase in resistance to UV light exposure compared to their wild-type counterparts, together with the increased silicification of cell walls, which, in turn, was usually associated with the oval morphotype. GPCRs are the largest family of membrane proteins and mediate many physiological functions through their interaction with heterotrimeric G proteins, also mediating most cellular responses to hormones and neurotransmitters, important cell recognition and communication processes.

A genome-enabled bioinformatic approach was also applied in *P. tricornutum* to identify putative molecules for cell-substratum adhesion (PDC) (Willis et al., 2014). The analyses detected the presence of molecules that diatoms may use for cell–substratum adhesion: PDC4, an arabinogalactan fasciclin-like protein that is putatively a cell-surface molecule involved in secretion; PDC7, a c-type lectin, is a molecule involved in carbohydrate binding and cell–cell adhesion; PDC8, involved in cell–substratum adhesion by increasing the adhesive molecules that are exposed on the surface of the mucilage; PDC9, a homolog to fasciclin proteins involved in interactions within the mucilage; PDC10, a fasciclin-like molecule with a role in cross-linking. The fasciclin-like proteins in

Arabidopsis thaliana play a role in maintaining proper cell expansion under salt-stressed conditions (Johnson et al., 2011).

Benthic diatoms represent key primary producers in coastal environments, with important ecological functions in nutrient cycling and sediment stabilization. A reference genome for the marine biofilm-forming diatom *Seminavis robusta* D. B. Danielidis & D. G. Mann, 2002 showed that genes that were differentially expressed within diatom–bacteria interactions were strongly conserved in other benthic diatoms, while many species-specific genes were strongly upregulated during sexual reproduction (Osuna-Cruz et al., 2020). The vonWillebrand factor, type D domains (vWDs) was found in several extracellular proteins, and is usually involved in protein multimerization and maturation of the adhesive into multi-protein complexes. In fact, several adhesive proteins, such as zonadhesin, sea star foot protein and diatom adhesive trail proteins, contained vWDs (Hennebert et al., 2012; Lachnit et al., 2019; Subramanian et al., 2018) 61 of which also had the diatom-specific conserved C-terminal domain GDPH, which is important for diatom-secreted proteins involved in motility, mucilage pads and gamete fusion. The vWD family is abundant in raphid species and is differently expressed in *S. robusta*, with increases in the expression of genes related to bacterial interactions and recognition, motility and cell adhesion. This expansion of the vWD family may suggest an adaptation to highly heterogeneous benthic environments due to the presence of diverse and dense bacterial populations as compared to the water column (Nasrolahi et al., 2012). In addition, stress conditions can produce a von Willebrand factor deficiency (Subramanian et al., 2018). Three proteins containing the ancient cell adhesion domain fasciclin (FAS1) were identified, as well as eight proteins with a peptidase C2, calpain domain, and calcium-responsive intracellular proteases involved in the regulation of cell migration, integrin-mediated adhesion and actin-based membrane protrusion, enabling cell movement by modifying the adhesion sites (Lachnit et al., 2019). These results indicate that the molecular aspects of the adhesion process may provide powerful tools to understand the ecology of epiphytic communities under the current environmental changes. In addition, a full comprehension of the relationships influencing the activation of specific genes will be critical under the forecasted climate changes to develop newer technologies able to reduce or retard the formation of diatom biofilms and EPS on man-made submerged structures.

Algal Colonization

Marine rocky bottoms and other submerged natural surfaces are rapidly colonized by micro- and macro-algae (Lebret et al., 2009). Several organisms, including macroalgae, seaweeds, seagrasses, and even sessile and motile invertebrates, may provide additional space for colonization when the competition for space increases on non-living substrates. The propagule pools of algal colonizers are diverse and are influenced by environmental factors (Clare & Aldred, 2009). Their probability of encountering a surface is strongly dependent on their abundance in the water mass. In epibenthic communities, including secondary surfaces produced by foundation organisms such as seagrasses, algae are usually predominant on sunlit substrates, while animals dominate shaded surfaces. Two main factors cause this pattern: (a) the more competitive nature of algae and (b) the evolved differentiation of niches that results in animals avoiding competition with algae, which is also due to chemical interactions. Organisms belonging to those pools are suspended in the water column and transported by fluid–dynamic forces. Once the propagules are in close proximity to the available surface, physical–chemical forces act upon cells, particularly electrostatic forces and van der Waals forces (van Loosdrecht et al., 1989). Furthermore, the metabolic products exudated and retained by living substrates such as seagrasses potentially promote adhesion and and/or retard colonizing propagules (Michael et al., 2008). Bacteria and diatoms, which are present in the propagule pools, may represent the earliest colonizers of these substrates. However, they are ubiquitous components of encrusting communities because they can quickly spread on any submerged surface (Michael et al., 2008). They start to settle on the surfaces as primary colonizers, promoting the so-called periphyton formation. Their interaction promotes the formation of microbial consortia, resulting in biofilm formation (Michael et al., 2008), which can be observed even on the youngest seagrass leaves (Sterrenburg et al., 1995). The colonization and the biofilm formation on seagrasses are mainly affected by light, temperature and nutrient availability (Lee et al., 2008; Prado et al., 2007). However, their productivity is largely influenced by the intensity and quality of light (Drake et al., 2003; Mazzella & Alberte, 1986). Primary colonizers alter the initial surface properties after the formation of a biofilm complex, thus influencing the secondary colonization. Further, the surface characteristics of both the type of substrate and the cell complex play an important role in the subsequent settlers (Michael & Smith, 1995). Seagrasses and seaweeds represent secondary substrates for the colonization of other encrusting organisms because marine seagrasses and macroalgae are prone to epiphytism.

The colonization of their surface may lead to severe perturbations and sometimes to the death of the host, as in the case of some tropical seagrasses, which are severely impacted by the excessive growth of epiphytes when algal grazers are scarce. This demonstrates how the adhesion process, involving the features of the primary surface (e.g., live or inorganic substrates), the first colonizers (e.g., bacteria and micro-algae) and further colonizers (as macroalgae and sessile animals), is complex and dramatically influenced by chemical relationships and the presence of grazers. Consequently, to protect themselves against settling and/or competing organisms, many algae developed chemical defenses against further colonizers by producing a wide variety of bioactive metabolites (Callow & Callow, 1998).

However, the surface topography may also play a critical role in the colonization of submerged surfaces (both alive and inorganic) through the colonizing stages of micro- and macro-algae. The surface topography dramatically influences the attachment of zoospores of the green macroalga *Ulva linza* Linnaeus, 1753 and cells of the diatom *Navicula salinicola* (formerly Navicuna *incerta*) Hustedt, 1939 on inorganic substrates (Xiao et al., 2018). Indeed, the preferred location of cells of *N. incerta* was dominated by a positive correlation between the density of cell adherence and the amount of available attachment points, while the settlement of spores of *U. linza* was mainly regulated by both

Wenzel roughness (relationship between the roughness and wettability of a surface) and the local binding geometry.

Seagrasses and Other Living Substrates for Epiphytes

Seagrasses are among the most relevant available natural surfaces for epiphytic organisms in the marine environment (Borowitzka et al., 2006; Buia et al., 1992; Mateo et al., 2006; Mazzella et al., 1993; Reyes et al., 1998; Uku & Björk, 2001). They provide a wide range of substrates, which are structurally different depending on the leaf morphology of individual species (Kuo & Hartog, 2006). Sea grasses are colonized by a multitude of epiphytic organisms that broaden their ecological role. For this reason, they can be considered as “models” used to investigate the dynamics of colonization of micro- and macro-organisms (Onuf, 2006). The lifetime of their above-ground parts (leaves, stems, rhizomes) varies within species and seasonality, ensuring a temporary but continuously renewed substrate (Borowitzka et al., 2006). The process of colonization of a seagrass leaf is easily sketched by comparing differently aged leaves of *P. oceanica,* a seagrass endemic to the Mediterranean that is characterized by its long persistence and high sensitivity to environmental triggers. Often, the organisms that settle on the leaf surface may persist throughout its lifespan. Due to this process, the lower (i.e., younger) portions of leaves show an early colonization stage, whose age increases according to the distance from the base, reaching the maximum age at the leaf tips. Consequently, the mechanisms facilitating the anchoring to the leaf surface are more efficient at the edge of leaves and near their apex, where a thicker film of first colonizers is already present. This is due to the higher turbulence that characterizes this portion of the leaves, which favors the chances that cells and propagules encounter the available leaf surface for attachment (Trautman & Borowitzka, 1999). The leaves of seagrasses, as hosts of epiphytes, differ according to various aspects, such as anatomy, morphology, surface roughness and chemistry. All these characteristics may affect the presence, distribution and abundance of epiphytes in various areas of the world (Sekar et al., 2004).

As soon as they encounter the surface of a seagrass leaf, algae become the most abundant and diverse encrusting organisms on seagrasses, with a wide variety of forms ranging from unicellular diatoms and dinoflagellates, which are found on almost all seagrasses, to macro-algae, especially crustose coralline ones. Epiphyte algae play a crucial role in the whole ecosystem, enhancing the primary production (Borowitzka et al., 2006). Furthermore, the high generation rate and growth of epiphytic biomasses strongly contribute to food webs, providing the main food source for the associated fauna (Buia et al., 1992). As noted above, the diversity and biomass of epiphytes is highest in the upper portions of leaves (apico–basal pattern), especially on the oldest leaf and near the leaf apex (Mateo et al., 2006; Uku & Björk, 2001). In most marine angiosperms, the rhizomes of seagrasses generally carry a low epiphyte load compared with the leaves, with the exception of *P. oceanica*, where a larger species richness and higher biomass on the exposed and long-lived rhizomes has been reported (Mateo et al., 2006). The low abundance of epiphytes on the rhizomes is probably due to the fact that the rhizomes of almost all seagrasses are continuously buried. Epiphytes, as part of the seagrass ecosystem, are also considered bioindicators for factors that could be causal in seagrass decline, being sensitive to environmental perturbations (Michael et al., 2008). The process of colonization and the biofilm formation of seagrasses seems to be crucial in terms of the selection and composition of species, as well as biomass production.

Environmental perturbations may negatively affect all stages of the community and biofilm development, which represent the most vulnerable targets. In fact, global change stressors can alter beneficial interactions in structurally complex systems, leading to dramatic changes in the dynamics of the succession of seagrass epiphytes (Pillay & Waspe, 2019).

Global stressors such as OA influence both bacterial and eukaryotic epiphytes, which form distinct communities at the CO_2-impacted sites compared with control sites (Hassenrück et al., 2015). Both bacterial and eukaryotic epiphytes form distinct communities at low-pH sites as compared with control sites, and a site-related CO_2 effect was demonstrated in the succession pattern of microbial epiphytes on seagrasses and close stands of corals. These authors further found an increased relative sequence abundance of bacterial types associated with coral diseases at the CO_2-impacted site, whereas certain crustose coralline algae commonly related to healthy reefs exhibited lower diversity. These trends in the epiphytic community suggest that seagrasses play a role as potential vectors of pathogens for closed coral reefs and may support previous predictions of a decrease in the reef health and prevalence of diseases under future ocean acidification scenarios (Heitzman et al., 2022). Consequently, global stressors might have strong effects on the succession of epiphytes on seagrasses, which may have further consequences for other environments in the same area.

In the case of seagrasses, which are considered “foundation species” due to their role in structuring complex communities, the composition of associated epiphytic communities is also influenced by the nature of the first colonizers. In fact, the epiphytic community associated with the leaf stratum of seagrasses is seasonally variable (Michael et al., 2008). From this perspective, the overall density and distribution of seagrass meadows, along with seasonal and global environmental changes, may be more important than the seagrass species identity itself (Moore & Duffy, 2016).

As a general rule, in addition to the concepts reported above, the structure of the epiphytic community on seagrass leaves is mainly due to: (a) the rate of leaf growth; (b) the availability of nutrients; (c) the local effect of grazer communities; (d) the effect of various stressors, such as the OA and the warming of oceans. The diversity of the associated invertebrates can be a key driver of ecosystem functioning, in addition to the still uncertain factors influencing local biodiversity (Zhang & Silliman, 2019). In both marine and terrestrial systems, facilitation cascades where the primary foundation and/or autogenic ecosystem engineering species (as seagrasses) promote the settlement and survival of a secondary foundation/engineering species have been shown to enhance local biodiversity and ecosystem functioning. The functioning of seagrass ecosystems may be enhanced in scenarios where secondary foundation species (e.g., epiphytic organisms) specifically increase the diversity of key functional groups, such as epiphyte grazers (Elven et al., 2004).

A considerable amount of the literature demonstrates that secondary foundation species are important drivers of local biodiversity in marine ecosystems and, considering the dramatic importance of the first colonizers in determining the anchoring of secondary foundation species, the role of organisms such as bacteria, micro-algae and cyanobacteria emerges because they prime a facilitation cascade (Zhang & Silliman, 2019). Several investigations demonstrated that epiphytic communities on seagrasses were mostly influenced by nitrogen (N) and phosphorus (P) additions (Frankovich et al., 2009). The variability in the epiphytic community structure was mainly related to the uncontrolled temporal and spatial environmental heterogeneity. However, P additions increased the relative abundance of the red algae, cyanobacteria and green algae, while diatoms in these conditions showed a concomitant decrease. In particular, red algae and cyanobacteria produce a complex that can facilitate the further anchoring of other epiphytes, representing the key first colonizers of seagrass leaves. Within the diatom community, species relative abundances, species richness and diversity seem to respond weakly to nutrient additions. Phosphorus additions produce changes in the structure of the diatom community, especially in warmer conditions (Frankovich et al., 2009) and, consequently, global warming produces stronger changes. Other studies, conducted in warm areas, reported contrasting results and different triggers. For example, according to Campbell & Fourqurean (2014), a lower pH induces declines in the abundance of coralline algae, along with increases in filamentous algae, but the nutrient enrichment induced negligible effects on epiphyte community structure or the overall epiphyte loading.

In addition, OA alters the relationships between epiphytes, seagrasses and animal grazers. It has been demonstrated that the deterrent compound contents may change in both the seagrass leaves and in the epiphytic layer, and that the wound-activated infochemicals produced by epiphytes are modified by changes in the pH of the medium (Mutalipassi et al., 2020). Leaves at normal pH (8.1) sites showed, according to Berlinghof et al. (2022), a 25% higher total epiphytic cover compared to acidified sites. In normal conditions, encrusting red algae dominated the epiphytic community (32% cover) of leaves. In contrast, leaves of *P. oceanica* living in acidified conditions were dominated by hydrozoans (21%), probably due to the negative effects of OA on calcifying epiphytes. Leaves with and without epiphytes, collected from a low-pH meadow, produced and respired significantly more oxygen than leaves from the normal pH, showing an average increase of 47 ± 21% (mean ±SE) in epiphytes to net primary production (NPP) and 50 ±4% in the respiration, respectively. Epiphytes, however, made little contribution to the increase in respiration. In contrast, their contribution to NPP was quite relevant (56 ±6% of the total flux). In addition, the volatile organic compounds (VOCs) produced by individual diatoms are likely to trigger contrasting reactions in invertebrates according to pH (Mutalipassi et al., 2022). The perception of epiphyte VOCs varies due to alterations in species' ability to perceive and/or interpret chemical cues as infochemicals or due to changes in the structure of the VOCs themselves. Consequently, OA alters the fine-tuned chemical cross-talks between seagrass epiphytes and associated invertebrates, with consequences for the structure of communities and food webs of seagrass ecosystems (Guan et al., 2019; Mutalipassi et al., 2020). These variations induce dramatic differences in the colonization patterns because they represent an indirect influence of the biodiversity in the epiphytic layer, over-imposed on the direct effects of OA on the survival of individual species of micro- and macro-algae (Frankovich et al., 2009).

However, we should consider that seagrasses may defend against epiphytic colonizers by adopting mechanisms of chemical mimicry (Jüttner et al., 2010). The seasonal dynamics of chemical defenses against micro-organisms in seagrasses and the drivers of these dynamics have been investigated, demonstrating that the seagrass *Zostera marina* Linnaeus, 1753 is prone to micro-colonizers, although this seagrass, as all marine spermatophytes, can chemically defend against settlers and grazers (Guan et al., 2019). Besides these biotic factors, the seasonal changes in environmental factors such as nitrogen availability and temperature regimes also affect the defense levels in this seagrass, either directly or through indirect effects on its microbial settlers. The relationships between seagrass leaf surface and micro-epiphytes may be useful, therefore, in identifying newer compounds that retard the accumulation of encrusting organisms, because the release of several toxic antifouling chemicals into seawater has led to marine environmental degradation. From this perspective, the development of new methods based on biomimicry, as sketched by seagrass leaf ecology, could provide nontoxic or less toxic solutions for the deterrence of unwanted settlers (Sammarco & Coll, 1992). In addition, natural mechanisms developed by various seagrasses and algae are often highly dynamic, and biotechnological applications may need to mimic not only defensive compounds but also their dynamics in order to exploit the full potential of biomimicry for marine applications. For example, the effectiveness of zosteric acid, a natural antifoulant extracted from the marine seagrass *Z. marina*, in preventing the attachment of several encrusting organisms was demonstrated by previous research (Haslbeck et al., 1996). Accordingly, understanding the primary and interactive effects of the first colonizers and the abundance of algal epifaunal assemblages in relation to global stressors such as ocean acidification and warming is important for seagrass ecology and management, especially in fragmented habitats (Källén et al., 2012), as well as for the development of new technologies.

Ocean Acidification Effects on Epiphytes

The uptake of carbon dioxide by the oceans, enhanced by its rising concentrations in the atmosphere, mostly due to anthropogenic emissions, is a global environmental concern known as ocean acidification (OA). This phenomenon is drastically changing the carbonate chemistry and reducing the pH of the oceans (Caldeira & Wickett, 2003; Doney et al., 2009; Feely et al., 2004; Gattuso & Hansson, 2011; Hönisch et al., 2012; Orr et al., 2005; Tans, 2009), with far-reaching consequences for marine communities and ecosystem dynamics. Ocean acidification, at a global scale, has deleterious effects on various taxa of marine organisms (Table 1), negatively affecting the correct function and services of the ecosystems (Hall-Spencer & Harvey, 2019).

Several species respond to the acidification of the seawater (Table 1), but the direction and magnitude of their responses will ultimately depend on the interspecific and ontogenetic variations in their physiology and the importance of calcification (e.g., for the bioconstruction of skeletons).

Factors influencing the ecology of the first colonizers will likely modify both the structure and the diversity of benthic communities (Brown et al., 2016). Seawater acidification significantly alters the community structure by influencing the relative abundance of several species and triggering a reduced community variability (Figure 2). This results in a “flattening” of benthic communities in acidified environments (Speights & McCoy, 2017).

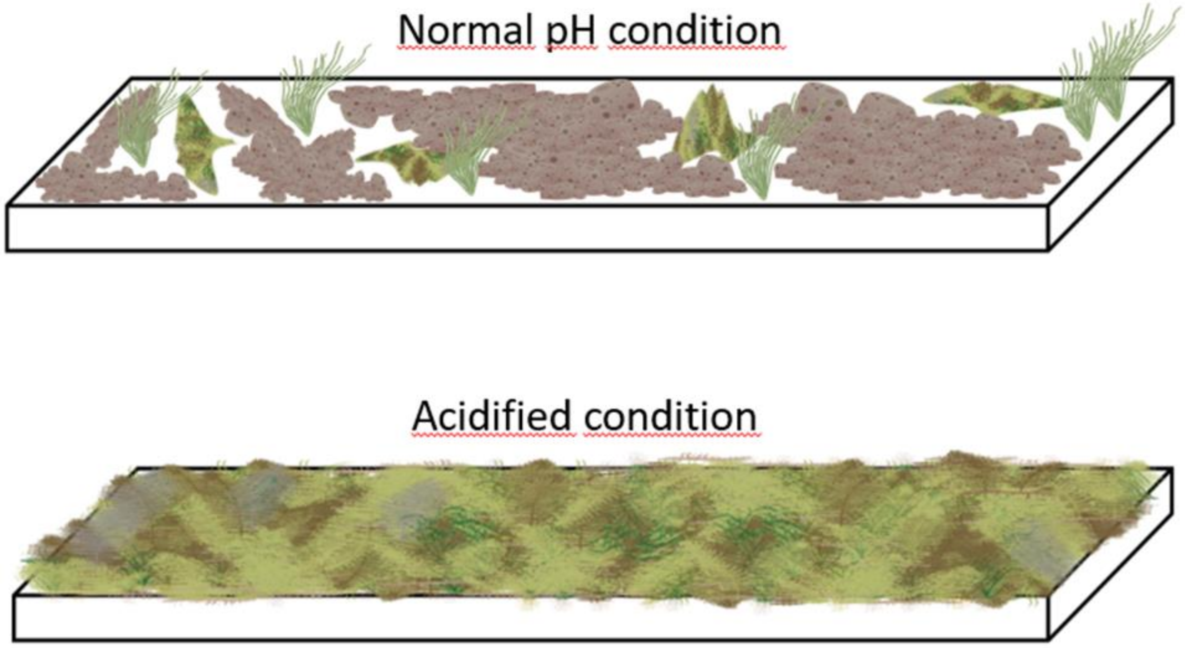

Figure 2. The alterations in epiphytic communities triggered by OA induce a reduction in the first basal colonizers and a modified conditioning film, leading to an impoverished mature community. Such variations result in a “flattening” of communities in acidified environments and an altered ecological stability, with consequences for biodiversity (lower levels) and the abundance of some invasive and stronger organisms (larger biomass).

However, the responses of individual organisms may be contrasting. OA does not affect the growth of calcified organisms such as adult mussels and barnacles, while the growth of non-calcifying organisms, such as some hydroids, is significantly reduced in elevated CO_2 treatments (Berlinghof et al., 2022). Conversely, the percentage cover of some adult bryozoan colonies may be higher under acidified conditions. On the overall, these changes lead to a decrease in diversity (41% lower) in the acidified treatment relative to ambient conditions, according to Brown et al. (Brown et al., 2016).

This topic reaches paramount importance when seagrass meadows are considered, because they represent stable but sensitive environments and produce habitats for ecologically and economically important coastal communities (Campbell & Fourqurean, 2014). Seagrasses are among the most diverse ecosystems in the world. Severe natural and anthropogenic changes such as ocean warming (Dobretsov et al., 2019), OA (Ragazzola et al., 2012), the physical impacts of the fishery, the higher presence of particles (due to industrial or urban pollution), and microplastics are extremely detrimental to these ecosystems (Hale et al., 2011). In particular, the effects that OA has on the physiology of the first colonizers will have a remarkable effect on the composition of seagrass-associated communities.

The obtained results will also be useful for a comprehensive approach to various biotechnologies, because the shifts in the colonization consortia induced by global changes require newer approaches to the field of anti-fouling coatings for several marine applications (Speights & McCoy, 2017). It is forecasted that OA will reduce the biodiversity of marine communities by acting on important ecological processes that ultimately determine the community structure (Hale et al., 2011; Teixidó et al., 2018). Consequently, OA will increasingly interact, in the future, with the ecology of encrusting organisms, posing large threats to coastal ecosystems. Overall, acidification-driven changes in the benthic community might produce important implications for ecosystem functions and food-web dynamics in various environments. Seagrass meadows are likely to be significantly impacted as well, due to the importance of relationships (Zupo et al., 1997) between the leaf growth and the succession of associated organisms building a complex epiphytic layer. In addition, the indirect effects of OA will alter the chemical relationships between epiphytes and various animal consumers. For example, OA remarkably influences the production patterns of various algal infochemicals, and this produces dramatic changes in the chemical relationships among species within the same community (Campbell & Fourqurean, 2014; Duncan et al., 2022; Mutalipassi et al., 2020).

However, the fate of highly invasive encrusting species in an acidified ocean remains poorly understood, due to lack of information on the changes in successional stages and at various levels of biological organization. Previous investigations (Brown et al., 2016) demonstrated that the ecological succession in an acidified site was initially delayed and the community remained in an early stage for at least 8 weeks. The changes observed in the succession led to the homogenization of communities in acidified conditions and altered the community structure, reflecting both short- and longer-term acidification histories. The observed shifts in the biodiversity and structure of the communities are a result of interspecific variability in response to increased $p\mathrm{CO}_2$ and shifts in key species interactions (Doney et al., 2009). It is worth considering that OA altered the development of initial biofilms, allowing for some polychaetes to perform best under acidified conditions, although the early negative effects of acidification were demonstrated in the recruitment stages of various worms (Hadfield et al., 2014; Kroeker et al., 2013).

In contrast, the effect of OA on initial biofilms facilitates the adhesion of the ascidians *Diplosoma sp*. and *Botryllus sp*. They settled later, but were more tolerant to acidification, probably due to the absence of calcareous structures (Lau et al., 2005; Ross et al., 2011).

Our knowledge about the effects of OA relies on studies about the biology, physiology and behavior of various marine species (Asnicar et al., 2022; Duncan et al., 2022; Foo & Byrne, 2017; Kroeker et al., 2013; Milazzo et al., 2016; Ross et al., 2011). Unfortunately, assessment of the effects of OA on marine communities is difficult; for this reason, it is necessary to disentangle and clarify the effects of acidification on the succession and development of marine communities, as highlighted by Brown et al. (2018). Moreover, since the saturation state of calcium carbonate falls due to the decrease in pH, and the solution equilibrium shifts towards higher dissolved concentrations of calcium ions, research has predominantly focused on primary producer calcifying organisms (Martin & Gattuso, 2009; Ragazzola et al., 2012, 2021) because their skeletons are naturally corroded by low pH levels (Dobretsov et al., 2019; Langer et al., 2009).

As shown above, most studies involved manipulative laboratory experiments (Asnicar et al., 2022) and long-term mesocosm observations (Hale et al., 2011). In order to facilitate the comprehension of the effect of OA on marine communities, scientific research is now moving towards in-situ $p\text{CO}_2$ manipulations (Albright et al., 2018; Brown et al., 2016) and studies exploiting natural CO_2 vents (Hall-Spencer et al., 2008; Porzio et al., 2011, 2013). The latter provides insights into the effects of seawater acidification at community and ecosystem levels (Brown et al., 2018). Many studies carried out in those natural laboratories show an alteration in the community composition, which led to a reduction in the ecological complexity and a decrease in biodiversity (Teixidó et al., 2018; Vizzini et al., 2017).

In order to increase our understanding of the direct and indirect effect of OA on coastal benthic ecosystems, it is essential to determine what kinds of changes might occur at the primary producers' level (Table 1). Despite this, the impact of high CO_2 concentration on benthic ecosystems, especially on the microphytobenthic assemblage dynamics, remains poorly studied and explored. Changes in the community composition are represented by a clear pattern (Agostini et al., 2018; Harvey et al., 2021; Peña et al., 2021; Short et al., 2014), triggering a shift from calcifying organism (e.g., coralline algae) towards non-calcified species (especially brown algae and diatoms). Similar changes in the patterns of colonization were also observed by the study conducted by Porzio et al. (2013) of the early colonizers in the CO_2 vents off the Island of Ischia (Bay of Naples, Italy), which highlighted a progressive decrease in the number of species along the pH gradient. Harvey et al. (2021) also supported this evidence, asserting that OA can set the course of successional development in the phytobenthos. This facilitates turf algae formation at the expense of the biomass and diversity of the whole benthic community, supporting the concept that the OA may lock the algal community at an earlier stage, dominated by r-selected species (Smith et al., 2020).

Due to the high solubility of their skeleton, which has a high magnesium content (Schwartz, 1976), encrusting red algae (Corallinales) show strong vulnerability to the phenomenon of acidification (Donnarumma et al., 2014). Under normal conditions, they act as pioneer species in the colonization of the leaf surface of *P. oceanica* that, once established, help to condition the adhesion and colonization of other groups of organisms (Casola et al., 1987). While corallinales often represent the dominant group of epiphytic colonizers along the leaves of *P. oceanica*, in acidified sites the algal epiphytic assemblage is mainly composed of flashy algae, both encrusting and erect brown and green algae. In those environments, the community structure of epiphytic assemblages undergoes a strong reduction in terms of taxonomic diversity due to the decrease/absence of corallinales, leading a community dominated by flashy and turf algae. The resulting epiphytic community faces a trivialization of its biodiversity, appearing more homogeneous and strongly impoverished. After the initial colonization, the assemblages of epiphytes present in natural acidified areas seem not to evolve, remaining in their initial state (Mecca et al., 2020). The disappearance of the coralline red algae could affect the subsequent stages of colonization, causing cascade effects on the trophic web associated with the canopy and the functioning of the entire ecosystem (Martin et al., 2008). Furthermore, the scarce or totally absent corallinales cover may induce a great palatability of the leaves, enhancing the grazing activity, especially by some fish, such as *Sarpa salpa* (Linnaeus, 1758), whose abundance tripled close to the vents compared to ambient pH sites (Mirasole et al., 2021). The increased grazing led to evident modifications in the leaf canopy of *P. oceanica* in acidified areas, which could suggest a possible correlation between the phenology of the seagrass and the structure of the epiphytic community (Mecca et al., 2020).

Although the canopy presents a high shoot density in acidified zones, the *Posidonia* leaves appear narrower and shorter than under normal pH conditions, resulting in a decrease in available surface area for epiphyte colonizers.

Table 1. Summary of the effects of OA on the early stages of plant and animal colonizers.

Colonizer organism	Effect on colonization	Environment/Substrate	References
Calcified epiphytes	Strong reduction in the cover	*P. oceanica* leaves in shallow volcanic CO_2 vents in Ischia island	(Hall-Spencer et al., 2008)
Calcareous algae Non-calcified algae Turf algae	Disappearance within the acidified site Become dominant in acidified site Declined the most in diversity and abundance	Rocky shore in Ischia island shallow volcanic CO_2 vents	(Porzio et al., 2011)
Algal species with calcareous structures Turf algae	Decline and disappear along the pH gradient Increase cover along the pH gradient	Rocky reef in CO_2 shallow volcanic vents in Ischia Island	(Teixidó et al., 2018)
Cover of coralline algae Large canopy forming macroalgae Low-profile algae Turf algae	Significantly reduced Significantly reduced abundance Increased in cover Increased in cover	Shikine island in Japan CO_2 seeps in shallow waters	(Agostini et al., 2018)
Coralline algae Non-calcified macroalgae	Sharp decline in coralline species diversity in both sites More abundant	Seeps off the volcanic coasts of Italy (Vulcano Island, Mediterranean Sea) and Japan (Shikine Island, North-Western Pacific)	(Peña et al., 2021)
Coralline algae Filamentous turfs cover	Significant negative effect of high pCO_2 on calcification with partial tissue mortality at very high pCO_2 Increase	Laboratory experiment - mesocosm	(Short et al., 2014)

Coralline algae cover	Declines consistently with decrease of pH	Great Barrier Reef World Heritage Area (Australia)	(Smith et al., 2020)
Taxonomic diversity	Strong reduction	*P. oceanica* orthotropic shoots collected by CO_2 shallow volcanic vents in Ischia Island	(Mecca et al., 2020)
Encrusting red Algae (Corallinales)	Disappearence with the increasing acidification of seawater		
Encrusting and erect brown and green algae (Fleshy and Turf algae)	Become dominant with acidification		
Epiphytic coralline algal cover	Significant reduction with increased acidification of seawater	*P. oceanica* blades collected by CO_2 shallow volcanic vents in Ischia Island	(Martin et al., 2008)

It is also appropriate to note that all these variations in terms of the abundance and biodiversity of epiphytes are seasonally dependent and the pathways of colonization vary according to the physical and chemical features of the substrates. In addition, multi-stressor approaches indicate multiple effects of OA when coupled with temperature increases, which could facilitate the introduction of invasive species. Many studies showed negative (Torstensson et al., 2012) or irrelevant effects (Tortell et al., 2000) on diatoms after high CO_2 exposure in laboratory experiments, even under multi-stressor scenarios. In contrast, some other research works demonstrated that diatoms benefit from the increase in pCO_2, especially through the reduction in the energetic costs of their "CO_2 Concentrating Mechanisms" (CCMs) (Brown et al., 2018; Hopkinson et al., 2011). In their study, Johnson et al. (2013) also demonstrated that high CO_2 levels stimulate primary productivity in the periphyton, as proven by the increase in the photosynthetic standing crop of the above-mentioned benthic assemblages, mainly due to the increased presence of diatoms. The same study also showed a non-uniform response of benthic diatoms to OA.

Variations in the chemistry of the seawater due to the higher $p\mathrm{CO}_2$ have different effects on the periphyton communities, causing increases in some populations and decreases in some others. A reduction in diversity in the assemblages occurs when moving along the $p\mathrm{CO}_2$ gradient: as pH decreases, diatoms characterized by larger size become dominant in the biofilm community. Under the present $p\mathrm{CO}_2$ conditions, smaller cell-size genera maximize the uptake of carbon thanks to the higher surface:volume ratio (SVr). With the increase in cell size and the consequent reduction in SVr, a limitation starts to occur in the diffusion path (Kiørboe, 1993). The actual incremental trend of CO_2 levels might provide an advantage to larger species by inverting the competitive skills of different size diatom classes.

These community shifts are likely a result of interspecific variability in response to OA and changes in species interactions. High $p\mathrm{CO}_2$ altered biofilm development, with detrimental consequences on whole ecosystems, triggering a dramatic cascade of effects. In benthic communities, where the time stability of plant and animal associations is a key factor to assure the resilience and conservation of species, pH changes in the seawater may have dramatic consequences for the assemblages of species. In these environments, well-known time successions lead to stable associations that assure the functioning of ecosystems (Zunino et al., 2019). Slight changes in the assemblages of first colonizers may totally change the time evolution of benthic communities, leading to still-unknown variations in their composition and functioning. The synergistic actions of several factors of global change (OA and human disturbances, such as nutrient increases) could lead to a rapid acceleration of the whole process, causing winners and losers among species in marine benthic communities. Those variations in phenology and epiphyte communities can be considered relevant aspects of the effects of OA and could represent drastic modifications that seagrass systems are going to face.

Global Warming Modifies Epiphytic Communities

Another key stressor is the well-known increase in temperature affecting all oceans in the last five decades (Chapin et al., 2000). In terrestrial systems, the influence of warming on community dynamics has been largely investigated through field manipulations. However, only a few warming experiments were conducted in the marine environment to determine models for the adhesion and progression of communities (Table 2). In many ecosystems, it is still unclear which interactions among surface colonizers are most likely to be altered under increasing thermal stress and what impacts, if any, such altered interactions will have on the structure of mature communities. An increased temperature is arguably one of the most important facets of global climate changes, because temperature influences various processes across all the biological scales, including the survival and recruitment of plants and invertebrates (Wahl et al., 2010). Global warming can alter the multifunctionality of plant and animal communities associated with structurally complex ecosystems by modifying the coexistence of engineers and the diversity of the functional traits present in a landscape (Moore & Duffy, 2016).

Table 2. Summary of the effects of rising temperature of seawater at the early stages of plants and animals.

