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# Assessing the effects of long-term ocean acidification on benthic communities at CO<sub>2</sub> seeps

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ASSESSING THE EFFECTS OF LONG-TERM OCEAN  
ACIDIFICATION ON BENTHIC COMMUNITIES AT CO<sub>2</sub> SEEPS

by

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A thesis submitted to Plymouth University and Bremen University in  
partial fulfilment for the degree of

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# **Assessing the effects of long-term ocean acidification on benthic communities at CO<sub>2</sub> seeps**

Cecilia Baggini

## **Abstract**

Ocean acidification has the potential to profoundly affect marine ecosystems before the end of this century, but there are large uncertainties on its effects on temperate benthic communities. Volcanic CO<sub>2</sub> seeps provide an opportunity to examine and improve our understanding of community responses to ocean acidification. In this thesis, two Mediterranean CO<sub>2</sub> seeps (Methana in Greece and Vulcano in Italy) were used to investigate the responses of macroalgae and their epifaunal communities to increased CO<sub>2</sub>. Changes in plant-herbivore interactions at elevated CO<sub>2</sub>, as well as adaptation potential of dominant macroalgae and responses of macroalgae and epifauna to concurrent exposure to elevated CO<sub>2</sub> and copper pollution, were also examined. Firstly, I determined that volcanic seeps off Methana (Greece) are suitable for ocean acidification studies as they do not have confounding gradients in temperature, salinity, total alkalinity, nutrients, hydrogen sulphide, heavy metals or wave exposure. Calcifying macroalgae abundance decreased as CO<sub>2</sub> increased both at Methana and at Vulcano, while furoid algae seemed to benefit from elevated pCO<sub>2</sub> levels. Seasonality greatly affected macroalgal responses to increasing CO<sub>2</sub>, according to the annual cycles of dominant species. Epifaunal communities of dominant furoid algae changed at elevated pCO<sub>2</sub> as well, with calcifying invertebrates decreasing and polychaetes increasing near the seeps. Herbivore control of macroalgal biomass did not greatly change at elevated pCO<sub>2</sub> levels, as limpets had a minor role in controlling macroalgal biomass off

Vulcano (Italy) and sea urchins were replaced by herbivorous fish near seeps off Methana. The two macroalgal species examined for signs of long-term acclimatisation (*Cystoseira corniculata* (Turner) Zanardini and *Jania rubens* (Linnaeus) J.V.Lamouroux) to ocean acidification using reciprocal transplants did not appear to have permanently acclimatised to elevated pCO<sub>2</sub> levels, but changed their physiology in four to nine months depending on the local environment. Furthermore, when exposed to a 36-hour copper pulse at elevated pCO<sub>2</sub> levels both seaweed species accumulated more copper in their tissues compared to those exposed to copper in reference pCO<sub>2</sub> conditions, and this resulted in altered epifaunal assemblages on *C. corniculata*. These observations suggest that benthic communities will significantly change as CO<sub>2</sub> levels increase, and that long-term acclimatisation is not likely to play a significant role; this would have profound consequences for benthic ecosystems and the services they provide.

# **Untersuchungen zu den Langzeiteffekten der Ozeanversauerung auf benthische Lebensgemeinschaften an natürlichen CO<sub>2</sub>-Quellen**

Cecilia Baggini

## **Zusammenfassung**

Ozeanversauerung hat das Potenzial, Meeresökosysteme noch vor dem Ende dieses Jahrhunderts nachhaltig zu verändern. Große Unsicherheiten gibt es allerdings bislang über die Auswirkungen auf Benthosgemeinschaften der gemäßigten Breiten. Natürliche vulkanische CO<sub>2</sub>-Quellen bieten die Möglichkeit, unser Verständnis der Reaktionen natürlicher Lebensgemeinschaften auf die Versauerung der Ozeane zu erweitern. In dieser Arbeit wurden zwei Standorte natürlicher CO<sub>2</sub>-Quellen im Mittelmeer CO<sub>2</sub> untersucht (Methana in Griechenland und Vulcano in Italien), um die Reaktionen von Makroalgen und deren Aufwuchsgemeinschaften auf erhöhte CO<sub>2</sub>-Konzentrationen zu untersuchen. Veränderungen von Pflanze/Herbivor-Wechselwirkungen bei erhöhtem CO<sub>2</sub>, sowie das Anpassungspotential von dominanten Makroalgen und die Reaktionen von Makroalgen und Epifauna auf die gleichzeitige Exposition unter erhöhtem CO<sub>2</sub> und Kupferverschmutzung wurden erfasst. Zunächst stellte ich fest, dass die natürlichen CO<sub>2</sub>-Quellen vor Methana (Griechenland) gut für Versauerungsstudien geeignet sind, da sie nicht wesentlich durch zusätzliche Schwankungen in der Temperatur, im Salz- und Gesamtalkaligehalt, in der Nährstoffverfügbarkeit, im Gehalt von Schwefelwasserstoff und Schwermetallen oder der Wellenexposition beeinflusst sind. An beiden Standorten verringerte sich die Abundanz kalkbildender Makroalgen mit zunehmender CO<sub>2</sub>-Konzentration, während fucoide Algen von

einem erhöhten  $p\text{CO}_2$  profitierten. Abhängig von den spezifischen Jahreszyklen der jeweils dominanten Arten wurde ein starker saisonaler Einfluss auf die Reaktionen von Makroalgen auf steigende  $\text{CO}_2$ -Konzentrationen ermittelt. Die Epifauna-Gemeinschaften auf dominanten fucoiden Algen veränderten sich ebenfalls mit steigendem  $p\text{CO}_2$ , indem die Abundanz kalkbildender Wirbelloser abnahm und Polychaeten in der Nähe der Quellen zunahm. Die Kontrolle der Großalgen-Biomasse durch Herbivore veränderte sich bei erhöhtem  $p\text{CO}_2$  nur unwesentlich: Napfschnecken spielten eine untergeordnete Rolle bei der Kontrolle der Makroalgenbiomasse vor Vulcano (Italien) und am Standort Methana wurden in der Nähe der  $\text{CO}_2$ -Quellen Seeigel durch herbivore Fische ersetzt. Die beiden Makroalgen-Arten (*Cystoseira corniculata* (Turner) Zanardini und *Jania rubens* (Linnaeus) J.V.Lamouroux), welche mittels reziproker Transplantationen auf Anzeichen langfristiger Anpassungen an Ozeanversauerung untersucht wurden, scheinen sich nicht genetisch an den erhöhten  $p\text{CO}_2$  angepasst zu haben, aber änderten ihre Physiologie innerhalb weniger Monate in Abhängigkeit von der lokalen Umgebung. Eine zusätzliche 36-stündige Kupferexposition bei erhöhtem  $p\text{CO}_2$  führte in beiden Algenarten zur verstärkten Akkumulation von Kupfer im Gewebe im Vergleich zu den Kontrollbedingungen unter normalem  $p\text{CO}_2$ . Dieser zusätzliche Faktor führte zu einer Veränderung der Epifauna-Gemeinschaft von *C. corniculata*. Diese Beobachtungen legen nahe, dass sich benthische Lebensgemeinschaften bei einem Anstieg des  $\text{CO}_2$ -Niveaus deutlich verändern, und dass eine genetische Anpassung vermutlich nicht erfolgt. Daraus könnten sich weitreichende Folgen für benthische Ökosysteme und ihre Dienstleistungen ergeben.

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At no time during the registration for the degree of Doctor of Philosophy has the author been registered for any other University award without prior agreement of the Graduate Committee. Work submitted for this research degree at the Plymouth University has not formed part of any other degree either at Plymouth University or at another establishment.

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Presentations at conferences:

- Using CO<sub>2</sub> vents as a natural analogue for ocean acidification (2011). Oral presentation, 4th Annual Plymouth Marine Science Conference, Plymouth, UK.

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- Effects of short-term copper exposure on seaweeds acclimatised to high CO<sub>2</sub> (2013). Pollution Responses in Marine Organisms symposium, Faro, Portugal.
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List of research chapters and declaration of own contribution

**Chapters 2 and 3:** the same contributions apply to both chapters

**Chapter 2: Assessing the suitability of a volcanic seep area off Methana (Greece) for ocean acidification studies**

**Chapter 3: Changes in subtidal macroalgal communities along pCO<sub>2</sub> gradients at Mediterranean volcanic seeps**

**Contributors:** Cecilia Baggini, Maria Salomidi, Emanuela Voutsinas, Laura Bray, Eva Krasakopoulou, Jason M. Hall-Spencer

**Contributions:** I designed and performed the sampling with help from MS, EV, LB and EK; I analysed all samples with help from EK, performed statistical analyses and wrote the manuscript with help from all authors; JMH-S supervised the thesis.

**Chapter 4: Canopy algal epifauna changes at elevated pCO<sub>2</sub> at two Mediterranean volcanic seeps**

**Contributors:** Cecilia Baggini, Renato Chemello, Marco Milazzo, Mariagrazia Graziano, Antonella Pancucci-Papadopoulou, Laura Ciriminna, Jason Hall-Spencer

**Contributions:** I designed the experiment with help from MG, RC and MM, and performed the sampling with help from LC, who also helped processing the samples; I identified all invertebrates with help from RC and AP, performed statistical analyses and wrote the manuscript; JMH-S supervised the thesis.

### **Chapter 5: Effect of herbivores on benthic communities at different pCO<sub>2</sub> levels**

**Contributors:** Cecilia Baggini, Mariagrazia Graziano, Marco Milazzo, Maria Salomidi, Yiannis Issaris, Marco Franzitta, Giuseppe Bruno, Jason Hall-Spencer

**Contributions:** I designed the experiment with help from MG and MM, and performed the sampling with help from MM, GB, MS and YI; I processed all samples with help from MF, performed statistical analyses and wrote the manuscript with help from all co-authors; JMH-S supervised the thesis.

**Chapters 6 and 7:** the same contributions apply to both chapters

### **Chapter 6: Seaweed acclimatisation to high pCO<sub>2</sub> at volcanic seeps**

### **Chapter 7: A short-term copper pulse affects macroalgal copper accumulation and indirectly alters epifaunal colonisation at elevated pCO<sub>2</sub>**

**Contributors:** Cecilia Baggini, Kai Bischof, Murray Brown, Jason Hall-Spencer

**Contributions:** I designed the experiment with feedback from MB during the transfer process, performed the sampling, processed all samples, performed

statistical analyses and wrote the manuscript; JMH-S and KB supervised the thesis and provided useful feedback on the manuscript.

## **DECLARATION/ ERKLÄRUNG**

Ich erkläre

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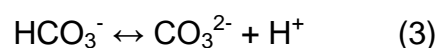
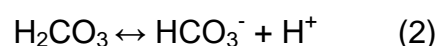
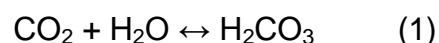
## **Chapter 1**

# **Community responses to ocean acidification on temperate rocky reefs: possible causes and adaptation potential**

## 1.1 Introduction

Since the beginning of industrial revolution, man has released huge quantities of carbon dioxide to the atmosphere. Atmospheric CO<sub>2</sub> concentrations reached 400 ppmv in April 2014 (NOAA, 2014) and are rising more rapidly than previously thought (Intergovernmental Panel on Climate Change, 2014). This increase is a concern, as atmospheric CO<sub>2</sub> was relatively constant over the last 800.000 years, with values ranging between 172 and 300 ppmv (Lüthi *et al.*, 2008). About one fourth of anthropogenic CO<sub>2</sub> emissions has been absorbed by the oceans, thereby reducing their effects in the atmosphere (Le Quéré *et al.*, 2009), but at the same time increasing the carbon dioxide concentration in seawater. A trend in increasing surface ocean pCO<sub>2</sub> parallel to the increase in atmospheric carbon dioxide is clearly detectable using long-term monitoring stations (Brewer *et al.*, 1997; Hofmann *et al.*, 2011).

Increased seawater pCO<sub>2</sub> causes changes in the ocean carbonate system, a process referred to as 'ocean acidification' (Caldeira and Wickett, 2003). When carbon dioxide dissolves in water it forms carbonic acid (H<sub>2</sub>CO<sub>3</sub>), a weak acid that is highly unstable in seawater (1). H<sub>2</sub>CO<sub>3</sub> readily dissociates to form bicarbonate ions (HCO<sub>3</sub><sup>-</sup>) and hydrogen ions (2). Bicarbonate ions can in turn dissociate to produce carbonate ions (CO<sub>3</sub><sup>2-</sup>) and hydrogen ions. However, increased [H<sup>+</sup>] causes reaction (3) to reverse and bicarbonate ions to become more stable in seawater.



Overall, ocean acidification causes a decrease in seawater pH (i.e. an increase in  $[H^+]$ ) and carbonate ion concentration and an increase in aqueous  $CO_2$  ( $CO_{2(aq)}$ ), dissolved inorganic carbon and  $[HCO_3^-]$ , with total alkalinity remaining constant. Decreased carbonate ion concentrations are leading to lower calcium carbonate saturation state ( $\Omega_{CaCO_3}$ ) in the world oceans, meaning that  $CaCO_3$  is becoming more likely to dissolve. This happens because  $[CO_3^{2-}]$  is one of the determinants of calcium carbonate saturation, as shown in equation (4).

$$\Omega_{CaCO_3} = [Ca^{2+}] [CO_3^{2-}] / K_{sp}^* \quad (4)$$

There are two forms of calcium carbonate: aragonite and calcite. These have different solubility product constants ( $K_{sp}^*$ ) and thus different solubility, with aragonite being less stable than calcite in seawater (Tyrrell, 2008). A third form of calcium carbonate, high-magnesium calcite, forms the carbonic skeleton of some organisms and is even more soluble than aragonite.  $K_{sp}^*$  varies with pressure and temperature, making calcium carbonate more likely to dissolve at low temperatures and high pressure. Deep polar waters are already undersaturated (i.e.  $\Omega_{CaCO_3} < 1$ ) with respect to aragonite, whereas superficial waters are predicted to become undersaturated in the next decades (Orr *et al.*, 2005; Steinacher *et al.*, 2009; Tittensor *et al.*, 2010).

