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Ocean acidification alters the responses of invertebrates to wound-activated infochemicals produced by epiphytes of the seagrass *Posidonia oceanica*

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ABSTRACT

Ocean acidification (OA) influences the production of volatile organic compounds (VOCs) by seagrass leaves and their associated epiphytes. We hypothesize that the perception of “odour” produced by seagrass leaf epiphytes will change with seawater acidification, so affecting the behaviour of seagrass-associated invertebrates. To test this hypothesis, we collected epiphytes from leaves of *Posidonia oceanica* growing at two pH conditions (7.7 and 8.1) and identified the most abundant genera of diatoms. We tested the VOCs produced at pH 8.1 by the epiphytic communities *in toto*, as well as those produced by selected diatoms, on various invertebrates. A complex set of species-specific and concentration-dependent chemotactic reactions was recorded, according to the pH of seawater. In particular, VOCs produced by individual diatoms triggered contrasting reactions in invertebrates, depending on the pH. The perception of epiphyte VOCs is likely to vary due to alteration of species ability to perceive and/or interpret chemical cues as infochemicals or due to changes in the structure of VOCs themselves. Thus, OA alters the fine-tuned chemical cross-talks between seagrass epiphytes and associated invertebrates, with potential consequences for the structure of communities and food webs of seagrass ecosystems.

Key words: ocean acidification; seagrass; climate change; diatoms; invertebrates

1. INTRODUCTION

Seagrasses produce stable and diverse environments, playing both structural and trophic roles (Coll et al., 2011; Duarte et al., 2010; Fourqurean et al., 2012). They are "ecosystem engineers", generating physical modifications of their environment that influence the structure and the dynamics of associated communities (Sanders et al., 2014; Stingl, 2018) with beneficial or harmful consequences on the engineer itself (Blake and Duffy, 2010; Whalen et al., 2013). Seagrasses create, in cooperation with associated epiphytic and vagile communities, a stable and complex framework (Unsworth et al., 2015) that promptly responds to extrinsic environmental perturbations (Castejón-Silvo et al., 2012). The stable coexistence (Michel et al., 2015) of plant and animal communities influences food webs (Farina et al., 2009), characterized by seasonal variations of energy flows (Prado et al., 2013; Dattolo et al., 2014).

Posidonia oceanica (L.) Delile is the most stable, long-living and widely distributed seagrass in the Mediterranean (Procaccini et al., 2003). The complex trophic relationships between *P. oceanica*, its epiphytes and the associated animal communities are known (Mazzella et al., 1992). However, the importance of chemical interactions that facilitate the functioning of these ecosystems and the flow of information within the meadows is only recently emerging (Vos et al., 2006; Zupo et al., 2016). Trophic and non-trophic interactions may affect the stability of associated communities (Hammill et al., 2015), as exemplified by the shrimp *Hippolyte inermis,* a common inhabitant of *Posidonia* meadows (Zupo, 2000). Chemical information is often mediated by volatile organic compounds (VOC), released from wounded micro-algae and critical for various invertebrates, to detect the presence of predators and identify food sources (Jüttner et al., 2010; Zupo et al., 2016). Recent laboratory experiments demonstrated that invertebrates are able to recognize the "odours" of microalgae present in their own environment and to decipher their meaning, while they are unable to understand chemical signals produced by the wounding of microalgae living in external environments (Maibam et al., 2014). In addition, it has been demonstrated that several invertebrates recognize the VOCs produced by leaves of *P. oceanica* and they are attracted or repelled by these chemical signals, depending on their habitat preferences (Zupo et al., 2014).

However, anthropogenic environmental pressures are producing a widespread degradation of ecosystems and the services they provide (Harfoot et al., 2014), causing alterations of animal's behaviour in response to chemical signals (Munday et al., 2009; Zupo et al., 2016, 2015). It is forecasted that the pH of oceans will drop by 0.3-0.4 units over the next century (Caldeira and Wickett, 2005; Pachauri et al., 2014) due to anthropogenic CO_2 emissions into the atmosphere (Campbell and Fourqurean, 2013). The pH decrease of oceans will have deleterious consequences for several benthic organisms and communities (Gambi et al., 2016; Guerrero-Meseguer et al., 2017; Hall-Spencer et al., 2008) and it is responsible for impacts on ecosystem services of seagrasses (Zunino et al., 2019). At lower pH, calcareous organisms including algae suffer from higher decalcification rates (for reviews, see Foo et al., 2018; Gonzalez-Delgado and Hernández, 2018; Porzio et al., 2013). Consequently, calcareous algae are almost absent on leaves of *P. oceanica* under acidified conditions (Hall-Spencer et al., 2008; Martin et al., 2008; Nogueira et al., 2017).

Nevertheless, as mentioned above, chemical information flowing through ecosystems may play a vital role for several organisms (Briffa et al., 2012). Thus, besides the physiological effects of seawater acidification that directly hamper the survival of various species (Porzio et al., 2018), its effect on chemical communications must be considered (Wyatt et al., 2014; Zupo et al., 2014). While several organisms might adapt to acidification by over-expressing some genes involved in the calcification process (Porzio et al., 2017; Tynyakov et al., 2015), they might not survive if their responsiveness to critical chemical signals decreases (Dixson et al., 2010; Zupo et al., 2014). Hence, the answers of key invertebrates to the "odours" produced by micro- and macro-algae, tested in acidified conditions, permit to forecast their potential resilience (Ricevuto et al., 2012; Zupo et al., 2015). Wound-activated plant VOCs include key information-transmitting compounds (so-called *semiochemicals*; Dicke and Sabelis, 1988) as demonstrated in diatoms (Nappo et al., 2009), acting as mediators of organismal interactions or pheromones and allelochemicals involved in induced and activated defences (Kuhlisch and Pohnert, 2015; Thoms and Schupp, 2008). Their recognition is crucial for invertebrates (Dicke and Sabelis, 1988) to detect the presence of food sources (Maibam et al., 2015) or the activity of predators (Fink, 2007; Moelzner and Fink, 2014). Volatile organic compounds are quickly delivered to target organisms, even at a long distance from the source of the "odour" (Kaasik et al., 2011; Lewis et al., 2012). Wound-activated VOCs may indicate the presence of consumers (Moelzner and Fink, 2015; Pohnert et al., 2007), thereby eliciting an active reaction by the receiver organisms. Ocean acidification can alter these communications and, accordingly, invertebrate behavioural responses to infochemicals (Briffa et al., 2012; Roggatz et al., 2016), leading to dramatic modifications of invertebrate communities (Garrard et al., 2014). Such 'misunderstandings' in chemical communications can be due to changes in the infochemical metabolism of 'producers' or to the ability of 'receivers' to identify and correctly interpret signals (Roggatz et al., 2016).

This study fundamentally aims at deciphering plant-animal chemical communication in a range of environmental conditions, taking into account the effect of pH on the present assemblage of epiphytes, as indirect effects of acidification on the physiology of key invertebrates. Algal epiphytes settled on *Posidonia* leaves naturally growing either at pH 8.1 or 7.7, which respectively represent current and predicted (end of the century) values of sea surface acidity, were taxonomically identified. In addition, to investigate invertebrate's chemotactic responses in a changing ocean, VOCs were extracted from the whole epiphytic community and offered to a number of target invertebrates in normal and in acidified conditions, to compare their behavioural responses. We also tested the chemotactic reactions of invertebrates to the VOCs produced by two of the main epiphytic microalgal taxa, to analyse their individual effects.

2. MATERIAL AND METHODS

2.1 Collection sites

Leaves of *Posidonia oceanica,* along with their epiphytes, were collected in spring, at a site off Castello Aragonese d'Ischia, in the north-eastern sector of the Island of Ischia (Bay of Naples, Italy; 40°43.853'N, 13°57.698'E). Along the coast, a shore islet is present, characterized by the presence of several submerged areas characterized by volcanic emissions of CO_2, both on the north and south sides (Tedesco, 1996). In particular, on the south side, according to the intensity of CO_2 emissions the pH of the seawater drops along a gradient from normal pH (8.1, control station) down to an intermediate acidified zone (7.8), until an exceptionally low value of 6.4 (Foo et al., 2018; Kerrison et al., 2011). From 2 to 3 m depth, where the pH varies from 8.1 to 7.7, the area is characterized by the presence of a *Posidonia oceanica* meadow (Garrard et al., 2014; Hall-Spencer et al., 2008; Lauritano et al., 2015; Ravaglioli et al., 2017; Scartazza et al., 2017). A plethora of studies approached the vents at Aragonese Castle of Ischia, as proxies to assess the potential impacts of climate-driven OA (Foo et al., 2018). pH at south sites of the Castle was assessed by means of various deployments. The data referred to our sampling sites are collected by the National Bureau of Standards pH (pHNBS) logged hourly everyday by means of a SeaFETTM (Foo et al., 2018). These vents provide a model system to assess impacts of decreased pH on organism and species interactions.

2.2 Collection of epiphyte pools

Five shoots of *Posidonia oceanica* were collected by scuba divers at each selected pH. Shoots were randomly collected over a surface area of about 20 m^2 and immediately transferred to the laboratory. Shoots collected in the acidified area were shorter than those living at normal pH, due to strong grazing activity operated by such herbivorous fish as *Sarpa salpa*. Thus, all leaves collected in the acidified area had lost most of their apical portion and tips (Donnarumma et al., 2014). Due to these morphological differences and to avoid comparisons between different portions of leaves, only epiphytes growing on the central parts of leaves were considered for our experiments. In particular, the lowest 10 cm of intermediate and adult leaves were discharged; the next 20 cm were scraped and used for VOC extractions; the remaining apical portion and tips, when present, were again discharged. Epiphytes present on the surface of leaf portions (central parts) were collected by means of a steel razor blade gently dragged (several times) over the leaf. The epiphytes collected from leaves at normal pH were weighed (fresh weight) and immediately frozen (-20°C), to be used for the extraction of VOCs a few hours prior to starting choice tests. In addition, three subsamples of scraped epiphytes were weighed (fresh weight), wrapped in aluminium foils and dried in an oven (65 °C) until constant weight (dry weight). The fresh weight/dry weight ratio was calculated, to be used for evaluation of the actual biomass of epiphytes present at both pH conditions. Finally, three small portions (1 cm long; 3 replicates for each site) of leaves from both pH zones were cut and conserved for taxonomic analyses of epiphytes. These samples were immediately fixed in 4% glutaraldehyde and stored at 5°C.