Colonizer organism	Effect on colonization	Environment/Substrate	Reference
Macroalgae cover (*Gracilaria gracilis*)	Increase	Seaweed beds and unvegetated sandflats	(Beltrand et al., 2021)
Seagrass cover	Reduction		
Macrofaunal abundance	Reduction		
P. oceanica abundance	Reduction, becoming steep when the temperature reaches 28°C	P. oceanica meadows of Cabrera Island (Algerian	(Marbà & Duarte, 2010)

		subbasin of the Western Mediterranean)	
Phytoplankton	Indirect reduction by increased grazing	Coastal regions	(Lewandowska et al., 2014)
Phytoplankton	Reduction and shift towards assemblage dominated by smaller species	Nutrient-limited water (oligotrophic oceans)	(Li et al., 2009; Morán et al., 2010)
Hydroides elegans	Production of stronger tubes more resistant to simulated predator attacks	Laboratory experiment - mesocosm	(Li et al., 2016)
Bacterial biofilm	Most diversified bacterial communities at high temperature conditions	Laboratory experiment - mesocosm	(Whalan & Webster, 2014)
Bacterial biofilm	Higher biomass production and higher diversity in the biofilm at higher temperature	Laboratory experiment - mesocosm	(Lau et al., 2005)
Crustose coralline algae	Lack of seasonality growth and high mortality	Marmion Lagoon, South-Western coast of Western Australia	(Short et al., 2015)
Kelp forests (*Nereocystis luetkeana*)	Clear decrease without full recovery	Coastline in northern California	(McPherson et al., 2021)
Diatom *Chaetoceros socialis*	Reduction of production and cell size; poleward shift	Laboratory experiment - mesocosm	(Li et al., 2017)

	in biogeographic distribution		
Coralline algae	Clear reduction of cover and negative effect on photosynthetic efficiency	Laboratory experiment - mesocosm	(Kelaher et al., 2022)

This effect is mediated by warming, favoring the retention of tolerant engineer traits over sensitive ones and the functional characteristics of residents nested within engineered patches. In situ comparisons of functional traits and community metrics of macrofauna inhabiting beds of seagrasses and macroalgae, as well as unvegetated sandflats, indicated unique assemblages in seagrass habitats and a dominance of surface-dwelling fauna due to global warming (Lawrence & Bolton, 2023). Macrofaunal abundance is reduced in seaweed beds and unvegetated sandflats, which are dominated by burrowing infauna (Beltrand et al., 2021). Experimental findings indicate that increases in temperature in temperate coastal systems may lead to declines in seagrass, but increased seaweed cover. This shift is likely to lower ecosystem heterogeneity due to the faunal similarity between unvegetated sandflats and seaweed beds and, in parallel, reduce the habitat for critically endangered specialist seagrass inhabitants (Beltrand et al., 2021).

However, community responses to short-term increases in temperature are variable, and they are influenced by the individual responses of key species (Smale & Wernberg, 2012). The importance of community-level, field-based manipulations of environmental change factors, which examine interactions between all specific members of the local species pools, should be recognize, because the strength and direction of interspecific interactions governing complex benthic communities are expected to change according to the increasing global temperatures.

The increased water temperatures associated with global changes led to the formation of diverse biofilm communities, affecting the subsequent settlement of other organisms. In their study, (Whalan

& Webster, 2014) demonstrated that the most successful larval settlement of the abundant Great Barrier Reef sponge *Rhopaloeides odorabile* Thompson, Murphy, Bergquist & Evans, 1987 occurred on biofilms that developed at higher temperatures. This is likely to be related to the more diversified communities that developed compared with the biofilms tthat developed at lower temperatures. Similar pathways are detectable in the study conducted by Lau et al. (2005), in which they tested the role of biofilm in the larval settlement of two species of barnacles (*Amphibalanus amphitrite* Darwin, 1854 – formerly *Balanus amphitrite* - and *Balanus trigonus* Darwin, 1854) and the polychaete *Hydroides elegans* (Haswell, 1883). Biofilms were qualitatively different in terms of their bacterial community composition, and also quantitatively differed in the density and total biomass produced. The settlement of the larvae of the two species of barnacles was strongly induced by the biofilm that developed under higher-temperature conditions, while the surfaces colonized by microbial communities exposed to lower temperatures inhibited (*B. trigonus*) or did not affect (*B. amphitrite*) the larval settlement (Lau et al., 2005). This response to the colonization process of the larvae coincides with the differences expressed in terms of bacterial community composition of the biofilm, rather than the biomass or the density produced.

Several studies have also investigated the effect of global warming on the survival and distribution of seagrasses, especially in *P. oceanica* (Ontoria et al., 2019; Savva et al., 2018). *P. oceanica* is endemic to the Mediterranean Sea, which is warming up three times faster than the global ocean (Burrows et al., 2011). Therefore, it has more significant effects on the marine organisms inhabiting this local area. According to Marbà & Duarte (2010), warming of the Mediterranean triggers *P. oceanica* mortality, and a further increase in mortality was observed when seawater surpassed 28 °C. Under these warming conditions, other seagrass species can occur. However, *P. oceanica* exhibits distinct structures and functions, including the contribution of such species as the mussel *Pinna nobilis* Linnaeus, 1758, which only occurs in its meadows (Richardson et al., 1999). Pillay & Waspe (2019) in another study, investigated a mesocosm to quantify the effects of ocean warming on the epiphytic organisms of the temperate seagrass *Zostera capensis* Setchell, 1933, using a sympatric specialist (*Siphonaria compressa* Allanson, 1958) and a generalist organism (*Fissurella mutabilis* G. B. Sowerby I, 1835) to exemplify two limpet models of grazers (Lebret et al., 2009). Their results indicated changes in epiphytes loads due to warming, which increased up to 15 times in the absence of grazers. Grazers differentially impacted the community, with the specialist limpet providing a stronger cleaning function at high temperatures. The specialist species exhibited higher survival rates at higher temperatures, while the survival rates of the generalist limpet reduced. These findings highlight the need for specialist, warm-tolerant grazers to be prioritized in conservation/restoration programs for seagrasses. Such consideration will produce positive feedback loops, benefitting both seagrasses and grazers under global change conditions, with positive community- and ecosystem-wide ramifications.

The warming of the oceans has also been related to a decline in phytoplankton biomass (Hofmann et al., 2011; Sommer et al., 2012; Steinacher et al., 2010). However, the effect of temperature may vary in different ecosystems. In coastal regions (nutrient-replete waters), phytoplankton is dominated by large diatoms (20–200 µm), which are the preferred food source for overwintering copepods (Sommer & Sommer, 2006). In these regions, temperature causes metabolic changes in plankton populations. Therefore, due to the warming of oceans, there is an increase in the grazing pressure caused by copepods, reducing phytoplankton abundance (Lewandowska et al., 2014). On the other hand, in nutrient-limited waters, temperature mainly influences plankton biomass through physical mechanisms (Lewandowska et al., 2014). In the water column, the growth of larger diatoms is limited under low-nutrient conditions, favouring small phytoplankton (<20 µm) and flagellates, while an increase in ocean temperature increases nutrient limitations and reduces phytoplankton biomass. This causes a shift towards an assemblage of phytoplankton, dominated by smaller species (W. K. W. Li et al., 2009; Morán et al., 2010), resulting in a shift in copepod food sources from large diatoms to ciliates (Stibor et al., 2004). These changes in the marine plankton community vary according to the surrounding nutrient regime; nonetheless, they can affect biogeochemical cycles, climate patterns, and, ultimately, the function and structure of benthic ecosystems (Lewandowska et al., 2014). Similar relationships might be hypothesized for benthic diatoms colonizing seagrass leaves, although no information is available from previous investigations as far as we know.

As mentioned above, multi-stressor scenarios are most likely to be important when forecasting future variations in the ecology of key early colonizers. Among others, Li et al. (2016) investigated the polychaete *H. elegans*, because the calcareous tube it builds can be used as an example to evaluate the individual and interactive effects of OA, temperature increases and reduced salinity, on the mechanical properties of its tube. Under OA (pH 7.8), polychaetes produced a mechanically weaker tube, exhibiting less resistance to simulated predator attacks. Seawater warming of up to 29 °C induced an increase in the tube volume, the tube mineral density and the tube resistance to a simulated predatory attack. The observed weakening effect by OA did not make the removal of tubeworms from the substrate easier, except for the earliest stage, when warming had a weak effect. Reduced salinity (down to 27 PSU) did not affect the structure and the resistance of the tubes. These investigations demonstrate that multi-stressor scenarios are needed to obtain realistic previsions, and that both mechanical analysis and computational modeling should be integrated to provide insights into how communities might develop in future conditions according to the effect of various stressors on the ecology of marine early colonizers.

Biotechnological Issues

The prevention of biogenic encrustations is one of the largest challenges faced by the maritime industry, but antifouling agents commonly impact marine ecosystems (Loeb et al., 1984). The development of antifouling strategies for the protection of submerged structures requires a clear understanding of how various organisms may respond to environmental stressors associated with climate changes (Brown et al., 2018). Climate changes facilitate invasions and induce shifts in community composition, especially in complex and stable ecosystems. Invasive alien species, whose introduction is facilitated by global warming, can tolerate significantly higher temperatures than native species and, consequently, climate changes are likely to have disproportionately negative impacts on native species (Li et al., 2016). Global change stressors can alter mutually beneficial interactions in structurally complex systems, leading to impaired functionality and service provision. Based on the temperature tolerance, survival, and growth results, Sorte et al. (2010) predicted that native species in a California Bay will decrease in abundance according to the increase in ocean temperatures, being substituted by introduced species. The facilitation of invasions by climate change is underway. Over the last 40 years, invasive dominance has increased concurrently with ocean warming (Brown et al., 2016) and this has had substantial ecological and economic impacts. From this perspective, the effects of the first colonizers on further anchoring processes may vary due to changes in the temperature and pH of the medium. As a general rule, higher recruitment rates are observed at warmer seawater temperatures, but the highest recruitment values may shift from resident species to alien species, dramatically modifying the trends of ecological successions observed on submerged surfaces according to a complex series of interactions among invasive and resident species as they relate to seawater temperature (Baragi & Anil, 2016).

In addition, modern approaches to the control of encrusting organisms should avoid the use of toxic compounds, which are largely demonstrated to impact key coastal and planktonic environments (Terlizzi et al., 2000). At present, several environmentally friendly approaches are being tested worldwide to reduce the toxic effects of biocides on non-target species. The use of natural products has had significant effects. For example, it has been demonstrated that the international ban on the toxic antifoulant tributyltin (TBT) had positive effects on "green tide" phenomena, in addition to the reduction in its toxicant power on natural stocks of mollusks and crustaceans in several water bodies. Advances also include the use of nanomaterials, but some physiological constraints are elicited by the increase in seawater temperature, reducing the effectiveness of anti-fouling coatings (Dugdale et al., 2005). Other approaches include the encapsulation/immobilization of commercially available biocides to achieve control over the leaching rate and the overall realization of low-adhesion surfaces that can retard the production of first colonization films, priming the further colonization of larger foulers (Swain et al., 2000; Terlizzi et al., 2000). However, several compounds used to obtain a non-polluting control of foulers may change their chemical properties under acidified or warm conditions. In this case, the mechanisms of oxidative stress, detoxification and neurotransmission in aquatic organisms might be impacted by the newer conditions imposed by global changes. Consequently, the antioxidant patterns that characterize the exposure of marine organisms to anti-fouling agents could vary. These effects indicate the need to identify newer candidate compounds and new strategies to allow for the activity of plant and animal foulers with an efficient and eco-friendly strategy.

Unfortunately, the impacts of OA on encrusting communities, which may be some of the initial colonizers of hard substrates, with a high economic importance, require further investigation. There are well-known effects of OA on first colonizers as diatom and algal communities, indicating that they are often poorly developed in low-pH conditions, which allows for the easy prediction that encrusting communities will face dramatic changes, with reductions in groups bearing exposed exoskeletons. However, our actual knowledge of the long-term changes in benthic communities according to the forecasted decreases of pH is almost null (Brown et al., 2018).

While the impact of ocean warming on single species has been revealed, its global impact on benthic communities is almost totally unknown (Khosravi et al., 2019). According to the abovementioned studies, the future warming of oceans will likely increase the pressure on encrusting organisms, while decreasing the diversity of communities due to the introduction of alien species, particularly in habitats where organisms exist at their upper tolerance limits in terms of temperature (Sekar et al., 2004). Overall, the combined effect of various stressors and their influence on the efficacy of protective coatings is little investigated. Consequently, investigations taking into account both the chemical ecology relationships and the features of foulers influenced by global changes are urgently needed.

Conclusions

Climate change at a global scale affects marine environments, with dramatic consequences at various levels of complexity (Hofmann et al., 2011). Both OA and rising temperatures are changing the benthic communities, primarily (but not only) acting on the settlement of early colonization stages. Several recent research papers have explained the effects of those stressors on individual species (Ross et al., 2011). Although the direct effects that OA has on calcified organisms are evident and very well detailed in various studies (Zupo et al., 1997), the indirect effects are less evident but might be deleterious as well. This review sheds light on the effects of two key stressors (higher $p\mathrm{CO}_2$/lower pH and higher temperature) on the primary colonizers, the pioneer organisms, paying special attention to seagrass meadows due to their critical role in the ecology of coastal areas. Environmental changes may affect whole ecosystems, interrupting or disturbing vital relationships among organisms, such as chemical communications (Mutalipassi et al., 2022). In benthic communities, where the time stability of plant and animal associations are key factors to assure the resilience and conservation of species, variations in the temperature or pH of the seawater may have dramatic consequences for the final species assemblages (Pawlik, 1992). In these environments, well-known time successions bring stable associations. Slight changes in the assemblages of first colonizers, and thus in the biofilm development and composition, may dramatically change the time evolution of benthic communities, producing still-unknown variations in the composition and functioning of the entire ecosystem (Terlizzi et al., 2000). The biodiversity of ecosystems is largely controlled by these successions (Michael et al., 2008). Furthermore, biodiversity helps determining the services provided by those environments, and their economical exploitation. Thus, the correct forecast of the indirect effects of global changes on local biodiversity may be crucial to allow for the sustainable management of marine biodiversity and its related resources (Hale et al., 2011; Moore & Duffy, 2016).

Several modern strategies to reduce the attachment of encrusting organisms partially lose their efficacy in the light of changes produced by global stressors, because both the chemistry of anti-fouling coatings and the physiology of biofoulers are impacted by key stressors, such as the OA and the warming of oceans (Brown et al., 2018). For this reason, further research will be needed to identify the factors priming the anchoring of biofoulers, also considering the physiology and molecular ecology of microorganisms such as bacteria and micro-algae, facilitating the further colonization of other organisms. The data reported here represent a first step towards the development of new strategies, taking into account the properties of diatoms and bacteria as pioneering adhesive organisms (Molino & Wetherbee, 2008). We indicated how they determine the final destiny of benthic communities on both man-made structures and in stable and structurally complex ecosystems such as seagrass meadows. The changes still occurring in the chemical and physical environment of oceans might impact and dramatically change the ecology of pioneer adhesive organisms (Asnicar et al., 2022; Duncan et al., 2022), priming the colonization of economically important ecosystems worldwide.

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CHAPTER 2

Paper in preparation

Diatom assemblages are influenced by Ocean Acidification

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Abstract

Among global environmental changes, ocean acidification (OA) represents one of the main threat for marine biodiversity and ecosystem functioning. Seagrass meadows are among the main habitat formers in the Mediterranean Sea and can be directly or indirectly influenced by OA. Also, the epiphytic species associated with seagrasses can be affected by OA. We focused to the benthic diatom assemblages leaving on seagrasses and naturally exposed to different pH conditions (acidified vs control sites). Our study revealed that diatoms assemblages associated with *Posidonia oceanica* leaves changed among pH conditions and seasons, but were more influenced by the seasonality than the pH. In particular, in spring the genera that characterized the acidified site (CO_2 vent) exhibited overall larger size than in the control site and in autumn. This result can be related to the environmental conditions (*i.e.*, nutrients availability, temperature and irradiance) that, together with a different pCO_2 concentration, influenced the abundance and the distribution of the diatoms. Benthic diatoms play a key role in determining the structure and the dynamics of the overlying benthic communities. Thus, changes in their abundance and diversity can have consequences in the future on the entire food web and trophic-dynamic associated with *Posidonia* meadows.

Keywords: assemblages, benthic diatoms, ocean acidification, *P. oceanica* meadows

Introduction

Ocean Acidification (OA) represents one of the main threats among the ongoing global environmental changes, resulting from the increasing atmospheric pCO_2 concentration, due to rising anthropogenic emissions (Gattuso et al., 2015). Oceans can absorb a large part of this carbon dioxide buffering the present increase, drastically changing the carbonate chemistry balance and reducing the ocean pH (Gattuso & Hansson, 2011; Hönisch et al., 2012; IPCC, 2021).

OA is widely recognised as a deleterious phenomenon for various marine taxa, negatively affecting the correct functioning and the ecosystems services (Hall-Spencer & Harvey, 2019). As a result, the oceanic uptake of CO_2 from the atmosphere is threatening marine biodiversity with far-reaching consequences on communities and ecosystems (Garrard et al., 2013).

Direct effects of OA are evident and well explored, especially for what concerns the physiological responses of marine species. For this reason, most part of studies and reviews focused on specific responses of various taxa (for example see Dupont et al., 2010; Kroeker et al., 2010; Zunino et al., 2017). Conversely, there is still a lack of knowledge regarding indirect impacts, such as at community level or ecosystem scale. This is probably due to the high complexity that characterises important ecosystems, such as seagrass meadows. Seagrasses, and among all *Posidonia oceanica* (L.) Delile, play a crucial role as habitat formers providing important ecosystem services. The complex and highly structured *Posidonia* habitat hosts a high biodiversity and represents a nursery area for several species of fish and invertebrates, being important for fisheries and many other human activities (Jackson et al., 2015). *Posidonia* meadows are highly productive ecosystems, with a net primary production equivalent to 14.8 tons CO_2 ha yr^{-1} (Duarte et al., 2013). In this context, epiphytic communities hosted on leaf blades, make a major contribution to primary production and biodiversity of the meadows, sustaining the associated food-webs (Borowitzka et al., 2006; Michael et al., 2008; Piazzi et al., 2016). Seagrasses are among the most relevant natural surfaces for epiphytic organisms in the marine environment (Buia et al., 1992; Mateo et al., 2006; Mazzella et al., 1993). A multitude of diverse epiphytic organisms settle and colonise *P. oceanica* leaves, broadening the ecological role of the entire habitat. In this purpose, *P. oceanica* leaf stratum is exploited as “models” used to investigate the dynamics of colonization of micro- and macro-organisms (Onuf, 2006). Normally, bacteria represent the very first colonisers, creating a conditioning biofilm on the surfaces. However, they are promptly followed by a widespread and ecologically important group of microalgae: the diatoms (phylum Ochrophyta, class Bacillariophyceae). Benthic diatoms are among the most frequent algal early colonizers of any artificial substrates, strongly modifying the surface properties and the ecological function (Molino & Wetherbee, 2008).

Benthic diatoms represent key organisms riding through primary and secondary biofilm formation, playing important roles in determining structure and dynamics of the overlying benthic communities (Terlizzi et al., 2000). For this reason, they may represent key elements in the determination of colonization patterns (Somma et al., 2023).

The aim of the present study was to reconstruct and analyse the assemblages of epiphytic diatoms that colonise the leaf blades of Mediterranean seagrasses, with a special focus on *P. oceanica* meadows. In particular, because of its ecological relevance, we wanted to stress the possible effects of ongoing climate changes on the settlement and selection of benthic diatom associations.

Material and methods

Sampling and laboratory analyses

Sampling was carried out using *ad-hoc* prepared panels bearing eight glass disks with low adhesive power, developed and set *in situ* to select highly adhesive benthic diatoms. Each panel consisted of two plastic frames, a mooring for anchoring to the bottom and a buoy that favoured its vertical position (see Fig. 1). The sampling panels were designed to be free to float holding the vertical position, emulating the conditions under which the epiphytes colonise seagrasses leaves. Each face of the panel was equipped with four glass slides (23.75 cm^2) coated with a silicone surface with low adhesive power, in order to select among the early coloniser, only the species that strongly adhere to the substrate. The collecting panels were assembled and deployed by scuba diver at 5 m depth close to seagrass meadows, with special focus on *P. oceanica* meadows.

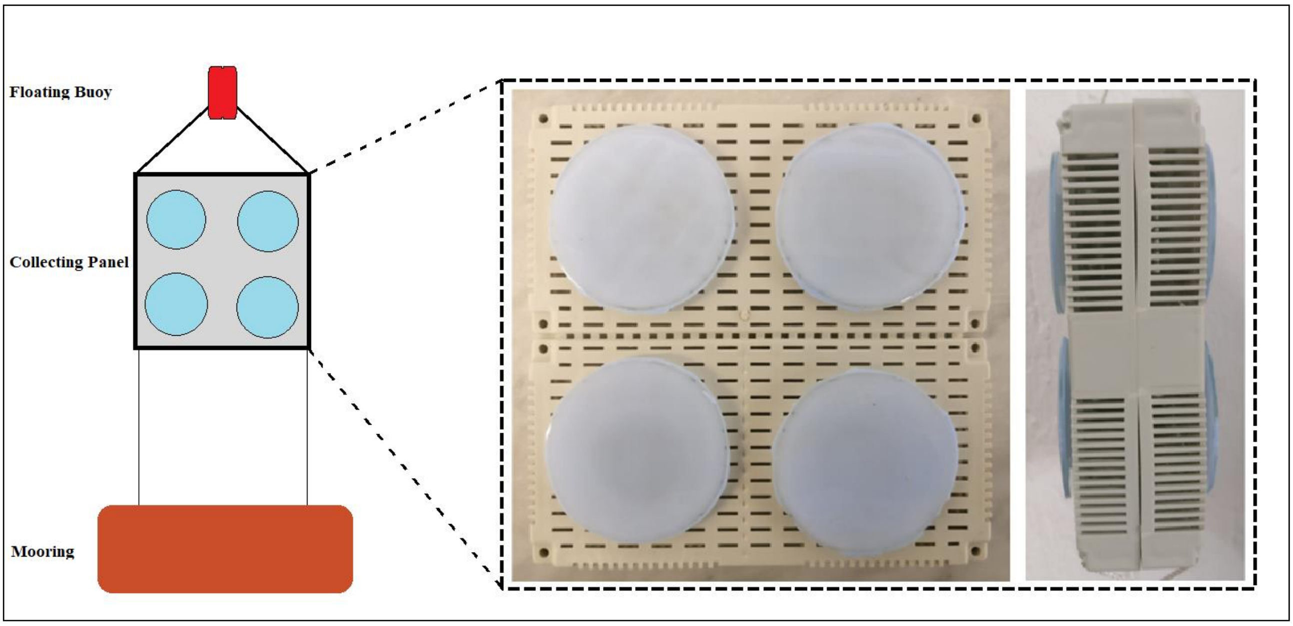

Figure 1. Schematic representation of the device used for sampling benthic diatoms (on the left). On the right the frontal and lateral view of the panel bearing the glass slides with smooth low-adhesion surfaces.

In order to assess the effects of temperature on the benthic diatoms assemblage associated with the seagrass *Cymodocea nodosa* (Ucria) Asch., we designed a sampling campaign following a latitudinal gradient (North-South Italy). For this purpose, three sampling sites characterised by different temperature regimes were selected along the Italian coasts: Gulf of Trieste (Northern Adriatic Sea), Ischia Island (Gulf of Naples, Central Tyrrhenian Sea) and Panarea Island (Aeolian Archipelago, Southern Tyrrhenian Sea). Unfortunately, the collecting panels deployed in Trieste were lost, while the panel deployed off Panarea Island was tampered with, preventing us from being able to samples benthic diatoms and to compare the sites along the latitudinal gradient.

For what concerns the effect of temperature on epiphytic diatom assemblage of *P. oceanica* meadows, we exploited as sampling area the peculiar 'Hot/Cold Points' site (38° 38'32"N; 15°

4′42"E) located off Panarea Island. This area is characterised by hydrothermal vents with a patchy *P. oceanica* “matte” interspersed with clearings of coarse sand affected by hot fluid emissions (Rogelja et al., 2016). Two sampling panels were deployed in the above-mentioned area, through which we collected epiphytic community that was fixed with 4% Glutaraldehyde and stored at 4°C. Despite we were able to collect epiphytes, it was not possible to analyse all the replicates and compare them due to the frequent issues with the Scanning Electron Microscope (SEM) that kept it out of order several times and for long periods.

For these reasons, we focused our work to the possible impacts of OA on the epiphytic diatoms that colonise *P. oceanica* leaf blades. In this regard, the panels were deployed in three sites around Ischia Island (Gulf of Naples, South Italy. See Fig. 2). Two panels were deployed in control conditions with ambient pH (~8.2): one located in Cartaromana bay, nearby Sant’Anna rocks (40°43'34.68" N, 13°57'40.92" E) and another in Lacco Ameno (40°45'23.90" N, 13°53'8.48" E). In order to collect epiphyte from a lower pH condition area, the shallow CO_2 vents site of Castello Aragonese was selected (40°43'50.62" N, 13°57'47.99" E). Here, the CO_2 bubbles emerging from the seabed acidifies the water to a pH around 7.7 (Hall-Spencer et al., 2008). The natural acidification of this area can be considered as a simulation of future scenarios due to the OA linked to present and past anthropic emissions. The panels were deployed two times in different season, in autumn (October 2020) and in spring (April 2021), and leaved underwater for 30 days. Unfortunately, the collecting panel deployed in Cartaromana bay during spring were lost. This event deprived us the possibility of a comparison between the three sites on a seasonal basis, forcing us to put aside the data relating to the Cartaromana bay site.

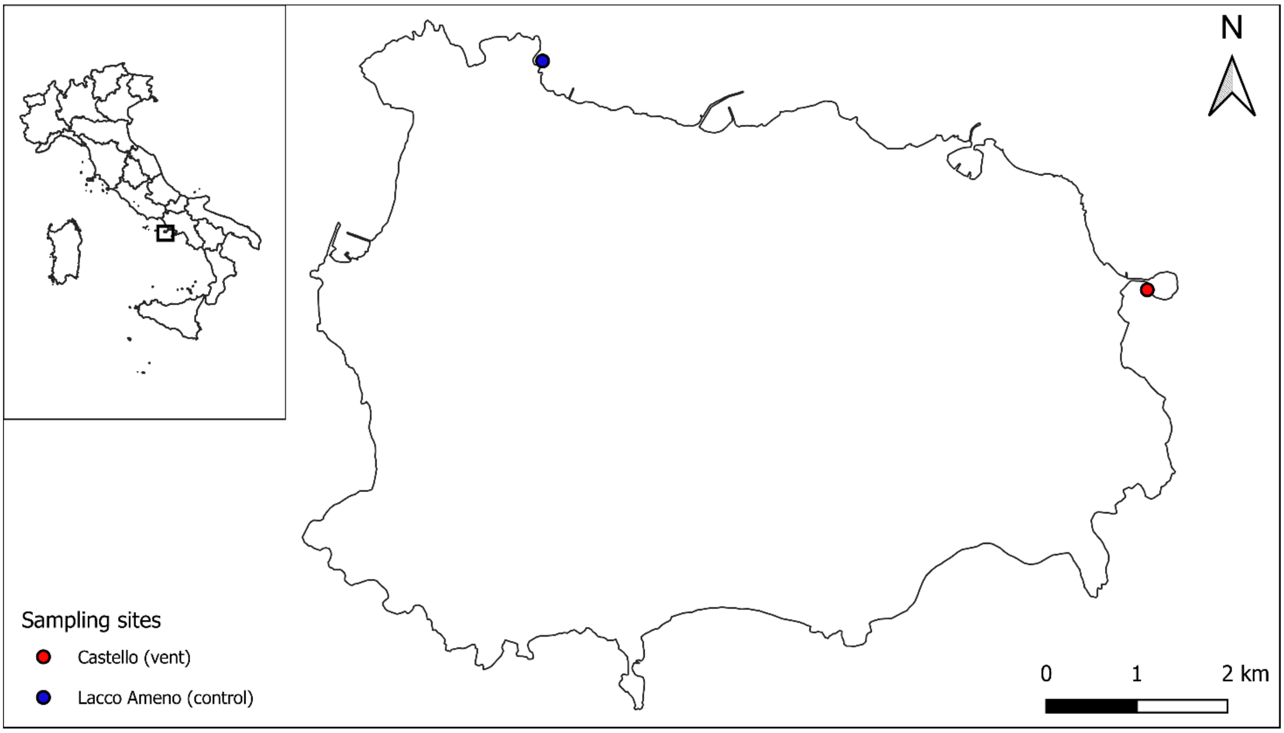

Figure 2 Map of the sampling sites: the control site (ambient pH condition) is located in Lacco Ameno (40°45'23.90" N, 13°53'8.48" E); the shallow CO_2 volcanic vent of Castello Aragonese (40°43'50.62"N, 13°57'47.99"E) was selected as Low pH site (naturally acidified condition). Spots indicate the acidified (in red) and normal pH (blue) sites.

After 30 days, the sampling panels were removed and brought to the laboratory to be processed and analysed. For each panel, five out of eight randomly chosen low-adhesion slides coated with silicone materials were removed, rinsed with 0.22 µm filtered (TPP vacuum filtration “rapid” - Filtermax) seawater and gently scraped with a sterile glass slide in order to collect all the organisms that colonised the smooth surface. The remaining 3 slides were used for benthic diatoms isolation purposes. The material obtained was fixed with a solution 4% Glutaraldehyde in filtered seawater and stored in 1.5 mL Eppendorf tubes.

Diatom identification was performed at genus level using a morphological approach, through the analysis of the ultrastructure of the frustule on images captured with Scanning Electron Microscope (SEM; see Annex I). In order to set up the stubs for the observation, a portion of biomass was collected and transferred in centrifuge glass tubes to undergo a strong acid-cleaning protocol.

The cleaning process allowed us to remove all the organic matter, while leaving intact the siliceous frustule of the diatoms for the morphological analysis of ultrastructure. The cleaning protocol we followed was the one proposed by von Stosch (1974) to which some modifications have been made in order to obtain a better final output. In particular, the samples were previously rinsed with distilled water and centrifuged at 4000 rpm for 10'; after each centrifuge cycle the supernatant was withdrawn and replaced with distilled water. After the removal of the supernatant, the volume of the sample was measured and an equal volume of HNO_3 [65%] was added in the tubes and left for 60' at a temperature of 60 °C. Once the action time of the nitric acid elapsed, three times the volume of H_2SO_4 [98%] were added. The tubes were then passed upon the reducing flame of a burner Bunsen until the pellet started to produce little bubbles, avoiding violent ebullition. This step was repeated several times, since the acid mixture in the tube stop to produce bubbles. In this way, the organic matter is degraded by acids, while the siliceous frustules remain intact. Therefore, the pellets were rinsed several times with distilled water until neutral pH was reached and thus dehydrated with Ethanol [90%] to favour the rapid evaporation of the liquid after deployment on the filters. Stubs were prepared applying the double-sided tape on the head and posing a special 0.5 μm filter (MilliporeTM, Isopore membrane filters); a drop of sample was placed on the filter, taking part of the pellet from the bottom of the tube. The stubs were left to dry for 24 hours under the chemical hood and then observed with the SEM (JEOL 6700 F) at the Microscopy Laboratory of Stazione Zoologica Anton Dohrn.

Statistical analyses

A multivariate approach was used to appraise the composition of diatom assemblages associated with *P. oceanica* leaves. A permutational analysis of variance (PERMANOVA, Anderson 2001a, 2001b, Anderson et al. 2008) was applied on a Bray-Curtis similarity matrix using square-root-transformed abundance data, in order to down-weight the most abundant species. The model included two crossed factors: Site-pH condition (fixed factor with two levels: Vent and Ambient) and

Season (fixed factor with two levels: autumn and spring). *Post hoc* pair-wise comparisons for all the combinations of ‘Season x Site-pH condition’ were also performed using t-tests and 9999 permutations of the raw data.

In order to validate PERMANOVA results, dispersion analysis test (PERMDISP) was carried out on the same resemblance matrix. PERMDISP analysis is commonly used to compare the sample dispersion of different groups measuring the average dissimilarity from individual observation units to their group centroid in a multivariate space. PERMDISP tests were set to 9999 permutations and significance to $p < 0.05$.

Data were graphically reported after performing a Principal Coordinates Analysis (PCoA). Finally, similarity percentage analysis (SIMPER, Clarke and Warwick 1994) was performed to identify the main species responsible for the similarity/dissimilarity within and between the benthic diatoms recorded in the different sites.

All the multivariate analyses were carried out by the software PRIMER v 6.1.11 with PERMANOVA + V 1.0.1 add-on package, developed by the Plymouth Marine Laboratory.

Results

Due to operational problems related to sampling (see section ‘Materials and Methods’), here we assessed the differences in the benthic diatom assemblages taking into account the data belonging to a control site (Lacco Ameno, ambient pH site) and a low pH area (Castello Aragonese, vent site) regarding two sampling seasons (autumn and spring).

Overall, twenty-six genera of diatoms belonging to fifteen orders and twenty families were recorded (See Table 1).Most of the genera sampled were represented in both the sites (vent and control), however, some genera (*Achnanthes*, *Campylodiscus*, *Cyclophora*, *Terpsinoe*) were recorded only in the vent site during spring season, while the genera *Rhopalodia*, *Dimeregramma*, *Bacillaria*, *Thalassiosira* were found only in the control site. The benthic diatom genera here recorded were also characterised according to their life forms in adnate, erect, motile and tube-dwelling. In particular, the genera most represented in our samples belong to adnate, followed by erect benthic diatoms.

Table 1. List of the genera recorded, here reported with respective families and orders. Information about life forms were added where available (N.A.: not available in literature)

Genera	**Family**	**Order**	**Life form**
Achnanthes	Achnanthaceae	Achnantales	Adnate
Amphora	Catenulaceae	Thalassiophysales	Adnate
Bacillaria	Bacillariaceae	Bacillariales	Motile
Berkeleya	Berkeleyaceae	Naviculales	Tube-dwelling
Biddulphia	Biddulphiaceae	Biddulphiales	N.A.
Campylodiscus	Surirellaceae	Surirellales	N.A.
Cocconeis	Cocconeidaceae	Achnantales	Adnate
Cyclophora	Cyclophoraceae	Cyclophorales	Erect
Cylindrotheca	Bacillariaceae	Bacillariales	Motile
Dimeregramma	Plagiogrammaceae	Triceratiales	N.A.
Diploneis	Diploneidaceae	Naviculales	Adnate
Fragilaria	Fragilariaceae	Fragilariales	Erect
Lampriscus	Triceratiaceae	Triceratiales	N.A.
Licmophora	Licmophoraceae	Licmophorales	Erect
Lyrella	Lyrellaceae	Lyrellales	N.A.
Mastogloia	Mastogloiaceae	Mastogloiales	Adnate
Navicula	Naviculaceae	Naviculales	Tube-dwelling
Nitzschia	Bacillariaceae	Bacillariales	Tube-dwelling
Pleurosigma	Pleurosigmataceae	Naviculales	Motile
Rhopalodia	Rhopalodiaceae	Rhopalodiales	N.A.

Surirella	Surirellaceae	Surirellales	Motile
Synedra	Fragilariaceae	Fragilariales	Erect
Terpsinoe	Biddulphiaceae	Biddulphiales	N.A.
Thalassiosira	Thalassiosiraceae	Thalassiosirales	N.A.
Toxarium	Toxariaceae	Toxariales	Erect
Tryblionella	Bacillariaceae	Bacillariales	Motile

Regarding the benthic diatoms diversity (Table 2, Fig. 3), for what concerns the number of genera, the results showed higher mean values in the control site in autumn (mean ±SD: 10.6 ±1.1) than in spring (mean ±SD: 8.4 ±2.9). In contrast, an inverted seasonality trend was highlighted for the low pH site (9.8 ± 2.6 recorded in spring *versus* 6 ±1.9 recorded in autumn).