With regards to the global ocean, mean pH has already decreased from ~8.2 to ~8.1 compared to pre-industrial times (Key *et al.*, 2004), probably the lowest value in the last two million years (Hönisch *et al.*, 2009). A further decrease of approximately 0.4 units is projected for the end of this century according to the “business as usual” scenario (Caldeira and Wickett, 2003), which underestimates the present rate of anthropogenic  $CO_2$  release into the atmosphere (IPCC, 2014). For the year 2300, different emission scenarios



depend on improvements in fossil fuel extraction techniques. In the “business as usual” scenario, the mean ocean pH is predicted to reach a value of 7.4, but if mining techniques improve the subsequent increase in anthropogenic emissions could lead to a mean ocean pH of 7.1. Moreover, methane hydrate exploitation could cause ocean pH to reach a minimum of 6.8 (Caldeira and Wickett, 2005). The predicted changes in seawater carbonate chemistry are illustrated in Figure 1.1; these changes will not be permanent, because increased dissolution of deep-sea carbonate sediments and weathering on land will eventually buffer them. Carbonate dissolution from rocks and sediments, however, takes thousands of years to equilibrate the ocean carbonate system (Caldeira and Wickett, 2003), whereas current rates of increase in atmospheric carbon dioxide are already causing dramatic changes in the ocean carbonate chemistry.

In Earth’s geological history, changes in seawater  $p\text{CO}_2$  occurred more slowly than today, allowing weathering and sediment carbonate dissolution to raise carbonate saturation states. Therefore, care should be taken when comparing current changes in carbonate chemistry with those geological periods in the past that had high  $p\text{CO}_2$ . Only periods with a similar rate and magnitude of change can help us predict the long-term effects of ocean acidification. In order to identify relevant episodes, it is necessary to reconstruct at least two seawater carbonate parameters, as high carbon dioxide concentration in seawater does not imply a low calcium carbonate saturation state (Zeebe and Ridgwell, 2011). An example is the Cretaceous, when very high seawater  $p\text{CO}_2$  was combined with high saturation state of calcium carbonate. This combination of factors was due to the slower increase of carbon dioxide compared to the current rates of increase and the fact that high concentrations of carbon dioxide were

maintained for millions of years. When changes in marine carbonate chemistry occur at these timescales, they can be buffered by dissolution of carbonate sediments. This situation had thus a lower potential to affect biological calcification than ocean acidification (Zeebe and Westbroek, 2003; Ridgwell and Schmidt, 2010).

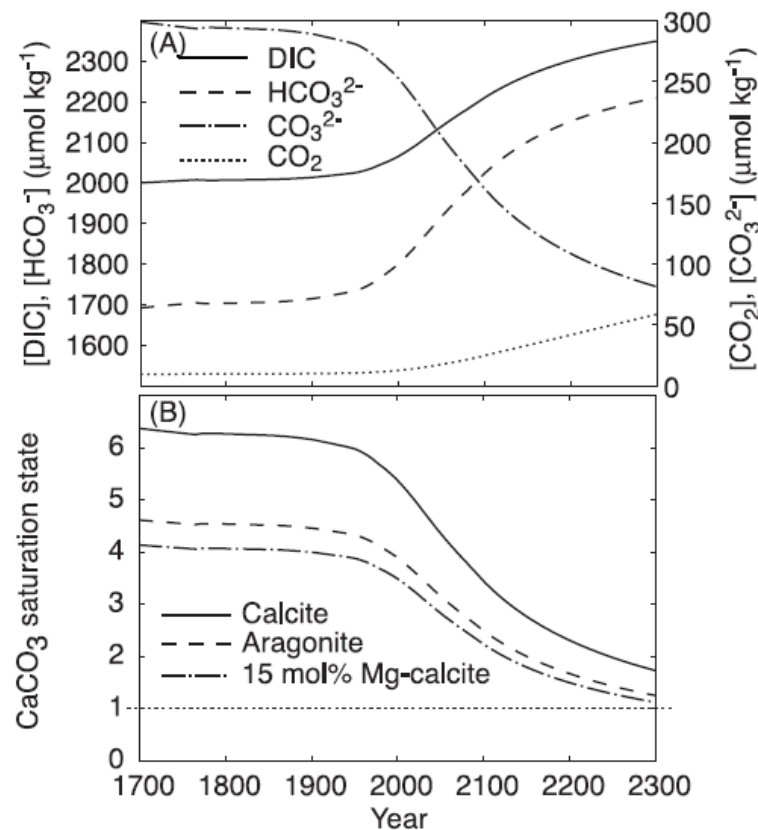


Figure 1.1. Surface water dissolved inorganic carbon chemistry between 1700 and 2300 calculated using a shallow-water ocean carbonate model at 25°C and 35 psu. (A) Total dissolved inorganic carbon concentration [DIC], [ $\text{CO}_2$ ], [ $\text{HCO}_3^-$ ], and [ $\text{CO}_3^{2-}$ ], and (B) surface water saturation state with respect to calcite, aragonite and 15 mol % magnesian calcite (Andersson *et al.*, 2005).

Suitable past ocean acidification analogues are periods of rapid (i.e. less than 10000 years) increase in seawater  $\text{pCO}_2$ , which would give no time for compensatory mechanisms to act. The best analogue for ocean acidification

with good geological records is probably the Paleocene-Eocene Thermal Maximum (PETM, ~55.8 million years ago). In this period a temperature increase of 5-9°C over a few thousand years was coupled with massive carbon release. The subsequent decline in seawater pH and CaCO<sub>3</sub> saturation state is similar to ocean acidification in many aspects, albeit rates of change were still slower than the current ones (Zachos *et al.*, 2005). This event did not appear to have significant effects on superficial nanoplankton (Gibbs *et al.*, 2006), but it caused a dramatic decrease in benthic foraminifera species (e.g. Thomas, 2007) and sediment fauna in general (Rodriguez-Tovar *et al.*, 2011). It is not clear, however, if extinctions were caused by changes in carbonate chemistry, oxygen reduction or temperature increase, although it could have been a combination of the above. All things considered, it seems that ocean acidification is an unprecedented phenomenon in our planet's history (Zeebe and Ridgwell, 2011).

Anthropogenic emissions are currently causing rapid changes in ocean carbonate chemistry that are well known and understood (Doney *et al.*, 2009; Orr, 2011), but ocean acidification also has the potential to affect various physical, biogeochemical and biological processes. Predictions of how these processes will respond to an increase in seawater pCO<sub>2</sub> are much less certain, and are an urgent priority for future research (Gattuso *et al.*, 2011). Ocean acidification can affect nutrient availability (Raven *et al.*, 2005; Doney *et al.*, 2009; Hutchins *et al.*, 2009) and trace metal speciation (Raven *et al.*, 2005; Tyrrell, 2008; Doney *et al.*, 2009; Millero *et al.*, 2009; Breitbarth *et al.*, 2010; Shi *et al.*, 2010), as well as sound wave diffusion through seawater (Hester *et al.*, 2008; Brewer and Hester, 2009). Furthermore, many biological processes may be affected, including all pH-dependent physiological functions (e.g. acid-base balance, protein activity) and those processes that use a carbon species as

substrate (e.g. photosynthesis, calcification) (Fabry *et al.*, 2008). Ocean acidification is then likely to affect benthic communities, but their responses are hard to predict because interspecific interactions can cause unexpected changes at the community level (Hale *et al.*, 2011)

## **1.2 Community effects of ocean acidification on temperate rocky reefs**

Laboratory experiments assessing responses to increased CO<sub>2</sub> determine species' relative sensitivities to high CO<sub>2</sub>. They can also reveal threshold tolerance values as well as physiological mechanisms involved in biological responses to ocean acidification. It is difficult, however, to scale up from such studies and predict how whole ecosystems will change as our oceans continue to acidify; this approach does not consider interactions between species, which dramatically influence community structure (Kroeker *et al.*, 2013a). Furthermore, the artificial conditions in a laboratory are probably very stressful for some taxa, and this could alter results (Widdicombe *et al.*, 2010).

Consequently, researchers have begun studying ocean acidification effects on community structure using mesocosm experiments. The need to test results obtained in laboratory conditions with simplified communities, however, has also led researchers to manipulate pCO<sub>2</sub> in the field or study areas with naturally high CO<sub>2</sub> concentrations. Such data are being used to develop models to predict how ecosystems will change as seawater carbon dioxide continues to increase.

### 1.2.1 Laboratory and mesocosm experiments

A community composed of long-lived organisms usually does not change its composition at the timescale of most laboratory and mesocosm experiments (i.e. days to months, rarely years). Therefore, only responses of single species and their interactions to increased pCO<sub>2</sub> can be observed using this approach. In this context, coral reef ecosystems are the most studied: mesocosm experiments lasting up to ten months reported a decrease in coral and coralline algae calcification and growth (Jokiel *et al.*, 2008; Küffner *et al.*, 2008), sometimes leading to net community CaCO<sub>3</sub> loss at low pH (Andersson *et al.*, 2009).

Temperate communities are much less studied, but there is evidence that ocean acidification will cause changes here as well. Macroalgal species show differential sensitivity to increased CO<sub>2</sub>; for instance, cover of the calcifying alga *Corallina officinalis* decreased, whereas the non-calcifying *Chondrus crispus* was more abundant as CO<sub>2</sub> increased (Hofmann *et al.*, 2012). Biomass and productivity decreased with increased CO<sub>2</sub> and temperature in communities associated with canopy-forming brown algae, but the invasive *Sargassum muticum* resulted more resistant to both stressors than the native *Cystoseira tamariscifolia* (Olabarria *et al.*, 2013). Community changes are therefore likely even when the dominant species are not calcifiers.

Some macroalgal species seem to be advantaged at elevated CO<sub>2</sub> because they are carbon-limited (Harley *et al.*, 2012). For instance, some turf algae have the potential of outcompeting kelp on Australian rocky shores when CO<sub>2</sub> and nutrients concurrently increase (Falkenberg *et al.*, 2013a). Shading from kelp,

however, reduces turf growth, meaning that a shift from kelp to turf algae is only likely if kelp cover is reduced by other stressors (Falkenberg *et al.*, 2012).

Invertebrate communities have been shown to change as well: for instance, Hale *et al.* (2011) found that organisms associated with turf algae generally reacted to increased pCO<sub>2</sub> consistently with predictions from single species experiments. However, reduced predation rates and competition for space caused nematode abundance to increase unexpectedly. These communities change from being dominated by calcareous organisms to being dominated by non-calcareous organisms at a pH between 7.2 and 7.8 (Christen *et al.*, 2013).

### **1.2.2 Field pCO<sub>2</sub> manipulation**

In recent years, various techniques to increase seawater pCO<sub>2</sub> in the field have been developed for the study of whole communities in their natural environment. In Chesapeake Bay (USA), CO<sub>2</sub> bubbling in open waters was used to assess changes in marine plants chemical defences (Arnold *et al.*, 2012). While this method allows for natural flow conditions, pCO<sub>2</sub> varies with current speed and direction.

Seawater mixed with CO<sub>2</sub> can also be injected into open chambers to better control carbonate chemistry variability while reproducing natural water movement (Campbell and Fourqurean, 2011). This system has been used to test the effects of ocean acidification on the seagrass *Thalassia testudinum*: its growth was not affected by elevated CO<sub>2</sub>, whereas nitrogen and phosphorous content significantly decreased with increased CO<sub>2</sub> (Campbell and Fourqurean, 2013). Seagrass epiphytes were more affected by increased CO<sub>2</sub> than by increased nutrients, with calcifying species being substituted by fleshy species (Campbell and Fourqurean, 2014).

A similar system, which uses closed or semi-enclosed chambers, is the Free Ocean Carbon Enrichment system (FOCE); similarly to the previous one, this system can be used to run long term experiments with entire communities exposed to natural levels of light, nutrients and temperature (Waz *et al.*, 2007; Kline *et al.*, 2012). FOCE systems have been successfully used in Australia, where pH was lowered by 0.4 units and dissolution of coralline algae increased at high CO<sub>2</sub> during a short-term experiment (Kline *et al.*, 2012). FOCE systems may be used to assess the effects of ocean acidification on many ecological processes, but are too small to influence some ecosystem properties, such as population connectivity or larval recruitment. So far, no results are available from temperate FOCE systems, even though experiments are being conducted in the Mediterranean Sea using eFOCE (European FOCE) and a shallow water FOCE (swFOCE) system is under development in California (Gattuso *et al.*, 2014).

### **1.2.3 Areas with naturally elevated pCO<sub>2</sub>**

Areas with naturally high CO<sub>2</sub> can be used to assess long-term community responses to ocean acidification. Hydrothermal vents are generally characterised by low pH and emit fluids that can contain various gases, especially carbon dioxide, hydrogen sulphide, methane and nitrogen (Tarasov *et al.*, 2005). Increased concentrations of heavy metals in the surrounding sediments are also common (e.g. Dando *et al.*, 2000; Hübner *et al.*, 2004), and macroalgae near such vents can have increased metal concentrations in their tissues (Couto *et al.*, 2010). Vents shallower than 200 m are generally populated by a subset species inhabiting the surrounding area. They are thus very different from deep-sea vents, where chemosynthetic vent-obligated species are abundant (Tarasov *et al.*, 2005).

In shallow areas, usually there is a notable reduction in biodiversity proceeding towards the vents (Melwani and Kim, 2008; Karlen *et al.*, 2010). Here, the most abundant species are those able to resist elevated temperatures and high concentrations of toxic gases and heavy metals. They also have to be resistant to reduced salinity if volcanic emissions are mixed with fresh water (Tarasov *et al.*, 2005). An exception to this trend was reported at vents off Milos (Greece), where sessile macroepibenthos had higher biodiversity near the vents than at the control sites (Morri *et al.*, 1999; Pansini *et al.*, 2000; Bianchi *et al.*, 2011). However, infauna showed an opposite trend, possibly because of the high sulphide concentration in the sediment near the vents (Thiermann *et al.*, 1997). Hot fluid emissions were also indicated as the cause for increased occurrence of warm-water seaweeds near the emissions (De Biasi and Aliani, 2003).

Although many of these areas have high seawater CO<sub>2</sub> concentration, there are very often confounding gradients in temperature, toxic gases, heavy metals or salinity, so their utility to understand community responses to ocean acidification is limited. However, it is possible to find shallow water seeps emitting almost exclusively carbon dioxide without confounding gradients in temperature, salinity, toxic gases and heavy metal contamination. There are only a few published examples of CO<sub>2</sub> seeps at temperate shores used as ocean acidification proxies, such as the two off Italy (Hall-Spencer *et al.*, 2008; Boatta *et al.*, 2013).

There biodiversity, especially that of calcifying taxa, is reduced as pCO<sub>2</sub> increases (Hall-Spencer *et al.* 2008; Martin *et al.*, 2008; Dias *et al.*, 2010; see Figure 1.2) and benthic communities are simpler and more homogeneous as CO<sub>2</sub> increases (Kroeker *et al.*, 2013b). Moreover, shifts in macroalgal dominance along a pCO<sub>2</sub> gradient have been reported (Porzio *et al.*, 2011), and



invertebrate recruitment is heavily influenced as well (Cigliano *et al.*, 2010). Benthic invertebrates also experience a reduction in diversity and biomass, and the community trophic complexity is lower at high CO<sub>2</sub> levels (Kroeker *et al.*, 2011).