2.3 Taxonomic identification of micro- and macroalgae

Fixed samples were dehydrated, gold sputtered and observed under Scanning Electron Microscope. Diatoms present on the internal surface of each leaf were identified in randomly located fields, up to the genus level, and the number of diatom cells of each genus was recorded to evaluate their abundance according to the leaf surface area. The most abundant genera were identified at species level and the numerical abundance of each species in each sector was recorded. Diatoms were also classified into three categories, according to growth-forms, taking into account their posture (prostrate or erect) and mobility (sessile, low and high mobility) as follows:

A) Erect: sessile species attached to the substrate by mucilage pads or stalks (Er);

B) Adnate: strongly adhering with the valve face to the substrate and having a scarce motility (Ad);

C) Biraphid: motile species (Mt).

In addition, nine adult leaves were randomly collected in each site for taxonomic identification of epiphytic macroalgae, and sections of 10 cm were cut in the middle part of each leaf. These sections were fixed in 2% buffered formalin and used for taxonomic analyses. Algae were collected from the surface with the aid of steel forceps, mounted on microscopy slides and observed to be identified under light microscopy; further, the total algal coverage (according to Boudouresque, 1971) was evaluated under a stereo-microscope. The percentage cover of each macroalgal species was recorded and total algal coverage (Mazzella et al., 1989; Panayotidis et al., 2004) was calculated.

2.4 Cultivation of diatoms

Two diatoms, found to be the most abundant epiphytic species on *P. oceanica* leaves, were isolated and axenically cultivated to test their individual effects as compared to the effect of the whole epiphytic community. Previous investigations (Maibam et al., 2014) indicated the informational role of some diatoms, e.g. *Cocconeis* spp., isolated from the epiphytic layer of *Posidonia oceanica*. Thus, monoclonal cultures of *Cocconeis scutellum* var. *posidoniae* and *Cocconeis neothumensis* var. *marina* were obtained. Both diatoms were grown in a thermostatic chamber (at 18°C) at an average irradiance of 100 µmol photons m^{-2} s^{-1} in sterile 14 cm diameter Petri dishes containing *f/2* medium (Raniello et al., 2007). After 15 days, when diatoms covered almost evenly the glass surface, the dishes were frozen (-20 °C) until the extraction of their VOCs, as indicated below.

2.5 Extraction of VOCs

Volatile organic compounds (VOCs) were extracted twice from samples of 3.0 g of frozen epiphytes (FW) collected over the leaves of *Posidonia oceanica* growing at normal pH (8.1). Epiphytic pools were ground in a mortar under liquid nitrogen, then hand-pottered and sonicated, to initiate the wound-activated enzyme cascade that leads to the liberation of VOCs (Pohnert, 2000). VOCs were concentrated by closed-loop stripping (Jüttner, 1988) performed at 22°C for 45 minutes. For this purpose, 3 g of ground and sonicated epiphytes were suspended in 40 mL of filtered (Millipore 0.25 µm) seawater and transferred to a 100 mL round bottom flask. After addition of 10 g of NaCl their VOCs were extracted and absorbed on a Tenax TA cartridge (Fink et al., 2006a, 2006b). The cartridge was removed and eluted with 6 mL diethyl ether. Ether was gradually evaporated using nitrogen gas (N_2, grade 5.0) and the residue was re-dissolved into 300 µL of pure ethanol. Controls were prepared according to the same procedure, but stripping was performed on filtered and sterilized seawater without the addition of epiphytes. VOC samples and controls were stored at -80°C, until the start of choice tests.

Similarly, VOCs were extracted twice from the diatom suspensions (2 × 40 mL) obtained for each species of diatoms, by scraping twice the bottom of each Petri dish by means of a steel blade. VOCs were concentrated by closed-loop stripping (Jüttner, 1988) performed at 22°C for 45 min. For this purpose, a suspension (40 mL) of the sonicated diatoms was transferred to a 100 mL round bottom flask and the VOCs extracted on a Tenax TA cartridge, after addition of 10 g NaCl (Fink et al., 2006a, 2006b) as above indicated for other epiphytes. In the case of diatoms, the biomass scraped off from 10 Petri dishes (about 5 mg of dry weight from each dish, on average) was extracted and tested in choice arenas at a single concentration, corresponding to about 5 cm^2 of leaf area surface (Buia et al., 1992), hypothetically covered by a single species of diatoms, simulating the scraping by a benthic grazer, as suggested by Maibam et al. (2014).

2.6 Collection of invertebrates

Invertebrates for behavioural tests were collected in a *Posidonia oceanica* meadow off Lacco Ameno (Bay of Naples), located 6 km far from the volcanic site (Zupo et al., 2006) and characterized by normal (8.1) pH (not exposed to seawater acidification; Garrard, 2013; Ricevuto et al., 2015). To this end, a circular plankton net (1 m frame diameter; mesh size 100 µm) was horizontally trawled from a boat over the plant leaves. Invertebrates were collected in a glass jar fixed to the end of the net. A preliminary sorting was performed on board and specimens of various invertebrates were pooled into plastic bags containing clean seawater, to be transported to the laboratory. All invertebrates were identified *in vivo* by specialists under a stereomicroscope and immediately moved into aerated vessels kept in a thermostatic chamber at 18 °C, in presence of small pieces of *Posidonia* leaves (for shelter and food) up to the day preceding the experiment. Only invertebrate species with at least 30 healthy individuals were considered for the choice tests. Thirteen species of *Posidonia*-associated invertebrates were selected. They all belonged to the main meso-faunal taxa present in seagrass environments, as polychaetes, gastropods molluscs, isopods, amphipods and decapod crustaceans (Zupo et al., 2016, 2015). All invertebrates were starved 24 hours prior to the behavioural tests. Specimens to be assayed at low pH were slowly acclimatised to acidified water (pH 7.7), starting the night before the experiment. After the experiments in acidified water, each specimen was kept at pH 7.7 in glass vessels prior to be slowly re-acclimatised to pH 8.1. The survival of invertebrates has been recorded (SM1) at the end of the experiments and all specimens still alive after the completion of the experimental procedures were returned to the sea.

2.7 Tests on invertebrates

Static chambers (Jüttner et al., 2010) were used to test the effect of epiphyte VOCs on invertebrates. For this purpose, VOCs were included into small agarose blocks (Maibam et al., 2014). Agarose gels were prepared by dissolving 1.2 g of agarose (Sigma A-9045) in 200 mL of filtered and sterilized seawater, so obtaining a concentration of 0.06%. The agarose was stirred at 80 °C until completely dissolved. An addition of 3.3 mL of 0.1 M NaOH was used to adjust the pH to a value close to 8.1, in order to assure chemical stability of infochemicals in their original form, up to the diffusion in the water and avoid degradation during storage. Three concentrations of ethanolic VOC extracts, *i.e.*, 250, 25 and 2.5 µL, were incorporated, respectively, into almost cold agarose, just before gelling, to obtain three doses of infochemicals, namely, “low”, “medium” and “high”. The low concentration simulates the VOCs released by the epiphytes growing on 5 mm^2 of *Posidonia* leaf, wounded by a small grazer. The medium concentration corresponds to epiphytes growing on 50 mm^2 of *Posidonia* leaf wounded by a large grazer. The high concentration corresponds to the epiphytes growing on 500 mm^2 of *Posidonia* leaf wounded by an herbivorous fish or a similar larger grazer (Maibam et al., 2014). To prepare controls, 250, 25 and 2.5 µL of the control extract were incorporated into still liquid warm agarose, just before gelling, to obtain three concentrations of control gels. The agarose solutions were then poured into Petri dishes and allowed to gel in a refrigerator at 5°C, 1 h prior to the start of assays. Finally, the agarose gel was cut into 0.5 cm^3 blocks to be used for the choice tests on invertebrates.

Thirteen invertebrate species, representative of various feeding habits and various levels of associations to *Posidonia oceanica* leaves (Table 1) were selected for the test. We expected that responsive organisms moved along the VOC gradient according to their preferences (Chase, 1982).

Table 1. Thirteen species of invertebrates tested for their responses to volatile produced by *P. oceanica* epiphytes and their trophic habits. The level of association to *P. oceanica* meadows is categorized in the last column (assoc.): 1, occasionally present; 2, typical; 3, generally abundant; 4, almost exclusive.

Taxon	**Species**	**Trophic habits**	**Association level**
Polychaete	*Platynereis dumerilii* (Audouin & Milne - Edwards, 1834)	herbivore	1
Isopod crustacean	*Dynamene bifida* Torelli, 1930	herbivore	1
Amphipod crustacean	*Caprella acanthifera* Leach, 1814	omnivore	3
Amphipod crustacean	*Gammarella fucicola* (Leach, 1814)	herbivore	1
Decapod crustacean	*Hippolyte inermis* (Leach, 1815)	herbivore/omnivore	4
Decapod crustacean	*Cestopagurus timidus* (Roux, 1830)	omnivore/carnivore	4
Decapod crustacean	*Calcinus tubularis* (Roux, 1830)	omnivore/carnivore	1
Gastropod	*Rissoa italiensis* Verduin, 1985	herbivore	3

Gastropod	*Rissoa variabilis* (Von Mühlfeldt, 1824)	herbivore	2
Gastropod	*Rissoa violacea* Desmarest, 1814	herbivore	2
Gastropod	*Bittium latreilli* (Payraudeau, 1826)	detritus feeder	1
Gastropod	*Gibbula umbilicaris* (Linnaeus, 1758)	herbivore	3

Assays were conducted in 14 cm diameter Pyrex Petri dishes, located over printed circular experimental arenas, according to the protocol suggested by Jüttner et al. (2010). Each arena consisted of five sectors, (-2, -1, 0, 1, 2) indicating the rate of repulsion or attraction, according to the movements of invertebrates (Jüttner et al., 2010; Zupo et al., 2016). Thereby, annotations refer to the distance from the "positive" target in sector +2 (*i.e.*, the one containing the agarose added with VOCs) while -2 is the one containing the control agarose; "0" is the central sector, intermediate between the positive target and the negative control.