Table 2. Mean (±SD) number of genera found in the sampling sites (Vent and Ambient) during the two seasons (spring and autumn). Replicates: N=5 (per sampling site in each season); N total=20.

	Number of Genera	
	Mean	**SD**
Vent spring	9.8	2.6
Vent autumn	6.0	1.9
Ambient spring	8.4	2.9
Ambient autumn	10.6	1.1

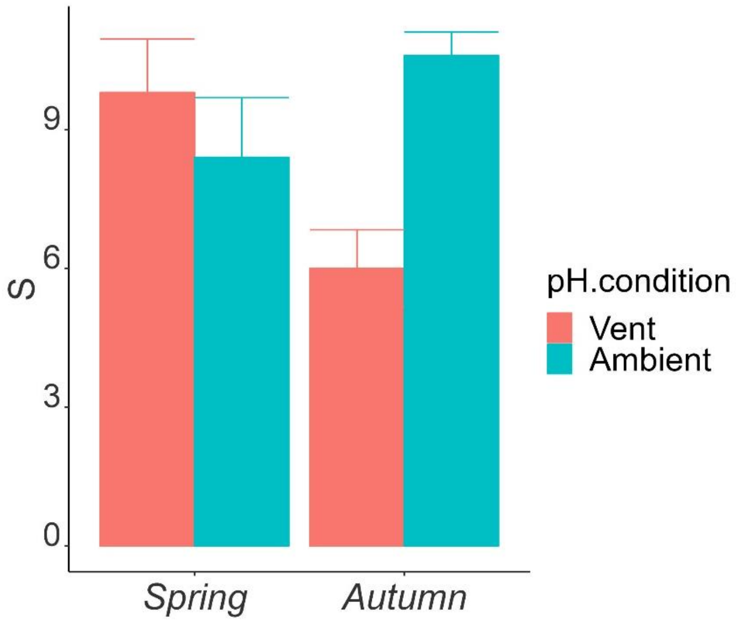

Figure 3. Number of genera (S, mean ± SD) sampled in the two pH condition sites in the two seasons.

According to the multivariate analyses, the abundance of benthic diatoms genera highlighted differences for the interaction between ‘pH conditions and Season’ (PERMANOVA: pseudo-$F_{1, 19}$ = 4.38, p(perm) < 0.05). Pair-wise *post hoc* tests revealed significant differences for all the levels of the two factors examined. The result of the test PERMDISP ($F_{1, 18}$=2.20, P(perm) > 0.05) revealed that the data are homogeneous and the samples distribution is not due to data dispersion.

Table 3. PERMANOVA results testing differences for the factors Site-conditions (pH) and Season (SE) and time in the epiphytic diatoms assemblages.

Source	*df*	MS	Pseudo-F	P(perm)
pH	1	2499	3.2135	0.0022
SE	1	3852.5	4.954	0.0004
pHxSE	1	3405.2	4.3788	0.0002
Res	16	777.66		

The ordination of Principal Coordinate Analysis (PCoA) explained on the first two dimensions 50.1% of the total variation, demonstrating a clear separation among seasons, while for what concerns the pH conditions the separation is not evident (Fig. 4). In particular, the PCoA highlighted a clear segregation in autumn especially for the vent site, with *Diploneis* and *Tryblionella* representing the genera that primary contribute to this separation.

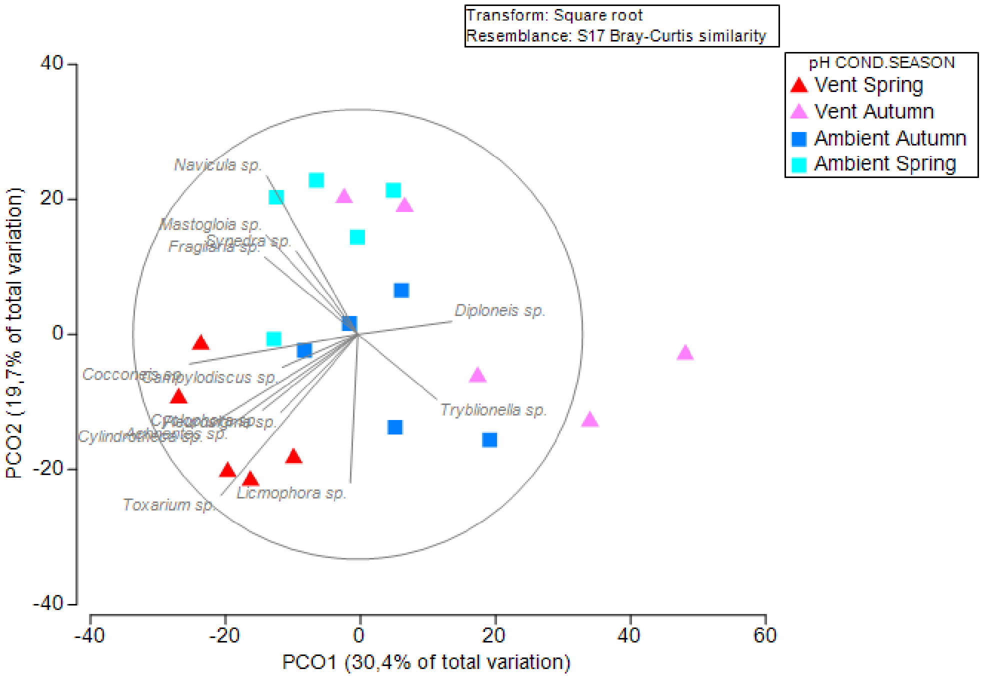

Figure 4: Principal Coordinate Analysis showing the distribution of the data belonging to the two sites (Vent and Control) and the two seasons (spring and autumn). Vectors of diatom genera recorded were overimposed (p of Pearson > 0.3).

Accordingly, dissimilarity of the assemblages was carried out through SIMPER analysis. Regarding the factor pH condition, the outcome of SIMPER analysis revealed an overall average dissimilarity of 48.50, with the genera that discriminate the two sites represented by *Toxarium*., *Cocconeis*., *Navicula*, *Fragilaria*, *Amphora*, *Mastogloia*, *Tryblionella*, *Achnanthes* and *Cylindrotheca* (Table 4).

Table 4. SIMPER analysis showing benthic diatom genera contributing to the dissimilarity among the two sites at different pH conditions (Vent and Control) with the relative average abundances (Av. Ab.) and the total percentage contribution (Contrib%); only genera that contribute more than 5% are reported here.

Genera	Vent Av. Ab.	Control Av. Ab.	Contrib%

Toxarium	0.97	0.4	8.92
Cocconeis	2.56	2.72	8.85
Navicula	0.48	1.32	7.96
Fragilaria	0.6	1.37	6.55
Amphora .	1.1	1.33	5.72
Mastogloia	2.16	2.59	5.65
Tryblionella	0.4	1.01	5.47
Achnanthes	0.77	0	5.11
Cylindrotheca	0.58	0.64	5.05
		Average dissimilarity = 48.50	

On the other hand, SIMPER analysis carried out on the season of sampling showed average dissimilarity of 50.75, with a discrimination mainly due to the genera *Toxarium*, *Cocconeis*, *Fragilaria*, *Cylindrotheca*, *Mastogloia*, *Achnanthes*, *Tryblionella*, *Navicula*, *Amphora* (Table 5).

Table 5. SIMPER analysis showing benthic diatom genera contributing to the dissimilarity among the two Season of sampling (spring and autumn) with the relative average abundances (Av. Ab.) and the total percentage contribution (Contrib%); only genera that contribute more than 5% are reported here.

Genera	**spring Av. Ab.**	**autumn Av. Ab.**	**Contrib%**
Toxarium	1.07	0.3	10.13
Cocconeis	2.95	2.33	9.84
Fragilaria	1.5	0.47	7.82
Cylindrotheca	0.88	0.34	6.83
Mastogloia	2.77	1.98	6.67
Achnanthes	0.77	0	6.2
Tryblionella	0.41	1	6.13

Navicula	1.07	0.73	5.96
Amphora	1.23	1.2	5.63
			Average dissimilarity = 50.75

Discussion

The decrease of ocean pH due to OA (Caldeira & Wickett, 2003; IPCC, 2021) is predicted to negatively affect biodiversity due to direct and indirect effects on marine biota (Cheung et al., 2009). While calcareous species are considered susceptible, also other organisms such as micro-phytobenthos can be affected directly or indirectly with consequences on their distribution and abundance (Porzio et al., 2013). The present study, dealing with the assessment of benthic diatom assemblages exposed to naturally high $p\mathrm{CO}_2$ / low pH conditions, revealed slight changes in the assemblage structure compared to those colonising surfaces in the control site with ambient pH conditions. Generally, the assemblages here recorded are mainly composed by the genera *Cocconeis* Ehr. (Achnanthales), *Mastogloia* Smith (Mastogloiales), *Amphora* Ehr. (Thalassiophysales), *Fragilaria* Lyngbye (Fragilariales), and *Navicula sensu lato*, in accordance with previous studies conducted by Mazzella & Spinoccia (1992) carried out in the same study area (Ischia Island). In particular, prostrate species of *Cocconeis* were the most frequent and abundant in both the pH conditions and in the two seasons, reflecting the results of Mazzella & Spinoccia (1992). In addition, the dominant genera of the leaf stratum (in particular *Cocconeis*, *Mastogloia* and *Amphora*) belong to the adnate life form, which strongly adhere to the susbtrate. Diatoms included in this categoryrepresent the very early colonisers and the more stable component of this benthic environment (Round et al. 1990). Furthermore, erect and motile life forms of benthic diatoms are also well represented (e.g. *Fragilaria*, *Toxarium*), indicating that the succession of benthic diatom communities is complex and well structured.

For what concerns the possible effect of OA, our results highlighted only slight differences in the pH conditions of sampling, demonstrating a more evident separation in the seasonality of the assemblages.

Several studies carried out in last years mainly addressed inter-specific influence of OA on growth and physiology of diatoms. Such influence produce responses that vary widely among different species (Gao & Campbell, 2014). These physiological differences triggered by pH sensitivity represent a relevant feature as this could alter the composition of benthic diatom assemblages. For instance, Wu et al. (2014) demonstrated a positive relationship between the cell size and the magnitude of the fertilization effect of higher CO_2 level on diatom growth, producing shift in the assemblage toward larger species. Despite the graphical ordination (PCoA) seems to not reveal such wide pattern of differentiation among the pH conditions, our results pointed out an interesting exclusive occurrence of the larger genera *Achnanthes*, *Campylodiscus*, *Cyclophora* and *Terpsinoe* in low pH condition, only during spring. Furthermore, the recorded genera *Licmophora*, *Toxarium*, and *Pleurosigma* were found more frequently under the same seasonal and pH conditions.

The comparison of OA effects in different environments revealed that diatom assemblages are affected more frequently by low pH condition in ocean than in coastal and benthic environments (Bach & Taucher, 2019). In this context, our results are in accordance with Duarte et al. (2013) who asserts that communities found near coasts might be adapted to larger carbonate chemistry variability, and therefore be better suited to deal with higher CO_2 levels.

Furthermore, multiple environmental drivers (such as temperature, nutrient availability, irradiance *etc.*) will further modify the different responses of diatoms to decreasing pH (Boyd et al., 2018; Gao et al., 2012). These additional drivers, together with OA, will also affect diatom assemblages leading to shifts in their composition. This concept might explain the evident seasonal separation of our results. Indeed, the different environmental conditions characterising the two seasons coupled with the effects of OA, produce a wide separation particularly within the low pH site.

In conclusion, the benthic diatom assemblages in the studied area showed a seasonal differentiation, highlighting evident separation in genera composition, especially for what concern the acidified area (vent). This led us to hypothesize that the effects observed in acidified area might be influenced by other combined environmental conditions, leading to wide separation of the assemblages. Due to their relevant ecological role both in the benthic succession and in the trophic relationship, epiphytic diatoms represent key elements in resilient habitats as seagrasses meadows. Consequently, also slight changes in the composition of the assemblages driven by global changes might produce a deleterious cascade effect at ecosystem level, negatively impacting the trophic web and the plant-animal chemical relationships (Mutalipassi et al., 2019).

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CHAPTER 3

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Identification of *Cocconeis neothumensis* var. *marina* (Bacillariophyceae) using a polyphasic approach and annotation of the 18S RNA ribosomal gene

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Abstract

Among the eukaryotic phytobenthos, several microalgae and above all diatoms significantly contribute to the primary production and the food webs of key marine ecosystems, such as the seagrass meadows. *Cocconeis neothumensis* Krammer has been suggested as a model organism for studying chemical ecology issues and the plant-animal chemical relationships within complex trophic relations, including some key grazers. Its genome is not yet sequenced and consequently the molecular identification of these diatoms, seasonally dominant in the epiphytic cover of *Posidonia oceanica* leaves, is presently impossible. The identification of molecular markers for this diatom is needed to provide an effective detection in key coastal environments as *P. oceanica* meadows. Here we report a polyphasic identification of *C. neothumensis*, using both ultra-morphological data and molecular conserved markers. In particular, for the molecular phylogenetic analyses, we sequenced the 18S ribosomal RNA gene (*18S rRNA*) and ribulose 1,5-biphosphate carboxylase *(rbcL)* genes. This comparative analysis allowed the suitability assessment of both genes as genetic markers for identification of closely related benthic diatom species and potential barcode genes. The molecular analyses performed on a strain isolated from *P. oceanica* leaves coincided with the morphological features detected by Scanning Electron Microscope (SEM) analyses, unambiguously identifying the species as *C. neothumensis*. This research will facilitate the identification of this model species using a multidisciplinary approach.

Key words: biotechnology, identification, microalgae, diatom, molecular markers, polyphasic, seagrass, SEM.

List of Abbreviations

coxI, mitochondrial cytochrome c oxidase I

ESTs, expressed sequenced tags

ITS, internal transcribed spacer

LM, Light Microscopy

OA, Ocean Acidification

rbcL, 1,5-biphosphate carboxylase genes

RVS, Raphe-sternum valve

SEM, Scanning Electron Microscope

SSU rDNA, small subunit ribosomal RNA genes

SV, Sternum valve

18S rRNA, 18S ribosomal RNA gene

28S rRNA, 28S rRNA large ribosomal subunit

Introduction

Cocconeis neothumensis Krammer, is a well-established model diatom (Zupo, 2000), and an excellent candidate for the extraction of apoptogenic compounds (Nappo et al., 2012), with promising activity for various biotechnological applications in the fields of aquaculture and medicine (Raniello et al., 2007), along with several congeneric species (Jüttner et al., 2010). Diatoms (Bacillariophyceae) are microalgae living both in freshwater and seawater, being a very diverse taxon of photosynthetic eukaryotic organisms that may become dominant both in benthic and in planktonic environments (Zupo et al., 2007). In these aquatic environments, diatoms may play crucial roles by promoting high ecosystem productivity (Falkowski et al., 1998) due to a very high turnover (Field et al., 1998), and assimilating more than 20% of the CO_2 from the Atmosphere, so contributing to reduce the Ocean Acidification (OA) process. They are unicellular, sometimes colonial, and may be found in almost every aquatic habitat as free-living photosynthetic autotroph, colourless, and even exhibiting a heterotrophic behaviour in special conditions. In some cases, they may play the role of photosynthetic symbionts and they may influence invertebrate physiology (Zupo et al., 2007) and behaviour (Jüttner et al., 2010). As a peculiarity, with respect to other micro-algae, they possess typical double-capsule shells (frustules) made of silica. This feature, as well, confers diatoms a great importance for industry, because several biotechnological applications have been set in the frame of microfiltration and production of micro-structures (Rabiee et al., 2021). In addition, several diatoms represent promising sources of bioactive compounds (Nappo et al., 2012) and many studies in the last decades have explored various species of planktonic and benthic diatoms to produce specific ecological effects (Saha et al., 2019) or extract compounds with anti-cancer or other medical activities (Riccio et al., 2020). Among other physiological peculiarities, diatoms are characterised by a typical peroxidase biosynthetic pathway (Ruocco et al., 2020), generally wound-activated, leading to the production of several volatile and lipidic compounds exhibiting specific biological activities (Ruocco et al., 2018) and biotechnological power (Nuzzo et al., 2018). It has been also demonstrated by means of transcriptomic investigations on a common diatom (*Nitszchia* sp.), which produces up to 50 % oil by weight under defined conditions, that environmental influences, as increased salinity, are able to induce a higher total lipid content mainly through a boosted biosynthesis of triacylglycerols (Cheng et al., 2014).

For this reason, several key species of diatoms have been investigated for their biological effects (Ruocco et al., 2019) and some of them have been further studied to obtain molecular information. For example, the genome of *Phaeodactylum tricornutum* Bohlin was sequenced (Bowler et al., 2008), showing that it is made of about 27.4 mega-bases and may contain 10,402 different genes. These authors reported on about 130,000 expressed sequenced tags (ESTs) that were further investigated and annotated (Maheswari et al., 2010). However, for most marine diatoms, a high-quality genome annotation is still missing, representing a major obstacle for a complete understanding of key molecular and cellular processes involved in ecological relationships and in biotechnological potential applications. In addition, modern techniques of DNA fingerprinting are increasingly adopted to speed-up the identification of diatoms in various environments and produce timely answers to blooms and other phenomena influencing the ecological status of coastal waters (Sison-Mangus et al., 2016). The described taxonomically significant microstructures of silica frustules observed under SEM are historically used for the determination of species (Jiang et al., 2015). However, some diatoms are often difficult to be identified especially when intra-genus differences should be evaluated (Romero & Riaux-Gobin, 2014). This is made even more difficult considering the increasingly common discovery of “cryptic” species (morphologically similar but genetically distinct) (Alverson, 2008). Consequently, molecular tools, complementing the traditional identification by morphological features, permit to precisely estimate the biodiversity of diatoms down at all the species level and various molecular analyses, based on DNA sequencing of specific of ribosomal genes, have been developed to classify these microorganisms. Molecular techniques are applied, with increasing frequency, to provide the identification of diatoms classically based on ultra-structural morphology, but a polyphasic identification, including morphological and molecular data, guarantees for absence of errors and improves the quality of ecological and biotechnological investigations. Molecular-assisted taxonomy, which combines molecular species delimitation with *post-hoc* morphological examinations, was proven to be effective for the classification of congeneric diatoms characterised by similar ultra-morphology and various molecular markers may delimit species boundaries, besides the results of morphological investigations (which may include evaluation of length, width, striation pattern, and allometric shape as described by the first axis in a geometric morphometric analysis). However, sufficient data for a molecular identification of diatoms are available only for a small bunch of species, especially for key phytoplanktonic taxa, while for most benthic species this task is impossible due to lack of annotated genomes (Mora et al., 2022). In particular, for the genus *Cocconeis*, some molecular markers of only a few species are currently present in The National Center for Biotechnology Information (NCBI) database (namely *C. placentula*, *C. stauroneiformis*, *C. euglypta*, *C. pediculus*) and it is impossible to determine at the species level such diatom as *C. neothumensis*, despite its demonstrated importance for the ecology of seagrass meadows (De Stefano et al., 2000; Majewska et al., 2014) and other benthic environments (N'Guessan et al., 2018) and its biotechnological power.

For this reason, here we isolated a strain of *Cocconeis* from the leaves of *Posidonia oceanica* (L.) Delile, and we produced monoclonal axenic cultures that were used both forfull taxonomical identification by means of SEM ultra-morphology observations and annotated 18S ribosomal RNA (*18S rRNA*) and ribulose 1,5-biphosphate carboxylase (*rbcL*) genes. This will provide new tools for taxonomy, molecular phylogenetics and eDNA metabarcoding, also to prime the production of data on other *Cocconeis* species(Nevrova & Petrov, 2019).

Material and methods

Sampling and Sites

Special panels bearing eight glass disks with low adhesive power were *ad-hoc* developed and set to sample benthic diatoms. Each panel consisted of two plastic frames, a mooring for anchoring to the bottom and a buoy that favoured its vertical position (see Fig. 1).

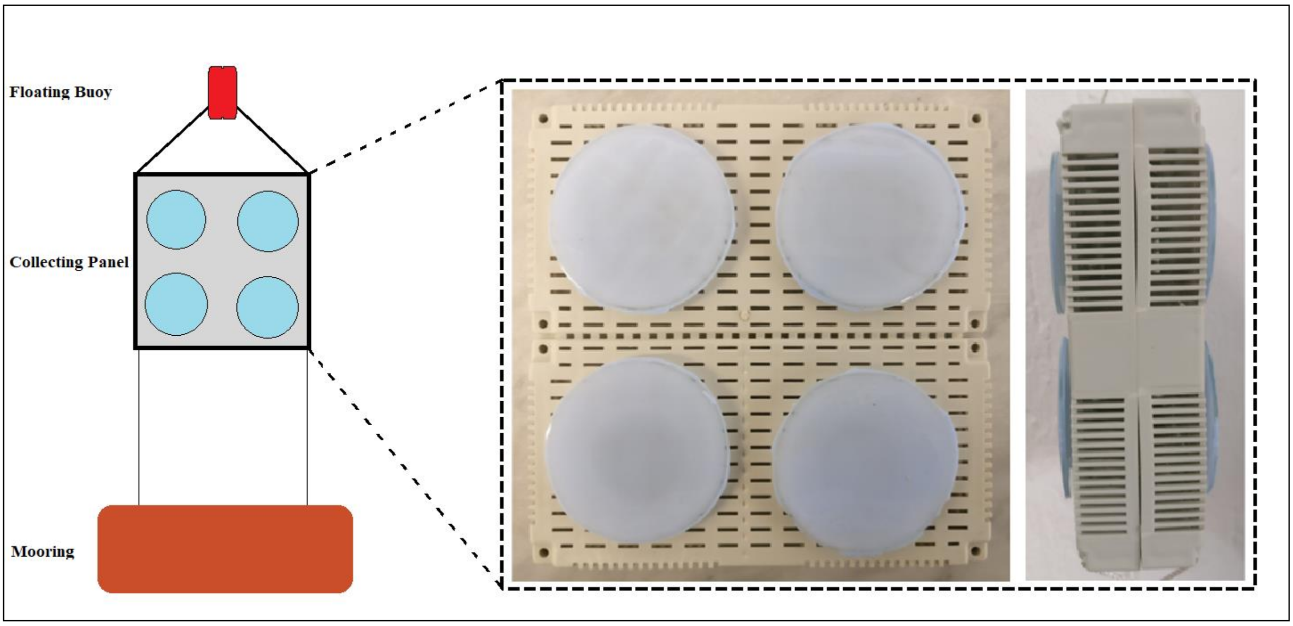

Figure 1. Schematic representation of the sampling system for the sampling of benthic diatoms (on the left). On the right the frontal and lateral view of the panel bearing the glass slides with smooth low-adhesion surfaces.

The sampling panels have been designed to hold a vertical position and to be free to float in order to emulate the conditions under which the epiphytes colonise *P. oceanica* leaves. On each face of the panels were applied four glass slides (23.75 cm^2) coated with a silicone surface with low adhesive power, in order to select only the species that strongly adhere to the substrate (*e.g.* diatoms of the genus *Cocconeis*). The collecting panels were deployed by scuba divers and fixed at the margin of the meadows of *P. oceanica* at 5 m depth. The sampling panel was deployed during the autumn

(October, 2020) in a site around Ischia Island (Gulf of Naples, Southern-Italy) located in the Cartaromana bay, near Sant'Anna rocks (see Fig. 2), where *P. oceanica* meadows represent the dominant ecosystem.

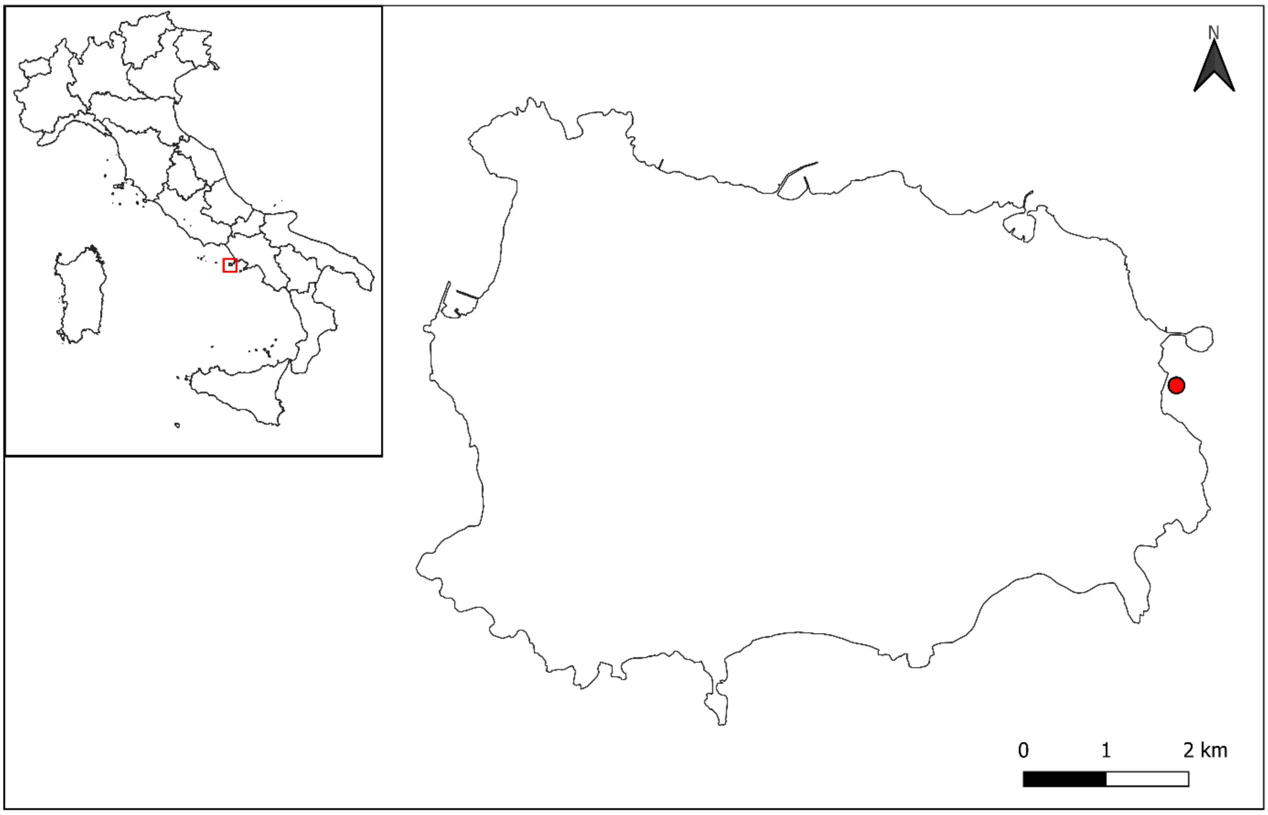

Figure 2. Map of the sampling site located in the Cartaromana Bay - Sant'Anna rocks (40°43'34.68" N, 13°57'40.92" E) on the East coast of the Ischia Island (Gulf of Naples, Italy).

Diatom isolation and mother culture maintenance

The sampling panels were left underwater for thirty days, after which they were removed and brought to the laboratory to be processed and analysed. Once in the laboratory, the low-adhesion slides coated with silicone materials were removed, rinsed with 0.22 µm filtered (TPP vacuum filtration "rapid" - Filtermax) sea water and gently scraped with a sterile glass slide in order to collect all the organisms that colonised the smooth surface. The epiphytes were collected in Petri dishes and analysed with an inverted microscope to isolate diatoms belonging to the genus *Cocconeis*. The entire sample was then divided into 6 sub-samples, each one in a well of Culture Multiwell Plates. From each sample, the diatoms of interest were isolated under a Leica inverted microscope, through sequential transfers of single cells, with the micromanipulator Leica microsystems, a Narishige syringe and a Pasteur glass pipette whose tip was towed over the flame of a Bunsen burner.

The collected and isolated diatoms were deployed in a well of a Multiwell Plate filled with 0.22 µm filtered and autoclaved sea water, in order to obtain axenic monoclonal cultures. Diatom isolation is complex for several reasons. The first issue concerns strictly the isolation of benthic diatoms, in particular those with strong adhesive power, *e.g.* diatoms of the genus *Cocconeis*. To isolate strong adhesive benthic diatoms, it was necessary to scrape the bottom of the well with the tip of the pipette withdrawing a single cell, being careful not to break the fragile siliceous frustule. Moreover, despite the very small volume of water taken, it is difficult to isolate a single diatom as searched. Often, other cells such as microalgae, spores and different species of diatoms are taken along. For this reason, isolated diatoms were observed daily to ensure that the culture was not contaminated by other microorganisms. Once monoclonal strains were obtained, the culture medium was prepared with sterile seawater, previously filtered at 0.22 µm and autoclaved at 121 °C for 25 minutes. After reaching room temperature, a volume equal to 10 mL/L of concentrated solution Guillard's *f/2* with silicates (Sigma-Aldrich) was added. All the operations of transferring or renewal of diatom culture were carried out under a laminar flow hood, using glassware previously sterilised in an oven (150 °C for 3 h), to minimise any type of contamination. In this way the mother-cultures of diatoms were obtained and then were kept under controlled conditions in a thermostatic chamber at 18 °C with 12:12 light:dark photoperiod. Light was provided by Sylvania GroLux (Osram Sylvania Inc., USA) at an irradiance of 140 $\mu E \cdot m^{-2} \cdot s^{-1}$. The diatoms were grown in sterile Culture Multiwell Plates of 6 wells (3.5 cm in diameter), each containing 8 mL of culture medium. The cultures were transferred approximately every 15-20 days to new sterile clusters, under a laminar flow hood, using sterile Pasteur pipette, taking care to change the pipette after each inoculation, in order to avoid any contamination.

Morphological identification

Diatom identification was performed using a morphological approach, through the analysis of the ultrastructure of the frustule on images captured with SEM (Scanning Electron Microscopy; JEOL 6700 F). For each mother-culture two SEM stubs were prepared using the same culture from which the material for the molecular analysis was obtained. In order to set up the stubs, a portion of biomass was collected and transferred in centrifuge glass tubes to undergo an acid-cleaning protocol for the removal of all the organic matter. The cleaning protocol followed the von Stosch (1974) method, to which some improvements have been made. The samples were previously rinsed with distilled water and centrifuged at 4000 rpm for 10 minutes; after each centrifuge cycle the supernatant was withdrawn and replaced with fresh distilled water. After the removal of the supernatant, the volume of the sample was measured and an equal volume of HNO_3 [65%] was added in the tubes and left for 60' at a temperature of 60 °C. Once the action time of the nitric acid had elapsed, three times the volume of H_2SO_4 [98%] were added. The tubes were then passed upon the reducing flame of a burner Bunsen until the pellet started to produce little bubbles. In this way all the organic matter is attacked and degraded by acids, while the siliceous frustules remain intact. Therefore, the pellets were rinsed several times with distilled water until neutral pH was reached and thus dehydrated with Ethanol [90%] to favour the rapid evaporation of the liquid after deployment on the filters. Stubs were prepared applying the double-sided tape on the head and posing a special 0.5 μm filter (Millipore^{TM}, Isopore membrane filters); a drop of sample was placed on the filter, taking part of the pellet from the bottom of the tube. The stubs were left to dry for 24 hours under the hood and then observed with the SEM at the Microscopy Laboratory of Stazione Zoologica Anton Dohrn. Terminology of the frustule morphology followed Hustedt (1933), Krammer (1990), and De Stefano et al. (2000).

Molecular identification

At the end of the grow-out phase (16 days), single diatom cell aggregates were collected by gently scraping off (using a sterile Pasteur pipette) the bottom of the Petri dishes and used for the amplification of *18S rRNA* and *rbcL* genes. In particular, the *Lyses & Bacteria/Fungi PCR-GO Kit* (DNA-TECH Spin off) was used to perform PCR directly from single cell aggregates. Single cell aggregates were added to the components of the kit (according to the manufacturer's instructions). Subsequently the PCR amplification reaction was directly carried out with a highly-processive lysis buffer, as well as a Hot Start Taq Polymerase, with a special buffer that allows the amplification of recalcitrant samples, as the case of diatom cells. The amount of total DNA extracted was estimated by measuring the absorbance at 260 nm, as well as the purity using 260/280 and 260/230 nm ratios, using a NanoDrop spectrophotometer (ND-1000 UV-vis Spectrophotometer; NanoDrop Technologies, Wilmington, DE, USA). The integrity of DNA was evaluated by agarose gel electrophoresis.

PCR was performed with specific primers for: i. 528F, 5'-3' GCGGTAATTCCA GCTCCAA and 1055R, 5'-3' ACGGCCATGCACCACCACCCAT, amplifying a *18S rRNA* fragment of 800 bp (Elwood et al., 1985; Kooistra et al., 2003); and ii. rbcL-F, 5'-3' ATGTCTCAATCTGTAWCAGAACGGACTC and rbcL-R, 5'-3' TAARAAWCKYTCTCTCCAACGCA, amplifying a *rbcL* fragment of 660 bp (Evans et al., 2007; Guo et al., 2015). The amplified fragments were purified from agarose gel (1.5%) using the *QIAquick Gel Extraction kit* (Qiagen, Milan, Italy). The purified PCR products were then sequenced on an Applied Biosystems 3730 DNA Analyzer 48 capillaries (Life Technologies) using BigDye® Terminator v3.1 Cycle Sequencing kit (Life Technologies). PCR product sequences obtained with both forward and reverse primers were firstly aligned with the software *MultiAlin* (http://multalin.toulouse.inra.fr/multalin/) for multiple sequence alignment (Corpet, 1988), in order to reconstruct the total *18S rRNA* and *rbcL* fragments, submitted to GenBank using *Basic Local*

Alignment Search Tool (BLAST; http://blast.ncbi.nlm.nih.gov/Blast.cgi) to identify the species, and then aligned to highly similar sequence using *MultiAlin*.

Results

The isolated benthic diatom strain is presently available in continuous axenic culture in 6-well Multiwells under controlled conditions of temperature and light. The mother culture is renewed every 16-20 days (when the growth plateau is reached), according to the method described by Raniello et al., (2007).

Molecular identification

BLASTn alignments on nucleotide collections of *18S rRNA* gene of the diatom analysed in the present work confirmed the results achieved with morphological analysis, displaying a high sequence similarity (97.9%) with the first hit *Cocconeis placentula* (Accession Number: AM502013.1, see Fig. 3 to see the phylogenetic relationship with the annotated diatoms belonging to the *Cocconeis* genus, based on *18S rRNA* gene).