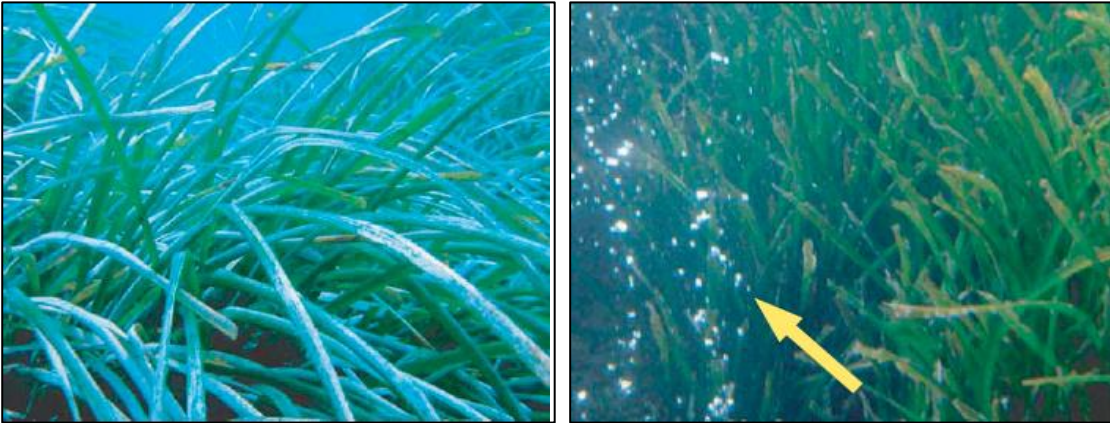


Figure 1.2. *Posidonia oceanica* covered in calcareous epiphytes at pH 8.2 at CO<sub>2</sub> vents off Ischia (left) and lacking Corallinaceae at pH 7.6 (right); yellow arrow points to CO<sub>2</sub> bubbles (Hall-Spencer *et al.*, 2008).

Experiments at seeps have revealed different sensitivities of sea urchin species to elevated CO<sub>2</sub>: *Paracentrotus lividus*, the main consumer of fleshy algae, is less resistant to elevated carbon dioxide (Calosi *et al.*, 2013a). Changes in herbivore densities have knock-on effects on fleshy macroalgae, such as the calcifying alga *Padina pavonica* (Linnaeus) Thivy. Even though these algae are less calcified at elevated CO<sub>2</sub> levels, their cover in fact increases because they are subject to less herbivore pressure (Johnson *et al.*, 2012). Experiments at seeps can also detect differences in responses to CO<sub>2</sub> as temperature increases: calcification rates in a Mediterranean coral was not affected by pCO<sub>2</sub> in spring, but strongly decreased at elevated CO<sub>2</sub> levels after a very warm summer (Rodolfo-Metalpa *et al.*, 2011).

At volcanic seeps, it is also possible to test differences in ecological processes along  $p\text{CO}_2$  gradients. For macroalgal communities, both early recruitment (Porzio *et al.*, 2013) and final community composition after one year (Kroeker *et al.*, 2013c) change greatly with increasing  $\text{CO}_2$  due to the direct effects of carbon dioxide and to changes in interspecific interactions. For instance, crustose coralline algae manage to recruit at elevated  $p\text{CO}_2$  levels, but their decreased growth rates near the seeps allow fleshy algae to outcompete them (Kroeker *et al.*, 2013c).

Carbon dioxide seeps are proving useful for ocean acidification research because they show us how whole communities change with long-term exposure to high  $\text{CO}_2$  levels. They can also be used to test hypotheses developed in laboratory-based experiments. However, seeps are not a perfect reproduction of  $\text{CO}_2$ -influenced ecosystems, since they are too small to host entire populations. Motile taxa such as fish are not likely to be as heavily influenced by  $\text{CO}_2$  as sessile taxa (Riebesell, 2008) and pelagic larvae settling near the seeps can originate from unaffected populations (Cigliano *et al.*, 2010). Moreover, if sites too close to the  $\text{CO}_2$  source are chosen, pH can fluctuate rapidly and be very different from what natural ecosystems experience. Hence, care must be taken to select sites appropriately, so that daily and seasonal fluctuations in pH remain as similar as possible to reference sites (Kerrison *et al.*, 2011).

Acidified estuaries are another possibility to study biological responses to ocean acidification in a natural environment. Two types of estuaries have been considered so far, those with high levels of biogenic  $\text{CO}_2$  produced by microbial respiration and those exposed to acid sulphate soils runoffs. In the first type pH is lower than in reference estuaries because of increased  $\text{CO}_2$  and can cause biological effects such as increased gastropod shell corrosion (Marshall *et al.*,

2008). However, in these areas oxygen levels are generally lower than reference values and nutrients have higher concentrations because of nutrient discharges, potentially confounding the effect of high CO<sub>2</sub> (Mucci *et al.*, 2011). Estuaries can also be subject to acidification episodes if acid sulphate soils are transported by rivers after heavy rainfall events. Although they are not proper ocean acidification analogues, since low pH is episodic and not due to increased CO<sub>2</sub> concentration, hypoxia is not an issue in these sites. Molluscs in these estuaries are less abundant than in control estuaries, although within the natural range of variation reported for that region (Amaral *et al.*, 2011), and mollusc shells are weaker as well (Amaral *et al.*, 2012).

Small-scale analogue systems are very useful to show community effects of ocean acidification, but they cannot be used to assess changes in large scale processes such as population connectivity and larval supply. These processes could be studied in larger high pCO<sub>2</sub> areas, such as upwelling regions, which could be a precious source of information on ecosystems subject to naturally high pCO<sub>2</sub>. In temperate regions, undersaturated waters rise from the deep sea along the east coast of USA and Canada and on the Chilean coast (Feely *et al.*, 2008; Mayol *et al.*, 2012). In some cases upwelling waters even penetrate in estuaries, where they have dramatic effects on the pH of waters lacking a carbonate buffer system (Feely *et al.*, 2010). Long-term monitoring of intertidal communities on the Eastern Pacific shores detected a decreased abundance of calcifying organisms when CO<sub>2</sub> is higher (Wootton *et al.*, 2008), and calcifying seaweed epibionts are also less abundant in upwelling areas (Saderne and Wahl, 2013). Elevated CO<sub>2</sub> levels in upwelling waters are compromising oyster aquaculture on eastern US shores (Barton *et al.*, 2012) as well as the fitness of other commercial species such as the Atlantic cod (Frommel *et al.*, 2012).

Some calcifying organisms seem to have adapted to the high pCO<sub>2</sub> recorded in upwelling areas: for instance, mussels are dominant in the Kiel fjord, despite being subject to upwelling episodes during their recruitment season (Thomsen *et al.*, 2010). However, at least some of them already live near their tolerance limit, such as sea urchin living on the USA east coast. Although their larval calcification is not impaired in present-day upwelling conditions, larvae are negatively affected by near future conditions (Evans *et al.*, 2013).

Using this approach could improve our understanding of large scale effect of ocean acidification, although with some limitations. In these systems it is often difficult to decide which environmental factor is driving the observed changes, as many factors usually co-vary with seawater CO<sub>2</sub> concentration. For instance, waters off Chile have high pCO<sub>2</sub>, but that is correlated with low oxygen concentration, making the effect of these two concurring factors difficult to disentangle and potentially leading to overestimation of high CO<sub>2</sub> negative effects (Mayol *et al.*, 2012). Furthermore, upwelling waters are generally low in temperature and rich in nutrients, whereas climate change scenarios predict opposite trends (Beardall *et al.*, 2009). Lower temperatures and higher food availability compared with future scenarios could reduce the biological effects of high CO<sub>2</sub>, thus leading to underestimation of the detrimental effects of ocean acidification (Thomsen *et al.*, 2010; Melzner *et al.*, 2011).

#### **1.2.4 Modelling approach**

Integrating results from single organisms and communities on an ecosystem scale could be done using a modelling approach. Models are widely used in climate change research for physical and chemical factors (e.g. Andersson *et al.*, 2005; Caldeira and Wickett, 2005; Orr *et al.*, 2005), and models involving

also biological factors are currently being developed. Policy makers need to know how marine ecosystems will react not only to ocean acidification, but to concurrent stressors such as increasing temperature, eutrophication and overfishing (Hilmi *et al.*, 2012). Models predicting ecosystem effects of multiple stressors, however, are more difficult to produce because of the system complexity and data scarcity on many factors, e.g. potential for adaptation (Blackford, 2010).

At the moment, there are only a few studies using models to assess ecosystem effects of ocean acidification, but some work has already been done on coral reefs. For instance, a recent study models the potential reef organisms have to influence carbonate chemistry on a local scale (Anthony *et al.*, 2011a). Their results show that the predicted increase in seaweed dominance could actually help calcification in communities downstream, as macroalgae decrease seawater pCO<sub>2</sub> through photosynthesis. These results are consistent with data from Manzello *et al.* (2012), who reported that seagrass beds in Florida take up carbon dioxide and raise the calcium carbonate saturation experienced by inshore coral reefs to pre-industrial levels, although this effect is seasonal because it depends on seagrass productivity.

Models can also be used to predict how interacting stressors will affect communities; for instance, it has been shown that overfishing and eutrophication will decrease coral reef resilience to ocean acidification and increasing temperature. The reason for this is that corals will be less competitive than macroalgae, which will grow faster and will be favoured by decreased fish grazing (Anthony *et al.*, 2011b). Similar results were obtained using a model to analyse how long-term fisheries exploitation and ocean acidification would interact to affect various functional groups (Griffith *et al.*,

2011). This study showed that increased seawater pCO<sub>2</sub> is likely to influence very heavily overfished areas and cause major regime shifts around 2040. It is already possible to see that although in their infancy, models are a promising approach because they can predict large-scale responses that are difficult to test experimentally.

### **1.3 Mechanisms driving community changes**

Community responses to ocean acidification result from the combination of direct effects of carbon dioxide on single species and indirect effects (i.e. biological interactions). While most ocean acidification research has focused on direct effects of elevated CO<sub>2</sub>, there is increasing evidence that indirect effects are extremely important in determining community changes (Alsterberg *et al.*, 2013). In the terrestrial environment, biological interactions have been proven to be more important than direct climate change effects (Ockendon *et al.*, 2014), but there is no such evidence for community responses to ocean acidification.

#### **1.3.1 Changes in organism physiology**

Most studies on the biological effects of ocean acidification are laboratory experiments that measure physiological responses, most commonly calcification, of a single species. A recent meta-analysis concluded that overall effects of high pCO<sub>2</sub> on organisms' survival, growth, calcification and reproduction are strong and negative (Kroeker *et al.*, 2013a). Moreover, high CO<sub>2</sub> levels have subtler negative effects, such as those on behaviour (Briffa *et al.*, 2012) and neuroreceptors (Munday *et al.*, 2014). Predictions of future ecosystems conditions are also complicated by high taxonomic variability in biological responses to ocean acidification. For example, Ries *et al.* (2009) measured net calcification rates in 18 animal and algal taxa. Calcification rates

decreased in most species, but a wide range of responses was recorded, from negative to parabolic (i.e. highest calcification rates at intermediate pCO<sub>2</sub> values) to positive.

Responses to ocean acidification will not only vary among taxonomic groups (Kroeker *et al.*, 2013a, see Figure 1.3), but in some cases will be species- (Miller *et al.*, 2009), sex- (Holcomb *et al.*, 2011), clone- (Pistevos *et al.*, 2011) or strain-specific (Langer *et al.*, 2009; Hoppe *et al.*, 2011). This high degree of variability in biological responses to ocean acidification has led to conflicting reports: for instance, calcification rates of the coccolithophore *Emiliana huxleyi* have been reported to decrease (De Bodt *et al.*, 2010; Riebesell *et al.*, 2000) or increase (Iglesias-Rodriguez *et al.*, 2008) when exposed to low pH. In the first instance, it was thought that this difference was caused by methodological issues (Iglesias-Rodriguez *et al.*, 2008). However, the two methods used to modify water chemistry in these studies cause very similar changes in the carbonate system (Schulz *et al.*, 2009). Evidence of strain-specific responses was given by Langer *et al.* (2009), and Hoppe *et al.* (2011) confirmed that methodological issues do not cause a variation in *Emiliana huxleyi* responses to ocean acidification.

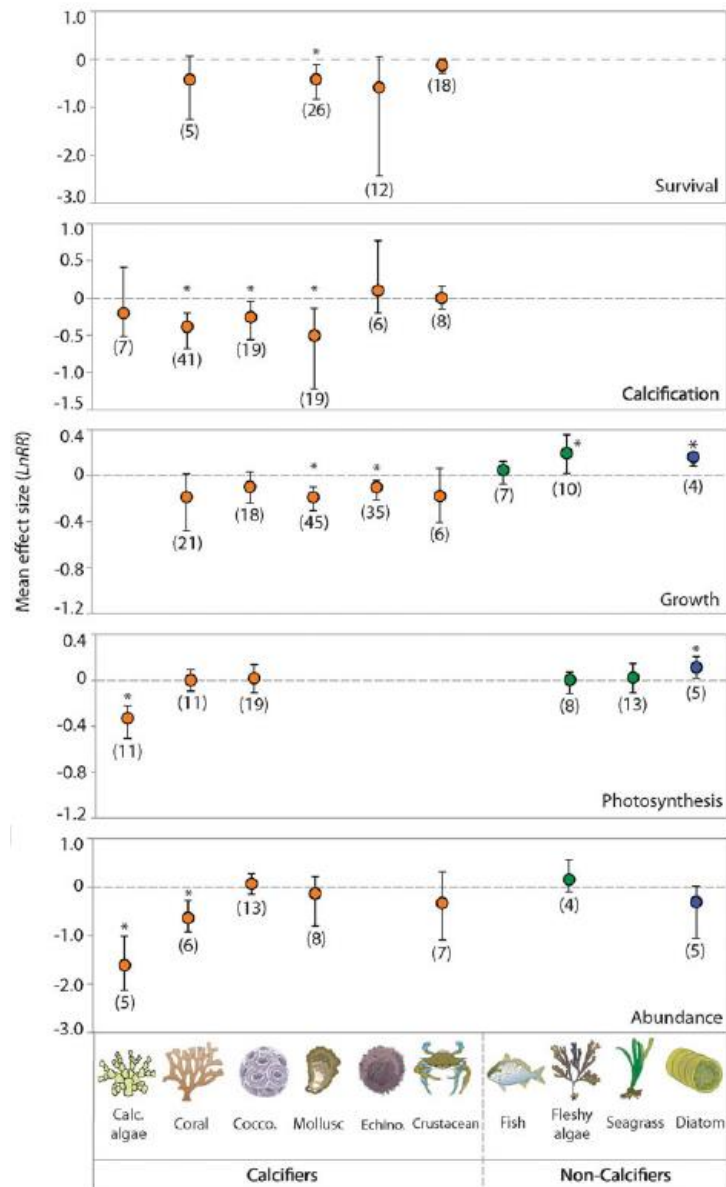


Figure 1.3. Taxonomic variation in effects of ocean acidification. Note the different y-axis scale for growth and photosynthesis. Mean effect size and 95% bias-corrected bootstrapped confidence interval are shown for all organisms combined (overall), calcifiers (orange) and noncalcifiers (green). The calcifiers category includes: calcifying algae, corals, coccolithophores, molluscs, echinoderms and crustaceans. The noncalcifiers category includes: fish, fleshy algae and seagrasses. The number of experiments used to calculate mean effect sizes are shown in parentheses. The mean effect size is significant when the 95% confidence interval does not overlap zero (\*); Kroeker *et al.*, 2013a.