Millipore (0.45µm) filtered seawater was enriched with CO_2 for the experiments in acidified conditions, using a Ferplast CO_2 Energy® reactor, which allows acidification through CO_2 additions without producing bubbling. Seawater pH was adjusted to 7.7 and checked prior to filling the experimental arenas and at the end of each test, using a pH meter "SG2 SevenGo" (Mettler Toledo GmbH). Due to the short time needed to complete each replicate test, the pH level inside arenas was stable during the experiment and the pH checked at the end of each test confirmed that variations due to respiration or influence of agarose gels were less than 0.1. In fact, Petri dishes were filled with 200 mL of seawater prior to start each replicate of the test and, after 20 minutes, each dish was emptied and refilled to avoid any stress for experimental organisms. Five individuals of each invertebrate species were released at the centre of each arena. Subsequently, the number of animals in each sector of the arena was recorded at four time intervals (5, 10, 15 and 20 minutes). To minimize external factors that could possibly influence the chemotactic reactions of animals during the experiment (*e.g.*, light, temperature, magnetism, etc.), all experiments were conducted at 18°C under a well-lit and diffused light, and each replicated two arenas were positioned in such a way that the positive targets opposed each other. Six replicate assays were conducted for each of 13 invertebrate species, each VOC source, three concentrations, at normal pH (8.1) and in acidified seawater (pH 7.7). The diffusion of VOCs into the experimental arenas and the gradient produced were calibrated using the method reported by Zupo et al. (2015).

2.8 Statistical treatment of data

Statistical differences in leaf length and total epiphyte coverage of *P. oceanica* and abundance of micro- and macro- algal epiphytes were assessed at different pH by multiple two-tailed Student's *t*- test, repeated for each variable. To this end, GraphPad Prism 5.0 for Windows (GraphPad Software, La Jolla California USA, www.graphpad.com) was used for the analyses. The significance of the spatial distributions observed at the end of each trial was evaluated by means of z-test on proportions. In particular, the proportion of individuals present in "+" sectors at the end of the experiment was compared to the proportion of individuals present in "-" sectors and the z-test indicated if the observed distribution was significantly different from a normal distribution (Sprinthall, 2011). In addition, a Preference Index (named "*Integer*" according to Jüttner et al. 2010) and the angular coefficient of each regression line (A.C, according to Jüttner et al. 2010) computed taking into account all readings at each time interval, in each sector of experimental arena, were evaluated for each species of invertebrate, referred to the VOCs of epiphytes tested under both experimental conditions (acidified and normal pH). The preference index (Jüttner et al. 2010) indicates the time spent by the experimental individuals in different sectors, at each time step of experiments. Positive values indicate a longer time spent in the positive sectors while negative values indicate a longer time spent in the negative sectors; values close to 0 indicate limited movements or random movements in both directions. The standard error of the means (SEM) was also evaluated for each odour concentration, according to James et al. (2008), to measure the response of each species avoiding the scattering of results due to natural random movements of invertebrates. Finally, a Cluster Analysis (Statistica 10, StatSoft) was performed on the matrix “treatments *vs*. arena sectors” to group invertebrate species according to their behavioural response patterns towards the epiphyte odours, tested in acidified and in normal pH seawater, respectively.

3. RESULTS

3.1 Epiphyte associations: macroalgae

The shape of *Posidonia oceanica* leaves and the taxonomical composition of their algal epiphytes exhibited marked differences between the two pH levels. At normal pH leaves were slightly longer (27.98 cm ±4.94, SEM) than those at the low pH site (21.03 cm ±1.85 SEM, Table 2), even though differences were not statistically significant. The difference is likely attributed to the fish-grazing pressure, as 100 % of leaves growing at low pH exhibited broken apices with typical bites indicating grazing activity of *Sarpa salpa* (Buia et al., 2004; Ferrari et al., 2008). The largest difference, however, was exhibited by total coverage (%) of the epiphytic assemblage, which reached more than 70 % of the leaf surface at pH 8.1 and only 12 % at pH 7.7 (two-tailed *t*-test: t = 6.57, d.f. = 16, P < 0.0001). This difference was even higher for calcareous algae (coralline algae); they covered 80 % of the leaf surface at pH 8.1, while they were absent at pH 7.7 (two-tailed *t*-test: t = 19.75, d.f. = 16, P < 0.0001, Table 2). In addition, leaves growing at normal (8.1) pH were characterized by a diverse macroalgal community dominated by *Pneophyllum fragile* and

Hydrolython spp., while the community living at the acidified site contained only two species of macroalgal epiphytes represented by *Myrionema orbiculare* (94 %), here significantly higher than at pH 8.1 (two-tailed *t*-test: t = 47.60, d.f. = 240, P < 0.0001) and *Sphacelaria cirrosa* (6 %, Table 2, Figure 1).

Table 2. Summary of the main features of *Posidonia oceanica* leaves and their Total epiphytic coverage in meadows studied at normal pCO_2 (pH 8.1) and at the acidified site (pH 7.7). n = 9; "+" indicates epiphytic presence estimate as a cover < 1%. Average data ±SEM are reported. It should be noted that the leaf coverage calculated for different algae can exceed 100%, due to the presence of several layers in the algal assemblages, as it is referred to the "total coverage" of algal assemblages, quantified as "the sum of % of surface covered by each species over the portion of leaf surface", as normally reported in phytobenthic research (Boudouresque, 1971; Mazzella et al., 1989; Panayotidis et al, 2004).

	Normal pCO_2/ pH (8.1)	**High pCO_2/low pH (7.7)**
	Mean ± SEM	**Mean ± SEM**
Leaf length (cm)	27.98 ± 4.94	21.03 ± 1.85
Broken apices	78%	100%
Total coverage (%) of the epiphytic assemblage	70.56 ± 3.48	12.00 ± 2.73
	of which	
Calcareous algae coverage (%)	79.78 ± 4.04	-
Macroalgal epiphytes (% cover):		
Acrothamnion preissii	+	-
Asperococcus sp	+	-
Castagnea irregularis	1.22 ± 0.60	-
Ceramium codii	1.00 ± 0.58	-
Ceramium flaccidum	+	-
Colaconema daviesii	+	-
Crouania attenuata	+	-
Dictyota dichotoma var. intricata	1.11 ± 0.56	-
Giraudya sphacelarioides	2.44 ± 1.17	-

Hydrolithon cruciatum	11.44 ± 2.11	-
Hydrolithon farinosum	31.67 ± 2.36	-
Laurencia sp.	+	-
Myrionema orbiculare	8.67 ± 2.05	93.89 ± 2.86
Pneophyllum fragile	35.00 ± 3.44	-
Polysiphonia scopulorum	2.44 ± 1.62	-
Sphacelaria cirrosa	2.89 ± 0.63	6.11 ± 2.86

3.2 Epiphyte associations: microalgae

Diatoms were abundant in the epiphytic layer of *P. oceanica* leaves, both at normal (8.1) and acidified (7.7) pH. In total, 21 genera of diatoms were identified, present at different abundances (Figure 2). *Cocconeis* was by far the most widely represented genus, reaching 92.7 % of the diatom flora in some samples (Figure 2 a) and on average, more than 75% of the total diatom coverage. Within the genus *Cocconeis*, *C. scutellum* var. *posidoniae* and *C. neothumensis* var. *marina* where the most frequent species and varied in abundance at sites with different pH. *C. scutellum* var. *posidoniae* was slightly more abundant at pH 8.1 compared to pH 7.7; in contrast, *C. neothumensis* var. *marina* was more abundant at pH 7.7 (two-tailed *t*-test: $t = 3.11$, d.f. = 24, $P < 0.005$, Figure 3). Other diatom genera did not significantly vary according to pH, except for the above mentioned *Cocconeis*, more abundant at pH 8.1 than at 7.7 (two-tailed *t*-test: $t = 2.14$, d.f. = 42, $P < 0.05$) and *Mastogloia*, among the rarest species, that also showed higher abundance at pH 8.1 than at 7.7 (two-tailed *t*-test: $t = 2.94$, d.f. = 49, $P < 0.01$; Figure 2). The abundance of "motile" (e.g. *Nitzschia* and *Navicula*) and "erect" (e.g. *Fragilaria* and *Grammatophora* spp.) diatoms was higher at the acidified site (although not significantly), while adnate diatoms, mainly represented by *Cocconeis* and some *Amphora* spp., were more abundant at normal pH (8.1).

3.3 Behavioural responses of invertebrates

VOCs extracted from the epiphytic layer of *P. oceanica* leaves grown at pH 8.1 produced behavioural responses in several invertebrates, depending on their concentration and pH, respectively (Figures 4). The preference indices characterizing each species at each condition indicate their responsiveness to VOCs (high positive or negative indices correspond to significant reactions as compared to random movements of animals in the experimental arenas; Figure 4). Positive indices indicate attraction, while negative indices indicate repulsion. The isopod *Dynamene bifida* is representative of the invertebrates most sensible to the presence of VOCs, since it showed high preference indices at low and intermediate concentrations, at both pH. In particular, at normal pH (pH 8.1), *Dynamene bifida* positively responded to the epiphyte odour at the medium concentration (Figure 4 b) and, to a lower degree, at the low concentration (Figure 4 a), while its response was not significantly different from random movements at the highest concentration (Figure 4 e). This species responded similarly at low pH, with significant attraction at medium concentration (Figure 4 d). However, its responsiveness at low and high concentration decreased (Figures 4 b, f), as compared to normal pH (Figure 4 a, e).