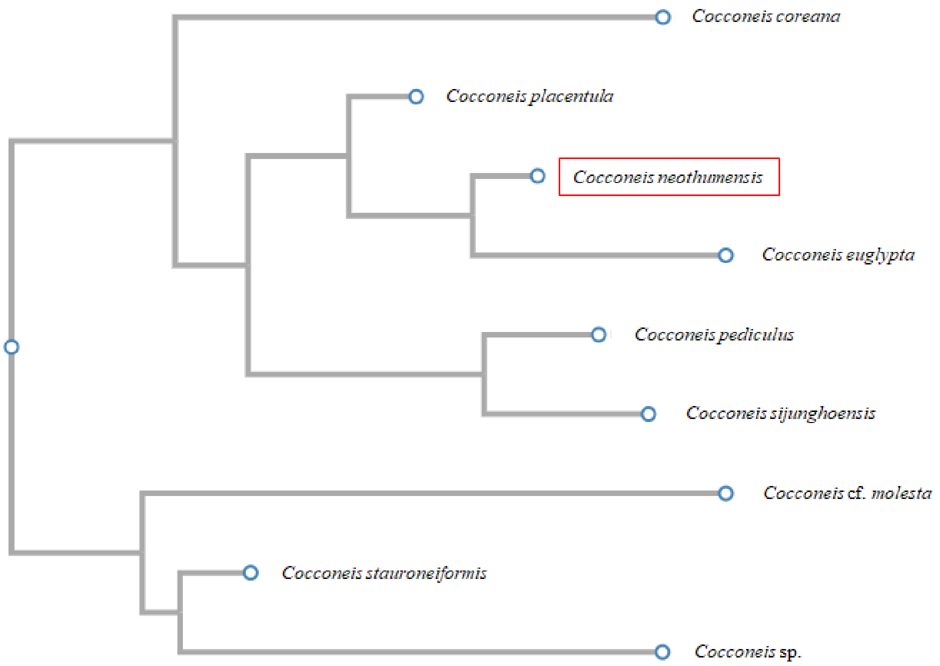

Figure 3. Maximum likelihood phylogenetic tree based on sequences of the *18S rRNA* gene, including *Cocconeis* sp. (HG993259.1), *Cocconeis coreana* (LR890011.1), *Cocconeis euglypta* (KM592933.1), *Cocconeis* cf. *molesta* (AJ535148.1), *Cocconeis pediculus* (FR873235.1), *Cocconeis placentula* (FR873239.1), *Cocconeis sijunghoensis* (KM592930.1), *Cocconeis stauroneiformis* (AB430614.1) and *Cocconeis neothumensis* identified in the present work (highlighted with the red box). Only bootstrap values above 50% are shown.

In the case of *rbcL* gene the alignment showed as first hit the diatom *Cocconeis euglypta* (Accession Number: LR890021.1) with 94.6% of pairwise sequence similarity (Fig. 4 to see the phylogenetic relationship with the annotated diatoms belonging to the *Cocconeis* genus, based on *rbcL* gene).

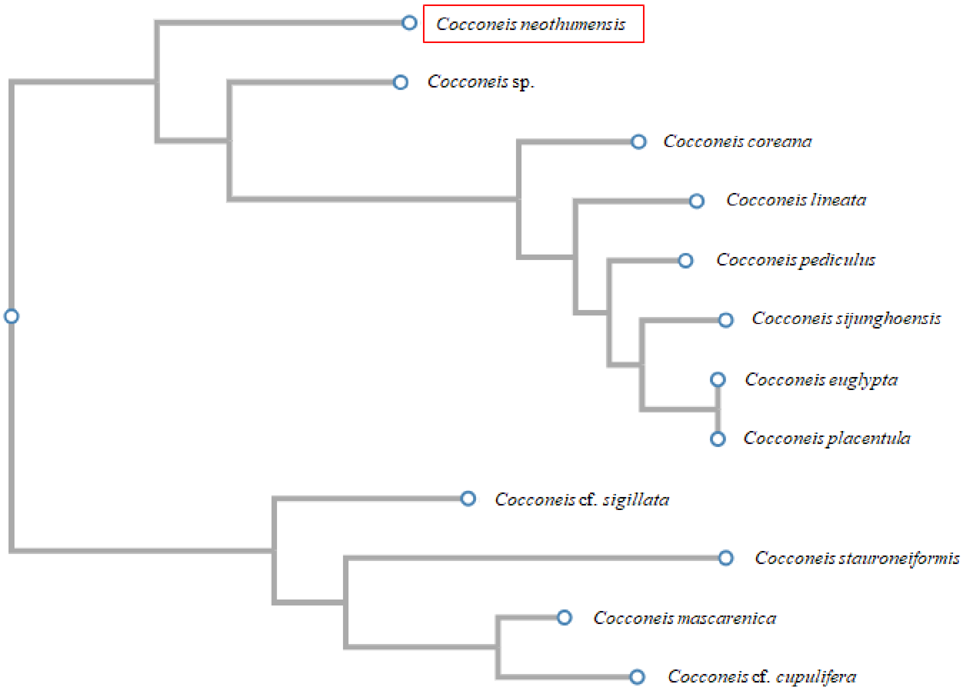

Figure 4. Maximum likelihood phylogenetic tree based on sequences of the *rbcL* gene, including *Cocconeis* sp. (LR890022.1), *Cocconeis coreana* (LR890017.1), *Cocconeis* cf. *cupulifera* (KT943680.1), *Cocconeis euglypta* (KT072907.1), *Cocconeis lineata* (LR890020.1), *Cocconeis mascarenica* (MK454988.1), *Cocconeis pediculus* (KM084991.1), *Cocconeis placentula* (MW484810.1), *Cocconeis* cf. *sigillata* (MT015687.1), *Cocconeis sijunghoensis* (LR890019.1), *Cocconeis stauroneiformis* (AB430694.1) and *Cocconeis neothumensis* identified in the present work (highlighted with the red box). Only bootstrap values above 50% are shown.

Morphological identification

Cocconeis neothumensis **var.** ***marina*** **M. De Stefano, D. Marino & L. Mazzella, (Figs. 5–6)**

Order: Achnanthales P. Silva

Family: Cocconeidaceae Kützing

Genus: *Cocconeis* Ehrenberg

Reference. De Stefano et al. 2000 (p. 233, figs 53–65).

Material examined. Ischia specimens were collected at Sant'Anna Rock, Cartaromana Bay (40°43'34.68''N, 13°57'40.92''E).

Description. The valves are lanceolate to elliptical-lanceolate almost circular with rounded apices (Figs 5, a d, g; and 6, a, c, d). The length varies from 14.2 to 10.8 µm, and the width from 8.3 to 6 µm. Transapical striae are radiate, from 20 to 30 in 10 µm.

Sternum valve (SV). Externally the valve is centrally slightly depressed toward the thin, straight sternum (Fig. 5, a, g). Longitudinal ribs are visible both externally and internally in valve view (Fig 5, a, b, d, arrowheads).

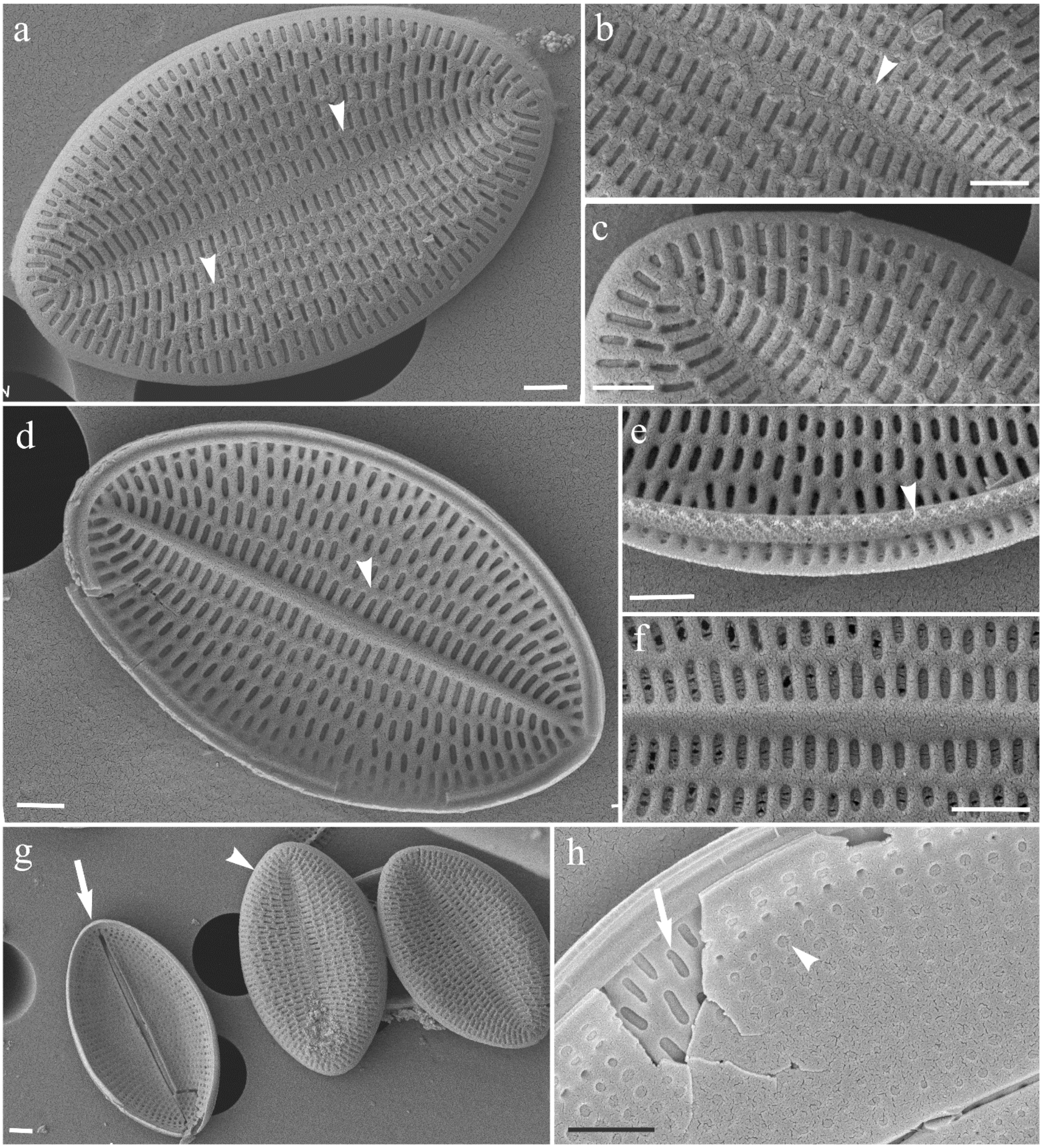

Figure 5. *Cocconeis neothumensis* var. *marina*, SEM. (a) External SV view showing shallow depression; (b) External SV view showing longitudinal ribs in the central valve zone (arrowhead); (c) External SV view of apex with transapical striae; (d) Internal SV view showing longitudinal ribs (arrowhead); (e) Broken cingulum (arrowhead); (f) Focus on SV areolae; (g) Panoramic on valves: SV (arrowhead) and RVS (arrow); (h) Broken frustule showing the different areolae between RSV (rounded areolae; arrow) and SV (rectangular areolae; arrowhead). Scale bars: 1 μm.

Raphe-sternum valve (RVS). Externally, the raphe consists of two straight branches ending centrally and distally in apically expanded pores (Fig. 6a). Internally, the straight raphe branches are bordered by ribs, ending centrally in simple pores gently bent toward the opposite side (Fig 6, b, c); the central nodule and the small helictoglossae at the poles are visible (Fig. 6c, arrowhead).

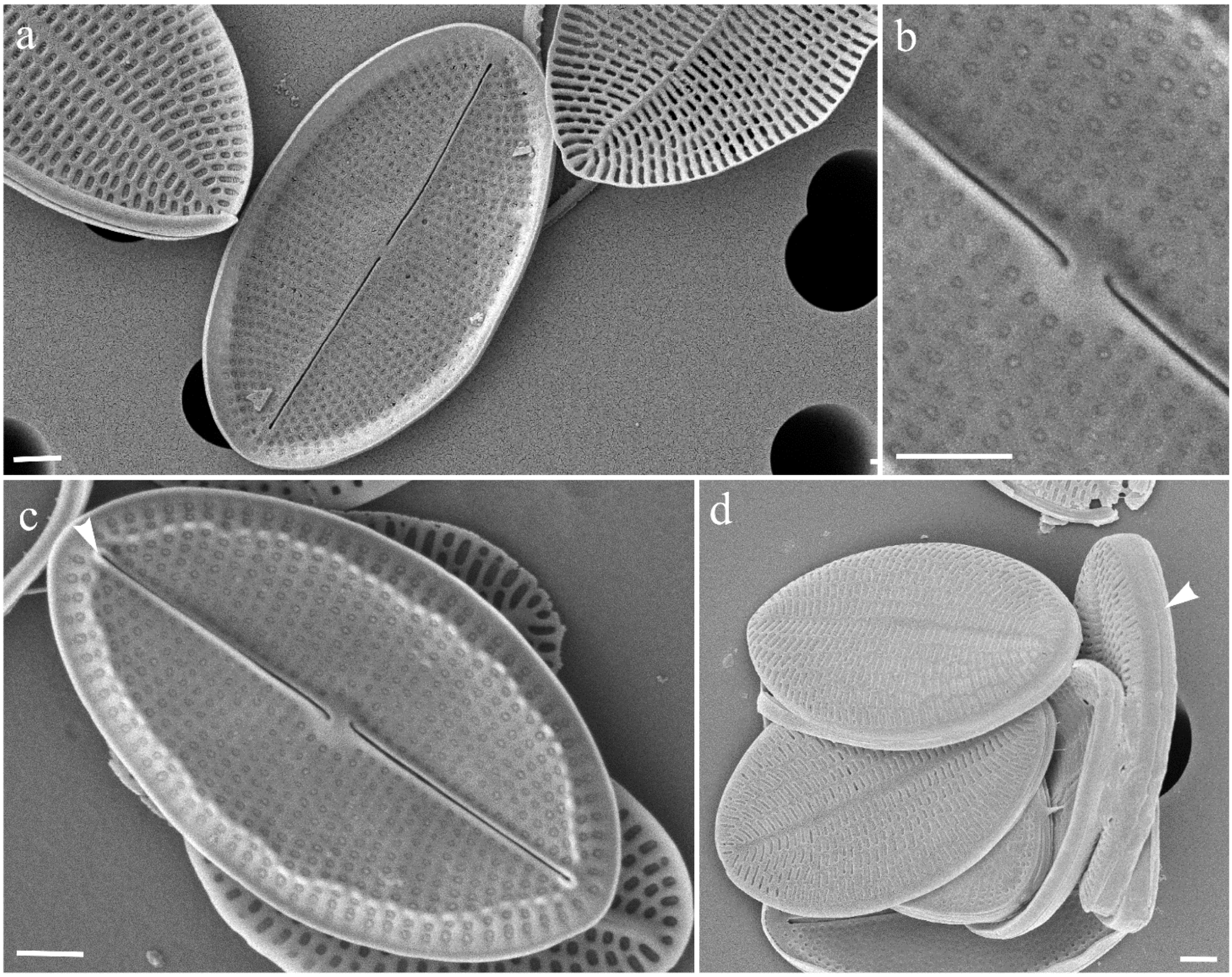

Figure 6. *Cocconeis neothumensis* var. *marina*, SEM. (a) External RSV view showing the straight branches of the raphe. (b) Internal RSV view showing the central nodule. (c) Internal RSV view showing raphe with small helictoglossae (arrowhead). Scale bars: 1 μm.

Discussion

Several strains of benthic diatoms were obtained as a result of the epiphyte collections and selection on low-adhesion surfaces. In particular, a strain further identified as *C. neothumensis* var. *marina* was isolated by micromanipulator and used to produce a monoclonal culture.

Despite the long history of taxonomic studies performed on benthic diatoms, the difficulties associated with their identification and classification are still a major challenge, due to small size and ultra-morphological similarities. In fact, several investigations avoided the identification at the species level, or dealt only with the community dynamics of benthic diatoms (Brotas & Plante-Cuny, 2003; Sullivan & Currin, 2000). The isolation of this strain represents a remarkable results because some communities of benthic diatoms are much more difficult to sample and quantify, than the planktonic diatoms, due to their strong adhesion to the substrates. To reveal easily and quickly the hidden diversity of adhesive diatoms, largely attributed to their very small and similar morphologies, the development of molecular barcoding techniques is urgently needed. Consequently, it is necessary to construct comprehensive genetic databases (Mann, 1989; Medlin et al., 1988). In fact, several researches investigated the ecology and the physiology of diatom genera characterised by medium adhesive power (for example *Skeletonema marinoi* Sarno & Zingone, 2005, Wang et al., 2020; *Nanofrustulum shiloi* (J. J. Lee, Reimer & McEnery) Round, Hallsteinsen & Paasche, 1999 and *Striatella unipunctata* (Lyngbye) A. Agarth, 1832, Glaviano et al., 2021) while a few researches took into account alive strains of *Cocconeis* spp. (Mora et al., 2022; Zupo et al., 2011) and *Mastogloia* spp. (Lobban & Pennesi, 2014; Pennesi et al., 2013) due to their strong adhesive power and also to the delicate structure of their frustules. Consequently, biologists and ecologists have largely disregarded such types of diatoms and only in the last decades a few data are available in literature (Kanjer et al., 2019; Mazzella & Spinoccia, 1992; Porzio et al., 2013) mainly taking into account the community assemblages and the taxonomy of benthic diatoms (Underwood & Barnett, 2006). Indeed, the isolation of benthic diatoms with strong adhesive power (such as *Cocconeis* spp.) is complex and time-consuming because they grow slowly and are generally overwhelmed by the growth of other benthic species, less adhesive and mobile (Correa-Reyes et al., 2001; Shishlyannikov et al., 2011). In our case, the process required the development of isolation techniques coupled with the processes of sequential transfers, observing daily the isolated cells to ensure that the culture was not contaminated by other microorganisms. Following these steps, this task required much effort up to final isolation and characterisation.

The frustule cleaning method (Von Stosch, 1974; De Stefano et al., 2000) was effective with the modification we developed (see the methods) and allowed to correctly identify the species based on the morphology of frustules and pores, as well as to obtain good SEM images for properly built up identification tables.

Molecular identification methods (Evans et al., 2007) are mainly based on conserved DNA sequences, such as *internal transcribed spacer (ITS)* which revolutionised our understanding of conventional taxonomy, but also partial small subunit ribosomal RNA genes (SSU rDNA). In fact, previous studies (An et al., 2017) suggest that the *rbcL* gene is the most appropriate genetic marker for the identification and classification of benthic diatoms. In addition, the 18S rRNA, *rcbL*, the large ribosomal subunit *28S rRNA*, the *mitochondrial cytochrome c oxidase I* (*coxI*), and the *internal transcribed spacer (ITS*) genes have been frequently used as genetic markers (Pniewski et al., 2010). The two internal spacers named ITS-1 and ITS-2 are located between genes encoding the 5.8S, 18S, and 28S nuclear ribosomal RNA (nrRNA) subunits. Along with the 5.8S rRNA, the ITS-1 and ITS-2 spacers are referred to as the ITS region (Baldwin, 1992).

Our strain of *Cocconeis neothumensis* var. *marina* closely recalls the original diagnosis provided by De Stefano et al. (2000) in most aspects, with the notable exception of their size. In fact, our specimens are slightly longer and wider compared to those observed by De Stefano et al. (2000). Moreover, the valves deriving from the samples collected in Ischia, in our study display a maximum number of longitudinal striae in 10 μm, amounting to 30, whereas De Stefano et al. (2000) reported 26 striae. Additionally, in figures 5 and 6, we have presented, for the first time, the SEM images of the external SV, which reveals the depression and longitudinal ribs that were previously only visible in the small Light Microscopy (LM) image in the original publication (see Fig. 53 in De Stefano et al. 2000).

The molecular techniques confirmed the results of the morphological identification, showing sequence similarity as first hit the diatom *Cocconeis placentula* Ehrenberg, 1838 and in the case of *rbcL* gene the alignment showed as first hit the diatom *Cocconeis euglypta* Ehrenberg, 1854. However, *Cocconeis neothumensis* var. *marina* has never been annotated in any repository or database (see Mann et al., 2010; Moniz & Kaczmarska, 2010) and its sequences are still unknown to science. This explains the results of the molecular investigations that identified the closest species already annotated as the presumptive targets (Ehara et al., 2000). In this view, it is interesting to note how this strain shares several morphological characters with the above species and or their varieties (De Stefano et al., 2000).

It is worth observing that, despite diatoms include at least 30.000 species (up to 100.000 according to some authors; Mann & Vanormelingen, 2013) at this moment the number of gene sequences deposited in GenBank are quite low. The quality of a database has a direct and absolute influence on the applicability and efficiency of DNA barcoding techniques (Lang & Kaczmarska, 2011). Currently, genetic information on most species could not be found in GenBank, indicating that the database is still insufficient, and that molecular taxonomic studies on benthic diatoms are limited.

We should stress the fact that the *18S rRNA* gene is characterised by low resolution (Mann et al., 2010; Moniz & Kaczmarska, 2010). Probably the *18S rRNA* gene of some benthic diatoms has undergone unusually rapid evolutionary changes (Beszteri et al., 2001; Jones et al., 2005). Thus, although *18S rRNA* has been widely used in phylogenetic studies on diatoms and has the largest database compared with other genetic markers, it could not solve all the identification confusions if not included into a polyphasic approach. In fact, due to a relatively low sequence distance within a genus, the *18S rRNA* might be not an appropriate genetic marker to differentiate diatom species clearly, exhibiting a low resolution to detect polyphyletic characteristics of several species (Sato et al., 2008).

In conclusion, despite the large ecological importance of benthic diatoms, their identification and classification systems still need to be improved. Here, we highlighted the importance and the need for a polyphasic approach and the possibility to speed up the process of complete identification using molecular data coupled with ultra-morphology information. A previous study (An et al., 2017) showed that *rbcL* gene represents a better genetic marker than *18S rRNA* to identify and phylogenetically classify benthic diatoms. The sequence identification herein provided, will represent a further step in the construction of a comprehensive database allowing a perfect identification of key species of benthic diatoms.

Author contributions

Emanuele Somma: Conceptualisation (equal); data curation (equal); sampling methodology and diatom isolation (lead); visualisation (lead); writing – original draft (lead). **Maria Costantini:** Analysis – molecular identification (lead); data curation (equal); writing – original draft (support). **Nadia Ruocco:** Analysis – molecular identification (support); writing – review and editing (support). **Chiara Pennesi:** Analysis – morphological identification (lead); data curation (equal); writing – original draft (support). **Antonio Terlizzi:** Funding acquisition (equal); project administration (support); resources (equal); supervision (equal); writing – review and editing (support). **Valerio Zupo:** Funding acquisition (equal); project administration (lead); resources (equal); supervision (equal); writing – original draft (support); writing – review and editing (lead).

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CHAPTER 4

In preparation

Ocean Acidification influences the selection of strains and the metabolism of the benthic diatom *Cocconeis neothumensis* var. *marina* M.De Stefano, D. Marino & L. Mazzella

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Abstract

The oceanic uptake of carbon dioxide (CO_2) is rapidly changing the seawater chemistry driving an alarming decrease of the mean pH. Ocean acidification is raising concerns for its negative effects on important ecosystems such *Posidonia oceanica* meadows, priority habitat in the Mediterranean Sea. *P. oceanica* produces high complex ecosystem and associated plant and animal communities are stabilized by chemical and trophic relationships. Epiphytes, and among all benthic diatoms, are at the very base of the ecological succession of the leaf stratum, playing a crucial role in the trophic web. In this context, OA threatens to endanger plant-animal relationships by acting on physiology of early colonisers, such as benthic diatoms. In the present study, we isolated two strains of *Cocconeis neothumensis* var. *marina* from site at different pH conditions. To this purpose, shallow CO_2 vent of Ischia Island were exploited as site under natural acidified condition. The two strains were cultured utilising *ad-hoc* constructed benthic photo-bioreactors, both in ambient (pH=8.2) and acidified (pH=7.6) conditions. The present study highlighted differences both in the growth and in the metabolic profiles of the two strains cultured at the two pH conditions. These results suggest that OA might influences the selection of unusual benthic diatom strains, with still unknown implications on trophic and chemical relationships.

Keywords: Benthic diatoms, growth-curves, metabolites, Ocean Acidification, *Posidonia oceanica*

Introduction

Diatoms are a widespread and ecologically important group of algae (phylum Ochrophyta, class Bacillariophyceae). They represent the most abundant group of eukaryotic algae (Bork et al., 2015), as well as the major constituents of benthic and planktonic algal communities worldwide, and also in terrestrial, freshwater and marine habitats (Mann & Droop, 1996; Zupo et al., 2007). Differently from other microalgae, a peculiar cellular structure characterises the diatoms, i.e., the frustule, a siliceous double-capsule shells. The frustule represents one of the most complex examples of natural micro- and nano-structured materials (Rogato & De Tommasi, 2020). This feature confers to diatoms a great importance, highlighting a multitude of industrial and biotechnological applications (Rabiee et al., 2021). In addition, the biotechnological relevance of diatoms is primarily due to the great amount and diversity of bioactive compounds they produce, particularly interesting for bio-medical and anti-cancer purposes (Riccio et al., 2020).

From an ecological point of view, diatoms play an essential trophic role, constituting the base of marine food web and contributing to the maintenance of oceanic geochemical cycles (Bowler et al., 2009, 2010). In particular, diatoms collectively account for 40% of the marine primary production (Clement et al., 2016; X. Li et al., 2017) and contribute up to 20% of the global carbon dioxide (CO_2) fixation, thereby playing a crucial role in regulating the global climate (Falkowski et al., 2004; Tréguer et al., 2018). It is therefore essential to study and thoroughly analyse the effects of climate change on physiology and ecology of these microalgae, especially in view of increasingly worrying future scenarios about Ocean Acidification (OA).

While direct effects are evident, OA may indirectly affect some peculiar stable habitats such *Posidonia oceanica* (L.) Delile meadows (Zunino et al., 2019). The resilience of these environments is assured by the complex chemical and trophic relationships that associated plant and animal communities contribute to build up. Rising pCO_2 level in the ocean may cause the interruption of plant-animal chemical relationships, leading to deleterious effects on the habitat.

Evidently, the sensitivity of diatoms to increasing pCO_2 is variable, likely being related to specific traits such as cell size or the carbon fixation pathway, as well as interactions with other environmental factors such as nutrient, temperature and light (Wu et al., 2014). In this regard, many studies highlighted irrelevant (Tortell et al., 2000) or negative (Torstensson et al., 2012) effects after exposure of diatoms at high pCO_2 in laboratory conditions. In contrast, more recent investigations demonstrated that an increase in CO_2 levels may benefit diatoms, especially through the reduction in the energetic costs of their highly efficient "CO_2 Concentrating Mechanisms" (CCMs). This mechanism, that permit diatom cells to maintain good photosynthetic performance under low CO_2 concentration (Matsuda et al., 2017), seems to be the result of the adaptation to gradual decrease in ambient CO_2 and increase in O_2 over geological times (Roberts et al., 2007). In this regard, the photosynthetic efficiency of diatoms is less affected then other microalgal groups under elevated pCO_2 (Cai et al., 2022). Furthermore, several studies demonstrated how cell-size could affect diatom responses to OA for what concerns photosynthetic capacity (Key et al., 2010; G. Li & Gao, 2013). In this context, Wu et al. (2014) highlighted how larger diatom species are more likely to be stimulated by the rising pCO_2 level. Indeed, the lower ratio surface area:volume leads to a lower diffusive flux of CO_2 relative to their carbon demand for growth. In their *in situ* studies, Tortell et al. (2008) pointed out the increase in diatom growth rate and the shift in diatom communities as response of OA.

Previous investigations demonstrated how physiological responses to CO_2 concentration are species-specific (Gao & Campbell, 2014). A few evidence arose from studies on diatoms dominating the epiphytic communities of *P. oceanica* leaves, in which high levels of pCO_2 alter the responses of invertebrates to wound-activated infochemicals produced (Mutalipassi et al., 2020). In particular, OA affects the metabolism of diatoms, acting on the production of Volatile Organic Compounds (VOCs) (Mutalipassi et al., 2022). These compounds play a role as infochemicals involved in important plant-animal communication responsible for the correct functioning of the habitat. In their studies, Mutalipassi et al. (2019), showed how increased pCO_2 level in the culture condition affects the metabolism of the benthic species *Cocconeis scutellum* var. *parva* (Grunow) Cleve, 1895. As already mentioned, diatoms belonging to the genus *Cocconeis* produce a cell death metabolite that triggers the apoptosis of the androgenic gland (A.G.) of *Hippolyte inermis* Leach, 1816, a protandric hermaphroditic decapod.

H. inermis is a shrimp mainly inhabiting *P. oceanica* meadows (Gambi et al., 1992) and it constitutes a key component of the leaf stratum food webs, linking primary producers, fishes and other carnivores (Zupo & Fresi, 1985). The early sex reversal due to the cell death of the A.G. in males of *H. inermis* represents a stabilizing factor for natural populations (Zupo 2000) by triggering an increase of ovigerous females during the autumn reproductive season. Mutalipassi et al. (2019) demonstrated how OA negatively affects the metabolism modifying their patterns of production of secondary metabolites. This could lead to the disruption of vital plant-animal relationships, which in turn, may influence the whole marine food web related to peculiar habitats such as *P. oceanica* meadows.

In this regard, a well-established model diatom is represented by *Cocconeis neothumensis* Krammer (Zupo, 2000), belonging to a genus that seasonally dominate the leaves of the above mentioned seagrass (Zupo et al., 2007). Furthermore, the promising biotechnological activity of *Cocconeis* spp. arouses significant interest (Raniello et al., 2007) especially for what concerns extraction of apoptogenic compounds (Nappo et al., 2012). In the present study, we collected, isolated and cultured two strains of *C. neothumensis* from meadows of *P. oceanica* located in two sites characterised by different environmental pH conditions (ambient vs acidified), exploiting shallow volcanic vents located around Castello Aragonese in the Island of Ischia (Gulf of Naples, Italy). In particular, this research work aimed to assess whether (and how) OA might influence the growth and the metabolism of this diatom species, involved in such particular plant-animal chemical relationships. Changes in communities' composition and in metabolic profiles might interrupt those communications with deleterious consequences, leading to negative effect not only on the *H. inermis* population, but also on the entire *P. oceanica* food web.

Materials and Methods

Diatom isolation

Ad hoc devised sampling panels were developed to collect epiphytic diatoms, by selecting mainly species that strongly adhere to the substrate (e.g. genus *Cocconeis*). The panels consisted in a plastic frame bearing 8 glass disk (23.75 cm^2) coated with a silicone surface with low adhesive power. The sampling system was designed to hold a vertical position and to be free to float, in order to emulate the conditions under which the epiphytes colonise *Posidonia* leaves. For this reason, the panel was anchored to the bottom through a mooring and maintained in vertical position with a buoy on the top.

The collecting panels were deployed by scuba divers at the margin of the *P. oceanica* meadows at a depth of 6 m around the island of Ischia (Gulf of Naples, Italy; Fig.1) and leaved for 30 days in October 2020. The first panel was located in Cartaromana bay, nearby Sant'Anna rocks (40°43'34.68"N, 13°57'40.92"E), as control site in ambient pH conditions (Control site). In order to collect diatoms from an area characterised by lower pH, the second panel was deployed near the Castello Aragonese (40°43'50.62"N, 13°57'47.99"E) exploiting the shallow CO_2 volcanic vents (Low pH site). Here the CO_2 bubbles emerging from the seabed acidifies the water to a pH of 7.7 (Hall-Spencer et al. 2008). The natural acidification of this area can be considered as a simulation of future scenarios due to the ocean acidification linked to present and past anthropic emissions.

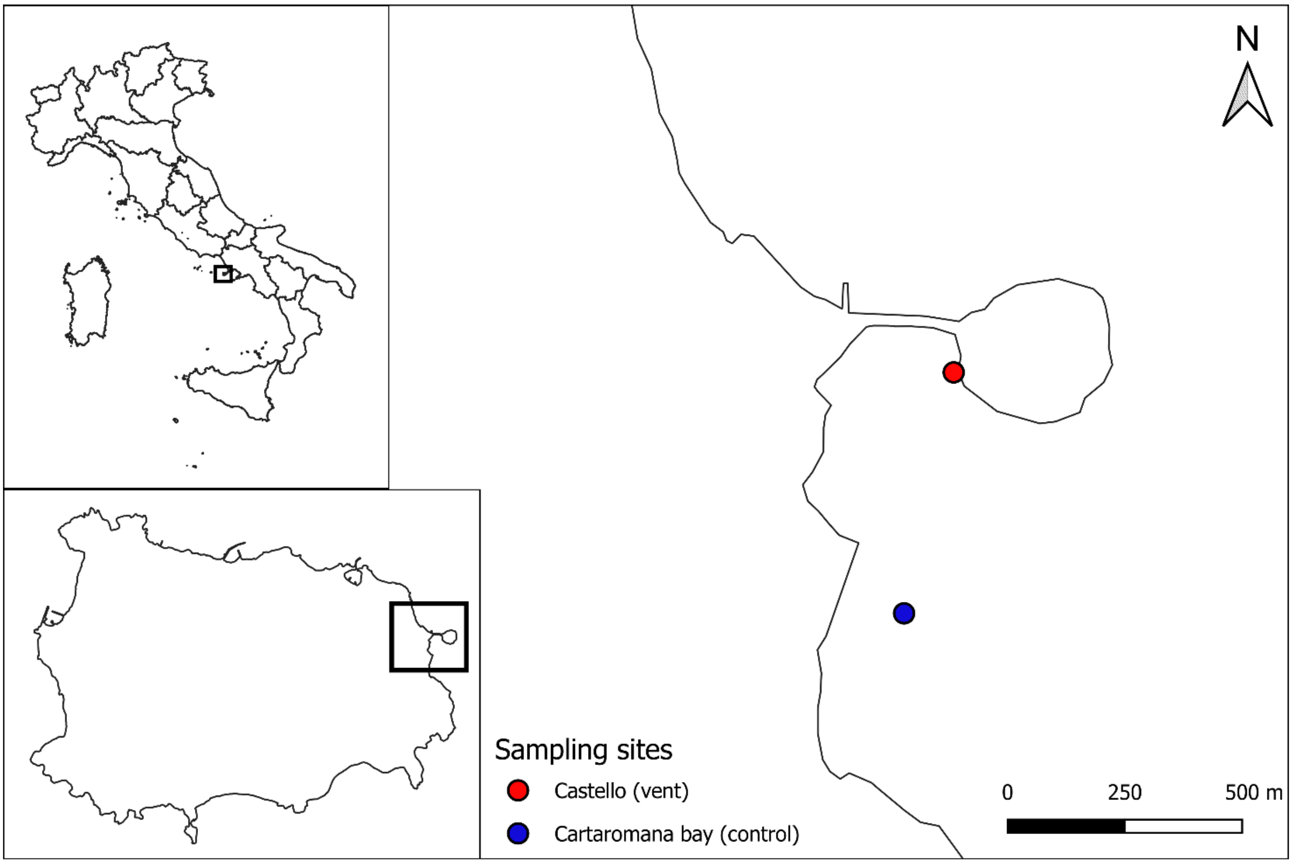

Figure 1. Map of the sampling: the control site (ambient pH condition) is located in Cartaromana bay, nearby Sant'Anna rocks (40°43'34.68"N, 13°57'40.92"E) indicated by a blue dot; the shallow CO_2 volcanic vent of Castello Aragonese (40°43'50.62"N, 13°57'47.99"E) was selected as Low pH site (naturally acidified condition) and is indicated by a red dot.