Predicting how species and their populations will react to increased CO<sub>2</sub> is complex but necessary if we want to prepare for marine ecosystem changes in the near future. Fortunately, certain traits are proving useful in determining how an organism may respond to ocean acidification. Firstly, very mobile taxa with high metabolic rates usually have efficient mechanisms to regulate pH (Melzner *et al.*, 2009), and are thus likely to be less affected directly by lowered pH compared to non-regulators. A meta-analysis on studies on mobile taxa with good acid-base regulation such as fish and brachyuran crustaceans did not show significant negative responses to high CO<sub>2</sub> levels on survival and growth (Kroeker *et al.*, 2013a), although there is evidence that some tropical fish species have their sensory abilities impaired when carbon dioxide increases (Munday *et al.*, 2014). Negligible effects of elevated CO<sub>2</sub> on growth and calcification were also related to good acid-base regulation in the cephalopod *Sepia officinalis* (Gutowska *et al.*, 2010). In contrast with these results, the giant squid *Dosidicus gigas* has been reported to suffer metabolic depression following short-term exposure to elevated CO<sub>2</sub>, possibly because of the very high pH sensitivity of its oxygen-binding pigments (Rosa and Seibel, 2008). Thus, highly pH-sensitive blood pigments insure the rapid oxygen supply necessary to maintain very high metabolic rates, but they could be a disadvantage in a high-CO<sub>2</sub> world if pH regulation is not extremely efficient (Pörtner *et al.*, 2004).

For calcifying organisms, pH regulation at the calcification site will probably determine calcification responses to high CO<sub>2</sub> (Venn *et al.*, 2011). [H<sup>+</sup>] regulation at crystallisation sites has been detected in many taxa, including crabs (Cameron, 1985), coralline red and calcareous green algae (Borowitzka, 1987; McConnaughey & Whelan, 1997), foraminifera (Rink *et al.*, 1998) and

scleractinian corals (Al-Horani *et al.*, 2003; Venn *et al.*, 2011). Differential efficiency in calcification regulation may be one of the causes for the varied calcification responses to high CO<sub>2</sub> between taxa.

At intermediate pCO<sub>2</sub> levels calcification rates can increase (Wood *et al.* 2008; Gooding *et al.* 2009; McDonald *et al.*, 2009; Ries *et al.*, 2009; Findlay *et al.*, 2011; Rodolfo-Metalpa *et al.*, 2011). These responses may be due to the fact that calcification substrata used by many organisms are bicarbonate ions or aqueous carbon dioxide (Wilbur, 1964; Bubel, 1975; Decker & Lennarz, 1988; Al-Horani *et al.*, 2003), both of which will increase following ocean acidification. Alternatively, increased calcification could be a defensive mechanism aiming to compensate for the increased dissolution rates of calcified structures at high CO<sub>2</sub> (McDonald *et al.*, 2009). There are several examples of increased shell dissolution at elevated CO<sub>2</sub> levels in molluscs, echinoderms and corals (Nienhuis *et al.*, 2010; Findlay *et al.*, 2011; Rodolfo-Metalpa *et al.*, 2011). These studies all conclude that the decrease in net calcification rates with increased pCO<sub>2</sub> levels is mostly caused by an increase in dissolution rather than a decrease in the organism's calcification. Organisms that build their skeletons using Mg-calcite, the most soluble form of calcium carbonate, suffer the most from increased dissolution rates as pCO<sub>2</sub> increases (Johnson *et al.*, 2014).

Given the significant increase in carbonate dissolution rates when calcium carbonate saturation state is low, species protecting their shells or skeletons with an organic layer will probably be favoured in an ocean acidification scenario. Rodolfo-Metalpa *et al.* (2011), for instance, showed that coral skeleton and mollusc shells were significantly more damaged in species without or with very limited organic cover. In some cases the organic layer is damaged at high

CO<sub>2</sub> levels, exposing carbonate structures to acidified seawater (Thomsen *et al.*, 2010; Rodolfo-Metalpa *et al.*, 2011)

Even species with very efficient acid-base regulation will require more energy to maintain extracellular pH values in a range compatible with the organism's needs as seawater pH decreases (Thomsen and Melzner, 2010). An organism may manage to maintain most of its physiological functions, but have to down-regulate some others, as reported for the swimming crab *Necora puber* (Small *et al.*, 2010) and in the barnacle *Semibalanus balanoides* (Findlay *et al.*, 2010). In other cases, changes in energy allocation such as compensatory increases in calcification rates could reduce the energy available for other essential physiological processes (Bradassi *et al.*, 2013). Energy can also be allocated depending on the individual; for instance, in the coral *Astrangia poculata* calcification rates decreased with increasing CO<sub>2</sub>, but this was more evident in females, probably because they needed energy for egg production (Holcomb *et al.*, 2011). However, up-regulation of physiological processes is not always sustainable in the long term (Lombardi *et al.*, 2011).

Energy availability is extremely important in determining biological responses to ocean acidification, so food supply has an essential role. Increased nutrient or food supply can counter the effects of ocean acidification in corals (Cohen *et al.*, 2009; Holcomb *et al.*, 2010; Chauvin *et al.*, 2011) and molluscs (Thomsen *et al.*, 2010; Thomsen *et al.*, 2013). Increased food availability does not always have a significant effect, but this probably happens when food is not limiting (Holcomb *et al.*, 2011); in some cases, energy supply even outweighs the effects of carbon dioxide (Thomsen *et al.*, 2013).

Photosynthetic organisms could be positively influenced by ocean acidification if they are able to fix more inorganic carbon, and thus have more energy available. Many algal species have carbon concentrating mechanisms (CCMs), so they are less likely to be limited by inorganic carbon concentration (Raven *et al.*, 2012). However, higher seawater pCO<sub>2</sub> would give them a significant advantage, because a greater proportion of their inorganic carbon uptake could derive from passive CO<sub>2</sub> diffusion instead of the energetically expensive CCMs (Cornwall *et al.*, 2012; Harley *et al.*, 2012). The increased energy availability, however, may not be enough to counter the negative effects of ocean acidification in calcifying species faced with the increased energetic costs of calcification at high pCO<sub>2</sub> levels (Bradassi *et al.*, 2013).

The natural pCO<sub>2</sub> variability a species experiences also contributes to its tolerance to ocean acidification. Species exposed to high levels of CO<sub>2</sub> in their habitat could be more resistant to the negative effects of ocean acidification (Maas *et al.*, 2012; Moulin *et al.*, 2011). In addition, short-term experiments are often not representative of the full acclimation potential of a species to ocean acidification. For instance, even a species considered very sensitive to ocean acidification such as the deep-sea coral *Lophelia pertusa* can acclimate to high-CO<sub>2</sub> conditions after some months, while undergoing strong negative effects in the short term (Form and Riebesell, 2011). On the other hand, some short-term responses to high CO<sub>2</sub> could require too much energy to be maintained in the long term (Lombardi *et al.*, 2011).

Most factors considered above can vary during an organism's life cycle, and larvae are more vulnerable than adults in many cases, since their regulatory system is usually less developed than that of adults (Ellis *et al.*, 2009; Melzner *et al.*, 2009). A recent meta-analysis seems to confirm that echinoderm larval

stages are more sensitive to ocean acidification than adults (Dupont *et al.*, 2010). In general, however, differences in responses between life stages are probably less pronounced than those between taxonomic groups, and the most sensitive life stage can vary between taxa (Kroeker *et al.*, 2013a). It is therefore important to consider the whole life cycle of a species before drawing conclusions regarding its sensitivity to increased seawater CO<sub>2</sub>.

### **1.3.2 Changes in biological interactions**

Changes in biological communities caused by elevated CO<sub>2</sub> will depend on the responses of single species and the subsequent changes in their interactions. Although only a few studies examine the effect of ocean acidification on biological interactions, there is evidence that competition will be influenced. For instance, elevated CO<sub>2</sub> levels can increase coral mortality due to enhanced competition from seaweeds (Diaz-Pulido *et al.*, 2011) or negatively influence kelp recruitment through increased turf cover (Connell and Russell, 2010). Furthermore, coralline algae with high-Mg calcite skeletons have their fitness reduced when seawater pCO<sub>2</sub> is high, causing them to be outcompeted by non-calcifying algae, commonly fast-growing species (Russell *et al.*, 2009; Hofmann *et al.*, 2012; Kroeker *et al.*, 2013c; Short *et al.*, 2014).

CO<sub>2</sub>-related changes in plant-herbivore interactions are poorly known. However, it seems that grazers' consumption rates do not change only because of variations in the animal's metabolic rates, but also as a consequence of the alteration in algal palatability. Since a wide range of reactions to high CO<sub>2</sub> is expected in algal taxa, it is not surprising that gastropod herbivores have increased (Falkenberg *et al.*, 2013b) and decreased (Swanson and Fox, 2007; Russell *et al.*, 2013) feeding rates at high CO<sub>2</sub> when grazing on primary

producers. In one study, the detected decrease in feeding rates is probably caused by an increase in kelp phlorotannins, a class of substances involved with herbivore deterrence in seaweeds (Swanson and Fox, 2007). Seagrasses show an opposite trend since their phenolic protective substances concentrations decrease as CO<sub>2</sub> increases, with potential positive effects on grazing organisms (Arnold *et al.*, 2012). Accordingly, sea urchins have been found to increase their seagrass consumption when pCO<sub>2</sub> increases (Burnell *et al.*, 2013). In other cases, change in pH alone has no significant effects on algal palatability, and changes in feeding rates only occur when interaction between temperature and pH is examined (Poore *et al.*, 2013).

There are also very few studies dealing with the response of predator-prey interactions to increased CO<sub>2</sub> levels. An experiment on four species of reef fish larvae and one of their predators (Ferrari *et al.*, 2011) found that at high CO<sub>2</sub> small fish recruits of all species suffered higher predation rates compared to the control. At the same time, predator's preferences seem to switch from two of the species to the other two. As for the cause of these changes, there is evidence to suggest that high CO<sub>2</sub> impairs some fish sensory perceptions (Munday *et al.*, 2014), but an effect on predator behaviour could not be excluded. CO<sub>2</sub>-driven changes in the predator-prey interaction between the intertidal snail *Littorina littorea* and the green crab *Carcinus maenas* have also been recently studied (Bibby *et al.*, 2007; Landes and Zimmer, 2012). Although crabs had reduced claw strength (Landes and Zimmer, 2012) and snails exhibited weaker shells and increased avoidance behaviour with increased CO<sub>2</sub> (Bibby *et al.*, 2007; Landes and Zimmer, 2012), the overall interaction strength between the two species was not affected by pCO<sub>2</sub> (Landes and Zimmer, 2012). Predator-prey

interactions are thus likely to show a wide range of responses to ocean acidification, which are rarely predictable from single-species studies.

## 1.4 Adaptation potential

Most experiments on ocean acidification effects so far have tested whether present populations of marine organisms can cope with future seawater CO<sub>2</sub> levels. Although this approach gives essential information on the physiological mechanisms involved in coping with ocean acidification, it excludes the possibility an organism could adapt (i.e. genetically change) to increased CO<sub>2</sub>. Experiments to test directly for adaptation to ocean acidification have mostly been performed on unicellular phytoplankton due to their extremely rapid generation time (reviewed in Collins *et al.*, 2014). Results so far suggest that taxa negatively affected by ocean acidification, such as calcifying coccolithophores, are under strong adaptive pressure and evolve to partially restore calcification and growth rates (Lohbeck *et al.*, 2012; Benner *et al.*, 2013). On the other hand, non-calcifying taxa such as the diatom *Thalassiosira pseudonana* were able to reduce their usage of energy-expensive carbon-concentrating mechanisms (CCMs), but no genetic adaptation seemed to take place (Crawford *et al.*, 2011).

For organisms with a longer life cycle, experimental evolution is generally not feasible, and alternative approaches need to be used. Variation in responses to ocean acidification among genotypes can hint at a species' potential for adaptation. Variability in fitness with increased CO<sub>2</sub> have been found in bryozoans (Pistevos *et al.*, 2011), oysters (Parker *et al.*, 2012), amphipods (Calosi *et al.*, 2013b) and coccolithophores (Langer *et al.*, 2009). It is also possible to measure within-population genetic diversity as a proxy for their

potential of undergoing rapid evolution (Kelly *et al.*, 2013). Results from these studies revealed that species with shorter generation times do not always have higher adaptation potential than species with longer generation times, but greater genetic diversity (Sunday *et al.*, 2011).

Another approach to assess adaptation or acclimation/acclimatisation potential in marine organisms (for definitions see Box 1) is using natural pCO<sub>2</sub> gradients, such as those found at volcanic seeps or in upwelling areas, to assess whether local populations have acclimated or adapted to elevated CO<sub>2</sub> levels (Reusch, 2014). Individuals from areas with different pH can be exposed to the same CO<sub>2</sub> conditions in the laboratory (“common garden experiments”) or reciprocally transplanted between sites to assess local adaptation (Sanford and Kelly, 2011). So far, both common garden experiments and reciprocal transplants have found significant inter-population differences in responses to pCO<sub>2</sub> in sea urchins, barnacles and polychaetes (Moulin *et al.*, 2011; Calosi *et al.*, 2013c; Evans *et al.*, 2013; Kelly *et al.*, 2013; Pansch *et al.*, 2014). Sea urchins naturally exposed to elevated pCO<sub>2</sub> have offspring that show increased resistance to ocean acidification (Moulin *et al.*, 2011; Evans *et al.*, 2013; Kelly *et al.*, 2013), while tolerant polychaete species transplanted between CO<sub>2</sub> levels can either adapt or acclimatise to high and variable CO<sub>2</sub> (Calosi *et al.*, 2013c). Short-term acclimation or acclimatisation to high pCO<sub>2</sub> can buffer populations against negative impacts of ocean acidification, giving them a chance to survive until adaptation takes place (Sunday *et al.*, 2014). This process, however, can be energetically expensive: barnacles populations not adapted to high and variable pCO<sub>2</sub> could cope with moderate carbonate dioxide levels, but only if food was abundant, whereas the adapted population was unaffected by food levels (Pansch *et al.*, 2014). The adaptation potential to ocean acidification of most



marine organisms is still unknown, and more evidence is urgently needed to fully determine which species will be able to adapt to the rapid environmental changes predicted for the next decades (Kelly and Hofmann, 2012).