Also the crustacean decapod *Hippolyte inermis,* tested under normal pH, exhibited a concentration-dependent behaviour, from repellence at the lowest VOC concentration, over non-responsiveness, at intermediate VOC concentrations, up to attraction, at the highest VOC concentration (Figure 4a, c, e). At low pH, however, the shrimp’s responsiveness to epiphyte VOCs decreased dramatically. As a consequence, its chemotactic responses were not significant at low and medium VOC concentrations (Figures 4 b, d), while the shrimp was repelled at the highest concentration of algal VOCs (Figure 4 f) in a shape comparable to that observed at normal pH and minimum concentration.

In parallel another crustacean decapod, *Cestopagurus timidus,* exhibited a strong negative response to low and intermediate VOC concentrations at normal pH (Figure 4 a, c), whereas no significant response was detectable at the highest concentration (Figure 4 e). When tested at pH 7.7 (Figure 4 b, d, f), *C. timidius* exhibited absence of chemotactic responses at all VOC concentrations. In contrast, the pagurid *Calcinus ornatus*, exhibited a negative response to the highest VOC concentration at normal pH (Figure 4 e). Its reactions were inverted in acidified water, where a positive response was observed at the lowest VOC concentration (Figure 4 b). Among other crustaceans, amphipods appeared less responsive to the epiphyte VOCs at all concentrations. Only at the highest concentration and low pH (Figure 4f) *G. fucicola* showed a stronger chemotactic reaction of repulsion, significantly different from random movements.

Molluscs, on the whole, exhibited less evident chemotactic reactions, especially at the highest concentration. Among the congeneric species of gastropod mollusks, *Rissoa italiensis* and *R. variabilis* exhibited significant positive reactions at the highest concentration, when tested at normal pH (Figure 4 e). They exhibited contrasting responses at low pH (Figure 4 b, d, f), which indicates an altered responsiveness to the VOCs of leaf epiphytes in acidified waters. In contrast, *R. violacea*, exhibited a significant positive reaction at the lowest concentration, while *R. variabilis* exhibited a negative reaction at the intermediate concentration (Figure 4 d). A different gastropod, *Gibbula umbilicaris*, showed an evident repulsion at the lowest concentration, at normal pH (Figure 4 a), but its responsiveness appeared lowered in acidified water (Figure 4 b, d, f). Finally, *Bittium latreilli* is a mollusc not showing any significant chemotactic reaction at any concentration (Figure 4). Last among reacting species, but even less responsive than most molluscs, appears the polychaete *Platynereis dumerilii*, exhibiting significant reactions only at medium concentration and normal pH (Figure 4c).

3.4 Behavioural response to VOCs released from dominant epiphytic diatoms

Experiments performed with VOC extracts of the most abundant epiphytic diatoms, *Cocconeis scutellum* var. *posidoniae* and *C. neothumensis* var. *marina* (Figure 5), indicated that the isopod *Dynamene bifida* was significantly attracted to the VOCs of *C. scutellum* var. *posidoniae* at low pH (Figure 5 c) resembling its chemotactic reactions towards the odour of epiphytes at intermediate concentration (Figure 4 d). However, it did not significantly respond to the VOCs of *C. neothumensis* var. *marina*. The amphipod *Caprella acanthifera* exhibited a strong avoidance reaction to the odours of *C. scutellum* var. *posidoniae* (Figure 5 a), but a positive response to the VOCs released from *C. neothumensis* var. *marina* at normal pH (Figure 5 b). However, *C. acanthifera* did not show any significant reaction to the odour of either diatom under acidified (pH 7.7) conditions.

The decapod *Hippolyte inermis* exhibited a slight (but non-significant) negative response to the VOCs of *C. scutellum* var. *posidoniae* at normal pH (Figure 5 a) and a positive reaction to the VOCs produced by *C. neothumensis* var. *marina* at low pH (Figure 5 d). The hermit crab *Cestopagurus timidus* exhibited avoidance behaviour to the odour of *C. scutellum* var. *posidoniae* at normal pH, comparable to the one exhibited towards the whole epiphytic community at the low concentration (Figure 4 a). It also avoided the odour of *C. neothumensis* var. *marina* at normal pH (Figure 5 b). The chemotactic response of the hermit crab towards both diatoms was weaker at low pH (Figure 5 c, d), as also observed for the whole epiphytic assemblage (Figure 4 b, d, f). Another hermit crab, *Calcinus ornatus*, avoided the odour of *C. scutellum* var. *posidoniae* at normal pH, in accordance with what observed for the whole epiphytic assemblage (Figure 4 a, c, e). However, it avoided the odour of *C. scutellum* var. *posidoniae* also at acidified pH, whereas it showed no reactions towards the odour of *C. neothumensis* var. *marina* at both pH.

As for gastropods, *Rissoa italiensis* exhibited an avoidance behaviour towards the VOCs produced by *C. scutellum* var. *posidoniae* at normal pH (Figure 5 a), that was opposed to the response to VOCs of the whole epiphytic layer (Figure 4). The closely related *R. violacea* exhibited avoidance behaviour only to the VOCs of *C. neothumensis* var. *marina* at normal seawater pH (Figure 5 b). Finally, *Gibbula umbilicaris* was attracted by VOCs extracted from *C. scutellum* var. *posidoniae* and tested under normal pH conditions (Figure 5 a), while its reactions were inverted in acidified seawater (Figure 5 c), as well as towards the VOCs produced by *C. neothumensis* var. *marina* and tested at low pH (Figure 5 d).

3.5 Analysis of behavioural patterns

The above-described complex patterns of chemotactic reactions exhibited by various invertebrates, at different pH and doses, were further analysed to identify common trends of responsiveness and the main effects of OA on the chemical communications within plant and animal communities. Behavioural relationships were summarized via a Cluster Analysis performed on the matrix of invertebrate responses to the whole bouquet of epiphytes, at various concentrations and at two pH regimes (Figure 6). This analysis highlighted the main factors influencing chemotactic responses and species-specific responsiveness of invertebrates to the epiphyte VOCs. Two main clusters are defined: one cluster (on the left of Figure 6) pooling the behavioural reactions of “responsive” (positive or negative chemotaxis) species and the other cluster (right side of Figure 6) containing less evident reactions (scarcely responsive species), to VOCs, depending on concentrations and pH.

Z-tests confirmed these results, indicating several species characterized by strong reactions at both pH (Table 3). The main relationships are also summarized by choice indices (Jüttner et al. 2010; Table 3 for both pH). On average and over most concentrations, some species (such as the polychaete *Platynereis dumerilii*, the isopod *Dynamene bifida*, the amphipod *Gammarella fucicola*, the decapods *Hippolyte inermis* and *Calcinus ornatus* and all rissoid mollusks) were attracted by the odour of epiphytes. In contrast, the decapod *Cestopagurus timidus* and the mollusk *Bittium latreilli* were repelled by the odour of epiphytes. Some invertebrates, as *Caprella acanthifera* and *Gibbula umbilicaris,* were scarcely responsive to the odour of wounded epiphytes. Contrasting reactions were exhibited by various species at two pH: *Caprella acanthifera* exhibited only a weak response to the smell of diatoms at normal (8.1) pH, but appeared much more attracted to the same diatom under acidified conditions. *Cestopagurus timidus* and *Bittium latreilli,* which were repelled by the odours of the epiphytes at normal pH of seawater, became attracted to their odours under acidified conditions. In contrast, *Rissoa italiensis* and *R. violacea,* which were attracted by the epiphytic layer at normal pH, were repelled by the odour of wounded algae in acidified seawater (Table 3).

Table 3. Summary of the differences in invertebrates behavioural reactions to the whole bouquet of leaf epiphytes, at two pH tested using z-test (n.s.: no significant differences between treatments, * p<0.05; ** p<0.01, *** p<0.005. “Integer” indicates an index of attraction (Jüttner et al., 2010) while the A.C indicates the angular coefficient of the regression line, as a mean to evaluate the tendency of each species to move towards positive (positive A.C.), negative (negative A.C.) or neutral areas (A.C. close to 0). Thus, a species characterized by a positive Integer (>1) and A.C. close to 0 is considered attracted (“+”) because it was prevalent in positive sectors of the experimental arena and did not change its position. Alternatively, a species characterized by a large negative Integer (<1) and a negative A.C. is considered repelled (“-“) because it was prevalent in negative sectors of the arena and moved towards the negative target.

	pH 8.1		pH 7.7			Normal pH	Acidified pH
Species	Integer	A.C.	Integer	A.C.	z-test	Attractiveness	
Platynereis dumerilii	2.30	0.01	1.10	-0.07	n.s.	+	+

Dynamene bifida	1.58	-0.29	5.42	-0.08	*	+	+
Caprella acanthifera	0.75	0.18	6.25	-0.06	*	0	+
Gammarella fucicola	4.33	0.33	6.42	-0.09	n.s.	+	+
Hippolyte inermis	2.75	0.04	0.58	0.38	n.s.	+	0
Cestopagurus timidus	-5.25	-0.24	3.83	0.05	**	-	+
Calcinus ornatus	4.25	0.20	0.63	0.51	*	+	0
Rissoa italiensis	2.00	-0.23	-6.17	-0.28	***	+	-
Rissoa variabilis	1.10	0.27	3.40	-0.06	n.s.	+	+
Rissoa violacea	1.20	-0.10	-1.40	-0.14	***	+	-
Bittium latreilli	-1.83	-0.27	0.33	0.17	n.s.	-	0
Gibbula umbilicaris	0.63	-0.44	0.25	-0.25	n.s.	0	0

1. DISCUSSION

4.1 Epiphytic composition and production of infochemicals

Seawater acidification influences the composition of macroalgae associations (non-calcareous filamentous and erect *vs* calcareous, coralline, encrusting, respectively; Table 2) and the reactions of the potential consumers to their VOCs (Table 3). Differences in the taxonomic composition of epiphytic communities between the site at “normal” pH and the “acidified” site were striking and coherent with the known evolution of plant-associated communities under acidified conditions, including previous observations on the same area (Cox et al., 2015; Donnarumma et al., 2014; Martin et al., 2008; Nogueira et al., 2017). *Posidonia oceanica* harbours an impoverished epiphytic layer in acidified environments, represented only by a few macroalgae, dominated by *Myrionema orbiculare*. This difference, along with a decrease in phenolic compounds of seagrass leaves

(Arnold et al., 2012), could be the reason for a higher level of fish grazing recorded on leaves of seagrasses in acidified conditions (Apostolaki et al., 2014). In addition, the C/N ratio of leaf tissues in *Posidonia* at Castello vents was found to be lower (Ricevuto et al., 2015), due to a larger proportion of N and a higher nutritional value, compared to leaf tissues under normal pH conditions (Scartazza et al., 2017). It is thus reasonable that volatile organic compounds released from wounded macro-algal epiphytes should also differ according to pH, due to variations in the composition of algal communities. Accordingly, they could convey contrasting “messages” to associated animal communities (Zupo et al., 2016).