Once in the laboratory, the low-adhesion slides were removed, rinsed with filtered sea water (0.22 µm TPP "rapid" Filtermax) and gently scraped with a sterile glass slide in order to collect all the organisms that colonized the smooth surface. The epiphytes were collected in Petri dishes and analysed with an inverted microscope to isolate diatom belonging to the genus *Cocconeis*. The entire sample was then divided into 6 sub-samples, each one in a well of Culture Multiwell Plates. From each sample, the diatoms of interest were isolated under Leica inverted microscope (Leica microsystem DM IL) through sequential transfers of single cells, with a micromanipulator (Leica microsystems, Leitz Micromanipulator), a Microinjector syringe (Narishige IM-5B) and a Pasteur glass pipette whose tip was towed over the flame of a Bunsen burner. Each isolated diatom was deployed in a well of a Multi-well Plate filled with sterile sea water, in order to obtain axenic monoclonal cultures. Those wells were observed daily to ensure that other microorganisms did not contaminate the culture. Once mother cultures of monoclonal strains were obtained, they were cultured and then kept under controlled conditions in a thermostatic chamber at 18°C with 12:12 light:dark photoperiod. Light was provided by Sylvania GroLux (Osram Sylvania Inc., USA) set at an irradiance of 140 $\mu E \cdot m^{-2} \cdot s^{-2}$. The culture medium was prepared with sterile seawater, previously filtered at 0.22 μm and autoclaved at 121°C for 25 minutes. After reaching room temperature, a volume equal to 10 mL/L of concentrated solution Guillard's *f/2* with silicates (Sigma-Aldrich) was added. All the operations of transferring or renewal of diatom culture were carried out under a laminar flow hood, using glassware previously sterilised in a forced air convection oven (150° C for 3 h), to minimise any type of contamination. The diatoms were grown in sterile Culture Multiwell Plates of 6 wells (3.5 cm in diameter), each containing about 8 mL of culture medium. The cultures were transferred approximately every 15-20 days to new sterile clusters, under a laminar flow hood, using a sterile Pasteur pipette. From the isolation effort 2 strains of *C. neothumensis* var. *marina* were obtained, one from each sampling site: CNN (from ambient pH condition site) and CNA (from acidified pH condition site).

Morphological identification

Diatoms identification was performed using a morphological approach, through the analysis of the ultrastructure of the frustule on images captured with SEM (Scanning Electron Microscopy). In order to setup the stubs, a portion of biofilm was collected from the mother culture and transferred in centrifuge glass tubes. For each mother-culture two stubs were prepared following a cleaning protocol with strong acids. The samples underwent the cleaning protocol with HNO_3 [65%] and H_2SO_4 [98%] aimed at eliminating all the organic material, leaving the siliceous frustules unaltered. The protocol applied was based on the method developed from von Stosch (1974) with some adjustment that allowed us to obtain a better result on the material to be observed.

After strong acid cleaning process, the samples were rinsed with distilled water several times in order to neutralize the pH. Once reached the neutrality, the samples were dehydrated with Ethanol [99%] in order to facilitate drying of the samples. Then the stubs were prepared applying the double-sided tape on the head and posing a special 0.5 μm filter; a drop of sample was placed on the filter, taking part of the pellet from the bottom of the tube. The stubs were left to dry overnight under the hood and then observed to the Scanning Electron Microscope (JEOL 6700 F) at the Advanced Microscopy Center (CeMA) of Stazione Zoologica Anton Dohrn.

Molecular identification

Lyses & Bacteria/Fungi PCR-GO Kit (DNA-TECH Spin off) was used to perform PCR directly from single colony from CNN and CNA, according manufacture instructions (see Somma et al., *submitted*). PCR was performed with specific primers rbcL-F, 5'-3' ATGTCTCAATCTGTAWCAGAACGGACTC and rbcL-R, 5'-3' TAARAAWCKYTCTCTCCAACGCA, amplifying a *rbcL* fragment of 660 bp (Evans et al., 2007; Guo et al., 2015). The purified PCR products using the *QIAquick Gel Extraction kit* (Qiagen, Milan, Italy) were then sequenced on an Applied Biosystems 3730 DNA Analyzer 48 capillaries (Life Technologies) using BigDye® Terminator v3.1 Cycle Sequencing kit (Life Technologies). The sequences obtained from CNN and CNA were submitted to GenBank using *Basic Local Alignment Search Tool* (BLAST; http://blast.ncbi.nlm.nih.gov/Blast.cgi) to identify the species, and then aligned to highly similar sequence using *MultiAlin* (http://multalin.toulouse.inra.fr/multalin/).

Massive culture in Photo-bioreactor at controlled pH conditions

Special photo-bioreactors adapted for benthic diatoms were ad-hoc designed and developed to perform at ambient (pH=8.2) and acidified (pH=7.6) culture conditions. Each photo-bioreactor was assembled using a Pyrex dish with a total volume of 2.4 L (300 mm x 200 mm x 40 mm). For each

Pyrex glass support a heat resistant glass plate was as lid, equipped with a central opening, where a pH probe was housed (InLab®Micro pH, Mettler Toledo). The InLab Micro probe is designed to work even in a reduced volume of water and up to a thickness of 3 mm. A pH controller (pH 201, Aqualight) was connected to the Inlab Micro Probe (via BNC cable) by an electronic valve which was connected to a CO_2 regulator (CO_2 Energy, Ferplast). A secondary opening was placed sideways, where a glass tube for the CO_2 flux and the cable of the water pump (Askoll Pure pump 300) were fixed. The centrifuge pump was added to avoid water stratification and the formation of any pH gradient along the photo-bioreactor. The CO_2 regulator and the centrifuge pump were designed and developed in order to operate simultaneously.

The two strains of diatoms CNN and CNA were both inoculated and cultured in photo-bioreactors at the two pH conditions, setting the controllers at 7.6 (low pH) and 8.2 (ambient pH). For each strain, three replicate were cultured at the above pH condition. To reconstruct the growth curves, in the plate of each photo-bioreactor 6 sterile square-shaped cover slides were deployed, in order to let the diatoms settle on them. After five days from the inoculation, and then each 2 days, a cover slide was collected from each plate and the cells settled was counted under the inverted microscope. Using an eyepiece with a grid, from each cover slides three random quadrats (0.2 mm x 0.2 mm) were photographed and the settled cells were counted. The data collected from the counting of cells were used to build up growth curves of the strains at the two pH culture condition (7.6 and 8.2). All the graphs concerning growth curves and the statistical analysis were obtained using the R version 4.3.1 (R Core Team, 2023). After 16 days the medium was removed and the plates were quickly rinsed with distilled water to remove residual salts. Emptied plates containing a diatom biofilm on their bottom were immediately frozen at -20 °C. Once completely frozen, the plates were placed in a freeze dryer (Lio 5P) and then dry diatoms were scraped off using a sterile iron blade.

^{1}H-NMR Metabolomic analysis

For each strain, three replicates at different pH culture condition were carried out: CNN pH 7.6 and 8.2, CNA pH 7.6 and 8.2. Diatoms were re-suspended in 170 µl of H_2O and 700 µl of methanol and were sonicated for 30 sec. Then, 350 µl of chloroform were added and samples were mixed on an orbital shaker in ice for 10 min. 350 µl of H_2O/chloroform (1:1, v/v) were added to each cell suspension and centrifuged at 4000 rpm for 10 min at 4 °C. Thereafter, the aqueous (polar) and lipophilic (apolar) phases were collected separately, transferred to a glass vial and dried under nitrogen flow. Samples were analyzed using Nuclear Magnetic Resonance (NMR) by a 600-MHz Bruker Avance DRX spectrometer with a TCI probe. The polar fractions were dissolved in 630 µl of PBS-D2O with the pH adjusted to 7.20, and 70 µl of sodium salt of 3-(trimethylsilyl)-1-propanesulfonic acid (1% in D2O) was used as the internal standard. On the other hand, the lipophilic fractions were dissolved in 700 µl of deuterated chloroform. All ^{1}H-NMR spectra on polar phases were acquired at 300 K with the excitation sculpting pulse sequence to suppress water resonance. A double-pulsed field gradient echo was used, with a soft square pulse of 4 ms at the water resonance frequency and with gradient pulses of 1 ms duration, adding 128 transients of 64 k complex points, with an acquisition time of 4 s/transient. Time domain data were all zero-filled to 256 k complex points and an exponential amplification of 0.6 Hz was applied prior to Fourier transformation.

Statistical and Pathway Analysis

The 0.50–8.60 ppm spectral region of the 1H-NMR spectra was integrated in buckets of 0.04 ppm using the AMIX package (Bruker, Biospin GmbH, Rheinstetten, Germany). The water resonance region (4.5–5.2 ppm) was excluded during the analysis and the bucketed region was normalized to the total spectrum area using Pareto scaling. Projections to Latent Structures discriminant analysis (PLS-DA) was used to compare the spectra obtained on the polar and apolar phases obtained from diatoms.

Results

Identification and growth curves

The isolation of diatoms resulted in fifteen strains of highly adhesive benthic diatoms epiphyte of *Posidonia oceanica* leaves. In fact, most of the cultured strains present in our collection belong to the genus *Mastogloia* and *Cocconeis*. Special interest has been paid to the two strains of the same species collected from two sites at different pH conditions (Control vs Low pH sites). The identification of the diatoms, carried out through the morphological analysis of the frustule ultrastructure, revealed that both strains object of our study may be morphologically identified as *Cocconeis neothumensis* var. *marina* (Fig. 2). This was also confirmed by molecular identification, showing that CNN and CNA were the same species (Fig. S1).

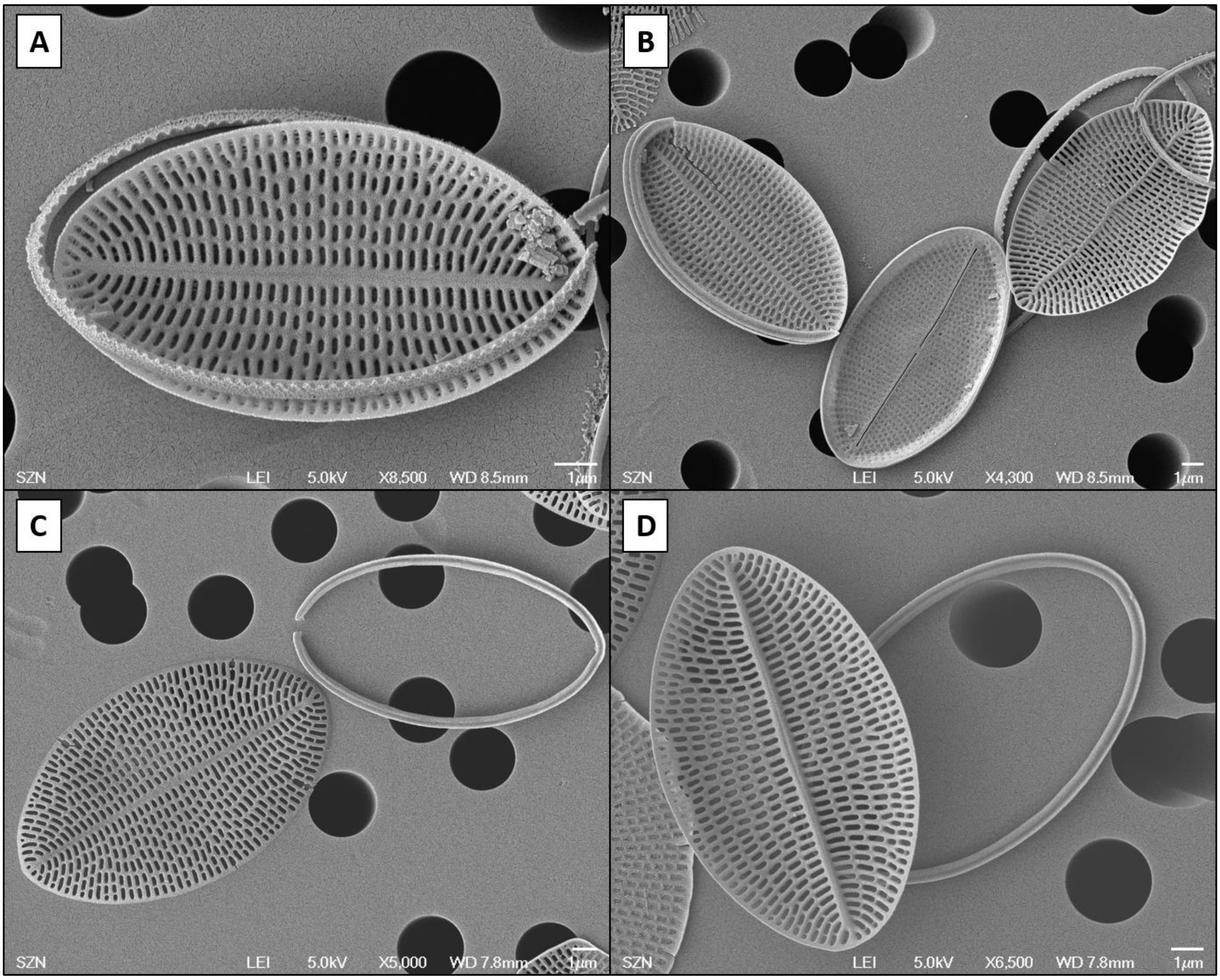

Figure 2. SEM images of the two strains of *C. neothumensis* var. *marina* isolated from the two different site: CNA collected from Low pH site (A and B) and CNN isolated from Control site (C and D).

A preliminary observation of the two strains in culture highlighted differences in the growth pathway. Indeed, the strain collected from Control site (CNN) presents a homogeneous growth forming a continuous biofilm. The strain CNA, belonging to the Low pH site, show a discontinuous and "patchy" growth, with the cells that tend to concentrate in small aggregates. These growth features of the two strains were maintained along the culture generations.

The growth data of the two strains fitted with the sigmoid Gompertz models:

$$y(t) = \alpha^{-\beta^{-k_t}}$$

as highlighted by the growth curves in Fig. 3. All the curves present high R^2 values that are indicative of the goodness of the data (see Table 1). The three replicates showed high variability due to the effect of patchiness in the cover pattern of the substrate.

Table 1. Parameters of the growth-curves represented in Fig. 3.

STRAIN	pH culture	α	β	k	R^2
CNN	7.6	33.12718	2.417448	0.2666841	0.8268
CNN	8.2	32.25347	3.578462	0.3829807	0.8529
CNA	7.6	37.33126	2.451702	0.186097	0.7707
CNA	8.2	36.64513	2.195439	0.2183115	0.8066

The curves showed differences in the growth of diatoms (see Fig.3). In particular, the strain CNN, in both culture pH conditions of, grows faster than the one collected from the vents. This strain reached a plateau earlier, as also supported by the highest growth rate values (k) in the equation of the model proposed (see Table 1). In addition, the differences in the growth of the two strains at the two conditions of culture also highlighted the abovementioned trend supported by statistic significance of the non-parametric test Kruskall-Wallis (see supplementary materials Fig. S2 and Fig. S3).

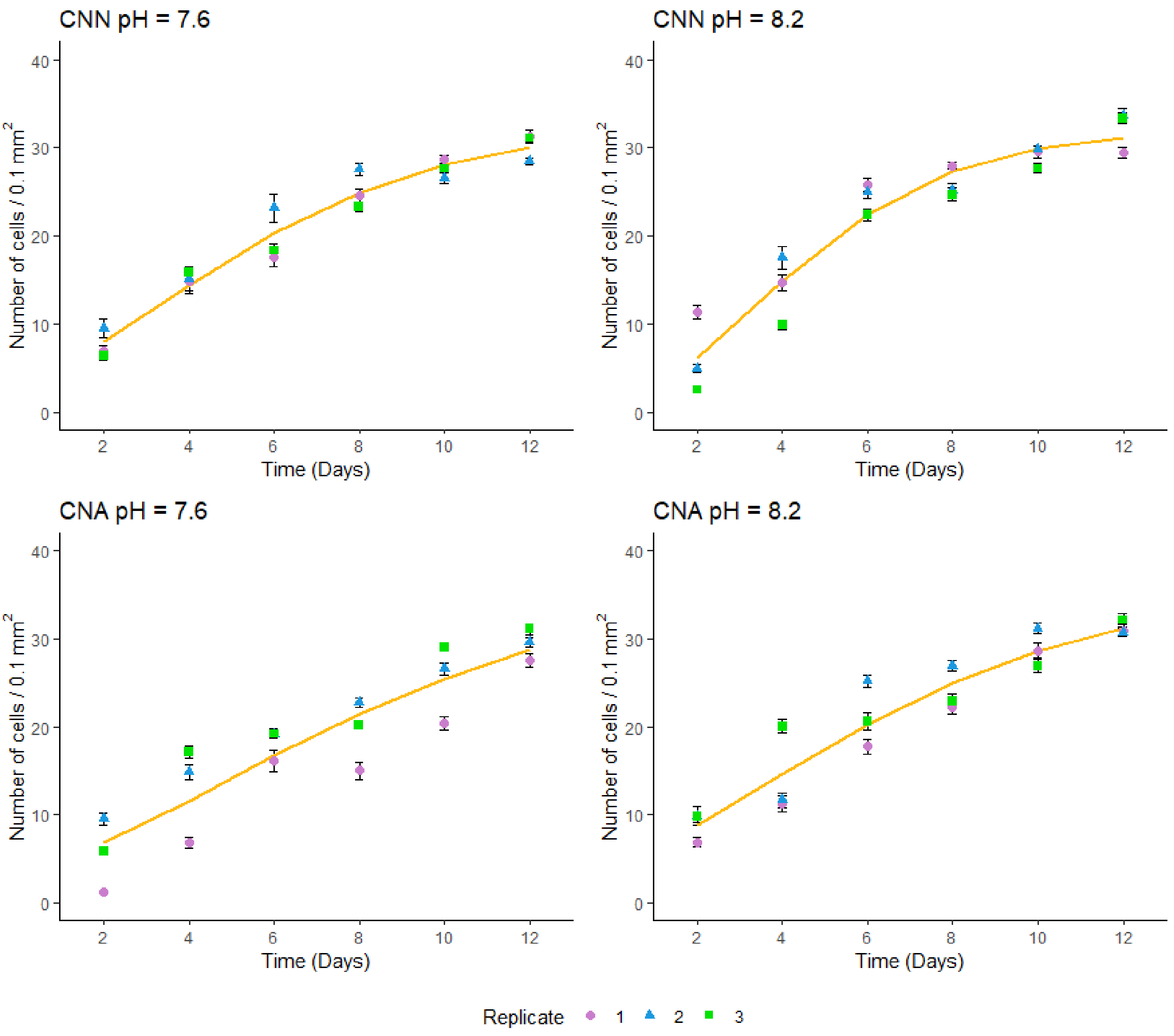

Figure 3. Growth-curves for the two strains (CNN and CNA) cultured at the two pH conditions (7.6 and 8.2).

^{1}H-NMR analysis of metabolites and lipids

^{1}H-NMR spectra were obtained from aqueous extracts of CNN pH 7.6 and 8.2, CNA pH 7.6 and 8.2. Considering polar metabolites from CNN pH 7.6 and 8.2, the Score Plot (Fig. 4A) showed that the two clustered in well separate classes, suggesting the presence of statistically different levels of metabolites (Fig. 4B). In particular: (i) the levels of lactate, 2-hydroxybutyrate, formate, glutathione and of some amino acids such as valine, glutamate, leucine and isoleucine were higher in CNN pH 7.6 group respect to the CNN pH 8.2 group. On the other hand, asparagine, phosphocoline, ornithine, glycine, choline, glycerophosphocoline and glucose were higher in CNN pH8.2 group.

Considering polar metabolites from CNA pH 7.6 and 8.2, the Score Plot (Fig. 5A) showed two well-separated groups, with higher variability in the pH 7.6 group. In particular, glucose, glycine, glycerophosphocholine, phosphocoline, lactate and choline were higher in CNA pH 7.6, whereas the aminoacids proline, arginine, leucine, valine, hydroxyproline were higher in CNA pH 8.2, as well as acetate and ATP (Fig. 5B).

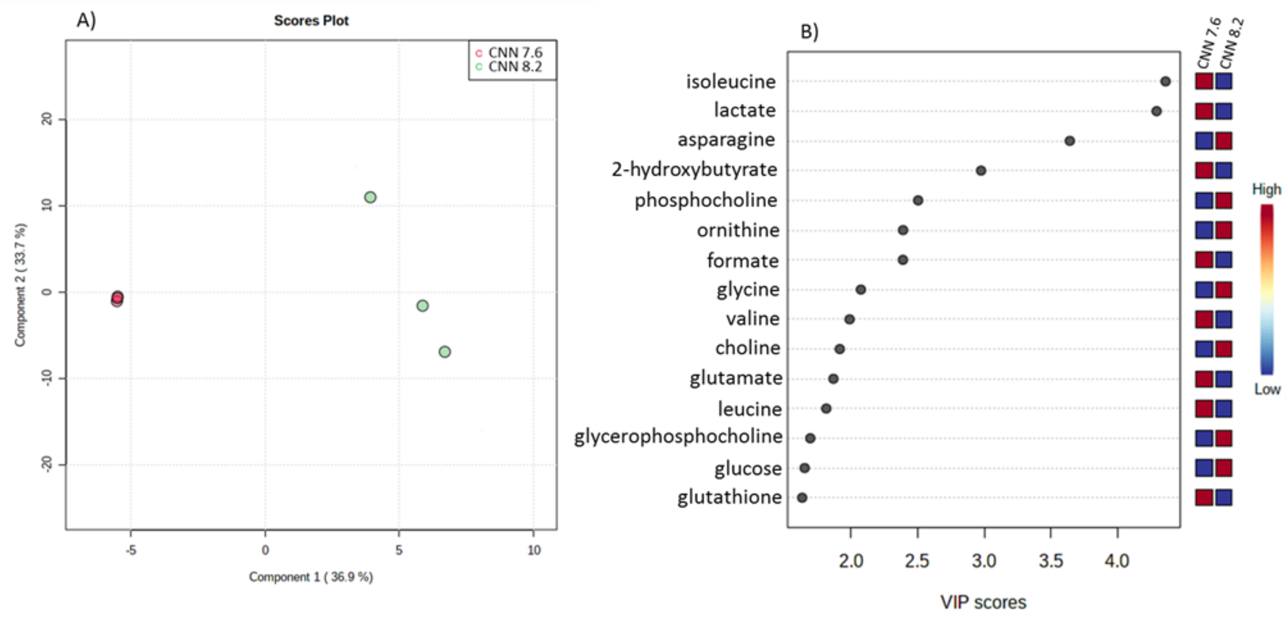

Figure 4. Score (A) and VIP (B) plots (where the metabolites increased or decreased) of aqueous extracts from CNN grown at pH 7.6 and pH 8.2.

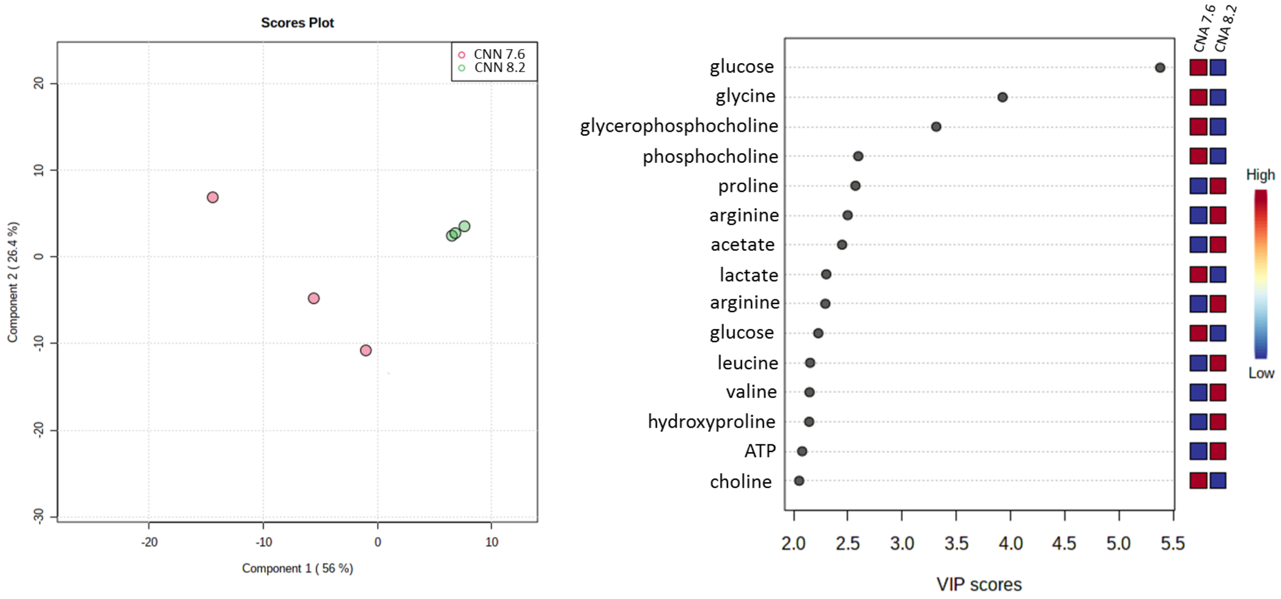

Figure 5. Score (A) and VIP (B) plots (where the metabolites increased or decreased) of aqueous extracts from CNA grown at pH 7.6 and pH 8.2.

^{1}H-NMR spectra were also obtained from lipophilic extracts of CNN pH 7.6 and 8.1, CNA pH 7.6 and 8.2. The Score Plot (Figs. 6A-7A) showed that the two clustered in well separate classes, suggesting the presence of statistically different levels of lipidic signals. These data suggested the presence in these two groups of statistically different levels of metabolites between the two groups. In fact, in the case of CNN pH 8.2 showed higher levels of some proton signals of cholesterol, omega 3, docosahexaenoic acid and arachidonic acid, whereas some proton signals of fatty acids and triglycerides were higher in CNN pH 7.6 (Fig. 6B). Also in the case of CNA, the largest amount of statistically different levels of several proto signals of phospholipids, linoleic acid and fatty acids (Fig. 7B) was observed at pH 8.2. CNA at pH 7.6 showed an increase of proton signals of cholesterol, arachidonic acids and fatty acids.

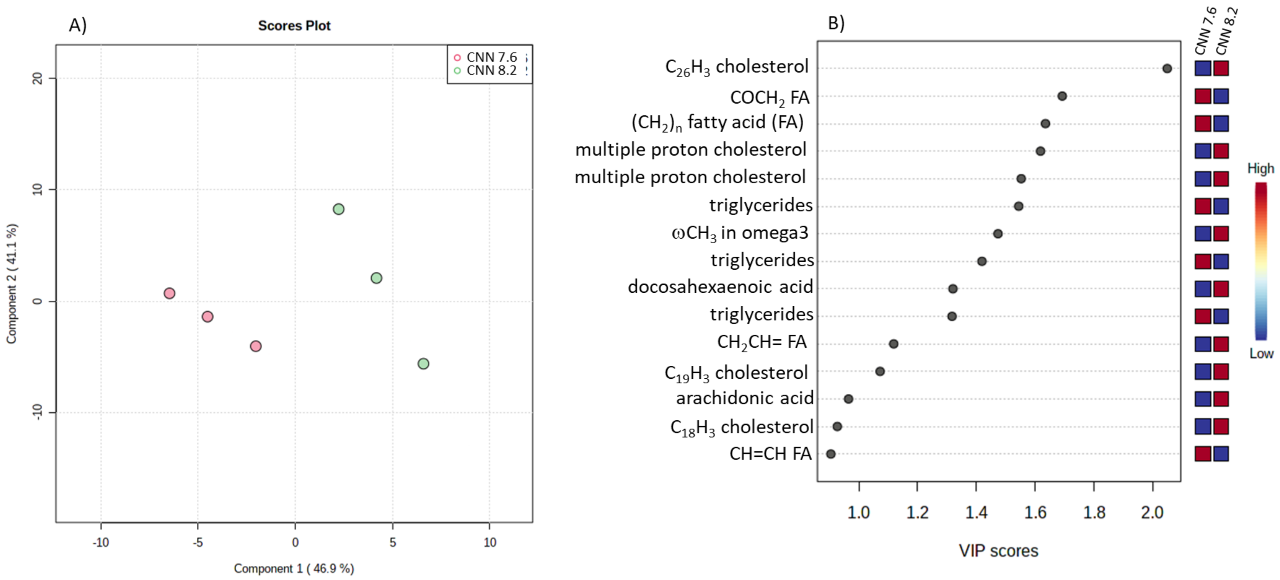

Figure 6. Score (A) and VIP (B) plots (where the metabolites increased or decreased) of lipophilic extracts from CNN grown at pH 7.6 and pH 8.2.

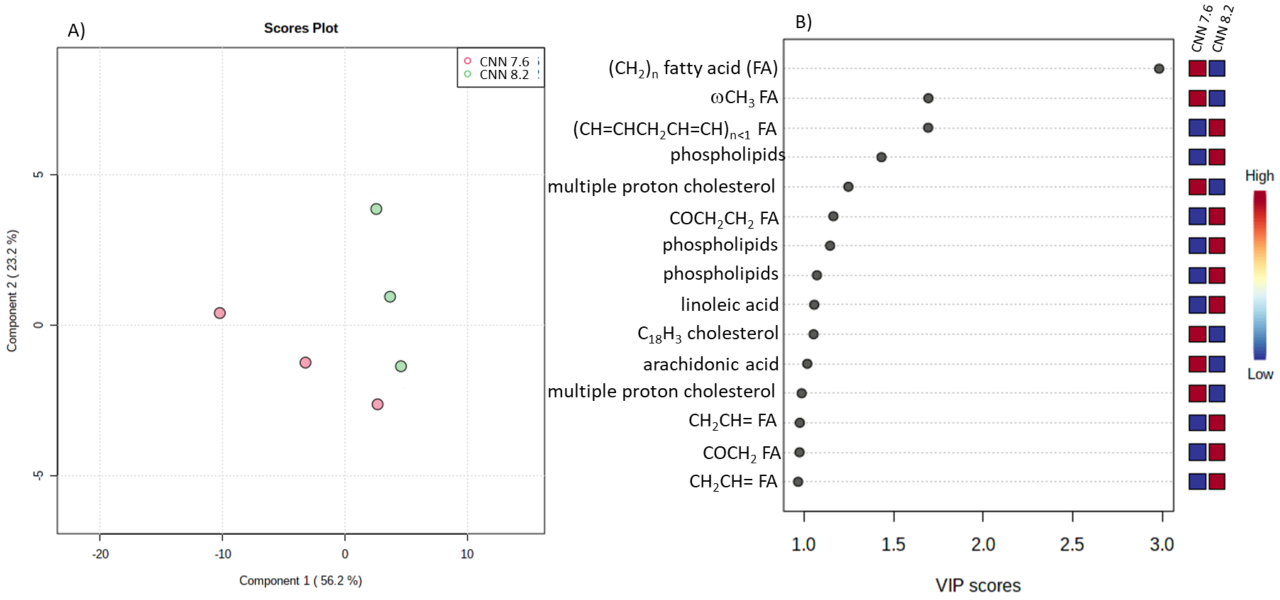

Figure 7. Score (A) and VIP (B) plots (where the metabolites increased or decreased) of lipophilic extracts from CNA grown at pH 7.6 and pH 8.2.

Discussion

Among global environmental changes, OA is known to treat marine life in many ways. Here we analysed the effect of low pH on the physiology of a benthic diatom, i.e., *Cocconeis neothumensis* var. *marina*. Our results revealed important differences between two strains collected from sites characterized by different pH conditions. The results highlighted important differentiations both for what concern the growth and the metabolites production. The growth of the strains CNN (isolated from natural pH site) and CNA (isolated from low pH site) in the two culture conditions produced different curves. In particular, both strains grew faster at pH 8.2 than at lower pH, showing significant differences especially in the exponential phase of the growth. Moreover, a further comparison of the two strains showed that CNN grows faster than CNA under both pH conditions. Even in this case, significant differences were revealed during the exponential growth phases.

These results are in accordance with previous studies that highlighted an increased growth rate of *Pseudo-nitzschia australis* Frenguelli at ambient pH as compared to the culture at lower pH (Ayache et al., 2021; Wingert & Cochlan, 2021). In contrast, Wingert and Cochlan (2021) demonstrated that pH decreases resulting from increased levels of dissolved CO_2 did not affect the specific growth rate of *P. australis* until the most extreme pH was reached. Indeed, culture of *P. australis* maintained at pH condition of 7.8, 8.0 and 8.1 showed statistically indistinguishable differences in growth rates, with a significant decrease for diatom cultured at pH 7.8. Similarly, (Cai et al., 2022), demonstrated how rising $p\mathrm{CO}_2$ did not produce significant effects on the growth of *Phaeodactylum tricornutum* Bohlin. On the other hand, OA seems to promote the growth of *Thalassiosira* sp. but only at the lower culture temperature (15°C).

Other studies, based on different strains of centric diatom demonstrated that OA might enhance the growth rates (Wu et al., 2014) but the study conducted by Wu et al. (2014) highlighted significant enhancement in the growth only in larger cell diatom species (diameter > 30 µm). Indeed, minor changes in the growth rate occur in diatom species with smaller cell size (diameter ranging from 4-30 µm). In particular, the strains of *C. neothumensis* var. *marina* here cultured had a total length of the apical axis of ~ 15 µm, size that are comparable with the one in the abovementioned study. This is probably due to the smaller surface:volume ratio of larger cell centric diatoms, that need higher CO_2 concentrations for the saturation of growth. Some studies on different diatom communities revealed that centric diatoms prove successful in OA scenario. Indeed, they are more favoured compared with pennate diatoms by higher pCO_2 (Cai et al., 2022; Feng et al., 2021; Tortell et al., 2002; Wu et al., 2014). It is likely that the highlighted differences in the response of various species of diatoms may be due mainly to cell size and morphology.

Strong differences were also highlighted from the H-NMR analysis on the metabolites produced by the diatom strains cultured here. In particular, polar metabolites of the two strains differs significantly, highlighting a higher production of the same metabolites in both the native isolation conditions. In particular, the strain CNN cultured at pH 8.2 and the strain CNA cultured at pH 7.6 both produced a higher amount of glycine, involved in the photorespiratory glycolate pathway (Kroth et al., 2008). Furthermore, also choline, phosphocholine and glycerophosphocholine maintain the same trend, with higher production in the native pH isolation conditions. For what concerns Valine and Leucine, the amount of these amino acids was higher for CNN cultured at pH 7.6 and CNA cultured at pH 8.2 (crossed pH compared to isolation conditions). Diatoms produce these amino acids from the cellular carbon (C) and nitrogen (N) metabolisms through glycolysis, gluconeogenesis, respiration and the citric acid cycle (Bromke, 2013). Anyway, generally there are evidences in favour of the species-specificity of essential and non-essential amino acids composition the diatoms within single taxonomic group (Kolmakova & Kolmakov, 2020; Khatuoon et al., 2009; Takano, 1968).

Regarding lipophilic compounds produced, significant differences arose from the H-NMR spectra obtained among the groups. In particular, cholesterol and arachidonic acid respect the abovementioned trend, showing higher content in CNN cultured at pH 8.2 and CNA cultured at 7.6 (native pH conditions of isolation).

The present study showed important differentiations among the two strains cultured in the pH conditions of 7.6 and 8.2. The results obtained from growth-curves and metabolomics analysis highlights the possible selection of diatom strains influenced by the OA effects. Due to their key role in marine food webs, selection of unusual diatom strains might undermine stability and resilience of such stable ecosystem as *P. oceanica* meadows. Furthermore, this OA effect involving changes in metabolic profiles might have negative implication on plant-animal trophic relationships and chemical communication. The modification or the interruption of such relevant relations in habitat specific communities, might critically affect the entire ecosystem with deleterious consequences.