**Box 1: Acclimation, acclimatisation and adaptation (Angilletta, 2009)**

**Acclimatisation:** the process by which an individual organism adjusts to a gradual change in its environment (such as a change in temperature or pH), allowing it to maintain performance across a range of environmental conditions.

**Acclimation:** similar to acclimatisation, but refers to changes occurring in response to an artificial or controlled situation.

**Adaptation:** a process involving the selection on genetic variation that shifts the average phenotype toward the fitness peak.

## 1.5 Interaction with other anthropogenic stressors

As well as ocean acidification, anthropogenic CO<sub>2</sub> emissions are causing an increase in seawater temperature (IPCC, 2014). Studying how these two factors will interact is essential to understand the future of marine ecosystems. Nonetheless, our knowledge of the combined effect of high pCO<sub>2</sub> and higher temperatures is currently lower than that of the effects of each factor alone. As with responses to changes in carbonate chemistry, interactions with temperature appear to have very variable effects on an organism's physiology, depending on which taxa it belongs to and which physiological responses are measured (e.g. Lischka *et al.*, 2010; Noiset *et al.*, 2013a), although there is a trend towards an increase in negative effects of ocean acidification when organisms are concurrently exposed to elevated temperature (Kroeker *et al.*, 2013a). One possible explanation for this pattern is that the energetic costs of coping with ocean acidification (e.g. maintaining intracellular pH or calcification rates) narrow an organism's aerobic scope (Kelly and Hofmann, 2012).

Hypoxic zones are also expanding in the world oceans, both in coastal areas and in the subsurface open ocean waters (Diaz and Rosenberg, 2008). As hypoxic zones are the result of microbial respiration, decreased oxygen levels are paired with increased pCO<sub>2</sub>. Carbon dioxide could then reach levels much higher than those predicted for the world oceans at the end of this century in hypoxic zones, threatening the survival of species otherwise resistant to ocean acidification (Melzner *et al.*, 2013). However, the effects of hypoxia and ocean acidification have mostly been studied in isolation, even though pteropods from hypoxic areas show increased resistance to increased pCO<sub>2</sub>, suggesting that carbon dioxide is an important stressor in hypoxic areas (Maas *et al.*, 2012). To date, the only study assessing the combined effect of hypoxia and ocean acidification on marine organisms found that the two stressors interact to affect organisms in ways not predictable from single-stressor experiments (Gobler *et al.*, 2014).

Climate change is also expected to increase the intensity of ultra-violet (UV) radiation in marine systems following depletion of the ozone layer (Austin *et al.*, 1992). Marine organisms utilise many mechanisms for photoprotection against excessive radiation, most of which are energetically expensive and could decrease their resistance to other stressors, including ocean acidification (Häder *et al.*, 2007). The combination of increased CO<sub>2</sub> and UV radiation seems in fact to promote production of protective compounds (phlorotannins) in some brown algae (Swanson and Fox, 2007), even though not all taxa show an enhanced sensitivity to the combination of those stressors (Beardall *et al.*, 2009). For calcifying algae, the synergistic effect of CO<sub>2</sub> and UV radiations could be due to the reduction of the calcified layer as CO<sub>2</sub> increases, which impairs its photoprotective function (Gao *et al.*, 2009; Gao and Zheng, 2010),

even though not all coralline algae are negatively affected by the interaction of elevated carbon dioxide and UV radiations (Yildiz *et al.*, 2013).

Local stressors such as eutrophication and heavy metal pollution can also worsen community responses to ocean acidification, and managing them is essential to improve the resilience of marine ecosystems (Ghedini *et al.*, 2013). While at the single species level increased nutrient levels can offset the negative impacts of ocean acidification (Chauvin *et al.*, 2011; Thomsen *et al.*, 2013; Pansch *et al.*, 2014), elevated energy input often alters the outcome of inter-specific interactions, with more opportunistic species becoming more competitive (Connell and Russell, 2010; Falkenberg *et al.*, 2013a). The interactive effects of ocean acidification and heavy metals have not been studied at the community levels so far, but evidence from single-species experiments suggests that copper will enhance the negative effects of ocean acidification in some marine organisms, such as amphipods and polychaetes (Lewis *et al.*, 2012; Roberts *et al.*, 2013).

## **1.6 Thesis aims and objectives**

Ocean acidification is a relatively new research field and studies on its community effects at temperate rocky reefs are in their infancy. Volcanic seeps are proving useful to study community responses of ocean acidification, but more sites are needed to build confidence in predictions developed using these systems. In addition, there is evidence that indirect effects of carbon dioxide could play a crucial role in the detected community changes, but our knowledge on the relative importance of direct or indirect effects of carbon dioxide is extremely limited. There is also little knowledge on the adaptation potential of

macroalgae and on possible interactions of ocean acidification with other stressors, such as copper pollution.

The overall thesis structure is illustrated in Figure 1.4: this project aimed to determine how benthic communities and their epifauna change along natural CO<sub>2</sub> gradients in the oligotrophic Mediterranean Sea, and investigate whether community changes were caused by direct or indirect effects; the interactive effects of CO<sub>2</sub> and a short-term copper pulse on macroalgae and their epifauna was also assessed. The specific objectives were to:

- Determine whether volcanic seeps off Methana (Greece) are suitable to study the effects of increased pCO<sub>2</sub> on benthic communities by monitoring carbonate chemistry, temperature, salinity, total alkalinity and measuring inorganic nutrients, hydrogen sulphide, as well as total and bioavailable heavy metals (Chapter 2).
- Determine how benthic communities and epifauna of main canopy-forming species change along Mediterranean pCO<sub>2</sub> gradients by assessing community structure and composition (Chapters 3 and 4).
- Determine whether changes in herbivores abundance contribute to the detected changes in benthic communities along pCO<sub>2</sub> gradients by excluding intertidal and subtidal herbivores (Chapter 5).
- Determine whether two abundant macroalgal species, one articulated coralline (*Jania rubens*) and one furoid (*Cystoseira corniculata*), have acclimatised to high pCO<sub>2</sub> by transplanting them within and between high CO<sub>2</sub> and reference sites off Methana (Chapter 6).
- Determine whether two abundant macroalgal species, one articulated coralline and one furoid, respond differently to copper exposure at

different  $p\text{CO}_2$  levels and how this affects the fucoid epifauna by exposing macroalgae to copper *in situ* (Chapter 7).

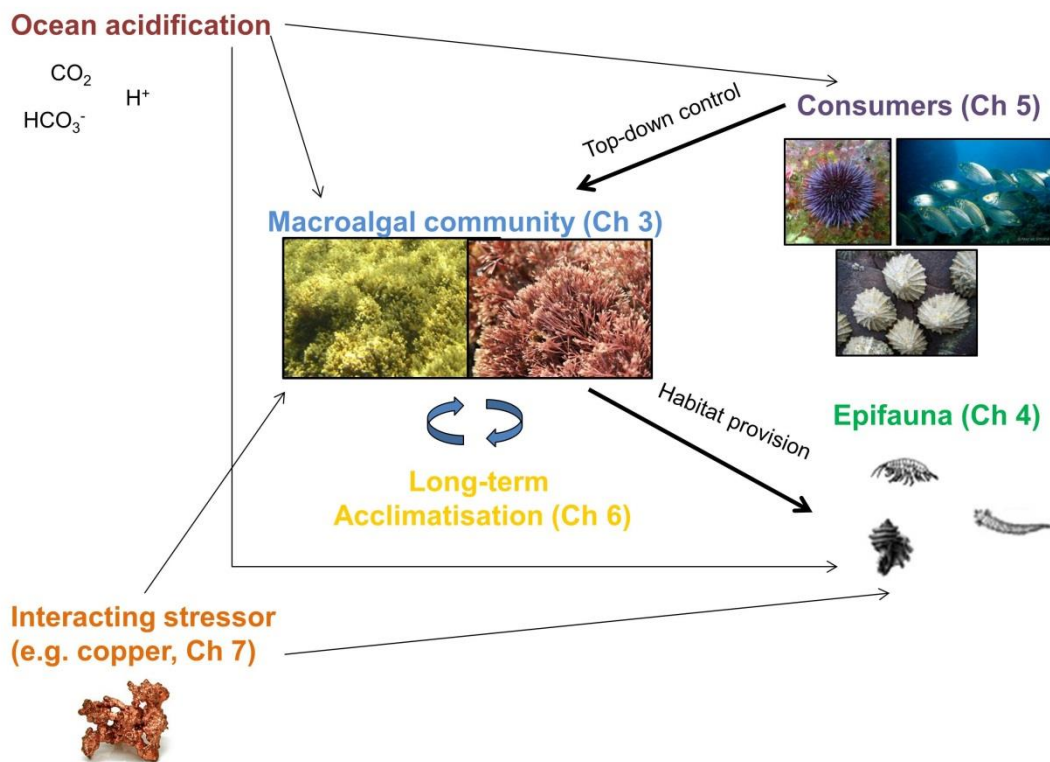


Figure 1.4. Visual abstract of thesis aims; using  $\text{CO}_2$  seeps as natural analogues for ocean acidification, I assessed how macroalgal communities (Chapter 3) and their epifauna (Chapter 4) change with increasing  $p\text{CO}_2$  levels, and whether long-term acclimatisation to elevated  $p\text{CO}_2$  increases adaptation potential of dominant macroalgal species (Chapter 6). I also examined how changes in herbivores densities affect the strength of top-down control on macroalgal communities (Chapter 5), and how interaction of ocean acidification with another stressor (short-term copper pollution) may influence dominant macroalgal species and their epifauna (Chapter 7).

Table 1.1. Summary of this thesis' surveys and experiments, with measured parameters and sample sizes. (A) Summary of sampling for Chapter 2, with sampling date, site, measured parameters and sample sizes: pH, temperature and salinity (pH/T/S), water samples for total alkalinity (TA), water samples for inorganic nutrients (nitrites, nitrates, ammonium, phosphates, silicates), water samples for free sulphides, sediment samples for heavy metals content, macroalgal samples for heavy metals content. (B) Summary of sampling for Chapter 3, with sampling date, sites and number of 20 x 20 cm visual quadrats (Methana) or 20 x 20 cm quadrats scraped from rocky substratum (Vulcano). (C) Summary of sampling for Chapter 4, with sampling dates, sites and species (only Vulcano) and number of 20 x 20 cm quadrats of *Cystoseira corniculata* scraped from the substratum (Methana) or number of collected thalli (Vulcano). (D) Summary of sampling for the two experiments described in Chapter 5. For the limpet exclusion experiment at Vulcano, the table shows sampling dates and number of pH, temperature and salinity measurements (pH/T/S), number of limpet abundance and length measurements, number of experimental plots per treatment where macroalgal cover or biomass were measured (C=controls, P=procedural controls, E=exclusions) and number of wax discs used to quantify limpet grazing. For the herbivore exclusion experiment at Methana, the table shows sampling dates and number of pH, temperature and salinity measurements (pH/T/S), number of transects for sea urchins (*Paracentrotus lividus* and *Arbacia lixula*) abundance and herbivore fish (*Sarpa salpa*, *Siganus luridus* and *Sparisoma cretense*) biomass and number of experimental plots per treatment where macroalgal cover or biomass were measured (C=controls, P=procedural controls, E=exclusions). (E) Summary of sampling for *Cystoseira corniculata* (01-05/10/2012 to 22-25/06/2013) and *Jania rubens* (22-25/06/2013 to 04-09/09/2013) transplants described in Chapter 6. The table shows macroalgal species, treatment (expressed as "Site of origin". "Site of transplant", for a detailed description of the experimental design see Chapter 6), number of thalli used to measure growth and epiphyte cover (\*=only epiphytes measured), photosynthetic parameters, pigments content, carbon, nitrogen and phosphorous (C,N,P) content (\*\*P not measured) and total phenols content. N.u.=not used because sample size was too small; lost=all thalli lost; N/A=not applicable. (F) Summary of sampling for the copper exposure experiments described in Chapter 7. For the

environmental monitoring, the table shows experiment dates, sites and number of pH, salinity and temperature measurements (pH/S/T) and number of water samples collected for total alkalinity measurements (TA). For the copper pulse experiments, the table shows macroalgal species (*Cystoseira corniculata* on 18-25/06/2013 and *Jania rubens* on 04-09/09/2013), treatment (expressed as “Site of origin”. “Site of transplant”. “Copper exposure”, for a detailed description of the experimental design see Chapter 7), number of plaster blocks used, number of thalli used to measure copper content, maximum quantum yield, pigments content and epifaunal communities. N.u. treatment not used because sample size was too small; N/A=not applicable.