Taking into account the fundamental stabilizing role of non-trophic interactions mediated by infochemicals (Hammill et al., 2015; Ranya et al., 2014), the alterations of their recognition and interpretation may impact the ecosystem stability. We know that some diatoms associated to the leaves of *P. oceanica* produce infochemicals that trigger chemotactic reactions in various invertebrates (Maibam, 2012; Zupo et al., 2019; Zupo and Maibam, 2011) and that the acidification of water may influence these relationships, thereby disturbing intricately evolved plant-animal and animal-animal relationships (Mutalipassi et al., 2019). In addition, diatoms have been demonstrated to be an important source of infochemicals both for benthic (Fink et al., 2006a; Jüttner et al., 2010; Maibam et al., 2014) and planktonic (Maibam et al., 2015) organisms.

Tissues of *P. oceanica,* in their turn, produce VOCs that are recognized by several seagrass-associated invertebrates and trigger variable reactions under normal and acidified conditions (Zupo et al., 2016). Evidently, also macroalgae living in the epiphytic layer might produce infochemicals triggering reactions in the associated fauna. Since this point was never experimentally demonstrated by previous investigations, we demonstrate here for the first time the existence of infochemicals triggering communications among epiphytic macroalgae, diatoms and invertebrates associated to the leaf stratum of *P. oceanica*. In fact, the present study demonstrates that VOCs of epiphytes collected from the leaves of *P. oceanica* trigger chemotactic reactions in selected macro-invertebrates and these reactions were distinct from the responses to diatom-derived VOCs alone. As we tested the whole epiphytic community from *P. oceanica* leaves, the influence of the most abundant macroalgae, like crustose calcareous epiphytes (*Hydrolython* spp. and *Pneophyllum* spp.) may be particularly important for the observed chemotactic reactions, although these forms are quite reduced in their coverage and diversity under acidified conditions. In addition, several macroalgae produce VOCs that may play roles as infochemicals (Bravo-Linares et al., 2010; Saha et al., 2012; Saha and Weinberger, 2019; Zupo et al., 2016). Brown algae mainly produce chlorinated and oxygenated compounds, while green algae contain greater amounts of brominated, sulphur compounds, aldehydes and non-methane hydrocarbons (Bravo-Linares et al., 2010). Consequently, VOCs produced by a range of plants, from diatoms to macroalgae and seagrasses, may consistently play ecological roles as infochemicals for invertebrates, driving information about the presence of food, predators, possible risks for consumers (Jüttner et al., 2010; Maibam et al., 2014) and triggering specific chemotactic reactions (Fink, 2007).

4.2 The effect of the epiphytic community on invertebrates

Complex behavioural responses of invertebrates are influenced by the concentration of individual infochemicals, that may trigger opposite reactions in target organisms (Maibam et al., 2015, 2014). The dose-dependent chemotactic reaction to VOCs is a well-known event, since the answers of invertebrates change according to occurrences possibly triggering the emission of infochemicals (Jüttner et al., 2010; Moelzner and Fink, 2015). In fact, different amounts of infochemicals produced by the wounding of a large portion of a leaf (e.g., due to the foraging by a large fish) or local grazing by a small invertebrate consumer, represent quite different “messages” for associated organisms, and their interpretation triggers opposite reactions (or no reactions), as also demonstrated by previous studies on *Posidonia oceanica* VOCs (Maibam et al., 2014). Additionally, OA may affect these complex informational relationships through various possible routes. It has been demonstrated that OA triggers an increased metabolic load in marine invertebrates and that low pH may disrupt behaviour through “info-disruption”, i.e., impairment of their ability to gather and assess information and to make decisions (Briffa et al., 2012). It was demonstrated that low pH may change the charge distribution of invertebrate’s receptors, or disrupt chemoresponsive behaviours by influencing the ionic state of the odour molecules themselves. Finally, rather than impairing detection, high CO_2 could harm animal cognition and affect a wide range of sensory functions and behaviours (Briffa et al., 2012).

In our case (see table 3), most sampled invertebrates recognized the “odour” of *Posidonia* epiphytes “*in toto*” at pH 8.1. They were primarily attracted by VOCs produced by *Posidonia* epiphytes at pH 8.1, although in the case of two rissoid mollusks (*R. violacea* and *R. italiensis*) an inversion of their chemotactic reactions was observed in acidified conditions. The difference could be due to the structure of rissoid receptors for epiphyte VOCs in acidified waters (Briffa et al., 2012), in resemblance of what was demonstrated in the case of other infochemicals, such as peptide signalling molecules (Roggatz et al., 2016), although in our case the chemical nature of VOCs bouquets is quite different and we tested a multitude of cues, generated by several epiphytes. The different reactions exhibited by various invertebrates indicate the need of research about mollusk’s chemical receptors (Moelzner and Fink, 2015, 2014) as compared to the receptors of crustaceans (Briffa et al., 2012) and polychaetes (Hardege, 1999). In our study, polychaetes exhibited comparable chemotactic reactions in normal and acidified conditions. In contrast, decapod crustaceans as *Hippolyte inermis* and *Cestopagurus timidus* exhibited a complex trend of reactions, that in acidified conditions was shifted to the highest concentrations, so indicating a reduced responsiveness to the same compounds. As well, the isopod *D. bifida*, which is known to be metabolically adapted to acidic environments (Turner et al., 2016), responded significantly at medium concentration and showed a dose-dependent reaction under both normal and low pH, possibly pointing to a compromise between predation risk (high VOC concentration due to large omnivore grazing) and poor food availability (low VOC concentration).

Experimentation of invertebrate chemotactic responses can never exclude long-term adaptation to decreasing pH, which cannot be simulated in the laboratory. However, the experimental simplification is worth to detect invertebrate's reactions to the odour of epiphytes and specie-specific homeostatic efficiency at low pH is confirmed by the high survival of most invertebrates subjected to our experimental procedures (SM1).

4.3 The effect of dominant species of diatoms

As demonstrated for some diatom species, algal growth can be enhanced by ocean acidification (Wu et al., 2014) and this is the case of *Cocconeis* spp. (Mutalipassi et al., 2019). Given their well-known role as producers of infochemicals recognized by benthic and planktonic invertebrates (Maibam et al., 2015, 2014), they are quite relevant in acidified meadows, where macroalgal epiphytic layers almost disappear. In fact, the effects of the two most abundant diatoms found in the epiphytic layer were strong and they were modulated by normal pH. Volatile organic compounds from *Cocconeis scutellum* var. *posidoniae* were repellent for most invertebrates, excluding *Platynereis dumerilii* and *Gibbula umbilicaris* at normal pH, while most reactions were inverted in acidified conditions. Similar differences between epiphytes tested in acidified and normal pH were highlighted by the reactions of organisms exposed to the VOCs of the whole epiphytic layer at the lowest concentration.

The chemotactic reactions exhibited by invertebrates in response to VOCs produced by *Cocconeis neothumensis* var. *marina* (Raniello et al., 2007) were more complex when tested at normal pH. This diatom triggered avoidance behaviour only in the amphipod *Gammarella fucicola*, and the decapod *Cestopagurus timidus*. These reactions are in line with those exhibited by the same species at normal pH and at the lowest concentrations of VOCs. Thus, at least for these invertebrates, the chemical cues from VOCs produced by a single dominant diatom are equivalent to those from the entire epiphytic community (low concentration). An inversion of the behavioural response was exhibited by several invertebrates, when tested in acidified water. While *G. fucicola* and *R. variabilis* consistently exhibited negative chemotaxis towards the diatom’s odours, decapods became attracted (positive chemotaxis) under acidified conditions. Differently, the gastropod *G. umbilicaris* inverted and strengthened at low pH its repellence to the VOCs produced by this diatom.

These pH-dependent behavioural responses of *Posidonia*-associated invertebrates are in line with those detected in previous studies (Maibam et al., 2014). Their complex patterns are part of a multi-dimensional system of chemical communications evolved by each invertebrate species.

4.4 Messages conveyed by micro- and macroalgae

The behavioural effects triggered by diatom VOCs were generally stronger at the lowest concentrations. This may be explained taking into account species-specific activity patterns on seagrass leaves. The effect of larger vertebrate grazing may be revealed by the massive release of VOCs from wounded diatom cells, according to the damaged area, and this message is received and interpreted by invertebrates. The effect of larger grazers, as fish, representing potential predators (Dobroslavić et al., 2013), is revealed by the wounding of larger portions of leaves and this is detected through the emission of higher quantities of VOCs, and the presence of macroalgae metabolites. Due to these ecological relationships (Ruocco et al., 2018; Silvo, 2011; Tomas et al., 2005a, 2005b; Zupo et al., 2001) microalgae (diatoms, in particular) trigger significant chemotactic reactions at low concentrations, whereas macroalgae trigger chemotactic reactions at higher concentrations.