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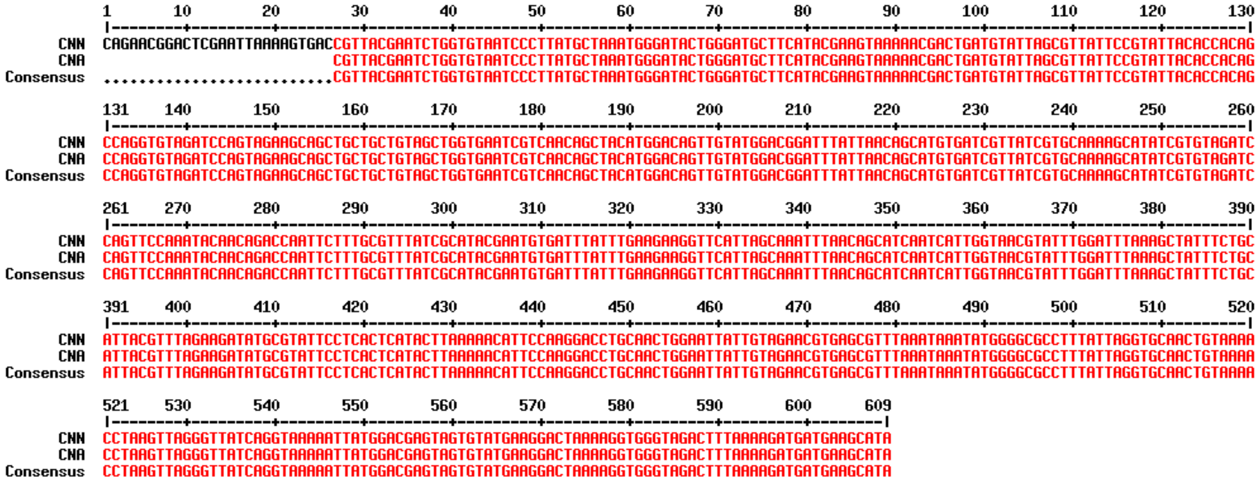
Supplementary material



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          1       10        20        30        40        50        60        70        80        90       100       110       120       130
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      CNN CAGAACGGACTCGAATTAAAAGTGACCGTTACGAATCTGGTGTAATCCCTTATGCTAAATGGGATACTGGGATGCTTCATACGAAGTAAAAACGACTGATGTATTAGCGTTATTCCGTATTACACCACAG
      CNA                           CGTTACGAATCTGGTGTAATCCCTTATGCTAAATGGGATACTGGGATGCTTCATACGAAGTAAAAACGACTGATGTATTAGCGTTATTCCGTATTACACCACAG
Consensus ..........................CGTTACGAATCTGGTGTAATCCCTTATGCTAAATGGGATACTGGGATGCTTCATACGAAGTAAAAACGACTGATGTATTAGCGTTATTCCGTATTACACCACAG

          131    140       150       160       170       180       190       200       210       220       230       240       250       260
          |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
      CNN CCAGGTGTAGATCCAGTAGAAGCAGCTGCTGCTGTAGCTGGTGAATCGTCAACAGCTACATGGACAGTTGTATGGACGGATTTATTAACAGCATGTGATCGTTATCGTGCAAAAGCATATCGTGTAGATC
      CNA CCAGGTGTAGATCCAGTAGAAGCAGCTGCTGCTGTAGCTGGTGAATCGTCAACAGCTACATGGACAGTTGTATGGACGGATTTATTAACAGCATGTGATCGTTATCGTGCAAAAGCATATCGTGTAGATC
Consensus CCAGGTGTAGATCCAGTAGAAGCAGCTGCTGCTGTAGCTGGTGAATCGTCAACAGCTACATGGACAGTTGTATGGACGGATTTATTAACAGCATGTGATCGTTATCGTGCAAAAGCATATCGTGTAGATC

          261    270       280       290       300       310       320       330       340       350       360       370       380       390
          |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
      CNN CAGTTCCAAATACAACAGACCAATTCTTTGCGTTTATCGCATACGAATGTGATTTATTTGAAGAAGGTTCATTAGCAAATTTAACAGCATCAATCATTGGTAACGTATTTGGATTTAAAGCTATTTCTGC
      CNA CAGTTCCAAATACAACAGACCAATTCTTTGCGTTTATCGCATACGAATGTGATTTATTTGAAGAAGGTTCATTAGCAAATTTAACAGCATCAATCATTGGTAACGTATTTGGATTTAAAGCTATTTCTGC
Consensus CAGTTCCAAATACAACAGACCAATTCTTTGCGTTTATCGCATACGAATGTGATTTATTTGAAGAAGGTTCATTAGCAAATTTAACAGCATCAATCATTGGTAACGTATTTGGATTTAAAGCTATTTCTGC

          391    400       410       420       430       440       450       460       470       480       490       500       510       520
          |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
      CNN ATTACGTTTAGAAGATATGCGTATTCCTCACTCATACTTAAAAACATTCCAAGGACCTGCAACTGGAATTATTGTAGAACGTGAGCGTTTAAATAAATATGGGGCGCCTTTATTAGGTGCAACTGTAAAA
      CNA ATTACGTTTAGAAGATATGCGTATTCCTCACTCATACTTAAAAACATTCCAAGGACCTGCAACTGGAATTATTGTAGAACGTGAGCGTTTAAATAAATATGGGGCGCCTTTATTAGGTGCAACTGTAAAA
Consensus ATTACGTTTAGAAGATATGCGTATTCCTCACTCATACTTAAAAACATTCCAAGGACCTGCAACTGGAATTATTGTAGAACGTGAGCGTTTAAATAAATATGGGGCGCCTTTATTAGGTGCAACTGTAAAA

          521    530       540       550       560       570       580       590       600      609
          |--------+---------+---------+---------+---------+---------+---------+---------+--------|
      CNN CCTAAGTTAGGGTTATCAGGTAAAAATTATGGACGAGTAGTGTATGAAGGACTAAAAGGTGGGTAGACTTTAAAAGATGATGAAGCATA
      CNA CCTAAGTTAGGGTTATCAGGTAAAAATTATGGACGAGTAGTGTATGAAGGACTAAAAGGTGGGTAGACTTTAAAAGATGATGAAGCATA
Consensus CCTAAGTTAGGGTTATCAGGTAAAAATTATGGACGAGTAGTGTATGAAGGACTAAAAGGTGGGTAGACTTTAAAAGATGATGAAGCATA
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Figure S1. Alignment of *rbcL* gene amplified for CNN and CNA using *Multialin*.

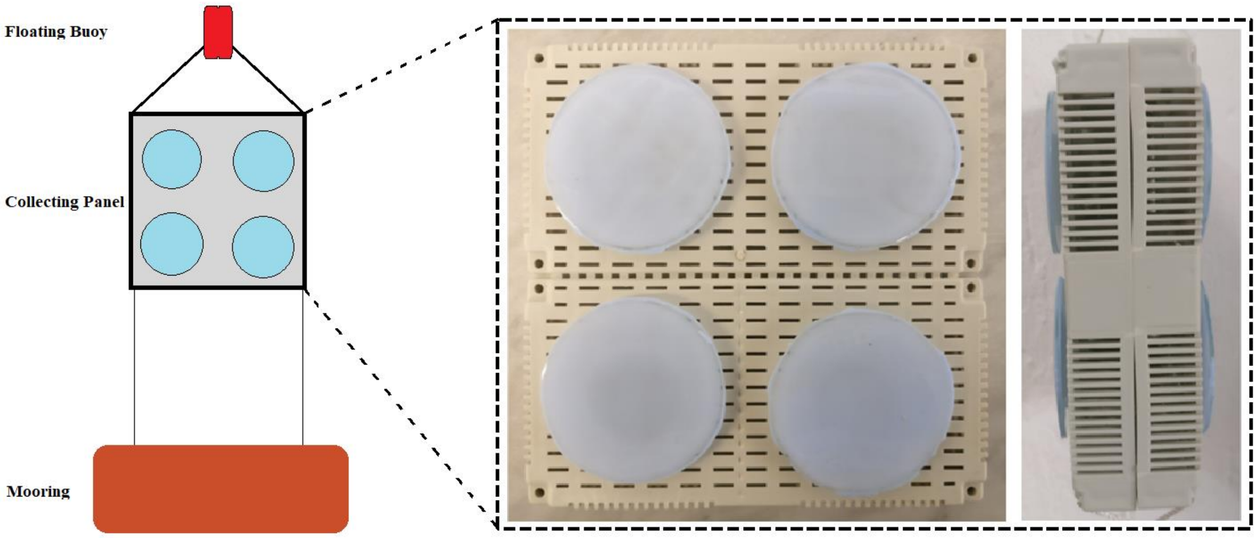

Figure S2. Special collecting panel with low adhesive power surfaces. On the right the schematization of the designed panel. On the right, a detail of the plastic frame (frontal and side view) with the smooth silicon surface glass disks

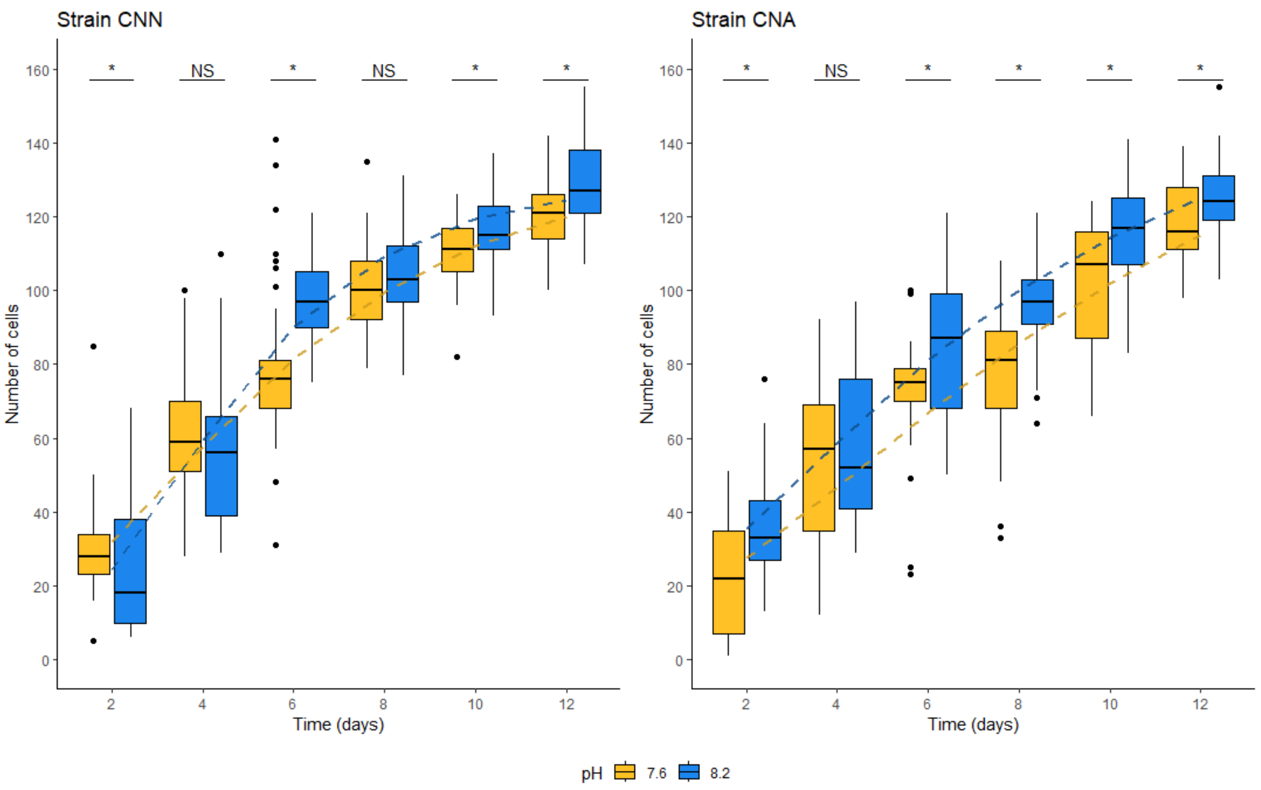

Figure S3. Boxplot comparison among pH culture conditions in photobioreactors.

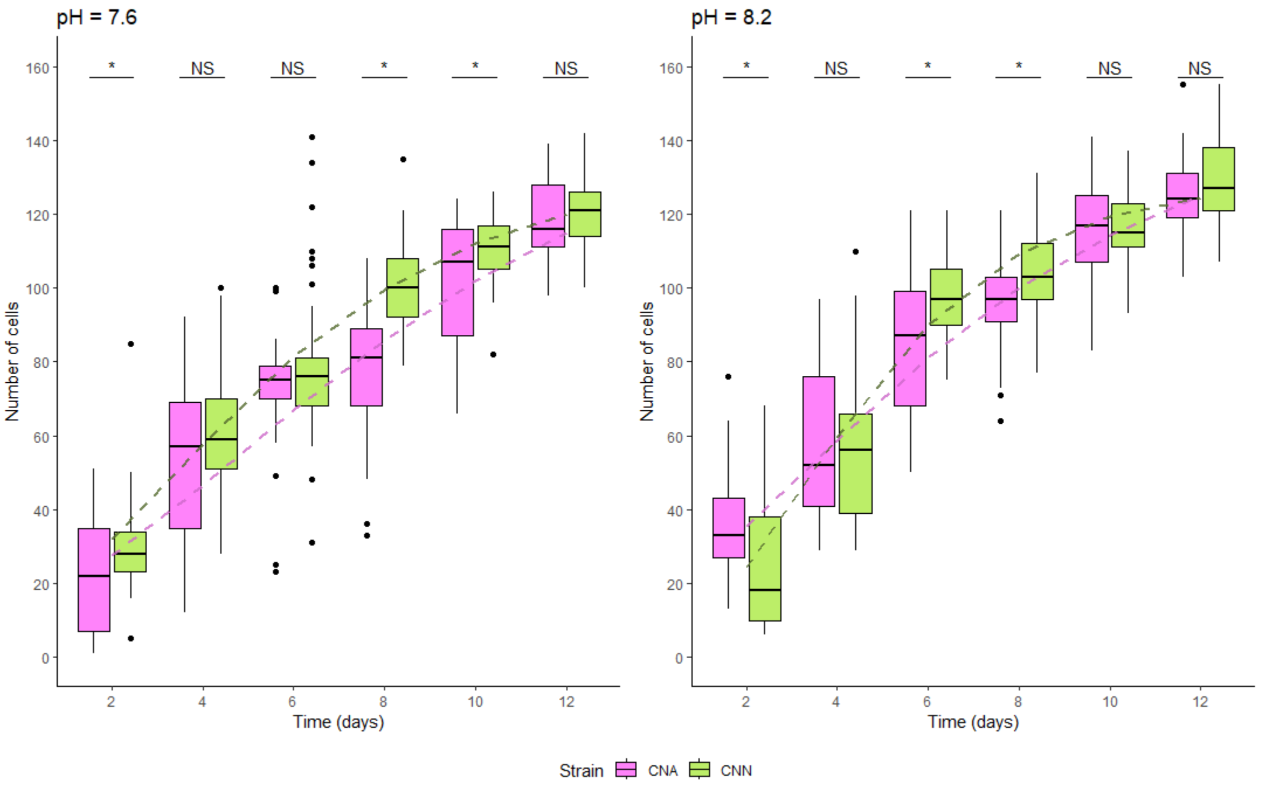

Figure S4. Boxplot comparison among the two isolated strains.

CHAPTER 5

In preparation

Effects of Ocean Acidification on plant-animal chemical relationships as illustrated by the diatom *Cocconeis neothumensis* var. *marina* isolated in *Posidonia oceanica* meadows

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Abstract

Ocean acidification (OA) is expected to affect marine ecosystems at different levels, influencing with cascade effects important habitat such as *Posidonia oceanica* meadows. Seagrass meadows, and in particular *P. oceanica* ones, are characterised by complex plant-animal trophic relationships that ensure stability and resilience of the ecosystem. These specific connections among communities, inhabiting *P. oceanica* meadows, are place at risk by the ongoing changes in seawater chemistry due to the pH lowering. Here we explored the peculiar chemical relationship among the protandric decapod *Hippolyte inermis* and its diatom food. In particular, we tested the effect of two benthic diatoms strains of *Cocconeis neothumensis* var. *marina* isolated from site under different pH conditions. Taking advantage from the available transcriptomic data, we performed Real-Time qPCR of seventeen gene of interest on cDNA extracted from 5-days post-larvae fed with *in vivo* diatom food. Our results demonstrated how diatom strain selected by OA show differences in the expression of genes belonging to key-pathway related to the early sex shift of the caridean decapod.

Keywords: apoptosis, benthic diatoms**,** hermaphroditism**,** Ocean Acidification, plant-animal relationships, *Posidonia oceanica*, sex-reversal.

Introduction

Climate changes on a global scale are largely due to rising anthropogenic pressure. They are causing a widespread alteration of ecosystems (Harfoot et al., 2014). The augmented human activities (such as burning of fossil fuel, deforestation, land-use changes etc.) led to an important increase of the partial pressure of carbon dioxide in the atmosphere (Friedlingstein et al., 2020; IPCC, 2021). This increase is buffered by the oceanic absorption (Zeebe, 2012), demonstrating how ocean represent an effective atmospheric CO_2 sink (Le Quéré et al., 2015). Indeed, among the CO_2 emitted in the last century about 50% has been absorbed by the oceans (Gruber et al., 2019; Watson et al., 2020) leading to a disruption in the seawater carbon chemistry (Orr et al., 2005), as well as the lowering of the mean pH (IPCC, 2021). This phenomenon, globally recognised as Ocean acidification (OA), represents an alarming consequence of climate change. The oceanic uptake of atmospheric CO_2 has already caused a 0.1 (pH units) decline of pH since the industrial revolution and it is likely to cause up to 0.3–0.32 unit decline by the 2100 (Gattuso et al., 2015). Although OA is recognised as a pervasive threat to marine biodiversity (Garrard et al., 2013; Hall-Spencer & Harvey, 2019; Hassoun et al., 2022), indirect effects at community and ecosystem level remain still poorly understood.

OA might severely affect specific environments, such as seagrass meadows. In particular, *Posidonia oceanica* L. Delile meadows are key coastal ecosystems due to their important ecological roles and also thanks to their remarkable resilience. Their well-structured associated communities are stabilised by specific chemical and trophic relationships (Buia et al., 2000; Zupo et al., 1997). The epiphytic communities living on the leaf blades of *P. oceanica* represent the basis of this complex structure. Seagrass, and in particular *P. oceanica*, are among the most relevant available natural surface for marine epiphyte (Buia et al., 1992; Mazzella et al., 1993; Uku & Björk, 2001). In past, several authors reported that complex communities of high degree of species-specificity colonise *P. oceanica* leaves (Scardi et al., 1986; Mazzella, 1983; Mazzella, 1999). In particular, algae are among the most abundant and diverse benthic organisms on seagrass leaves, with a wide variety of forms

(Somma et al., 2023). Epiphytic algae play a crucial role in the whole ecosystem, enhancing the primary production (Borowitzka et al., 2006) and contributing to food webs, providing the main food source for the associated fauna (Buia et al., 1992).

Benthic genera of pennate diatoms as *Cocconeis* sp. proliferate extensively on *P. oceanica* leaves, seasonally dominating the epiphyte community associated (Majewska et al., 2014; Zupo et al., 2007). In this view, *Cocconeis* is well-established model diatom genus because of its promising biotechnological power mainly due to the production of a still unknown apoptogenic compound (Nappo et al., 2012; Raniello et al., 2007). Furthermore, diatom belonging to the genus *Cocconeis* are involved in a peculiar plant-animal chemical relationship with *Hippolyte inermis* Leach, 1816, a marine caridean proterandric decapod. *H. inermis* is mainly inhabiting *P. oceanica* meadows (Gambi et al., 1992), of which it is a key component of the food webs, linking primary producers, fishes and other carnivores (Zupo & Fresi, 1985). In addition, besides its role as a key food item for *H. inermis*, *Cocconeis* sp is also responsible for the production of an apoptogenic metabolite that trigger the apoptosis of the androgenic gland (AG) of the shrimp (Zupo & Messina, 2007). In fact, *H. inermis* undergoes to a singular food-determined sex reversal (Reverberi, 1950) facilitating an increase of ovigerous females during the autumn reproductive season. In this context, the early sex reversal of *H. inermis* represents a stabilizing factor for their natural populations (Zupo, 2000).

The delicate equilibrium of this peculiar plant-animal trophic relation is being threatened by global changes. Indeed, Mutalipassi et al. (2019) demonstrated the deleterious effect of OA on the metabolism of *Cocconeis* sp., modifying the patterns of production of secondary metabolites. This could lead to the interruption of vital relationships, which in turn, might affect the food web associated to peculiar habitats such as *P. oceanica* meadows.

The present study is aimed at exploring how OA might indirectly affect the population of *H. inermis*, influencing the secondary metabolites production of their food. In this purpose, we tested two strains of *C. neothumensis* var *marina* isolated from *P. oceanica* meadows located in two sites under different environmental pH conditions (ambient and naturally acidified).

Taking advantage to recent transcriptomic data reported by Levy et al. (2021), we were able to set up a laboratory experiment that allowed us to detect the expression level of genes of interest belonging to three key gene pathways: ferroptosis, apoptosis and insulin-like hormone (IAG) production (Zupo et al., 2023). In particular, we performed RNA extraction from *H. inermis* individuals feed with *in vivo* culture of two strains of *C. neothumensis* var. *marina*: CNN (isolated from normal pH site) and CNA (isolated from an acidified coastal site). The expression levels of the genes were detected in five days old post-larvae (PL_5) through Real-Time qPCR and then compared with the results obtained in the transcriptome.

Materials and Methods

Diatom isolation, identification and culture

Diatoms were collected exploiting special low-adhesive sampling panels. This sampling system was *ad-hoc* developed in order to collect the early stages of colonization, selecting only species that strongly adhere to the substrate (*e.g. Cocconeis sp.*). The panels consisted in a plastic frame bearing 8 glass slides (23.75 cm^2) coated with a silicone smooth surface with low adhesive power. The sampling devices were anchored to the bottom through a mooring and maintained in a vertical position by means of a buoy fixed on the top. On this way the panels held a vertical position and were free to float, in order to emulate the conditions under which epiphytes colonise *P. oceanica* leaves. The panels were deployed in 6 m depth, at the margin of *P. oceanica* meadows in two different sampling sites (see Fig. 1): one located in the Cartaromana bay (Control site at ambient pH, 40°43'34.68"N, 13°57'40.92"E) and another located off the Castello Aragonese (Low pH site, 40°43'50.62"N, 13°57'47.99"E) exploiting the shallow CO_2 vents (Hall-Spencer et al., 2008). The natural acidification of this area is considered a simulation of future scenarios due to OA linked to present and past CO_2 emissions.

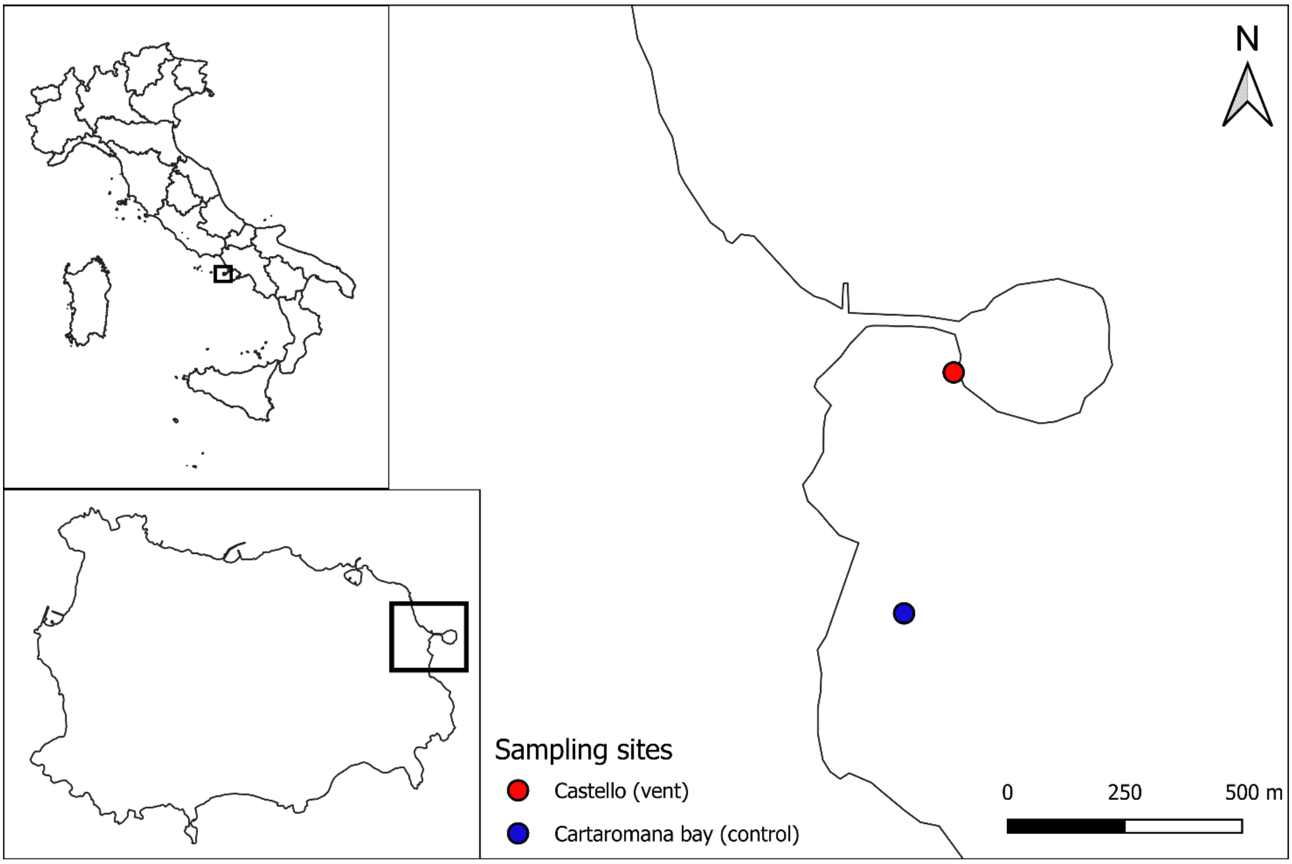


Figure 1. Map of the two sampling sites in which the collecting panel were deployed off the Ischia Island (Gulf of Naples, Italy). In red the Low pH site represented by the shallow CO_2 vent of Castello Aragonese (40°43'50.62"N, 13°57'47.99"E); in blue the Control site of Sant'Anna located in the Cartaromana bay (40°43'34.68"N, 13°57'40.92"E).

After 30 days from the deployment, the collecting panels were removed and transferred to the laboratory. Here, the low-adhesive silicone surfaces were rinsed with filtered seawater (0.22 µm TPP "rapid" Filtermax) and gently scraped with a sterile glass slide. The collected epiphytic biofilms were transferred in Multiwell-plates and diatoms shaped as *Cocconeis* spp. (to be further confirmed under the SEM) were isolated through sequential transfers of single cells. All the operations were conducted under a Leica inverted microscope (Leica microsystem DM IL), using a micromanipulator (Leica microsystems, Leitz Micromanipulator) and a Microinjector syringe (Narishige IM-5B) with a glass pipette whose tip was towed over the flame of a Bunsen burner. During each sequential transfer, single cells were transferred in a new well filled with sterile seawater, to obtain monoclonal mother-cultures. Once obtained, the mother cultures were stored in a thermostatic chamber under controlled condition of temperature (18°C) and light (with dark:light photoperiod 12:12 ; Sylvania GroLux, Osram Sylvania Inc., USA; irradiance set on 140 μE · m^{-2} · s^{-2}). The culture medium was prepared with sterile seawater, previously filtered at 0.22 μm and autoclaved at 121°C for 25 minutes. After reaching room temperature, a volume equal to 10 mL/L of concentrated solution *Guillard's* f/2 with silicates (Sigma-Aldrich) was added. The mother cultures were transferred approximately every 15-20 days to new sterile clusters, under a laminar flow hood, using a sterile Pasteur pipette.

In order to produce massive culture for experimental purposes, diatoms of interest were inoculated in Petri dishes. The Petri dishes were previously sterilised in a forced air convection oven (150° C for 3 h), and all the operation were carried out under laminar flow hood to avoid any type of contamination.

Diatom identification

The identification of the species was firstly carried out through morphological approach, analysing the ultrastructure of the frustule on images captured with SEM (Scanning Electron Microscopy). For each strain, a portion of biofilm was scraped from the mother-culture with a sterile Pateur pipette and transferred in glass centrifuge tube. All the samples underwent to a strong acids cleaning protocol with HNO_3 and H_2SO_4 for the removal of the organic matter. The cleaning protocol followed the von Stosch (1974) method, to which some improvements have been made. Once the cleaned samples have been obtained, stubs were prepared applying the double-sided tape on the head and posing a special 0.5 μm filter (Millipore^{TM}, Isopore membrane filters); a drop of siliceous sample was placed on the filter and left to dry for 24 hours under the hood. The samples were observed with the SEM (JEOL 6700 F) at the Advanced Microscopy Center (CeMA) of Stazione Zoologica Anton Dohrn.

In order to perform molecular identification, Lyses & Bacteria/Fungi PCR-GO Kit (DNA-TECH Spin off) was used to perform PCR directly from single colonies from the strains cultured, according manufacture instructions (see Somma et al., submitted). PCR was performed with specific primers *rbcL*-F, 5’-3’ ATGTCTCAATCTGTAWCAGAACGGACTC and *rbcL*-R, 5’-3’ TAARAAWCKYTCTCTCCAACGCA, amplifying a *rbcL* fragment of 660 bp (Evans et al., 2007; Guo et al., 2015). The purified PCR products using the *QIAquick Gel Extraction kit* (Qiagen, Milan, Italy) were then sequenced on an Applied Biosystems 3730 DNA Analyzer 48 capillaries (Life Technologies) using BigDye® Terminator v3.1 Cycle Sequencing kit (Life Technologies). The sequences obtained from CNN and CNA were submitted to GenBank using Basic Local Alignment Search Tool (BLAST; http://blast.ncbi.nlm.nih.gov/Blast.cgi) to identify the species, and then aligned to highly similar sequence using MultiAlin (http://multalin.toulouse.inra.fr/multalin/).

Hippolyte inermis collection and rearing

Shrimp females of *H. inermis* were collected in spring 2021 through trawling a plankton net upon *P. oceanica* leaves in Lacco Ameno, off the Ischia Island (Gulf of Naples, South Italy). All the female individuals were directly sorted on boat and transferred into plastic containers filled with clean seawater. Once in laboratory, all the individuals were taxonomically identifying on the base of morphological features, under a Leica MZ6 stereomicroscope (Zupo, 1994). The laboratory rearing of *H. inermis* was carried out following the protocol described by Zupo & Messina (2007). The experiment was entirely conducted under controlled conditions, housing the samples in a thermostatic chamber at 18 °C with a 12:12 day:night photoperiod. These conditions, routinely applied to our cultures, emulate the recorded natural condition during the reproductive seasons of these shrimps. In particular, ovigerous female were maintained in 2 L Erlenmeyer flasks (containing 1.5 L of filtered seawater) until larval hatching. At this point, larvae were collected and transferred in 1 L Erlenmeyer flasks (containing 0.8 L of filtered seawater), in which they were reared according to the abovementioned protocol. After around 30 days, settled post-larvae were collected and transferred in Petri dishes massive culture of the two diatom strains CNN and CNA. After five days from the metamorphosis, PL_5 were sacrificed and individually fixed in RNA-later (RNA Stabilization Reagent, Quiagen, Germany).

RNA extraction and cDNA synthesis

The RNA extraction was carried out utilising the RNeasy Mini Kit (Quiagen, Austin, TX, US) on the entire body of single fixed individuals. The PL_5 underwent to lyses process with 350 µL Buffer RLT/2-ME (10 µL β-mercaptoethanol each 1 mL of Buffer RTL). Consequently, all the samples were homogenised through a TyssueLyser (Qiagen, Austin, TX, US) set on 20.1 Hz for 3 minutes. The extraction process followed the provided manufacturer protocol (Quiagen, Austin, TX, US) and the extracted RNA was eluted in 30 µL RNase-free water and stored at -80°C.

The total amount of RNA obtained from the extraction protocol was estimated by absorbance measurements using NanoDrop spectrophotometer (ND-1000 UV-Vis Spectrophotometer; NanoDrop Technologies, Wilmington, DE, USA). The measurements of absorbance were carried out at wavelength of 260 nm and 230/260 and 260/280 nm ratios, in order to exclude presence of contaminants. For the synthesis of the cDNA, 600 ng of each sample of extracted RNA was retrotranscribed with iScript cDNA Synthesis kit (Bio-Rad, Milan, Italy) following the manufacturer's protocol instructions.

Gene expression by Real-Time qPCR

The expression level of seventeen genes of interest and *housekeeping* gene (see Tab.1) were followed by Real-Time qPCR. The extracted cDNA was diluted and used as a template in a reaction containing a final concentration of 0.3 mM for each primer and 1 × FastStart SYBR Green master mix (total volume of 10 µL) (Applied Biosystems, Monza, Italy). PCR amplifications were performed through ViiATM7 Real Time PCR System (Applied Biosystems, Monza, Italy) thermal cycler. The thermal profile applied was the following: 95 °C for 10 minutes, 1 cycle for cDNA denaturation;

95 °C for 15 seconds and 60 °C for 1 minute, 40 cycles for amplification; 72 °C for 5 minutes, 1 cycle for final elongation; one cycle from 60 °C to 95 °C for melting curve analysis to verify the presence of a single product. The fluorescence was measured through ViiATM7 software (Applied Biosystems, Monza, Italy). The expression rate of each gene was elaborated as Cq and analysed using REST software (Relative Expression Software Tool, Weihenstephan, Germany). Only values of ±1.3 in the Cq results were considered significant.

Table 1. List of the selected seventeen genes, divided according to the pathways, and relative acronyms.

	Acronym	**Gene name**
Housekeeping	*Coi*	*Cytochrome oxydase subunit*
Ferroptosis	*Gshi*	*Gamma Gluthamylcysteine Synthase*
	Stea3	*Sterile alpha-motif domain-containing protein 3 metalloreductase*
	Gpx4	*Glutathione Peroxidase 4*
	Sat	*Spermidine/spermine N1-Acetyltransferase 1*
Apoptosis	*Cyt-C*	*Cytochrome C*
	Atfc	*Activating Transcription Factor*
	Htra2	*High-temperature requirement A serine peptidase*
	Dronc	*Death receptor-associated nemesis-like*
	Tspo	*Translocator protein*
Insulin-like hormone secretion	*Pclo*	*Piccolo Presynaptic Cytomatrix Protein*
	Ac	*Adenylyl cyclase*
	M3R	*Muscarinic acetylcholine receptor M3*
	Cckar	*Cholecystokinin A Receptor*
	Vamp3	*Vesicle-associated membrane protein 3*
	Plc	*Phospholipase C*

Snp25	*Synaptosome-associated protein 25*

Results

Diatom isolation and identification

As result of the isolation process, several strains of benthic pennate elliptical diatoms epiphyte of *P. oceanica* leaves were isolated. In particular, among the fifteen isolated culture two strains of *Cocconeis neothumensis* var. *marina* De Stefano, D. Marino & L. Mazzella, 2000 were obtained: the strain CNN (isolated from the control site, Fig. 2 C and D) and the strain CNA (isolated from Low pH site, Fig. 2 A and B). Both the morphological approach, through the analysis of frustule ultrastructure, and molecular comparison confirmed the identification at species level. The diatoms were grown in sterile Culture Multiwell Plates of 6 wells (3.5 cm in diameter), each containing about 8 mL of culture medium.