| <b>(A) Chapter 2: Assessing the suitability of a volcanic seep area off Methana (Greece) for ocean acidification studies.</b> |       |         |    |                     |           |                   |                     |
|---|-------|---------|----|---------------------|-----------|-------------------|---------------------|
| Date  | Site  | pH/T /S | TA | Inorganic nutrients | Sulphides | Metals - sediment | Metals - macroalgae |
| 01/09/2011  | SEEP  | 1       |    |                     |           |                   |                     |
| 01/09/2011  | 200 W | 1       |    |                     |           |                   |                     |
| 01/09/2011  | 200 E | 1       |    |                     |           |                   |                     |
| 11-12/02/2012   | SEEP  | 2       | 3  |                     |           |                   |                     |
| 11-12/02/2012   | 200 W | 2       | 3  |                     |           |                   |                     |
| 11-12/02/2012   | 200 E | 2       | 4  |                     |           |                   |                     |
| 22/05/2012 to 06/06/2012  | SEEP  | 11      | 5  |                     |           | 3                 | 5                   |
| 22/05/2012 to 06/06/2012  | 200 W | 11      | 6  |                     |           | 3                 | 5                   |
| 22/05/2012 to 06/06/2012  | 200 E | 11      | 3  |                     |           | 3                 | 5                   |
| 22/05/2012 to 06/06/2012  | REF A | 1       | 3  |                     |           | 3                 | 5                   |
| 22/05/2012 to 06/06/2012  | REF B | 4       | 3  |                     |           | 3                 | 5                   |
| 27/09/2012 to 05/10/2012  | SEEP  | 11      | 6  |                     |           |                   |                     |
| 27/09/2012 to 05/10/2012  | 200 W | 3       | 6  |                     |           |                   |                     |
| 27/09/2012 to 05/10/2012  | 200 E | 3       | 6  |                     |           |                   |                     |
| 27/09/2012 to 05/10/2012  | REF A | 8       | 6  |                     |           |                   |                     |
| 27/09/2012 to 05/10/2012  | REF B | 3       | 3  |                     |           |                   |                     |
| 18-25/06/2013   | SEEP  | 13      | 6  | 3                   | 7         |                   |                     |
| 18-25/06/2013   | 200 W | 7       | 6  | 3                   | 3         |                   |                     |
| 18-25/06/2013   | 200 E | 7       | 6  | 3                   | 3         |                   |                     |
| 18-25/06/2013   | REF A | 9       | 6  | 3                   | 3         |                   |                     |
| 18-25/06/2013   | REF B | 8       | 6  | 3                   | 3         |                   |                     |
| 04-09/09/2013   | SEEP  | 3       | 3  |                     |           |                   |                     |
| 04-09/09/2013   | 200 W | 3       | 3  |                     |           |                   |                     |
| 04-09/09/2013   | 200 E | 3       | 3  |                     |           |                   |                     |
| 04-09/09/2013   | REF A | 3       | 3  |                     |           |                   |                     |

|  |                          |                     |                      |               |       |  |
|--|--------------------------|---------------------|----------------------|---------------|-------|--|
| 04-09/09/2013  | REF B                    | 4                   | 3                    |               |       |  |
| <b>(B) Chapter 3: Changes in subtidal macroalgal communities along pCO<sub>2</sub> gradients at Mediterranean volcanic seeps</b> |                          |                     |                      |               |       |  |
| <b>Methana - visual quadrats of benthic communities</b>  |                          |                     |                      |               |       |  |
| Date   | SEEP                     | 200 W               | 200 E                | REF A         | REF B |  |
| 23-29/05/2012  | 7                        | 7                   | 7                    | 7             | 7     |  |
| 30/09/2012 to 05/10/2012   | 6                        | 6                   | 6                    | 6             | 6     |  |
| <b>Vulcano - destructively sampled quadrats</b>  |                          |                     |                      |               |       |  |
| Date   | REF A                    | Mid CO <sub>2</sub> | High CO <sub>2</sub> |               |       |  |
| 15/05/2010   | 4                        | 4                   | 4                    |               |       |  |
| <b>(C) Chapter 4: Canopy algal epifauna changes at elevated pCO<sub>2</sub> at two Mediterranean volcanic seeps</b>              |                          |                     |                      |               |       |  |
| <b>Methana - quadrats of <i>Cystoseira corniculata</i> scraped</b>   |                          |                     |                      |               |       |  |
| Date   | SEEP                     | 200 W               | 200 E                | REF A         | REF B |  |
| 26/05/2012 to 06/06/2012   | 3                        | 3                   | 3                    | 3             | 3     |  |
| <b>Vulcano - macroalgal thalli collected</b>   |                          |                     |                      |               |       |  |
| Date   | Species                  | 600 ppm             | 1200 ppm             |               |       |  |
| 04-08/06/2013  | <i>Cystoseira</i> spp.   | 15                  | 14                   |               |       |  |
| 04-08/06/2013  | <i>Sargassum vulgare</i> | 9                   | 10                   |               |       |  |
| <b>(D) Chapter 5: Effect of herbivores on benthic communities at different pCO<sub>2</sub> levels</b>                            |                          |                     |                      |               |       |  |
| <b>Vulcano - limpets exclusion experiment</b>  |                          |                     |                      |               |       |  |
| Date   | Measured parameter       | Treatment           | 600                  | 1200          |       |  |
| 08/05/2012   | pH/T/S                   | N/A                 | 1                    | 1             |       |  |
| 18-19/07/2012  | pH/T/S                   | N/A                 | 2                    | 2             |       |  |
| 22-23/09/2012  | pH/T/S                   | N/A                 | 2                    | 2             |       |  |
| 27/10/2012   | pH/T/S                   | N/A                 | 1                    | 1             |       |  |
| 08/05/2012   | Limpets abundance/length | N/A                 | 7                    | 9             |       |  |
| 18-19/07/2012  | Limpets abundance/length | N/A                 | 18                   | 18            |       |  |
| 22-23/09/2012  | Limpets abundance/length | N/A                 | 9                    | 9             |       |  |
| 27/10/2012   | Limpets abundance/length | N/A                 | 9                    | 9             |       |  |
| 08/05/2012   | Macroalgal cover         | C                   | 3                    | 3             |       |  |
| 08/05/2012   | Macroalgal cover         | P                   | 3                    | 3             |       |  |
| 08/05/2012   | Macroalgal cover         | E                   | 6                    | 6             |       |  |
| 18-19/07/2012  | Macroalgal cover         | C                   | 3                    | 3             |       |  |
| 18-19/07/2012  | Macroalgal cover         | P                   | 3                    | 3             |       |  |
| 18-19/07/2012  | Macroalgal cover         | E                   | 6                    | 6             |       |  |
| 22-23/09/2012  | Macroalgal cover         | C                   | 3                    | 3             |       |  |
| 22-23/09/2012  | Macroalgal cover         | P                   | 3                    | 3             |       |  |
| 22-23/09/2012  | Macroalgal cover         | E                   | 6                    | 4             |       |  |
| 27/10/2012   | Macroalgal cover         | C                   | 3                    | 3             |       |  |
| 27/10/2012   | Macroalgal cover         | P                   | 3                    | 0             |       |  |
| 27/10/2012   | Macroalgal cover         | E                   | 6                    | 4             |       |  |
| 20/11/2012   | Macroalgal biomass       | C                   | 3                    | 3             |       |  |
| 20/11/2012   | Macroalgal biomass       | P                   | 3                    | 0             |       |  |
| 20/11/2012   | Macroalgal biomass       | E                   | 6                    | 4             |       |  |
| 20/11/2012   | Limpets grazing rates    | N/A                 | 41                   | 34            |       |  |
| <b>Methana - herbivore exclusion experiment</b>  |                          |                     |                      |               |       |  |
| Date   | Measured parameter       | Treatment           | REF A                | SEEP          |       |  |
| 30/09/2012 to 05/10/2012 and 20-25/06/2013   | Sea urchins abundance    | N/A                 | 6 per species        | 6 per species |       |  |
| 08/09/2013   | Fish biomass             | N/A                 | 3                    | 3             |       |  |
| 20/06/2013   | Macroalgal cover/biomass | C                   | 4                    | 4             |       |  |
| 20/06/2013   | Macroalgal cover/biomass | P                   | 3                    | 3             |       |  |
| 20/06/2013   | Macroalgal cover/biomass | E                   | 4                    | 3             |       |  |



| <b>(E) Chapter 6: Seaweed acclimatisation to high pCO<sub>2</sub> at volcanic seeps</b>   |               |                      |                              |                             |                     |                   |
|---|---------------|----------------------|------------------------------|-----------------------------|---------------------|-------------------|
| Species   | Treatment     | Growth/<br>epiphytes | Photosynthetic<br>parameters | Pigments<br>content         | C, N, P<br>content  | Phenol<br>content |
| <i>C. corniculata</i>   | SEEP.SECP     | 5                    | 5                            | 5                           | 5                   | 5                 |
| <i>C. corniculata</i>   | REFB.SECP     | 8                    | 6                            | 8                           | 7                   | 7                 |
| <i>C. corniculata</i>   | REFA.SECP     | 3                    | 3                            | 3                           | 3                   | 3                 |
| <i>C. corniculata</i>   | REFA.REFA     | 5                    | 4                            | 5                           | 4                   | 4                 |
| <i>C. corniculata</i>   | SEEP.REFA     | 10                   | 9                            | 10                          | 9                   | 10                |
| <i>C. corniculata</i>   | REFB.REFB     | 8                    | 7                            | 8                           | 4                   | 4                 |
| <i>C. corniculata</i>   | SEEP.REFB     | 4                    | 3                            | 4                           | 4                   | 3                 |
| <i>C. corniculata</i>   | SEEP          | 7*                   | 6                            | 7                           | 7                   | 6                 |
| <i>C. corniculata</i>   | REFA          | 7*                   | 7                            | 6                           | 7                   | 7                 |
| <i>C. corniculata</i>   | REFB          | 7*                   | 4                            | 7                           | 7                   | 7                 |
| <i>J. rubens</i>  | SEEP.SECP     | 4                    | 4                            | 4                           | 4**                 | N/A               |
| <i>J. rubens</i>  | REFB.SECP     | 4                    | 5                            | 4                           | 3**                 | N/A               |
| <i>J. rubens</i>  | REFA.SECP     | n.u.                 | n.u.                         | n.u.                        | n.u.                | N/A               |
| <i>J. rubens</i>  | REFA.REFA     | lost                 | lost                         | lost                        | lost                | N/A               |
| <i>J. rubens</i>  | SEEP.REFA     | lost                 | lost                         | lost                        | lost                | N/A               |
| <i>J. rubens</i>  | REFB.REFB     | 5                    | 5                            | 5                           | 9**                 | N/A               |
| <i>J. rubens</i>  | SEEP.REFB     | 6                    | 5                            | 6                           | 4**                 | N/A               |
| <i>J. rubens</i>  | SEEP          | N/A                  | 7                            | 7                           | 5**                 | N/A               |
| <i>J. rubens</i>  | REFA          | N/A                  | 7                            | 7                           | 5**                 | N/A               |
| <i>J. rubens</i>  | REFB          | N/A                  | 7                            | 7                           | 5**                 | N/A               |
| <b>(F) Chapter 7: A short-term copper pulse affects macroalgal copper accumulation and indirectly alters epifaunal colonisation at elevated pCO<sub>2</sub></b> |               |                      |                              |                             |                     |                   |
| <b>Environmental monitoring</b>   |               |                      |                              |                             |                     |                   |
| Date  | Site          | pH/T/S               | TA                           |                             |                     |                   |
| 18-25/06/2013   | SEEP          | 13                   | 6                            |                             |                     |                   |
| 18-25/06/2013   | REF A         | 9                    | 6                            |                             |                     |                   |
| 18-25/06/2013   | REF B         | 8                    | 6                            |                             |                     |                   |
| 04-09/09/2013   | SEEP          | 3                    | 3                            |                             |                     |                   |
| 04-09/09/2013   | REF A         | 3                    | 3                            |                             |                     |                   |
| 04-09/09/2013   | REF B         | 4                    | 3                            |                             |                     |                   |
| <b>Copper pulse experiments</b>   |               |                      |                              |                             |                     |                   |
| Species   | Treatment     | Plaster              | Copper<br>content            | Maximum<br>quantum<br>yield | Pigments<br>content | Epi<br>fauna      |
| <i>C. corniculata</i>   | REFA.REFA.Cu- | 4                    | 5                            | 5                           | 5                   | 4                 |
| <i>C. corniculata</i>   | REFA.REFA.Cu+ |                      | 5                            | 5                           | 5                   | 5                 |
| <i>C. corniculata</i>   | SEEP.REFA.Cu- |                      | 5                            | 5                           | 5                   | N/A               |
| <i>C. corniculata</i>   | SEEP.REFA.Cu+ |                      | 5                            | 5                           | 4                   | N/A               |
| <i>C. corniculata</i>   | REFB.REFA.Cu- |                      | n.u.                         | 3                           | n.u.                | N/A               |
| <i>C. corniculata</i>   | REFB.REFA.Cu+ |                      | n.u.                         | 3                           | n.u.                | N/A               |
| <i>C. corniculata</i>   | REFA.SECP.Cu- | 4                    | 5                            | 5                           | 4                   | N/A               |
| <i>C. corniculata</i>   | REFA.SECP.Cu+ |                      | 5                            | 5                           | 5                   | N/A               |
| <i>C. corniculata</i>   | SEEP.SECP.Cu- |                      | 4                            | 4                           | 4                   | 5                 |
| <i>C. corniculata</i>   | SEEP.SECP.Cu+ |                      | 5                            | 5                           | 5                   | 5                 |
| <i>C. corniculata</i>   | REFB.SECP.Cu- |                      | n.u.                         | 4                           | n.u.                | N/A               |
| <i>C. corniculata</i>   | REFB.SECP.Cu+ |                      | n.u.                         | 5                           | n.u.                | N/A               |
| <i>J. rubens</i>  | REFA.REFA.Cu- | 4                    | 4                            | 4                           | 5                   | N/A               |
| <i>J. rubens</i>  | REFA.REFA.Cu+ |                      | 5                            | 4                           | 5                   | N/A               |
| <i>J. rubens</i>  | SEEP.REFA.Cu- |                      | 5                            | 5                           | 4                   | N/A               |
| <i>J. rubens</i>  | SEEP.REFA.Cu+ |                      | 5                            | 5                           | 4                   | N/A               |
| <i>J. rubens</i>  | REFB.REFA.Cu- |                      | 4                            | 3                           | n.u.                | N/A               |
| <i>J. rubens</i>  | REFB.REFA.Cu+ |                      | 4                            | 4                           | n.u.                | N/A               |
| <i>J. rubens</i>  | REFA.SECP.Cu- | 4                    | 5                            | 4                           | 4                   | N/A               |
| <i>J. rubens</i>  | REFA.SECP.Cu+ |                      | 5                            | 5                           | 3                   | N/A               |

|                  |               |  |   |   |      |     |
|------------------|---------------|--|---|---|------|-----|
| <i>J. rubens</i> | SEEP.SECP.Cu- |  | 5 | 5 | 4    | N/A |
| <i>J. rubens</i> | SEEP.SECP.Cu+ |  | 5 | 5 | 5    | N/A |
| <i>J. rubens</i> | REFB.SECP.Cu- |  | 5 | 5 | n.u. | N/A |
| <i>J. rubens</i> | REFB.SECP.Cu+ |  | 5 | 3 | n.u. | N/A |

## Chapter 2

### **Assessing the suitability of a volcanic seep area off Methana (Greece) for ocean acidification studies**

Aspects of this chapter have been published as:

**C. Baggini**, M. Salomidi, E. Voutsinas, L. Bray, E. Krasakopoulou, J.M. Hall-Spencer (2014). Seasonality affects macroalgal community response to increases in pCO<sub>2</sub>. *PLoS ONE*, 9: e106520.