Further investigations will clarify the role of individual macroalgae in the ecology and the behaviour of invertebrates, but the present study demonstrates for the first time that macroalgae and diatoms, both present in the epiphytic layer of *P. oceanica*, are recognized by invertebrates by means of volatile infochemicals that trigger specific chemotactic reactions and may be further modulated by pH. Our results also indicate that some invertebrates invert their behaviour at lower pH, probably due to changes in the charge distribution of their receptors (Briffa et al., 2012;) or to molecular changes of infochemicals (Wyatt et al., 2014). The “meaning” of chemical cues originating from micro- and macro-algae is expressed at different concentrations and triggers specific reactions for the survival of marine invertebrates. Consequently, some diatoms produce VOCs that are recognized by the most responsive invertebrates at low concentrations, and their effects may be equivalent to those triggered by the whole epiphytic community, so demonstrating their importance as carriers of essential chemical messages, when wounded by small consumers. In contrast, the presence of larger predators is signalled by the emission of higher doses of VOCs, especially those produced by macroalgae that colonize the tips of *Posidonia* leaves.

However, as also observed in fishes (Porteus et al., 2018; Velez et al., 2019), ocean acidification may disrupt the ability of invertebrates to detect and correctly interpret chemical cues, by changing the functions of chemical receptors (Briffa et al., 2012). In addition, signals from plant tissues wounded by large grazers may be not recognized by smaller invertebrates at low pH, which become easier prey for their consumers. This process may, at least partially (Garrard, 2013; Garrard et al., 2014), explain the different assemblages of invertebrates associated to *P. oceanica* stands under acidified conditions.

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Captions to figures

Figure 1. Total epiphytic macroalgae coverage (± SEM, n = 9) recorded during this investigation at pH 8.1 and 7.7 on leaves of *Posidonia oceanica*.

Figure 2. Main genera of diatoms recorded at pH 8.1 and 7.7 on the leaves of *Posidonia oceanica* in the period of investigation, reported as percent total coverage. a) most abundant species (abundance from 1 to 100%); b) species present at an intermediate abundance (from 0.2 to 0.99% coverage); c) least abundant species (% coverage from 0 to 0.19%). Data are averages ± SEM, $n \geq 3$.

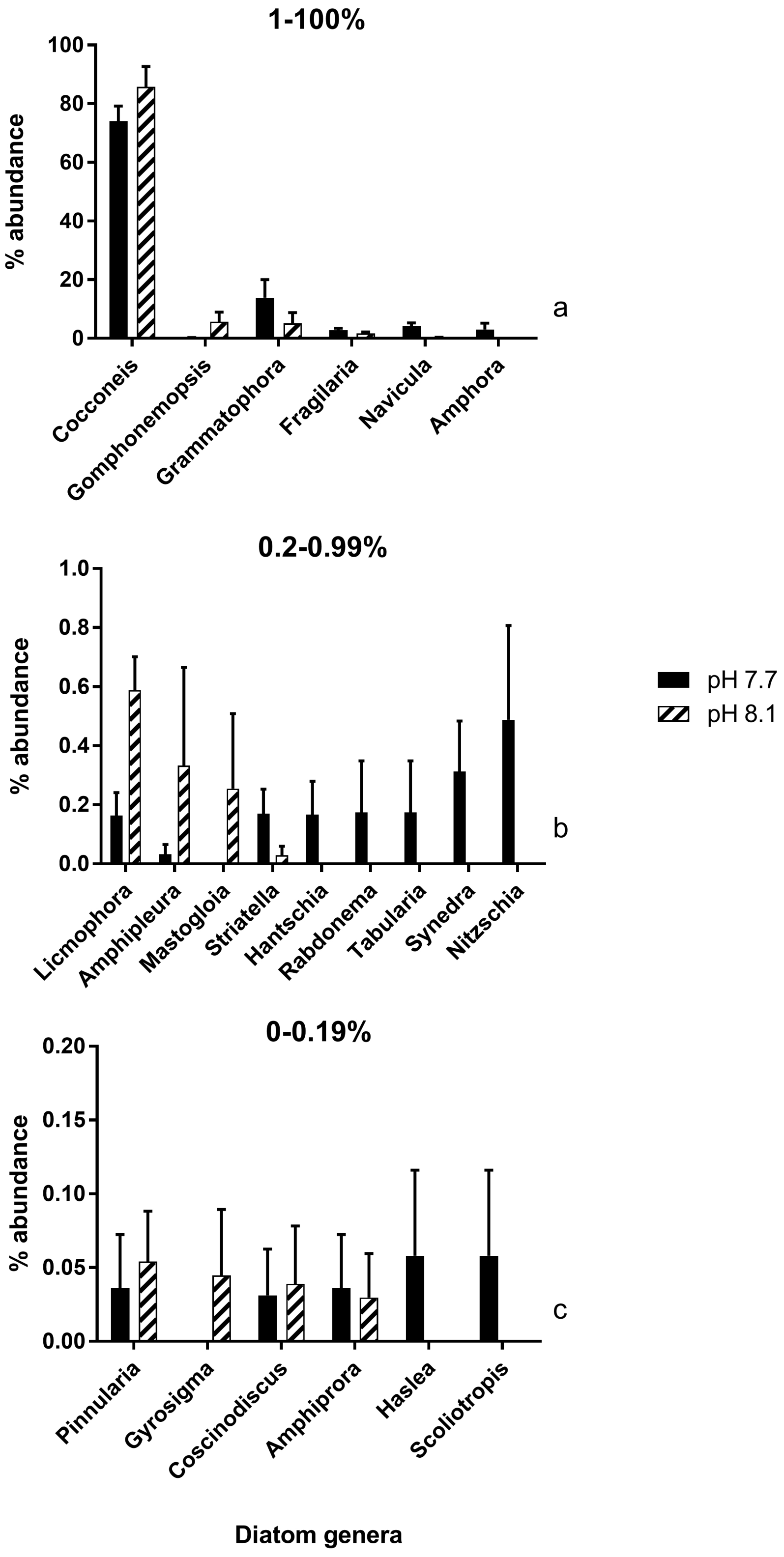
Figure 3. Average abundance expressed as percent total coverage (± SEM, n = 7) of the two main species of *Cocconeis* on leaves of *Posidonia oceanica* at normal (8.1) and low (7.7) pH.

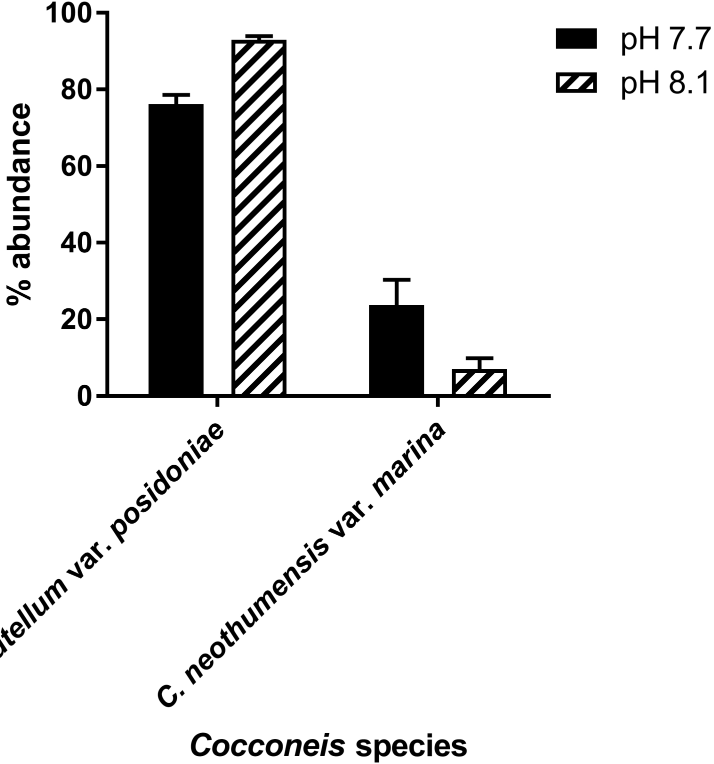
Figure 4. Behavioural responses of invertebrates to VOCs extracted from epiphytes collected "*in toto*" from leaves of *Posidonia oceanica* and tested at pH 8.1 at a) low concentration, c) medium concentration and e) high concentration and at pH 7.7 at b) low concentration, d) medium concentration, f) high concentration. Preference index indicates the distribution of individuals in the space of experimental arenas. Positive values indicate attraction towards VOC targets, while negative values indicate repulsion. Bars represent standard errors on the mean (SEM). The significance of differences in spatial distributions between the proportions of individuals present in positive and negative sectors at the end of experiments was analysed by z-test. Significant differences are marked by asterisks next to the species names (* $p<0.05$; ** $p< 0.01$; *** $p<0.001$).