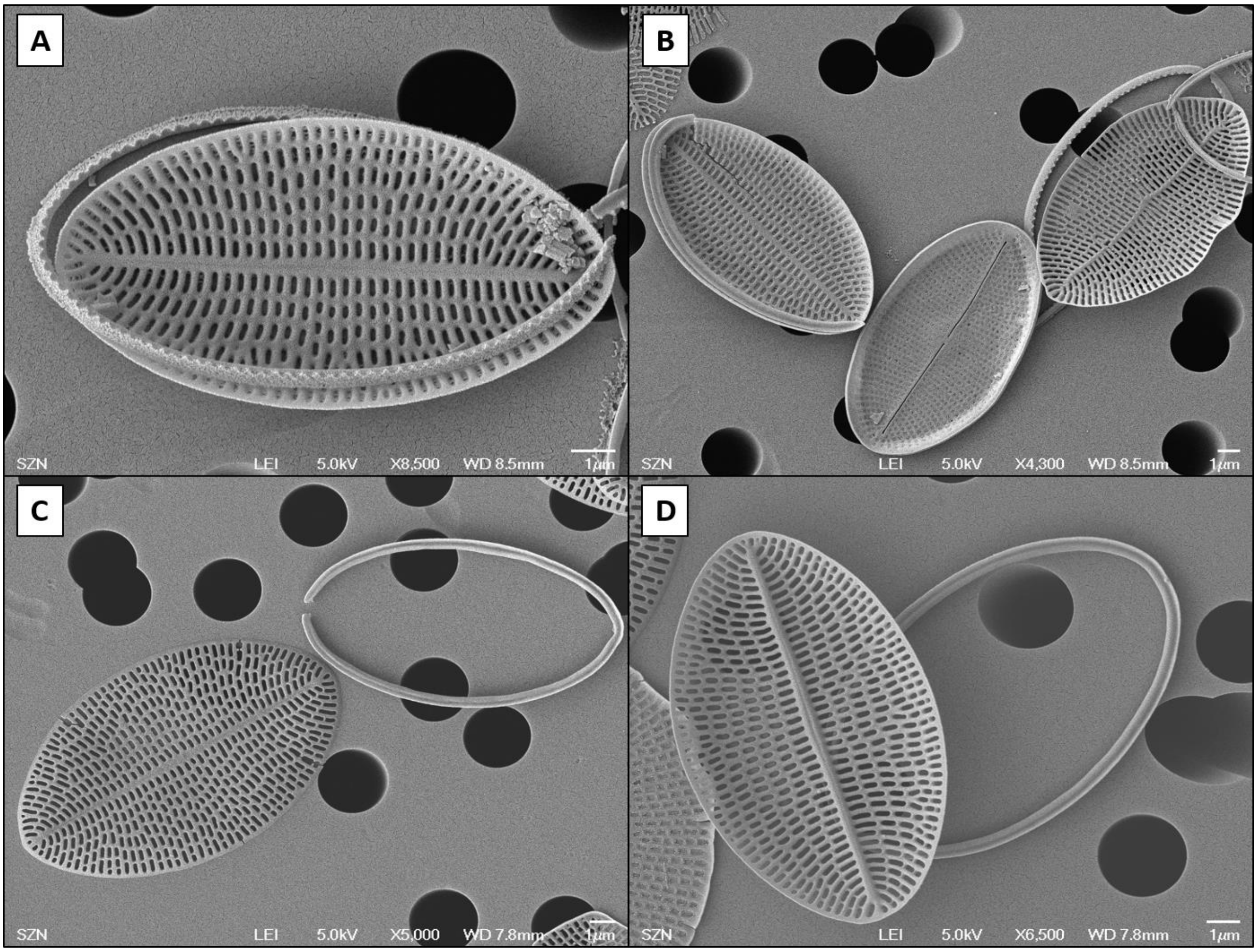


Figure 2. SEM images of the two strains of *C. neothumensis* var. *marina* isolated from the two different sites: CNA collected from Low pH site (A and B) and CNN isolated from Control site (C and D).

Gene expression

The post-larvae of *H. inermis* were reared 5 days while supplying two living strains of *C. neothumensis* var. *marina* isolated from different pH conditions, as food. To detect any changes in the early sex reversal process of the shrimp, the expression levels of seventeen selected genes were followed by Real-Time qPCR. The genes here explored belong to three key gene pathways involved in the sex differentiation of *H. inermis*, in particular: ferroptosis of the AG, apoptosis of the teste buds and Insulin-like hormone secretion. The expression of each gene was analysed and internally normalised against a positive control compared to the transcriptome data available (Levy et al. 2021). In particular, the results of gene expression of PL_5 fed with the diatom strain CNN were used as reference after comparison with the positive control of the treatment used in the transcriptome (Levy et al., 2021). The results obtained from the expression of the gene of PL_5 fed with the strain CNA highlighted some differences in the regulation of some genes of the abovementioned pathways.

In detail, for what concerns the ferroptosis pathway, all genes involved resulted up-regulated, and thus similar to the results obtained from the comparison with the transcriptome data. Regarding the genes involved in apoptosis processes, the gene expression resulted up-regulated as compared to the control (similar to the transcriptome data), with the exception of the *Htra2* and *Aftc4* genes that resulted down-regulated compared to the reference values.

Remarkable outcomes arose from the analysis of the gene expression of the Insulin-like hormone secretion pathway. In detail, most of the genes were found to be up-regulated from the comparison with the control, in contrast with the data carried out from Levy et al. (2021). The only exception was represented by the genes *Cckar*, that resulted down-regulated, and *Vamp*, up-regulated in comparison to the control. The expressions of these two genes resulted in accordance with the data emerging from the analysis of the transcriptome (Levy et al., 2021).

Table 2. Expression of the genes involved in the three pathways under analysis and comparison with the results obtained by the transcriptome (Levy et al., 2021). The result referred to the 5 individuals analysed. In red are indicated the up-regulated genes, while in blue the down-regulated genes.

Pathway	**Gene**	Ind. 1	Ind. 2	Ind. 3	Ind. 4	Ind. 5	**Transcriptome**
Ferroptosis	*Sat*	0.7	1.4	3.9	-0.2	0.8	🟥
	Stea3	2.9	3.9	5.1	1.9	3.1	🟥
	Gpx4	6.7	7.4	8.8	10.0	7.1	🟥
	Gshi	3.9	2.9	6.7	1.5	-0.13	🟥
Apoptosis	*Cyt*	0.4	5.3	6.7	5.8	4.5	🟥
	Htra2	-3.0	-1.0	-1.5	-0.3	-4.3	🟥
	Dronc	1.9	1.7	3.3	2.0	1.7	🟥
	Tspo	2.7	3.0	3.9	3.3	3.0	🟥

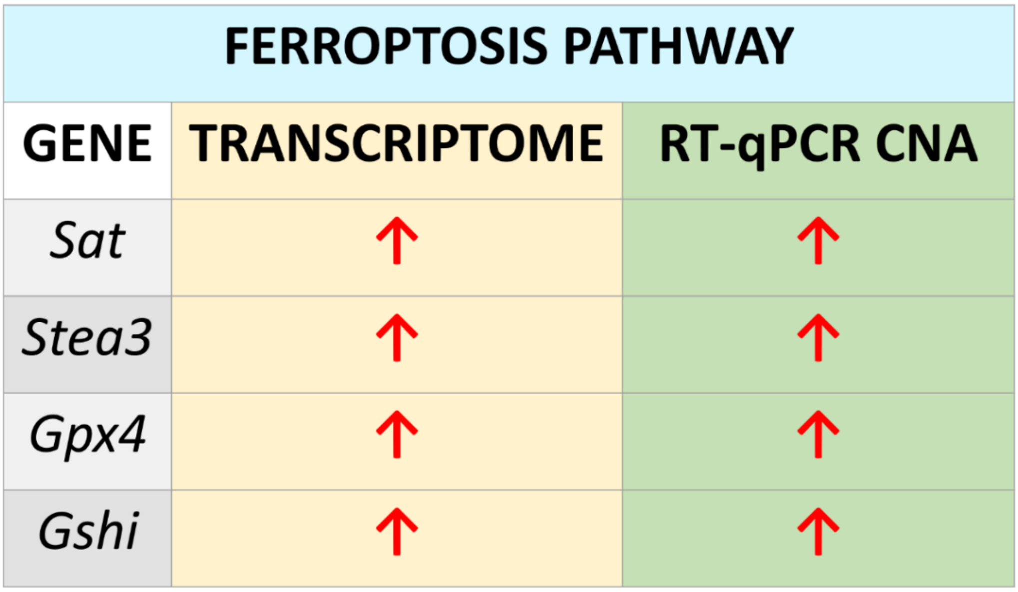
	Atfc4	-0.8	-3.3	-2.3	-6.7	-11.1	🟥
Insulin-like hormone	*M3R*	7.2	7.8	2.9	6.8	6.6	🟦
	Snp25	6.9	7.9	7.5	10.6	10.7	🟦
	Pclo	2.8	3.4	4.9	3.6	3.0	🟦
	Ac	3.1	4.0	5.1	4.6	4.1	🟦
	Cckar	0.9	-6.2	-6.8	-12.1	2.1	🟦
	Plc	0.8	-1.9	3.7	-0.2	-3.4	🟦
	Vamp	-3.9	6.7	6.7	6.2	5.3	🟥

Discussion

Here we aimed at exploring how OA might affect complex trophic relationships, such the one ruling the physiology of the shrimp *Hippolyte inermis* as influenced by its diatom food. In light of recent evidences arose by previous studies (Mutalipassi et al. 2019), the lowering of pH may influence these delicate interactions. According to the outcomes deriving from the analysis of the transcriptomic data (Levy et al. 2021), seventeen genes were selected (see Tab. 1) among the ones that showed significant differences in the expression induced by the ingestion of the diatom from the caridean decapod. These genes play a crucial role in three key-pathways involved in *H. inermis* early sex shift (Zupo et al., 2023).

Ferroptosis is a particular form of cell death only recently discovered in the nematode *Caenorhabditis elegans* (Maupas, 1899) Dougherty, 1953 (Deline et al., 2015). Ferroptosis cell death may be considered a highly conserved mechanism and it has been proposed that may play a crucial role in leading additional apoptosis in invertebrates (Deline et al., 2015; Zupo et al., 2023). Furthermore, apoptogenic activity was proposed to be responsible in the premature destruction of teste buds of *H. inermis* as selective mechanism of programmed cell death (Zupo, 1994). In parallel, insulin production pathway has been indicated to be fundamental for the regulation of the metabolism of insulin-like hormone, secreted by the AG, controlling sexual differentiation of the Crustaceans (Ventura et al., 2012).

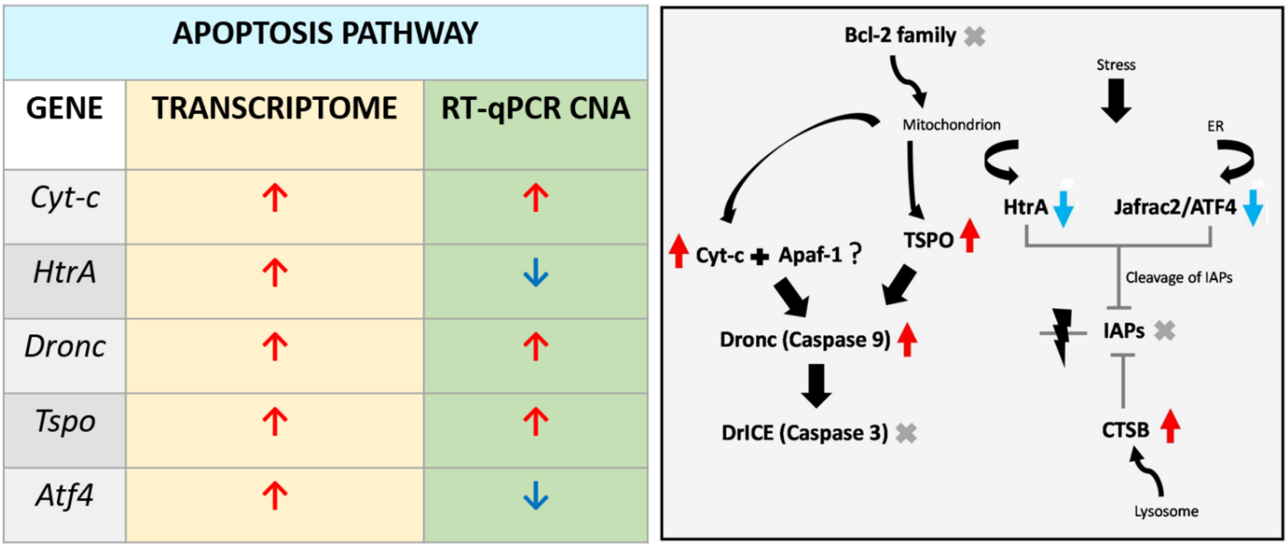
Here, we tested two different strains of *C. neothumensis* var *marina* isolated from different pH condition sites. The diatoms were supplied as a live food to *H. inermis* for the first five days after metamorphosis of larvae. Based on our results, the strain CNA produced different gene expression in the shrimps. In particular, PL_5 fed on the diatom strain isolated from acidified areas (CNA) showed the same expression in the genes related to the ferroptosis pathway (Fig. 3).



FERROPTOSIS PATHWAY		
GENE	**TRANSCRIPTOME**	**RT-qPCR CNA**
Sat	↑	↑
Stea3	↑	↑
Gpx4	↑	↑
Gshi	↑	↑

Figure 3. Summary of the results obtained on the expression levels of the genes involved in ferroptosis pathway compared with the data belonging to the transcriptome (Levy et al. 2021). All the genes are upregulated, as indicated by the red arrows pointing up.

Different results arose from the expression of the genes belonging to apoptosis pathway (Fig. 4), which exhibited discrepancy in the genes *Htra2* and *Atfc4*, both involved in the pathway referred to stress conditions state.



APOPTOSIS PATHWAY		
GENE	TRANSCRIPTOME	RT-qPCR CNA
Cyt-c	↑	↑
HtrA	↑	↓
Dronc	↑	↑
Tspo	↑	↑
Atf4	↑	↓

Figure 4. On the left summary of the results obtained on the expression of the genes related to apoptosis pathway and comparison with the data belonging to the transcriptome (Levy et al. 2021). On the right schematic representation of the apoptosis gene pathway and the gene expression resulted from the present experiment (modified from Zupo et al., 2023). Red up-arrows indicate up-regulated genes; blue down-arrows indicate down-regulated genes.

On the other hand, the expression of most genes belonging to the insulin-like hormone secretion pointed out an inverted trend (Fig. 5). Indeed, the genes *Vamp* and *Cckar* resulted respectively down- and up-regulated and thus in accordance with the result of the transcriptome. All the other genes, specifically *M3R*, *Snp25*, *Pclo*, *Ac* and *Plc* showed higher expression compared to the transcriptome data. The only gene that maintained the same expression as compared to the transcriptomic data is *Cckar*, which occurs in the initial phase of the pathway (Fig. 5). In light of this consideration, we can hypothesize a reduction in the apoptogenic compound in the diatom strain selected by low pH. In this context, OA might affect metabolism of the diatom food of the shrimp, causing a retard or worst the interruption in the sex-reversal process.

INSULIN-LIKE HORMONE PATHWAY		
GENE	**TRANSCRIPTOME**	**RT-qPCR CNA**
M3R	↓	↑
Snap25	↓	↑
Pclo	↓	↑
Ac	↓	↑
Cckar	↓	↓
Plc	↓	↑
Vamp	↑	↑

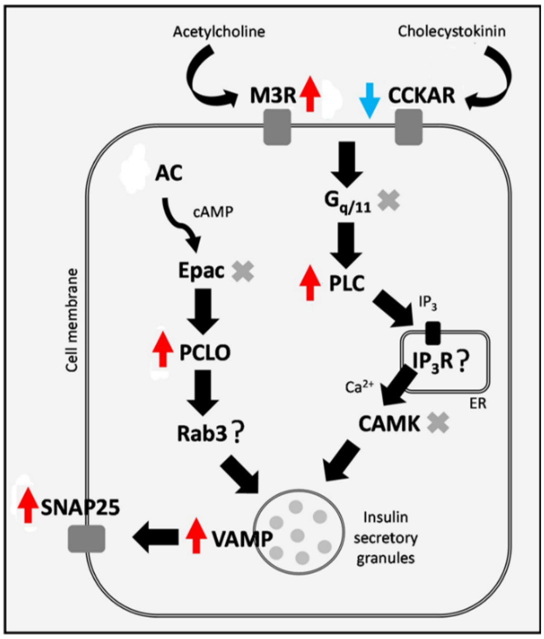


Figure 5. On the left summary of the results obtained on the expression of the genes related to insulin-like hormone pathway and comparison with the data belonging to the transcriptome (Levy et al. 2021). On the right schematic representation of the insulin-like hormone production gene pathway and the gene expression resulted from the present experiment (modified from Zupo et al., 2023). Red up-arrows indicate up-regulated genes; blue down-arrows indicate down-regulated genes.

In conclusion, our results indicate that OA selects unusual strains of *Cocconeis* diatoms, with different physiology and metabolic profile, as suggested by Somma et al. (*unpublished data*). Nevertheless, both strains of *C. neothumensis* var. *marina* here isolated and tested produced effect on the gene expression of *H. inermis*, highlighting some differences especially for what concerns the gene pathways referred to insulin-like hormone. This research demonstrates that the diatom strain CNA, selected under low pH conditions, affect the well-known early sex shift of the protandric shrimp *H. inermis*. Furthermore, the expression of genes referred to the ferroptosis pathway suggests that the consumption of the diatom CNA still triggers the ferroptotic cell death of the AG. In contrast, the expression of genes related to the apoptosis pathway showed some differences, mainly in the genes involved to the response to stress conditions. Remarkably, the results obtained on the genes related to insulin-like hormone production suggest that the production of the hormone is not interrupted, at least in this stage; this may indicate that the early sex reversal process is not completed even if the ferroptosis is triggered. A possible interpretation could be that a lower amount of active compounds is produced, and consequently the process of cell death interesting the AG was delayed, leading to a retarded block of the production of IAG. However, once started the process of cell death involving the AG, the destruction of the testes is triggered (according to Ventura et al., 2011) and consequently, it is necessary to hypothesize that a block in the production of IAG will be completed, even later, as above mentioned.

Further investigations will clarify the actual mechanisms ruling the metabolic pathways and the effects of the selected *Cocconeis* sp. strains on the physiology of *H. inermis*, according to the pathways already shown in previous investigations (Zupo et al., 2023). This will shed light on the finding of this research, indicating that the diatom strain selected under low pH conditions slow down the sex shift in this peculiar decapod. These changes, in an OA future scenario, might influence the delicate plant-animal relationship, with impact on the population ecology of various species of invertebrates for which microalgal food may influence their physiology. Furthermore, due to the key trophic role of *H. inermis* within its animal community (Zupo & Fresi, 1985), the food webs associated to the leaf stratum of *P. oceanica* might be impacted with unknown consequences.

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GENERAL DISCUSSION AND CONCLUSIONS

Ocean acidification (OA) is likely to produce detrimental effects on many specific ecosystems and to induce a decline in their associated biodiversity (Gaylord et al., 2015; Sunday et al., 2017), although this issue is still debated (Garrard et al., 2013). However, the habitat-forming seagrass *Posidonia oceanica* is expected to be vulnerable to OA, with dramatic consequences on the functioning of this ecosystem and loss of services (Zunino et al., 2019). In particular, the ecosystem stability of such important habitat is assured by complex trophic and chemical relationships established among the inhabiting organisms (Buia et al., 2000; Mazzella et al., 1993; Zupo & Fresi, 1985). Microphytobenthos is at the base of the food web and thus it is essential to assure correct succession and relationships. For this reason, the present PhD project aimed at stressing the possible effects of OA on this compartment under various points of view. In particular, we dealt with the assessment of the effects of low pH conditions on the structural and functional organization of benthic diatoms assemblages in a Mediterranean shallow CO_2 vent. Taking advantage of the unique features of a shallow CO_2 vent located off the Ischia Island (Gulf of Naples, Italy), it was possible to evaluate the effects of OA not only on benthic diatom assemblages, but also on the relationships they have with consumers and other microalgae.

In this view, after an effort in reviewing the literature available about this topic (Chapter 1), we planned a study aimed at reconstructing the assemblages of epiphytic diatoms within the *P. oceanica* leaf stratum, by sampling benthic diatoms in two seasons (autumn and spring) from sites characterised by different pH conditions. Overall, the benthic diatoms genera found in our study (Chapter 2) reflected the assemblages previously recorded by Mazzella & Spinoccia, (1992) in the same study area (Ischia Island, Gulf of Naples). Regarding the differences among the two sites (low pH and control conditions), our results showed only slight differences between the assemblages. In this view, some genera of large size diatoms were mainly recorded in the acidified area (*Achnanthes* sp., *Toxarium* sp., *Licmophora* sp., *Pleurosigma* sp). This is in accordance with Wu et al. (2014) which demonstrated shift in the assemblages toward larger species due to a positive relationship between the cell size and the magnitude of the fertilization. Evidently, a higher abundance of CO_2 may produce a kind of fertilization on diatoms growth. Nevertheless, our results did not reveal a clear differentiation among the pH conditions, highlighting a wide separation based on seasonality. It is worth to note that this separation is particularly evident in the acidified area of Castello, leading to hypothesize that OA effects might be influenced by other combined environmental conditions. The present hypothesis is also corroborated by several evidences demonstrating how additional stressors (*e.g.* temperature, nutrient availability, irradiance), together with OA, might also affect diatom communities leading to shifts in their composition (Boyd et al., 2018; Gao et al., 2012). In this view, the different environmental conditions characterising the two seasons, coupled with the effects of OA, might produce a wide separation particularly within the low pH site.

As already explained, benthic diatoms represent key organisms in the microphytobenthos associated with *P. oceanica* leaf stratum. They ride through primary and secondary biofilm formation (Molino & Wetherbee, 2008), representing the base in the food web. Furthermore, several groups of benthic diatoms are responsible for the production of important chemical signal that ensure the stability of animal communities associated to this environment. In some cases, in fact, diatom may affect invertebrate physiology (Zupo et al., 2007) and behaviour (Jüttner et al., 2010). In particular, among all the genera recorded, species belonging to the genus *Cocconeis* are well-established model diatom (Zupo, 2000), and an excellent candidate for the extraction of apoptogenic compounds (Nappo et al., 2012). They also showed promising activity for various biotechnological applications in the fields of aquaculture and medicine (Raniello et al., 2007). In this view, several strains of benthic diatoms were obtained as a result of the epiphyte collections and selection through micro-isolation of new benthic diatom strains. In particular, a strain further identified as *C. neothumensis* var. *marina* was isolated and maintained in culture. Due to their ecological and biotechnological relevance, in the present thesis we adopted a polyphasic identification approach, using both ultra-morphological data and molecular conserved markers (Chapter 3). The molecular analyses performed on the isolated strains, led to conclusions that coincided with the morphological features detected by the ultrastructural analysis of the frustule, unambiguously identifying the species as *C. neothumensis* var. *marina*. Remarkably, this research allowed a better identification of this model species using a multidisciplinary approach.

The isolation effort allowed us to obtain strain of the same species (*C. neothumensis* var. *marina*) from two sites characterised by different pH conditions: CNN (isolated from a control site under ambient pH condition) and CNA (belonging to the low pH site of CO_2 vent). Diatoms belonging to the genus *Cocconeis* are well-known to be involved in a peculiar plant-animal chemical relationship within *P. oceanica* meadows. In particular, *Cocconeis* sp. are responsible for the production of a cell death metabolite that triggers the apoptosis of the AG of *H. inermis* (Zupo, 2000), a protandric decapod mainly inhabiting *P. oceanica* meadows (Gambi et al., 1992). Previous studies demonstrated how decreased pH in the culture condition influence the metabolism of the congeneric benthic species *Cocconeis scutellum* var. *parva* (Mutalipassi et al., 2019).

In this regard, we dealt with the possible effects of low pH culture condition on growth and physiology of *C. neothumensis* var. *marina* (Chapter 4). In order to assess the possible differences between the two strains, we cultured the diatoms at both pH conditions (7.6 and 8.2) using *ad-hoc* constructed photobioreactors. Relevant differences arose both from the growth and from the metabolic profiles. Firstly, the culture of the two diatom strains revealed different growth-curves. In particular, both CNN and CNA growth faster at pH 8.2 than at lower pH. The analysis of the growth-curves highlighted significant differences especially in the exponential phase. Moreover, further comparisons of the two strains showed that the strain isolated from the control site (CNN) grows faster than CNA (isolated from low pH site) under both pH culture conditions.

In accordance with previous studies, that showed an enhanced growth under normal pH conditions or no effect of OA, our results highlighted that *C. neothumensis* var. *marina* growth faster at normal pH than under acidified culture condition (Ayache et al., 2021; Cai et al., 2022; Wingert & Cochlan, 2021). The present results are also in accordance with Wu et al. (2014) who demonstrated that only minor changes in the growth rate occur in diatom species with smaller cell size compared with significant enhancement in the growth in larger cell diatom species (diameter > 30 µm).

Wide differences also arose by the H-NMR analysis on the metabolites produced by the two diatom strains cultured. In particular, the spectra of both polar and lipophilic compounds differ significantly, revealing higher production of the same metabolites in both the native isolation conditions. In conclusion, this study suggests that OA might select unusual strain of benthic diatoms, with still unknown impacts on the upper trophic levels.

As already mentioned, *Cocconeis* sp. plays an important role as key food item for *H. inermis*. Moreover, it is also responsible for the production of apoptogenic metabolite, which trigger the early sex shift of the caridean decapod through the apoptosis of the AG (Zupo & Messina, 2007). In this context, the early sex reversal of *H. inermis* represents a stabilizing factor for their natural populations (Zupo, 2000). This delicate equilibrium risks being threatened by OA, as suggested by Mutalipassi et al. (2019). In light of our results on the two C. *neothumensis* var. *marina* strains (Chapter 4), we explored how OA might indirectly affect the population of *H. inermis*, influencing the secondary metabolites production of their food (Chapter 5).

Taking advantage of recent transcriptomic data (Levy et al., 2021) we were able to detect the expression level of seventeen genes of interest belonging to three key gene pathways: ferroptosis, apoptosis and insulin-like hormone (IAG) production (Zupo et al., 2023). In particular, RNA extraction was performed on five days old post-larvae (PL5) of *H. inermis* feed with in vivo culture of the two strains of *C. neothumensis* var. *marina*. The expression levels of the genes were detected through Real-Time qPCR and then compared with the results obtained in the transcriptome. Our results indicated that strains of *Cocconeis* diatoms, with different physiology and metabolic profile (Chapter 4), produce different responses in the gene expression. In particular, PL_5 fed with CNA revealed wide discrepancy in the expression of gene related to IAG production, suggesting that the production of the hormone is not interrupted, at least in this stage. This result might point out that the early sex reversal is not completed, even if the ferroptosis of the AG is triggered. Consequently, taking into account the key trophic role of *H. inermis* within the associated food webs, OA is likely to affect the entire *P. oceanica* ecosystem with still unknown consequences.

Furthermore, this study confirms the importance to exploit naturally acidified environments (as CO_2 vents) to test ecological hypotheses on the effects of OA on specific communities and ecosystems. Nevertheless, much caution must be paid to discuss and to contextualise the results obtained, as they can be bias by confounding multiple environmental variables that might occur in vents areas.

Finally, an integration of experiments is recommended from different research approaches to better understand and forecast the effects of the ongoing pH decrease on marine life. At the same time, we should be able to invert the trajectory of global CO_2 emissions, maintaining under control our carbon footprint and to follow choices towards a more sustainable life style. In particular, descriptions on how and where most species on Earth settle, live, and die according to the pH of seawater, could be an investment worth to retard the overwhelmingly rapid destruction of Nature.

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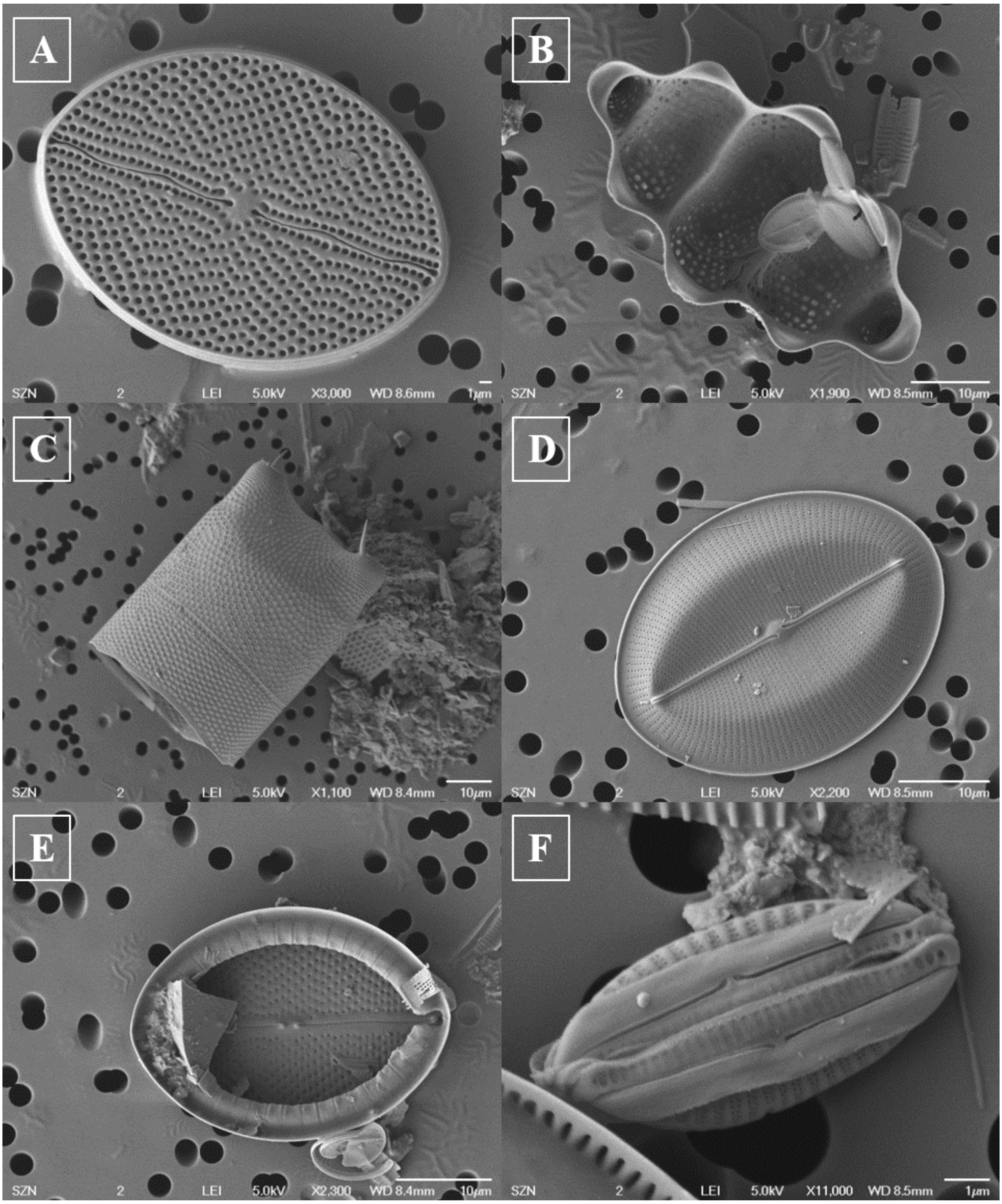
ANNEX I

Diatoms collected and analysed at the Scanning Electron Microscope


A
SZN 2 LEI 5.0kV X3,000 WD 8.6mm 1µm
B
SZN 2 LEI 5.0kV X1,900 WD 8.5mm 10µm
C
SZN 2 LEI 5.0kV X1,100 WD 8.4mm 10µm
D
SZN 2 LEI 5.0kV X2,200 WD 8.5mm 10µm
E
SZN 2 LEI 5.0kV X2,300 WD 8.4mm 10µm
F
SZN 2 LEI 5.0kV X11,000 WD 8.5mm 1µm