## Abstract

Ocean acidification poses a threat to a wide range of marine systems, but little work has been carried out at the ecosystem level due to logistical constraints. Work in areas with naturally high CO<sub>2</sub> is starting to show community effects of ocean acidification. Replication of these observations across a range of settings is needed to build confidence in predictions developed using these systems as ocean acidification analogues. The aim of this study was therefore to assess whether seeps off Methana, in the oligotrophic Aegean Sea, are appropriate for studying the community effects of ocean acidification. Monitoring of the gradient from 2011 to 2013 showed that median seawater pH decreased from present day values at reference sites (median pH = 8.12) to levels predicted for the end of this century at the seep sites (median pH = 7.69) with no confounding gradients in total alkalinity, salinity, temperature or wave exposure. Most nutrient levels were similar along the pH gradient; silicate levels increased significantly with decreasing pH, but they were high enough at all sites not to limit algal growth. Metal concentrations in sediment and seaweed tissues varied between study sites but did not consistently increase with increasing pCO<sub>2</sub>. Methana seeps have the same limitations as other seeps used for ocean acidification studies, i.e. variable pCO<sub>2</sub> and relatively small area influenced. Seeps off Methana, however, influence a relatively large area (~10 km of shore) compared to other seeps used for ocean acidification studies, which may limit the amount of mobile organisms and larvae coming from reference areas. It is therefore concluded that seeps off Methana are suitable for studies into the effects of ocean acidification, provided the limitations of using seep systems in ocean acidification studies are taken into account.

## 2.1 Introduction

Early work on the effects of ocean acidification involved experiments that focused on single species in laboratory conditions, where pH variability was minimised, for periods of up to 18 months (Kroeker *et al.*, 2013a). This body of work has rapidly advanced our knowledge of the relative sensitivity of different species, which can be used to formulate hypotheses about community responses. Nevertheless, surprising and unpredicted community responses to increased levels of pCO<sub>2</sub> can occur because of interactions between species. For instance, Hale *et al.* (2011) reports that most invertebrate taxa in a community mesocosm experiments responded to increased pCO<sub>2</sub> as expected from single species experiments. Nematodes, however, unexpectedly increased in abundance, probably because of the decreased competition with, and predation by, taxa sensitive to ocean acidification.

Community responses to ocean acidification will also depend on indirect effects of carbon dioxide, such as those which alter animal behaviour (Briffa *et al.*, 2012) and affect their neuroreceptors (Munday *et al.*, 2014). Thus, physiology and ecological niche cannot fully predict a species' susceptibility to environmental changes (Spicer, 2014). Moreover, laboratory and mesocosm experiments are usually too brief to ascertain the effect of increased carbon dioxide on climax communities comprising long-lived organisms (Kroeker *et al.*, 2013a). Hypotheses formulated using data from short-term single-species laboratory experiments thus need to be tested in complex communities, ideally in real marine ecosystems (Garrard *et al.*, 2013).

Areas chronically exposed to high pCO<sub>2</sub> can be used to assess long-term community responses to ocean acidification (Hall-Spencer *et al.*, 2008;

Fabricius *et al.*, 2011; Boatta *et al.*, 2013; Inoue *et al.*, 2013). Although in their infancy, there are widespread opportunities for such studies since hydrothermal seeps characterised by low pH and high pCO<sub>2</sub> levels occur worldwide (Tarasov *et al.*, 2005). However, many of these areas also have gradients in temperature, salinity, total alkalinity, inorganic nutrients, toxic gases and metals (Dando *et al.*, 1999; Karlen *et al.*, 2010), which could confound the ecological effects of carbon dioxide. As a consequence, geochemical baseline surveys are needed to check the extent to which seep systems can be used as natural ocean acidification laboratories (Kerrison *et al.*, 2011; Boatta *et al.*, 2013).

Only a few CO<sub>2</sub> seeps have so far been located that are suitable for use as ocean acidification analogues, namely seeps off Italy (Hall-Spencer *et al.*, 2008; Kerrison *et al.*, 2011; Boatta *et al.*, 2013), Papua-New Guinea (Fabricius *et al.*, 2011) and Japan (Inoue *et al.*, 2013). These studies have shown that increasing levels of seawater pCO<sub>2</sub> reduce benthic biodiversity, especially that of calcifying organisms (Martin *et al.*, 2008; Dias *et al.*, 2010; Fabricius *et al.*, 2014). Replication of such studies in a wider range of settings would strengthen the evidence for the ecosystem effects of increasing pCO<sub>2</sub> at the landscape scale. Data from a natural ocean acidification analogue in the Eastern Mediterranean would be useful due to its extremely low nutrient levels, which could exacerbate the effects of high pCO<sub>2</sub> due to the increased metabolic costs of coping with ocean acidification (Holcomb *et al.*, 2010; Melzner *et al.*, 2011; Kletou and Hall-Spencer, 2012).

The aim of this chapter is to assess the suitability of volcanic seeps off Methana (Saronikos Gulf, Aegean Sea, Greece) for ocean acidification studies. Temperature, pH, salinity, total alkalinity and the concentrations of heavy metals, hydrogen sulphide and major nutrients (nitrite, nitrate, ammonium, phosphate

and silicate) were monitored. In addition, wave exposure was determined using effective fetch (see section 2.2.8) as it affects the distribution of Mediterranean fucoid algae (Spatharis *et al.*, 2011).

## 2.2 Methods

### 2.2.1 Study area

The Methana peninsula is the westernmost volcanic system of the northern Aegean Volcanic Arc, derived from the subduction of the African tectonic plate beneath the Eurasian plate. The last volcanic eruption dates back to 230 BC, but the system is still hydrothermally active (Dando *et al.*, 1999). The seeps are shallow (0-5 m depth) and situated on the NE part of the peninsula (Figure 2.1). They appeared shortly after the last volcanic eruption, and the Pausania thermal baths nearby have been used since late Roman times (Bowden and Gill, 1997).

Geothermal fluids rise very slowly from the geological reservoir below Methana (2-3 km underground), where temperatures could be as high as 210 °C; they mix with shallow fluids in the process (D'Alessandro *et al.*, 2008). The released gases are dominated by CO<sub>2</sub>, as in most Mediterranean hydrothermal systems (Dando *et al.*, 1999), and are mainly derived from limestone. The estimated CO<sub>2</sub> flux from the whole peninsula is about 0.03 kg s<sup>-1</sup>, well below the range generally measured worldwide in volcanic/hydrothermal areas (0.2-450 kg s<sup>-1</sup>; Pecoraino *et al.*, 2005) and lower than those of the rest of the Aegean Volcanic Arc (0.2 kg s<sup>-1</sup> at Nea Kameni, Chiodini *et al.*, 1998; 0.6 kg s<sup>-1</sup> at Sousaki, D'Alessandro *et al.*, 2006; and 1.0 kg s<sup>-1</sup> at Nisyros, Cardellini *et al.*, 2003).

Gas concentrations have been recently measured in Pausanias baths, which are extremely close (<20 meters) to the seeps studied here. Gas emissions

were measured on two occasions, and gas composition was relatively stable over time (D'Alessandro *et al.*, 2008). Gas bubbles in the baths are mostly carbon dioxide, with small amounts of nitrogen, carbon monoxide and methane (Table 2.1). Methane concentrations (17-26 ppm) are much lower than those detected at ocean acidification analogues in Ischia (200 - 800 ppm; Hall-Spencer *et al.*, 2008), Vulcano (1700 ppm; Boatta *et al.*, 2013) and Papua New Guinea (87 - 4360 ppm; Fabricius *et al.*, 2011).

Table 2.1. Composition of gases bubbling at Pausanias baths in ppm (D'Alessandro *et al.*, 2008). These baths are adjacent to the submarine seeps. Carbon dioxide (CO<sub>2</sub>) accounts for over 90% of the emitted gases, with smaller percentages of nitrogen (N<sub>2</sub>), oxygen (O<sub>2</sub>), methane (CH<sub>4</sub>), carbon monoxide (CO), helium (He) and hydrogen (H<sub>2</sub>). Methane levels are much lower than in other seeps used as ocean acidification proxies.

| Date       | CO <sub>2</sub> | N <sub>2</sub> | O <sub>2</sub> | CH <sub>4</sub> | CO  | He | H <sub>2</sub> |
|------------|-----------------|----------------|----------------|-----------------|-----|----|----------------|
| 04/06/2006 | 991000          | 10700          | <400           | 26              | 1.6 | <5 | <5             |
| 23/06/2006 | 970000          | 30900          | 5600           | 17              | 1.7 | <5 | <5             |

The Pausanias bath water composition is moderately enriched in calcium and silicates due to the interaction of thermal waters and limestone and silicate rocks, respectively. Rock-water interactions were responsible for the detected enrichment in K, B and Li as well (D'Alessandro *et al.*, 2008).

### 2.2.2 Site descriptions

Two preliminary surveys were carried out in September 2011 and February 2012 to characterise carbonate chemistry and find areas with elevated pCO<sub>2</sub> as well as reference sites. These surveys showed that a relatively small area (approximately 20 m of shoreline) near a seep had a pH<sub>NBS</sub> constantly below 8.0 (Figure 2.1), whereas a much larger area was characterised by variable pH.



This area (shown in light grey in Figure 2.1) had a  $pH_{NBS}$  varying from 6.6 to 8.1, with lower values in autumn and higher in winter. Sediment Eh and dissolved oxygen analyses at the sediment-water interface confirmed that volcanic activity did not produce an anoxic layer in the sediment (Krasakopoulou *et al.*, unpublished data).

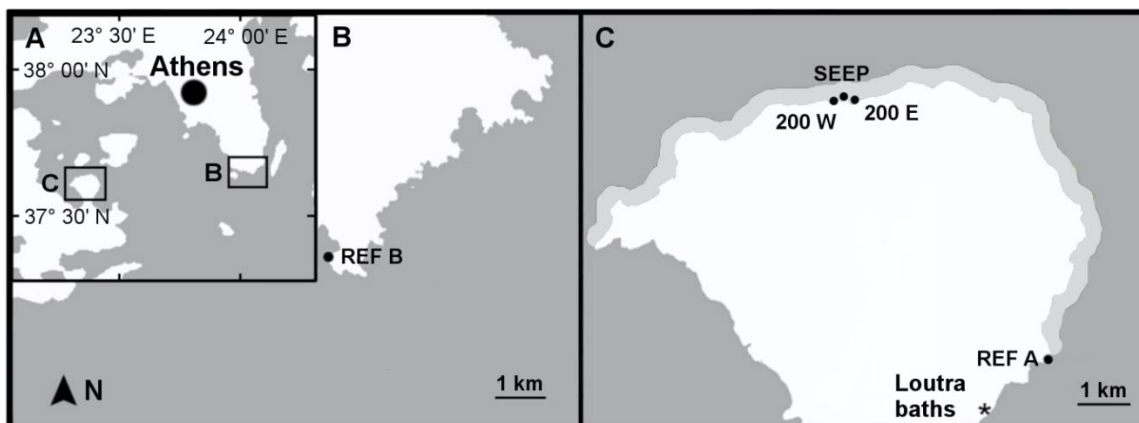


Figure 2.1. (A) Saronikos Gulf, Greece, with study areas marked by rectangles B and C. (B) Study site REF B (black point). (C) Study sites 200 W, SEEP, 200 E and REF A (black points), Loutra baths (\*) and area where pH was more variable than at reference sites (light grey).

Five sites with comparable habitats but different pH levels were selected: a site with  $pH < 8.0$  near the main seeps (SEEP), two sites with variable pH located approximately 200 m eastwards and westwards of the seep area (200 E and 200 W) and two reference sites, one just outside the variable pH area (REF A) and one at a more distant site unaffected by volcanic activity (REF B). Pictures of the typical benthic communities at SEEP and 200 E are shown in Figure 2.2. All sites had the same type of coastal morphology (large sparse boulders) and similar degrees of urbanisation, as only small villages and hotels were found in their vicinities. The dominant canopy-forming macroalgal species at all sites was *Cystoseira corniculata*, a fucoid alga characteristic of the Eastern

Mediterranean Sea (Taskin *et al.*, 2012). The genus *Cystoseira* indicates good environmental conditions (Ballesteros *et al.*, 2007) and *C. corniculata* is characteristic of sites with high wave exposure (Spatharis *et al.*, 2011).

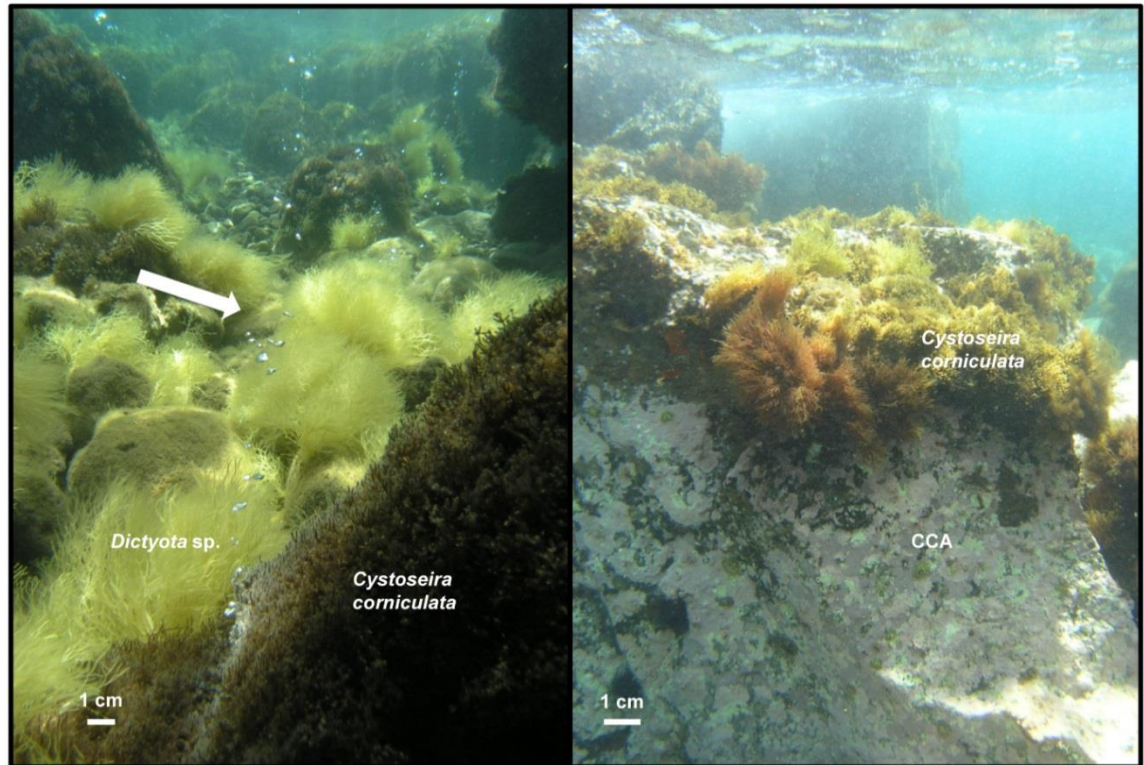


Figure 2.2. Typical appearance of benthic communities at SEEP (left) and 200 E (right) sites at 0.5 m depth in May 2012 with CO<sub>2</sub> bubbles seeping from the sea floor (arrow). Brown algae (e.g. *Dictyota* sp.) were dominant near the seeps; crustose coralline algae (CCA) became dominant as CO<sub>2</sub> levels decreased. Photos by Laura Bray (May 2012).