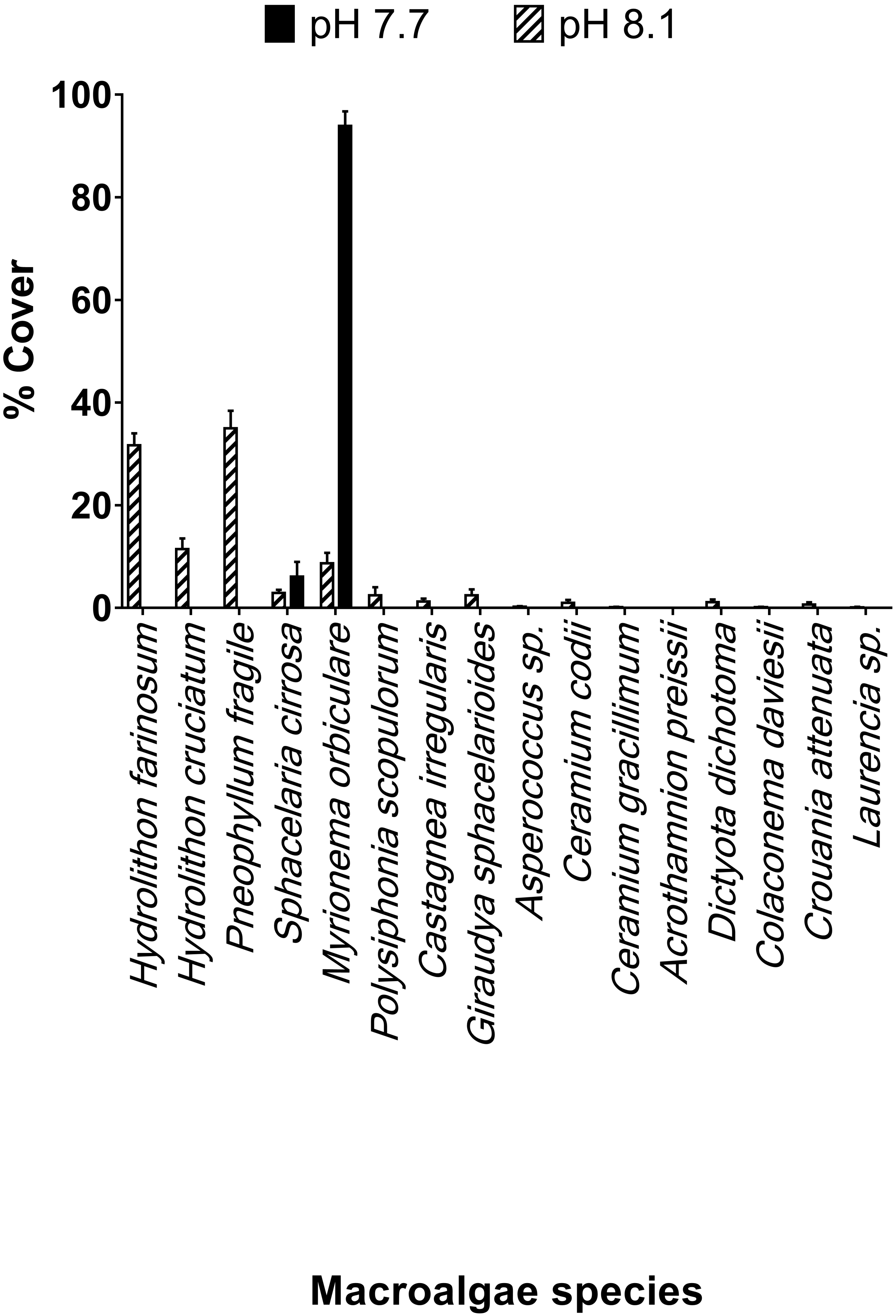
Figure 5. Behavioural response of Mediterranean benthic invertebrates elicited by the VOCs produced by the two most abundant diatoms present in the epiphytic layer of *Posidonia oceanica*: (a, c) *Cocconeis scutellum* var. *posidoniae* and (b, d) *Cocconeis neothumensis*

var. *marina*. Behavioural responses at normal pH (8.1) are shown in a and b, while the responses at acidified pH (7.7) are shown in c and d. Preference index indicates the distributions of individuals in the space of experimental arenas. Positive values indicate attraction towards VOC targets, while negative values indicate repulsion. Bars represent standard errors on the mean (SEM). The significance of differences in spatial distributions between the proportions of individuals present in positive and negative sectors at the end of experiments was analysed by z-test. Significant differences are marked by asterisks next to the species names (* $p<0.05$; ** $p< 0.01$; *** $p<0.001$).

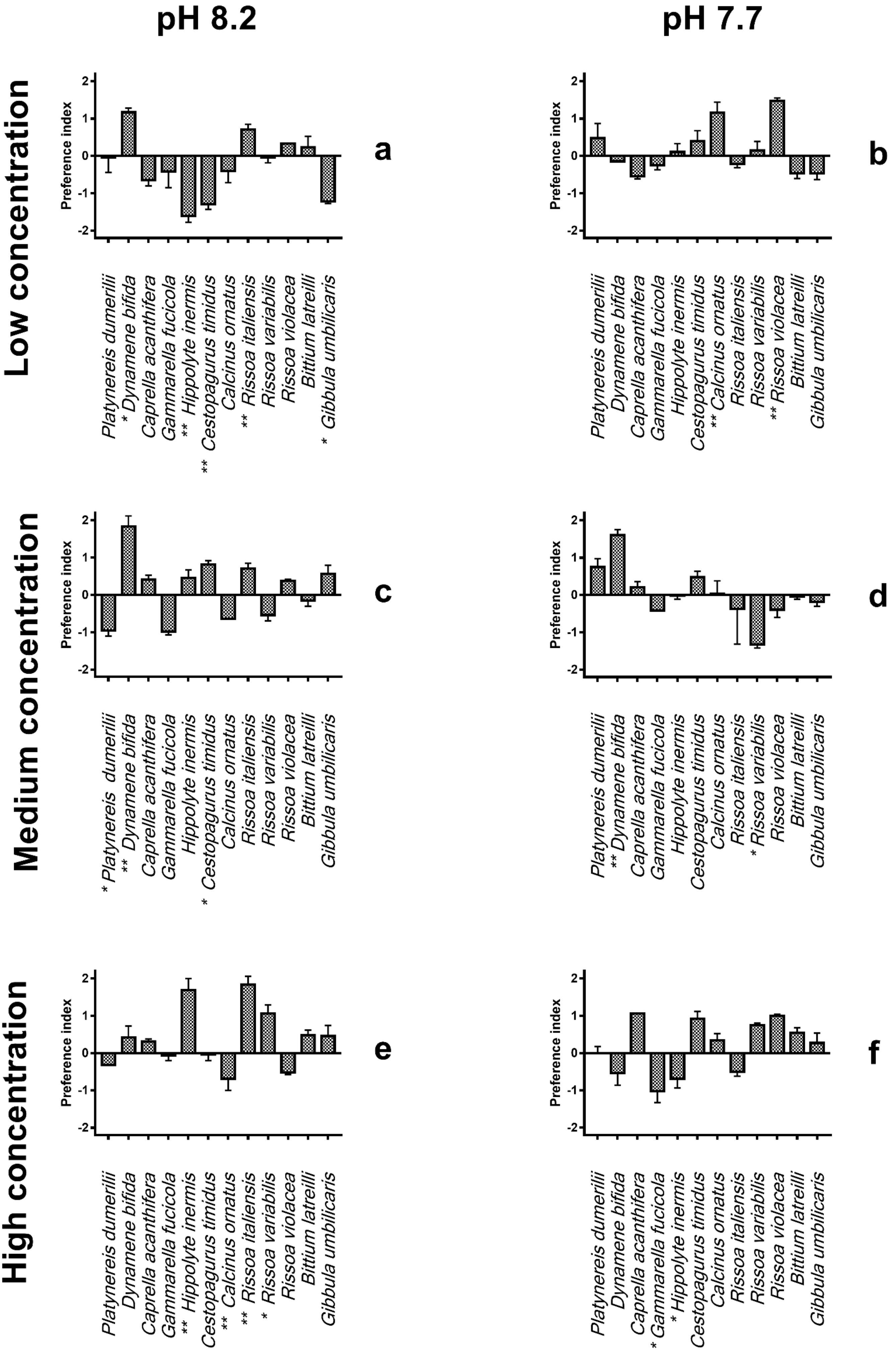
Figure 6. Cluster analysis (Complete linkage, Euclidean distance) tree diagram computed for 72 cases on the matrix “invertebrate species vs. chemotactic reactions” for all considered invertebrates. The analysis determines species-specific sensitivity of invertebrates to the epiphytes odour (pool of all epiphytes) for each testing condition (concentration and pH). The acronyms refer to the name of each invertebrate species, followed by the concentration (L for low, M for medium and H for high) and the pH regime (7 for acidified, 8 for normal). Pd, *Platynereis dumerilii*; Db, *Dynamene bifida*; Ca, *Caprella acanthifera*; Gf, *Gammarella fucicola*; Hi, *Hippolyte inermis*; Ct, *Cestopagurus timidus*; Co, *Calcinus ornatus*; Ri, *Rissoa italiensis*; Rv, *Rissoa variabilis*; Ro, *Rissoa violacea*; Bl, *Bittium latreilli*; Gu, *Gibbula umbilicaris*.