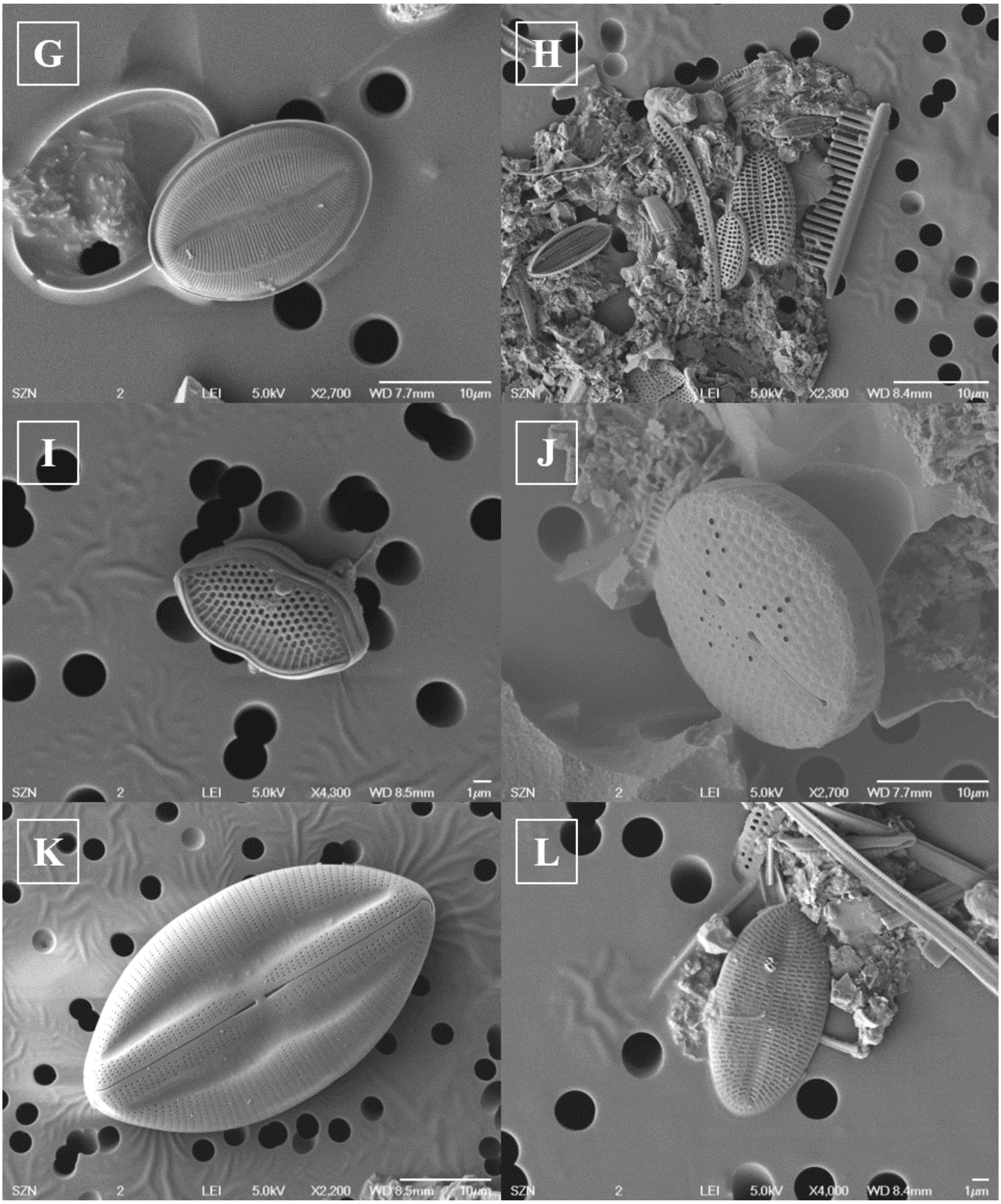
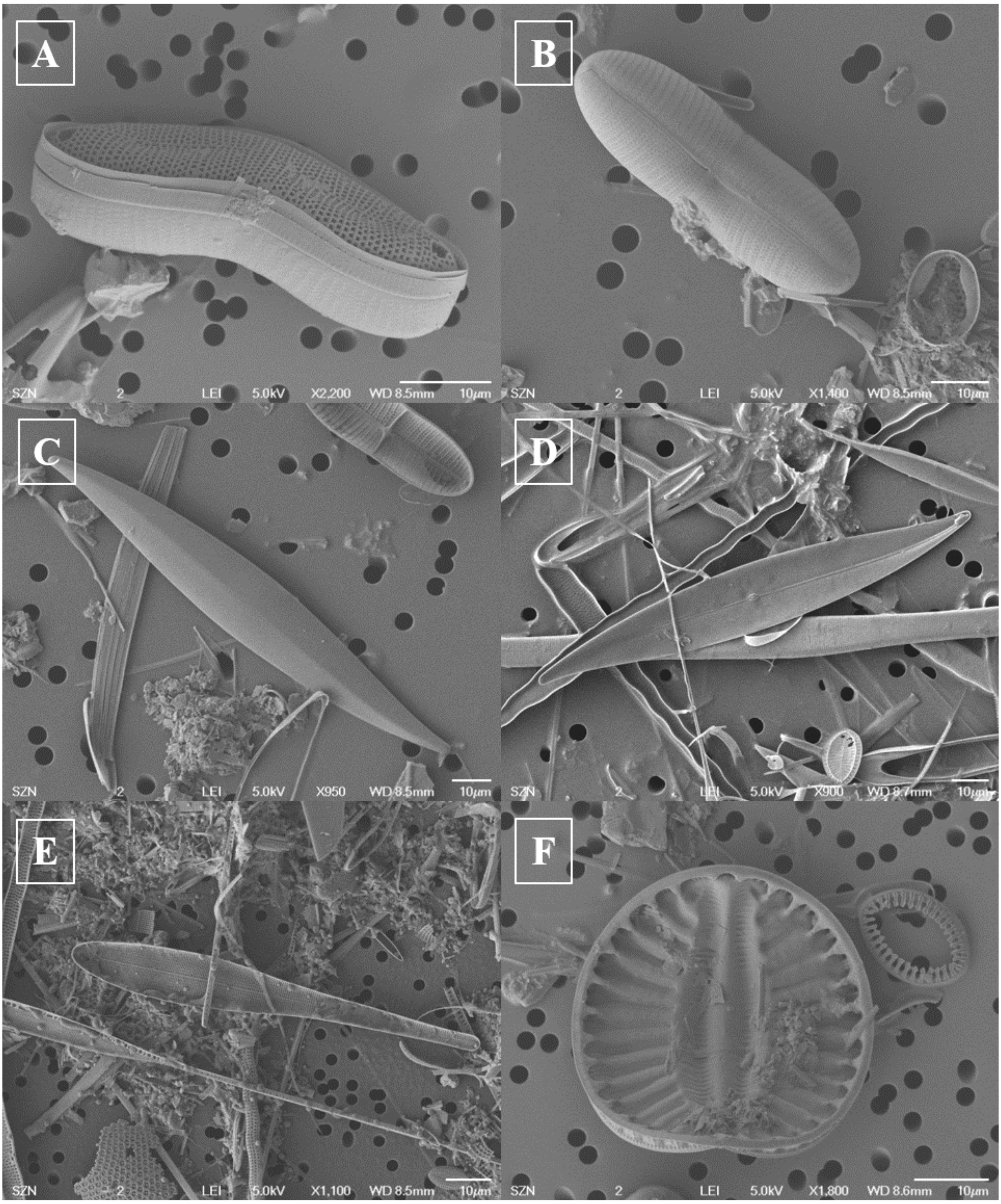


Figure 1. Example of images captured through SEM, belonging to the vent area of Castello Aragonese; sampling season: autumn. External view of valve of *Mastogloia* sp. (1-A); internal view of valve of *Terpsinoe* sp. containing some *Cocconeis* sp. (1-B); *Biddulphia* sp. (1-C); internal view of valve of *Cocconeis multiperforata* (1-D); internal view of valve of *Mastogloia* sp.(1-E); Amphora sp. (1-F); external view of valve of *Cocconeis* sp. (1-G, H); *Tryblionella* sp. (1-I); external view of valve of *Mastogloia fimbriata* (1-J); external view of valve of *Lyrella* sp. (1-K); external viex of sternum valve (SV) of *Cocconeis neothumensis* var. *marina.*


A
SZN 2 LEI 5.0kV X2,200 WD 8.5mm 10μm
B
SZN 2 LEI 5.0kV X1,400 WD 8.5mm 10μm
C
SZN 2 LEI 5.0kV X950 WD 8.5mm 10μm
D
SZN 2 LEI 5.0kV X900 WD 8.7mm 10μm
E
SZN 2 LEI 5.0kV X1,100 WD 8.5mm 10μm
F
SZN 2 LEI 5.0kV X1,800 WD 8.6mm 10μm

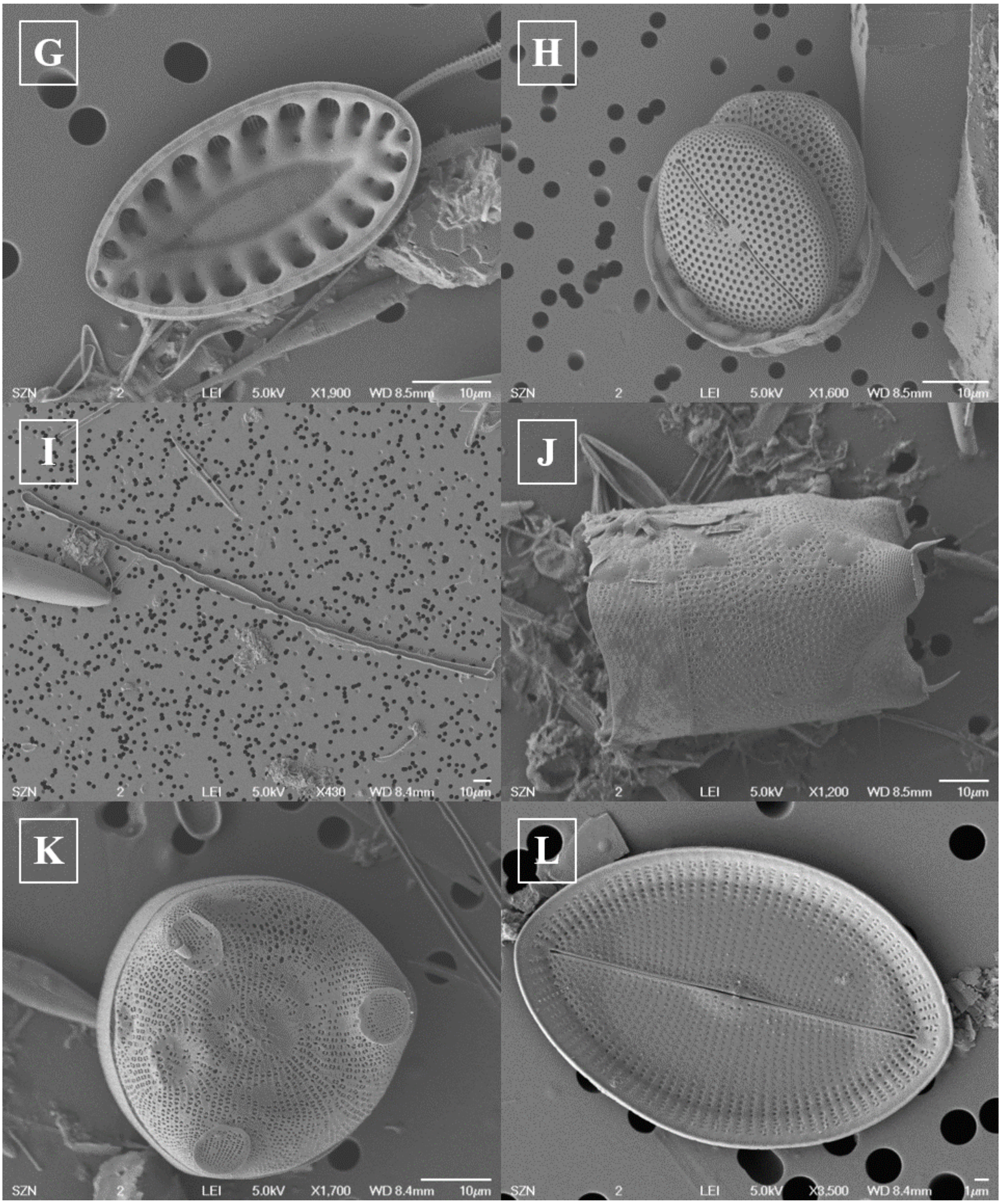
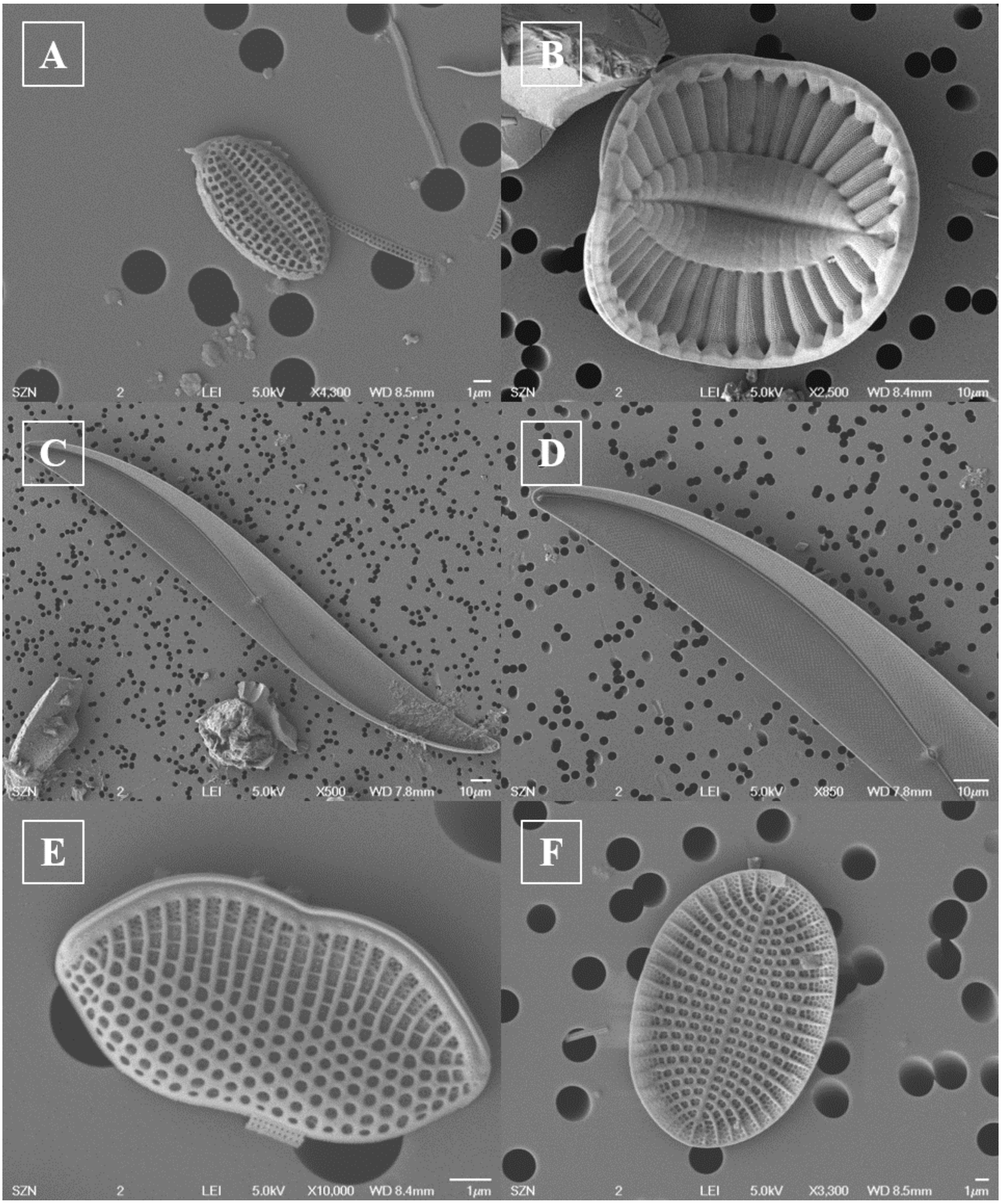


Figure 2. Example of images captured through SEM, belonging to the vent area of Castello Aragonese; sampling season: spring. Side view (2-A) and external view (2-B) of valve of *Achnanthes* sp.; external (2-C) and internal (2-D) view of valve of *Pleurosigma* sp.; internal view of valve of *Licmophora* sp. and *Cylindrotheca* sp. (2-E); internal valve of *Campylodiscus* sp. (2-F); *Surirella* sp. (2-G); external view of of raphe sternum valve (RSV) of *Mastogloia* sp. (2-H); internal view of valve of *Toxarium undulatum* (2-I); *Biddulphia* sp. (2-J); *Lampriscus* sp. (2-K); internal view of raphe sternum valve (RSV) of *Cocconeis* sp. (2-L).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B
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C
SZN 2 LEI 5.0kV X500 WD 7.8mm 10μm
D
SZN 2 LEI 5.0kV X850 WD 7.8mm 10μm
E
SZN 2 LEI 5.0kV X10,000 WD 8.4mm 1μm
F
SZN 2 LEI 5.0kV X3,300 WD 8.5mm 1μm

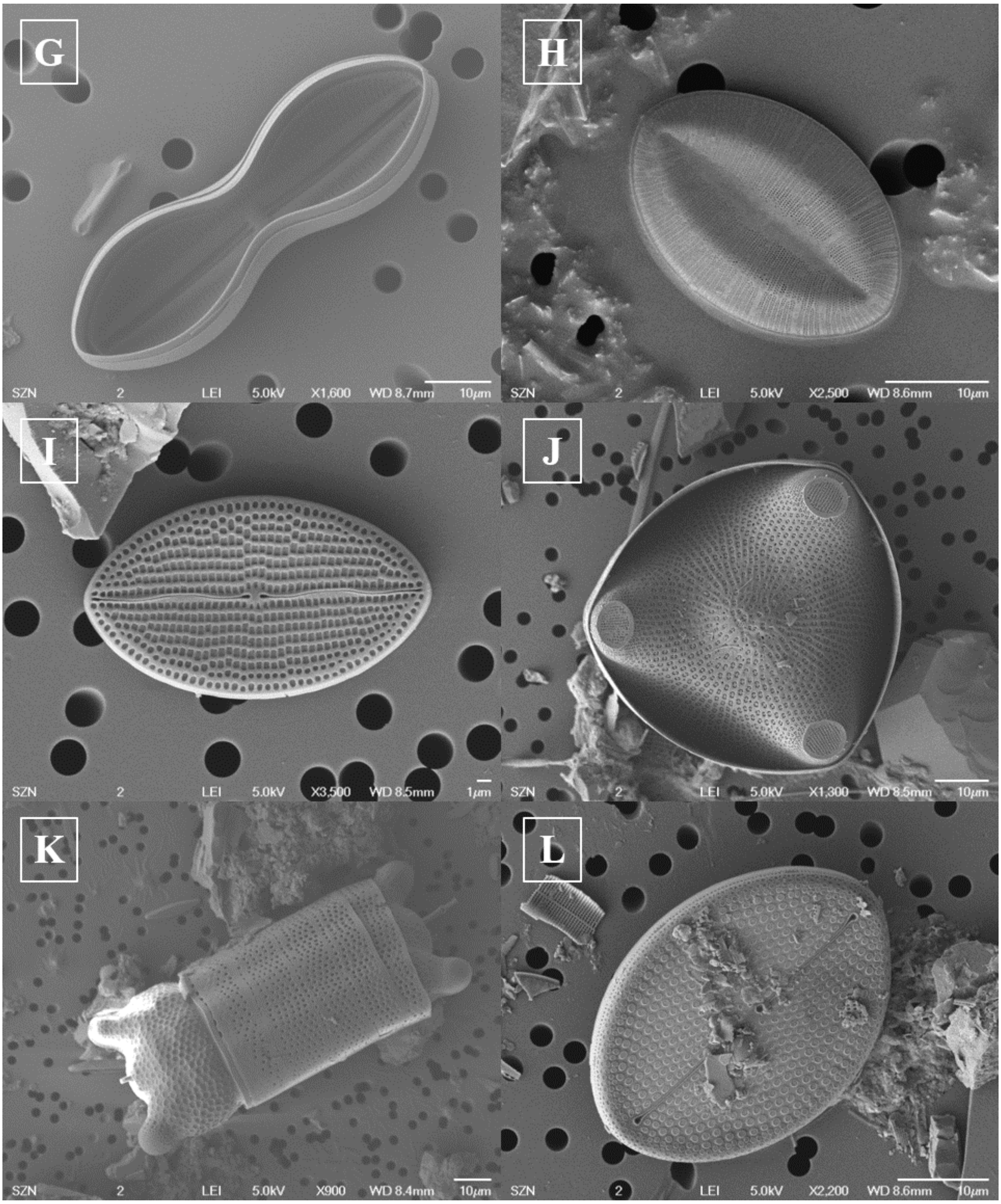
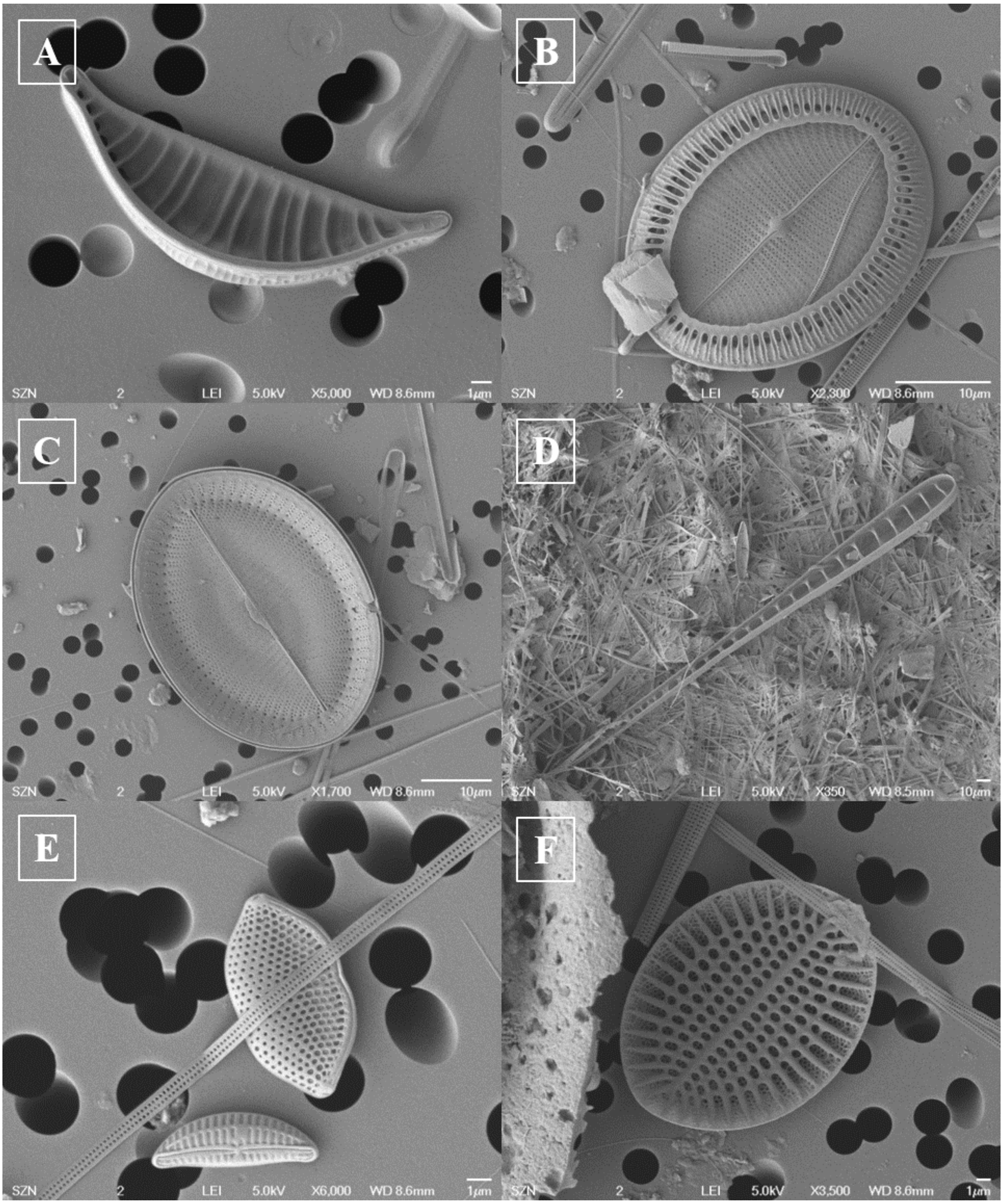


Figure 3. Example of images captured through SEM, belonging to the control area of Lacco Ameno; sampling season: autumn; external view of valve of *Cocconeis* sp. (3-A); *Campylodiscus* sp. (3-B); internal view and a detail of a valve of *Pleurosigma* sp. (3-C, D); *Tryblionella* sp. (3-E); internal view of a sternum valve (SV) of *Cocconeis* sp. (3-F); internal view of a valve of *Diploneis* sp. (3-G); external view of sternum valve (SV) of *Cocconeis multiperforata* (3-H); external view of valve of *Mastogloia mattheai* (3-I); *Lampriscus* sp. (3-J); Biddulphia sp. (3-K); external view of valve of *Mastogloia fimbriata* (3-L).


A
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B
SZN 2 LEI 5.0kV X2,300 WD 8.6mm 10μm
C
SZN 2 LEI 5.0kV X1,700 WD 8.6mm 10μm
D
SZN 2 LEI 5.0kV X350 WD 8.5mm 10μm
E
SZN 2 LEI 5.0kV X6,000 WD 8.6mm 1μm
F
SZN 2 LEI 5.0kV X3,500 WD 8.6mm 1μm

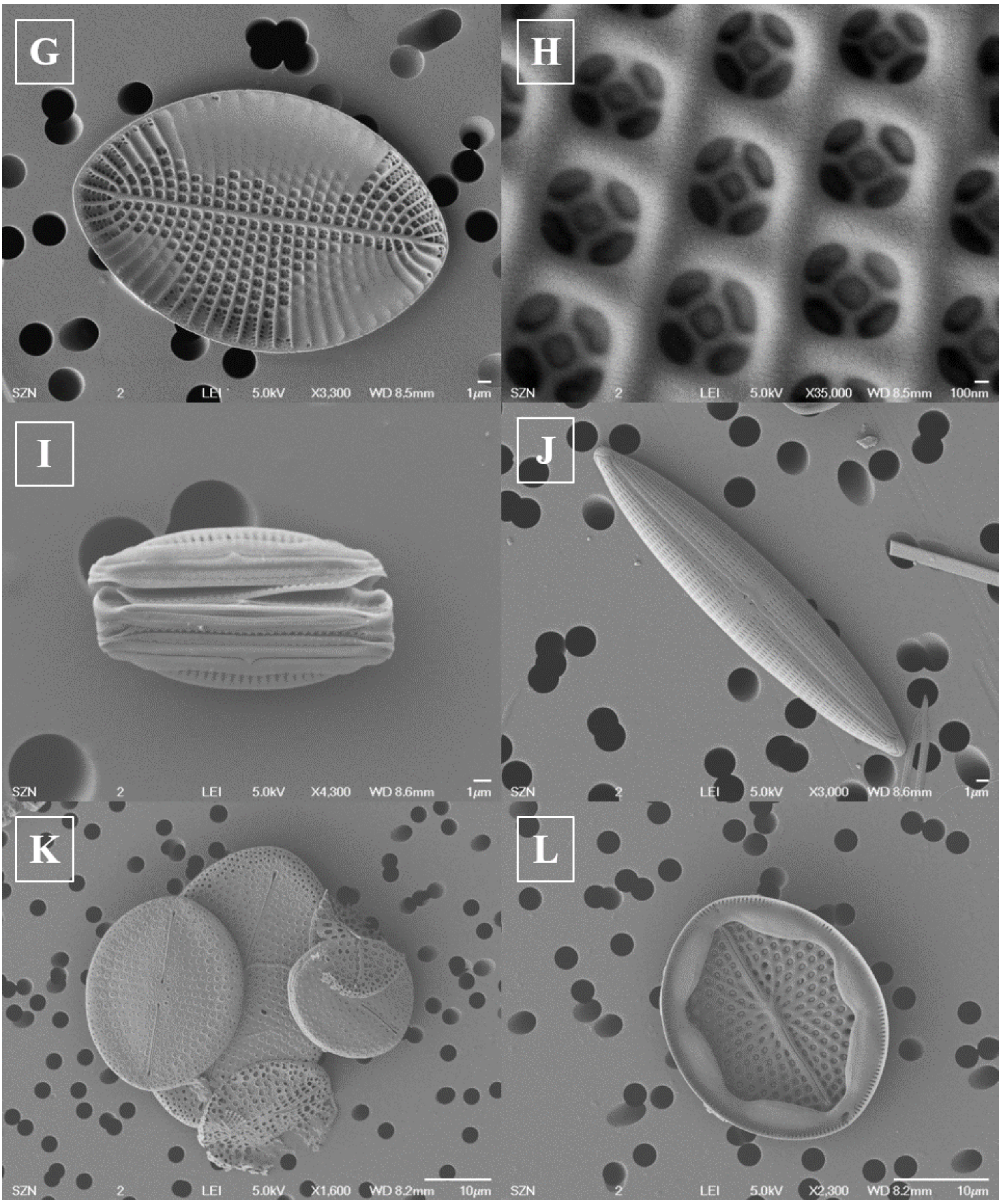

Figure 4. Example of images captured through SEM, belonging to the control area of Lacco Ameno; sampling season: spring; internal view of a valve of *Rhopalodia* sp. (4-A); *Cocconeis* sp. (4-B, C); internal view of a valve of *Licmophora* sp. (4-D); *Tryblionella* sp. and *Amphora* sp. (4-E); internal view of sternum valve (4-F,G) and detail of the pores (4-H) of *Cocconeis scutellum* var. *posidoniae; Amphora* sp. (4-I); external view of valve of *Navicula* sp. (4-J); external (4-K) and internal (4-L) view of valve of *Mastogloia fimbriata*.

ANNEX II

Papers published in collaboration and related to my PhD project

marine drugs

Article

Two Benthic Diatoms, *Nanofrustulum shiloi* and *Striatella unipunctata*, Encapsulated in Alginate Beads, Influence the Reproductive Efficiency of *Paracentrotus lividus* by Modulating the Gene Expression

Francesca Glaviano [1,2,†], Nadia Ruocco [1,†], Emanuele Somma [1,3,†], Giuseppe De Rosa [1,4], Virginia Campani [4], Pasquale Ametrano [1,2], Davide Caramiello [5], Maria Costantini [1,*] and Valerio Zupo [1,*]

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Abstract: Physiological effects of algal metabolites is a key step for the isolation of interesting bioactive compounds. Invertebrate grazers may be fed on live diatoms or dried, pelletized, and added to compound feeds. Any method may reveal some shortcomings, due to the leaking of wound-activated compounds in the water prior to ingestion. For this reason, encapsulation may represent an important step of bioassay-guided fractionation, because it may assure timely preservation of the active compounds. Here we test the effects of the inclusion in alginate (biocompatible and non-toxic delivery system) matrices to produce beads containing two benthic diatoms for sea urchin *Paracentrotus lividus* feeding. In particular, we compared the effects of a diatom whose influence on *P. lividus* was known (*Nanofrustulum shiloi*) and those of a diatom suspected to be harmful to marine invertebrates, because it is often present in blooms (*Striatella unipunctata*). Dried *N. shiloi* and *S. unipunctata* were offered for one month after encapsulation in alginate hydrogel beads and the larvae produced by sea urchins were checked for viability and malformations. The results indicated that *N. shiloi*, already known for its toxigenic effects on sea urchin larvae, fully conserved its activity after inclusion in alginate beads. On the whole, benthic diatoms affected the embryogenesis of *P. lividus*, altering the expression of several genes involved in stress response, development, skeletogenesis and detoxification processes. Interactomic analysis suggested that both diatoms activated a similar stress response pathway, through the up-regulation of *hsp60*, *hsp70*, *NF-κB*, *14-3-3 ε* and *MDR1* genes. This research also demonstrates that the inclusion in alginate beads may represent a feasible technique to isolate diatom-derived bioactive compounds.

Keywords: encapsulation; microalgae; modulated genes; sea urchin development

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Protandric Transcriptomes to Uncover Parts of the Crustacean Sex-Differentiation Puzzle

Tom Levy[1†], Valerio Zupo[2*], Mirko Mutalipassi[2], Emanuele Somma[2], Nadia Ruocco[2], Maria Costantini[2], Shai Abehsera[1], Rivka Manor[1], Vered Chalifa-Caspi[3], Amir Sagi[1,3*†] and Eliahu D. Aflalo[1,4*†]

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Hermaphrodite systems offer unique opportunities to study sexual differentiation, due to their high degree of sexual plasticity and to the fact that, unlike gonochoristic systems, the process is not confined to an early developmental stage. In protandric shrimp species, such as *Hippolyte inermis* and *Pandalus platyceros*, male differentiation is followed by transformation to femaleness during adulthood. The mechanisms controlling sexual differentiation have not been fully elucidated in crustaceans, but a key role has been attributed to the insulin-like hormone (IAG) produced by the androgenic gland (AG), a crustacean masculine endocrine organ. To uncover further transcriptomic toolkit elements affecting the sexual differentiation of *H. inermis*, we constructed eye and whole body RNA libraries of four representative stages during its protandric life cycle (immature, male, young female and mature female). The body libraries contained transcripts related to the reproductive system, among others, while the eye libraries contained transcripts related to the X-organ-sinus gland, a central endocrine complex that regulates crustacean reproduction. Binary pattern analysis, performed to mine for genes expressed differentially between the different life stages, yielded 19,605 and 6,175 transcripts with a specific expression pattern in the eye and body, respectively. Prominent sexually biased transcriptomic patterns were recorded for the *IAG* and *vitellogenin* genes, representing, respectively, a key factor within the masculine IAG-switch, and a precursor of the yolk protein, typical of feminine reproductive states. These patterns enabled the discovery of novel putative protein-coding transcripts exhibiting sexually biased expression in the *H. inermis* body and eye transcriptomes of males and females. Homologs to the above novel genes have been found in other decapod crustaceans, and a comparative study, using previously constructed transcriptomic libraries of another protandric shrimp, *P. platyceros*, showed similar sexually biased results, supporting the notion that such genes, mined from the *H. inermis* transcriptome, may be universal factors related to reproduction and sexual differentiation and their control in other crustaceans. This study thus demonstrates the potential of transcriptomic studies in protandric species to uncover unexplored layers of the complex crustacean sex-differentiation puzzle.

Keywords: androgenic gland, hermaphrodite, *Hippolyte inermis*, IAG-switch, *Pandalus platyceros*, protandry, reproductive physiology, sex-differentiation

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Research

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Ferroptosis precedes apoptosis to facilitate specific death signalling by fatty acids

Valerio Zupo[1,†], Maria Costantini[2,†], Eliahu D. Aflalo[3,5], Tom Levy[10,14], Vered Chalifa-Caspi[8], Olabiyi Obayomi[7,9], Mirko Mutalipassi[11,15], Nadia Ruocco[12], Francesca Glaviano[1], Emanuele Somma[1,13], Paola Nieri[6,8] and Amir Sagi[3,4]

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Cell death is physiologically induced by specific mediators. However, our power to trigger the process in selected cells is quite limited. The protandric shrimp *Hippolyte inermis* offers a possible answer. Here, we analyse a de novo transcriptome of shrimp post-larvae fed on diatoms. The sex ratio of diatom-fed shrimps versus shrimps fed on control diets was dramatically altered, demonstrating the disruption of the androgenic gland, and their transcriptome revealed key modifications in gene expression. A wide transcriptomic analysis, validated by real-time qPCR, revealed that ferroptosis represents the primary factor to re-shape the body of this invertebrate, followed by further apoptotic events, and our findings open biotechnological perspectives for controlling the destiny of selected tissues. Ferroptosis was detected here for the first time in a crustacean. In addition, this is the first demonstration of a noticeable effect prompted by an ingested food, deeply impacting the gene networks of a young metazoan, definitely determining its future physiology and sexual differentiation.

ANNEX III

Other contributions

Oral communication at congresses

Emanuele Somma, Francesca Glaviano, Maria Costantini, Antonio Terlizzi and Valerio Zupo (2022). Oral communication: "Effect of Ocean Acidification on the production of infochemicals and other important metabolites by the benthic diatom *Cocconeis neothumensis* in *Posidonia oceanica* meadows". 32nd ESCPB Congress *Comparative physiology and biochemistry in the Anthropocene: understanding the physiological and biochemical complexity of a changing world.*

Emanuele Somma, Valerio Zupo and Antonio Terlizzi (2021). Oral communication: "Microalgal associations on seagrass meadows: effects of global changes on the first colonization and plant-animal chemical relationships". *Incontro dei dottorandi e dei giovani ricercatori in Ecologia e Scienze dei Sistemi Acquatici*

Papers and book chapters in collaboration

Brundu, G., Cannavacciuolo, A., Nannini, M., **Somma, E.**, Munari, M., Zupo, V., & Farina, S. (2023). Development of an efficient, noninvasive method for identifying gender year-round in the sea urchin *Paracentrotus lividus*. Aquaculture, 564, 739082.

Zupo, V., & **Somma, E**. (2022). Crustacean Decapods are Models to Describe the General Trends of Biodiversity According to Ocean Acidification. In Crustaceans (pp. 109-120). CRC Press.

Esposito, R., Federico, S., Glaviano, F., **Somma, E**., Zupo, V., & Costantini, M. (2022). Bioactive Compounds from Marine Sponges and Algae: Effects on Cancer Cell Metabolome and Chemical Structures. International Journal of Molecular Sciences, 23(18), 10680.

Mutalipassi, M., Riccio, G., Mazzella, V., Galasso, C., **Somma, E**., Chiarore, A., ... & Zupo, V. (2021). Symbioses of cyanobacteria in marine environments: Ecological insights and biotechnological perspectives. Marine Drugs, 19(4), 227.

Gregorin, C., Albarano, L., **Somma, E**., Costantini, M., & Zupo, V. (2021). Assessing the ecotoxicity of copper and polycyclic aromatic hydrocarbons Comparison of effects on *Paracentrotus lividus* and *Botryllus schlosseri*, as alternative bioassay methods. Water, 13(5), 711.