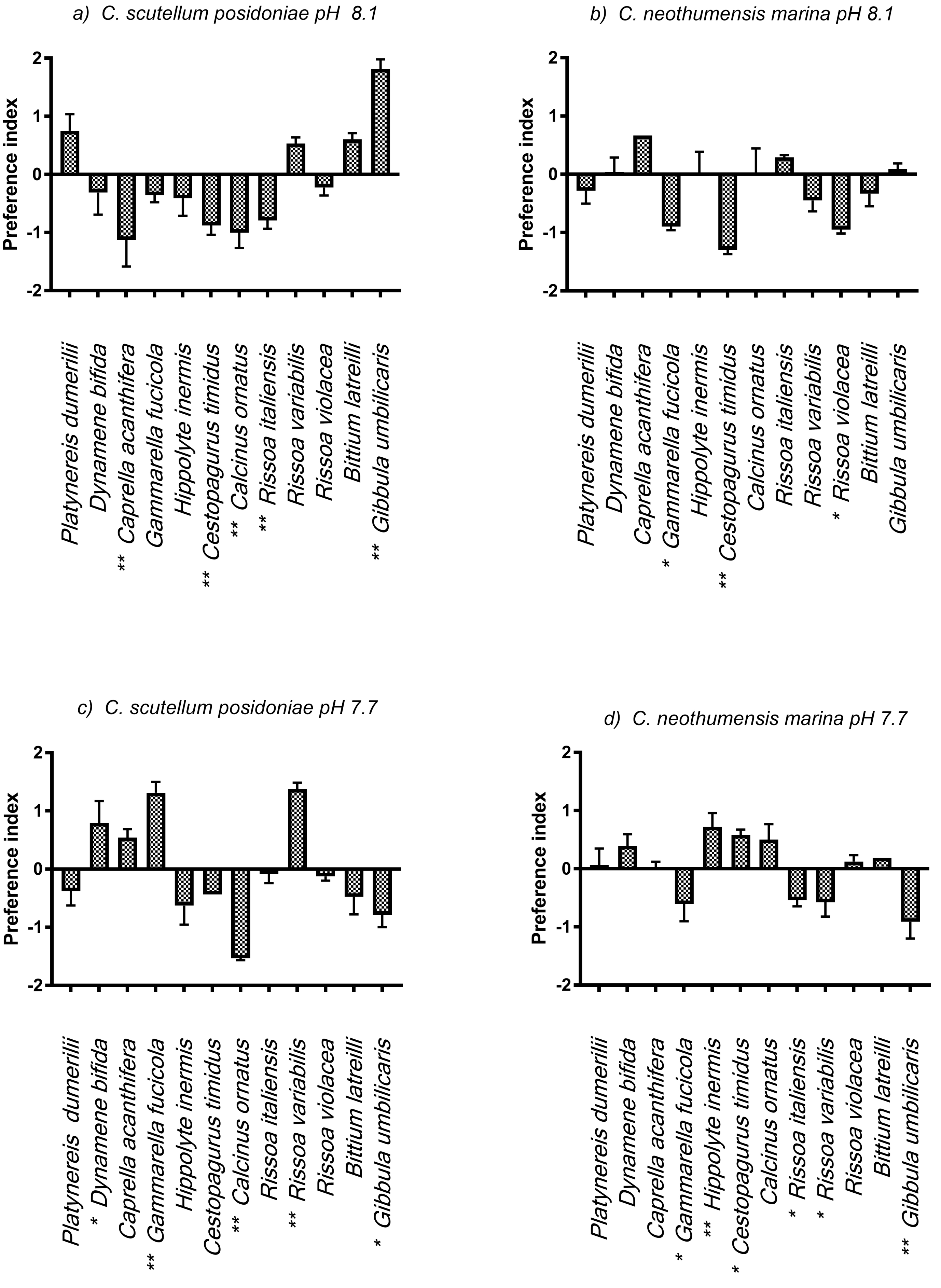

1-100%
% abundance
100
80
60
40
20
0
Cocconeis
Gomphonemopsis
Grammatophora
Fragilaria
Navicula
Amphora
a
0.2-0.99%
% abundance
1.0
0.8
0.6
0.4
0.2
0.0
Licmophora
Amphipleura
Mastogloia
Striatella
Hantschia
Rabdonema
Tabularia
Synedra
Nitzschia
b
pH 7.7
pH 8.1
0-0.19%
% abundance
0.20
0.15
0.10
0.05
0.00
Pinnularia
Gyrosigma
Coscinodiscus
Amphiprora
Haslea
Scoliotropis
c
Diatom genera

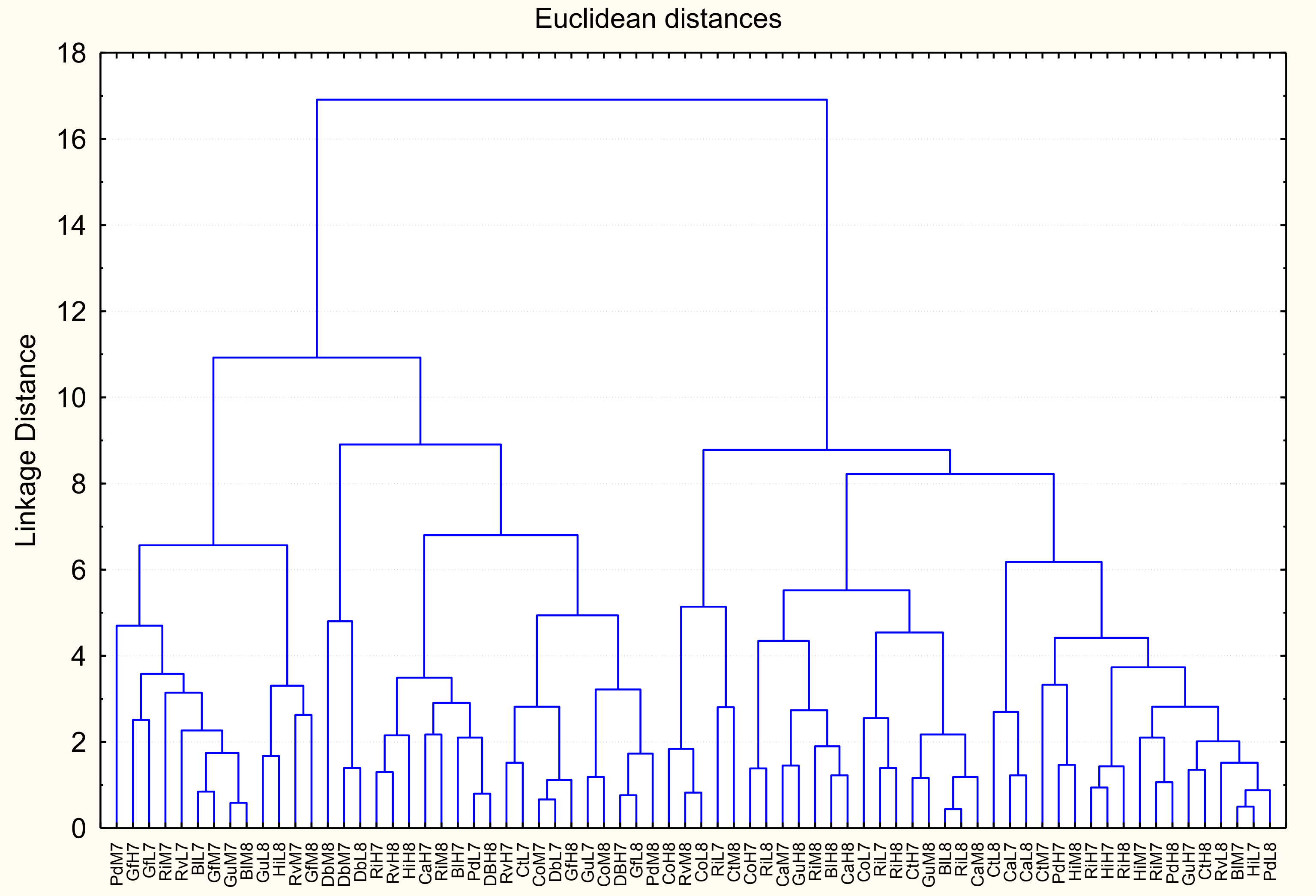

100
80
60
40
20
0
% abundance
pH 7.7
pH 8.1
C. scutellum var. posidoniae
C. neothumensis var. marina
Cocconeis species



pH 7.7
pH 8.1
100
80
60
40
20
0
% Cover
Hydrolithon farinosum
Hydrolithon cruciatum
Pneophyllum fragile
Sphacelaria cirrosa
Myrionema orbiculare
Polysiphonia scopulorum
Castagnea irregularis
Giraudya sphacelarioides
Asperococcus sp.
Ceramium codii
Ceramium gracillimum
Acrothamnion preissii
Dictyota dichotoma
Colaconema daviesii
Crouania attenuata
Laurencia sp.
Macroalgae species


pH 8.2
pH 7.7
Low concentration
Medium concentration
High concentration
Preference index
2
1
0
-1
-2
a
Platynereis dumerilii
* Dynamene bifida
Caprella acanthifera
Gammarella fucicola
** Hippolyte inermis
** Cestopagurus timidus
Calcinus ornatus
** Rissoa italiensis
Rissoa variabilis
Rissoa violacea
Bittium latreilli
* Gibbula umbilicaris
b
Platynereis dumerilii
Dynamene bifida
Caprella acanthifera
Gammarella fucicola
Hippolyte inermis
Cestopagurus timidus
** Calcinus ornatus
Rissoa italiensis
Rissoa variabilis
** Rissoa violacea
Bittium latreilli
Gibbula umbilicaris
c
* Platynereis dumerilii
** Dynamene bifida
Caprella acanthifera
Gammarella fucicola
Hippolyte inermis
* Cestopagurus timidus
Calcinus ornatus
Rissoa italiensis
Rissoa variabilis
Rissoa violacea
Bittium latreilli
Gibbula umbilicaris
d
Platynereis dumerilii
** Dynamene bifida
Caprella acanthifera
Gammarella fucicola
Hippolyte inermis
Cestopagurus timidus
Calcinus ornatus
Rissoa italiensis
* Rissoa variabilis
Rissoa violacea
Bittium latreilli
Gibbula umbilicaris
e
Platynereis dumerilii
Dynamene bifida
Caprella acanthifera
Gammarella fucicola
** Hippolyte inermis
Cestopagurus timidus
** Calcinus ornatus
** Rissoa italiensis
* Rissoa variabilis
Rissoa violacea
Bittium latreilli
Gibbula umbilicaris
f
Platynereis dumerilii
Dynamene bifida
Caprella acanthifera
* Gammarella fucicola
* Hippolyte inermis
Cestopagurus timidus
Calcinus ornatus
Rissoa italiensis
Rissoa variabilis
Rissoa violacea
Bittium latreilli
Gibbula umbilicaris


a) C. scutellum posidoniae pH 8.1
b) C. neothumensis marina pH 8.1
c) C. scutellum posidoniae pH 7.7
d) C. neothumensis marina pH 7.7
Preference index
2
1
0
-1
-2
Platynereis dumerilii
Dynamene bifida
Caprella acanthifera
Gammarella fucicola
Hippolyte inermis
Cestopagurus timidus
Calcinus ornatus
Rissoa italiensis
Rissoa variabilis
Rissoa violacea
Bittium latreilli
Gibbula umbilicaris
*
**


Euclidean distances
Linkage Distance
18
16
14
12
10
8
6
4
2
0
PdM7
GfH7
GfL7
RiM7
RvL7
BlL7
GfM7
GuM7
BlM8
GuL8
HiL8
RvM7
GfM8
DbM8
DbM7
DbL8
RiH7
RvH8
HiH8
CaH7
RiM8
BlH7
PdL7
DBH8
RvH7
CtL7
CoM7
DbL7
GfH8
GuL7
CoM8
DBH7
GfL8
PdM8
CoH8
RvM8
CoL8
RiL7
CtM8
CoH7
RiL8
CaM7
GuH8
RiM8
BlH8
CaH8
CoL7
RiL7
RiH8
CtH7
GuM8
BlL8
RiL8
CaM8
CtL8
CaL7
CaL8
CtM7
PdH7
HiM8
RiH7
HiH7
RiH8
HiM7
RiM7
PdH8
GuH7
CtH8
RvL8
BlM7
HiL7
PdL8