### 2.2.3 Seawater physicochemical parameters

The seeps were monitored from 2011 to 2013 (September 2011, January, February, May and September 2012, June and September 2013 - detailed sampling dates and sample sizes are reported in Table 1.1A); seawater physicochemical parameters were measured at different times of the day and in different meteorological conditions during each trip. Seawater pH, temperature and salinity were measured using a multiprobe (YSI 63) from shore. The probe was calibrated before use with pH 4.01, 7.01 and 10.01 NBS standards. Since

variations of up to 1 pH unit were detected over a few hours at the low pH site, the lack of precision in using the NBS scale for seawater measurements (approximately 0.05 pH, Riebesell *et al.*, 2010) was considered acceptable for this study. For pH, medians and interquartile ranges (IQ) were calculated from hydrogen ion concentrations before re-converting back to pH values following seep monitoring methods provided by Kerrison *et al.* (2011).

Seawater samples for total alkalinity determination were collected in 125 ml borosilicate glass bottles with Teflon caps. Three independent samples per site were collected at each visit, immediately poisoned with HgCl<sub>2</sub> and stored in the dark until analysis. Samples were analysed by Gran titration (AS-ALK 2, Apollo SciTech) and the reliability of the measurements was checked against standard seawater samples provided by A. Dickson (batches 112 and 121). The average total alkalinity value per site and individual pH measurements were used to calculate pCO<sub>2</sub>, HCO<sub>3</sub><sup>-</sup>, CO<sub>3</sub><sup>2-</sup>, Ω<sub>Ar</sub> and Ω<sub>Ca</sub> using the CO2SYS software (Lewis and Wallace, 1998).

#### **2.2.4 Seawater nutrient concentration**

In June 2013 three water samples per site were collected for nutrient analysis; the analyses were performed by the nutrient laboratory staff at the Hellenic Centre for Marine Research. Samples were stored frozen (-20°C), then analysed using a BRAN+LUEBBE II autoanalyser. Inorganic phosphate determination followed the colorimetric method of Murphy and Riley (1962) and nitrite ions (NO<sub>2</sub><sup>-</sup>) were measured colorimetrically according to Bendscheider and Robinson (1952). Determination of nitrate (NO<sub>3</sub><sup>-</sup>) was performed after its reduction to nitrite, which was then determined colorimetrically as above. Silicate was determined by adding a molybdate solution to the sample. The

silicomolybdic acid that formed was then reduced to an intensely blue-coloured complex by adding ascorbic acid as a reducing agent (Mullin & Riley, 1955). The determination of ammonium was performed according to Koroleff (1970) using a Perkin Elmer 25 Lambda spectrophotometer.

### **2.2.5 Free sulphides in seawater**

Free sulphides were determined using a method modified from Cline (1989). Three seawater samples per site were collected using plastic syringes to measure within- and between- site variability in sulphides contents; 2 ml of seawater were injected into a nitrogen-filled septum vial containing a small crystal of cadmium chloride. As the area near the seeps had the highest probability of having high sulphide concentrations, four additional samples were taken on two separate dates (22 and 25 May 2012). In order to validate the method, one sample was taken at the sulphide-rich Loutra thermal baths (location shown in Figure 2.1). For laboratory analysis, most of the water was removed by syringe after allowing the precipitate to settle. The samples were thus reduced to 0.8 ml volume, agitated to suspend all the precipitate and drawn up in a 1 ml disposable syringe which had been flushed with Ar.

Subsequently, 0.2 ml of a solution prepared using 400 mg of N,N-dimethyl-p-phenylene-diamine-dihydrochloride and 600 mg  $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$  dissolved in 100 ml 50% HCl were drawn into the same syringe. The argon bubble in the syringe was used to mix by inverting it a few times. The sample was left to stand for 20 minutes and then injected into a 1 ml semi-microcuvette and read in a Perkin Elmer Lambda 35 UV-VIS spectrometer at 670 nm. Standards were made using a 10 mM sodium sulphide stock solution (249 mg  $\text{Na}_2\text{S} \cdot 9 \text{H}_2\text{O}$  in 100 ml

degassed Milli-Q water). The stock solution was diluted immediately before use in degassed seawater to give a range of 0.1 to 100  $\mu\text{M}$ .

### **2.2.6 Heavy metals in sediment**

Sediments were examined for metals that can exhibit increased concentrations in volcanic areas (Al, As, Ca, Cd, Co, Cr, Cu, Fe, Li, Mg, Ni, Pb, Zn; Hübner *et al.*, 2004); S was also analysed to verify whether the area was contaminated by hydrogen sulphide emitted by the seeps, and Cr was analysed to determine sediment quality (see below). In May 2012, sediments were sampled for heavy metal analysis at the three sites where contamination from volcanic activity was likely (SEEP, 200 W and 200 E). Sediment was collected using plastic pots immediately closed with a lid. Three stations were selected for each site to assess within-site variability, and one sample per station was collected. Immediately after sampling, sediment was transferred to polypropylene bags and kept frozen until analysis.

Samples were oven-dried at 30°C until constant mass was reached. They were subsequently reduced to a fine powder and passed through a 180  $\mu\text{m}$  plastic sieve. Triplicate replicates for each station were prepared for analysis by digesting them with aqua regia. First, approximately 0.5 grams of sample were carefully weighed on an analytical balance (precision  $\pm 0.1$  mg) and transferred into a pre-cleaned and dry digestion tube (Tecator type). Subsequently, 7.5 ml of hydrochloric acid and 2.5 ml of nitric acid were added. One hour of pre-digestion allowed easily oxidised materials to be destroyed at low temperature. Temperature was then gradually increased in several steps: firstly samples were kept at 60 °C for 30 minutes, then at 85 °C for one hour, then at 105 °C for another hour, then at 120 °C for an hour and finally at 140 °C for a further hour.

After cooling, the digested material was transferred quantitatively to a 50 ml volumetric flask and diluted to volume with distilled water. The blank was prepared following the same steps as above, but without adding sediment. Three replicates of reference material (Harbour Sediment, LGC6156) were prepared following the same procedure.

Digested samples were then analysed for Al, Ca, Cr, Cu, Fe, Mg, Mn, Pb, S and Zn using inductively coupled plasma optical emission spectrometry (ICP-OES, Varian 725-ES; Melbourne, Australia) using a v-groove nebuliser and a Sturman-Masters spray chamber. The analysis parameters were as follows: forward power 1.4 kW, plasma gas flow 15 L min<sup>-1</sup>, auxiliary gas flow 1.5 L min<sup>-1</sup>, nebuliser gas flow 0.68 L min<sup>-1</sup>. Each sample was read three times, with four seconds of replicate read time and a viewing height of 8 mm. Every ten samples analysed, one of the standards was measured again in order to detect any deviation from the initial calibration.

As, Cd, Co, Li and Ni were present in very small quantities, so their concentrations were determined using an inductively coupled plasma mass spectrometry (ICP-MS, Thermo Scientific X series 2, Hemel Hempstead, UK) with a concentric glass nebuliser and a conical spray chamber with an impact bead. The analysis parameters were as follows: forward power 1.4 kW, plasma gas flow 13 L min<sup>-1</sup>, auxiliary gas flow 0.7 L min<sup>-1</sup>, nebuliser gas flow 0.8 L min<sup>-1</sup>. Each sample was read 50 times, with 10 ms read time. Every eleven samples, one of the standards was measured again to detect any deviation from the initial calibration. In addition, an internal reference (iridium) was used to correct for the density difference between standards and digested samples.

Biological effects of heavy metal enrichment of the sediments were examined using an index of ecological risk, the mean Sediment Quality Guidelines-quotient (SQG-Q). This index is an indicator of adverse biological effects caused by different concentrations of heavy metals. This type of numerical SQG can be used to obtain a first approximation of sediment toxicity (Long and MacDonald, 1998; Chapman and Wang, 2001). Mean SQG-Q using two sediment quality guidelines, ERM (effect range-median) and PEL (probable effect levels) was calculated for each site using the following equations (Long and MacDonald, 1998):

$$SQG - Q_{\alpha\_PEL} = \frac{\sum_{i=1}^n (PEL - Qi)\alpha}{n} \quad (1)$$

and

$$SQG - Q_{\alpha\_ERM} = \frac{\sum_{i=1}^n (ERM - Qi)\alpha}{n} \quad (2)$$

where

$$PEL - Q_{\alpha} = \frac{C}{PEL} \quad (3)$$

and

$$ERM - Q_{\alpha} = \frac{C}{ERM} \quad (4)$$

*PEL*-Q = Probable effect level quotient

*ERM*-Q = Effect range-median quotient

C- Heavy metal concentration in each station

*PEL* - Probable effect level of each heavy metal

*ERM* - Effect range-median of each heavy metal

*n* – Number of contaminants used.

PEL and ERM are the concentrations above which adverse effects frequently occur and although they have been calculated with slightly different methods, they both are reliable methods to predict sediment toxicity (Long *et al.*, 1998).

PEL and ERM values for the analysed elements are reported in Table 2.2.

Table 2.2. PEL and ERM threshold values used to calculate the potential biological effect of contaminants (Long *et al.*, 1998). They are concentration expressed as mg/g of dry sediment, and have been calculated from a database of toxicity tests.

|     | Cd   | Cr  | Cu  | Pb  | Ni   | Zn  |
|-----|------|-----|-----|-----|------|-----|
| PEL | 4.21 | 160 | 108 | 112 | 42.8 | 271 |
| ERM | 9.6  | 370 | 270 | 218 | 51.6 | 410 |

Each site can be assigned to one of the following impact level categories:

Category 1:  $SQG-Q < 0.1$  unimpacted - lowest potential for observing adverse biological effects;

Category 2:  $0.1 < SQG-Q < 1$  impacted - moderate potential for observing adverse biological effects;

Category 3:  $SQG-Q > 1$  highly impacted - potential for observing adverse biological effects.

### **2.2.7 Heavy metals in macroalgae**

A common phaeophyte, *Dictyota* sp., was analysed for heavy metal concentrations at all five sites. This macroalga was chosen as it was present at all sites and had low epiphyte load. Five individuals per species per site were



collected by snorkelers in May 2012, rinsed with fresh water to eliminate salt, gently brushed to remove epiphytes, kept frozen until transported to the laboratory and then freeze-dried. Freeze-dried macroalgae were ground with pestle and mortar and approximately 0.1 g of each sample was weighed in acid-washed Teflon tubes with a high precision digital scale (0.1 mg accuracy). Two ml of concentrated nitric acid were then added, and the tube containing the digestant was placed in a high-Throughput Microwave Reaction System Run (MARSXpress, CEM Corporation, Matthews, USA) and gently heated to boiling for at least 1 h to ensure full digestion. Samples were allowed to cool and then quantitatively transferred into pre-cleaned 10 ml volumetric flasks and diluted to volume with Milli-Q water. Blanks were prepared following the same procedure, but omitting the sample; a certified reference material (NIES Standard Reference Material No. 3, Chlorella) was simultaneously digested and analysed. Samples were then analysed for content of toxic metals (Al, As, Cd, Cr, Co, Cu, Pb, Ni, Zn; Baumann *et al.*, 2009; Mendes *et al.*, 2013; Khan *et al.*, 2015) and Fe, which had values higher than reference ones and showed between-site variability in the sediment samples. Analyses were performed using ICP-OES and ICP-MS following the procedure outlined in the previous section.

### **2.2.8 Wave exposure**

Wave exposure was estimated using a method from Howes *et al.* (1994), which uses modified effective fetch and maximum fetch to calculate a site-specific index of wave exposure, which is a first approximation of wave exposure. Other factors such as the associated local wind climate and wave refraction are ignored for simplifying the estimate. Modified effective fetch involves the measurement of three fetch distances: the normal to shoreline direction and the

two fetches at 45° to the left and to the right of the normal fetch. The effective fetch is then calculated with the formula:

$$Fe = \frac{\sum_{i=0}^n \cos \alpha_i * Fi}{\sum_{i=0}^n \cos \alpha_i} \quad (5)$$

Where Fe is the effective fetch measure in kilometres,  $\alpha_i$  is the angle between the shore normal and the direction i and Fi is the fetch distance in kilometres along direction i. The wave climate of a particular point cannot be characterized by effective fetch alone because waves may be generated in an area remote from the site and propagate into the area. These waves, normally referred to as swell, can be approximated using maximum fetch, which is the maximum fetch distance in kilometres that can be measured from the site. The two indexes are then combined in a matrix (Table 2.3) to determine the wave exposure category for each site. The wave exposure was only calculated for the sites SEEP, REF A and REF B, as the sites 200 W and 200 E are very close to SEEP and have a very similar shore orientation.

Table 2.3. Classification of a shore point wave exposure based on the combination of maximum fetch and modified effective fetch (both in km). Depending on the combination of these two factors, a point on the shore can be classified as very protected, protected, semi-protected, semi-exposed, exposed and very exposed (Howes *et al.*, 1994).

| Maximum Fetch (km) | Modified Effective Fetch (km) |                |                |              |              |
|--------------------|-------------------------------|----------------|----------------|--------------|--------------|
|                    | <1                            | 1-10           | 10-50          | 50-500       | >500         |
| <1                 | very protected                | n/a            | n/a            | n/a          | n/a          |
| <10                | protected                     | protected      | n/a            | n/a          | n/a          |
| 10-50              | n/a                           | semi-protected | semi-protected | n/a          | n/a          |
| 50-500             | n/a                           | semi-exposed   | semi-exposed   | semi-exposed | n/a          |
| 500-1000           | n/a                           | n/a            | semi-exposed   | exposed      | exposed      |
| <1000              | n/a                           | n/a            | n/a            | very exposed | very exposed |

### 2.2.9 Statistical analyses

Analysis of nutrient and metal concentration data was performed using separate multivariate analyses of variance (MANOVA) with one factor (site). Normality and homogeneity of variances were tested by visually examining boxplots and residual error plots and using Levene's test, and transformed when necessary. When the data did not meet Mauchly's test of sphericity, the degrees of freedom were corrected using Greenhouse-Geisser estimates of sphericity. Tukey HSD tests were used for multiple comparisons. Analysis of pH data was performed using a non-parametric analysis (Kruskal-Wallis ANOVA) followed by pairwise

multiple comparisons. All of the analyses above were performed using SPSS v. 19 (IBM, USA)

## 2.3 Results

### 2.3.1 Seawater physicochemical parameters

The seeps had the lowest median  $\text{pH}_{\text{NBS}}$  (7.69, IQ range 7.57 - 7.85,  $n=40$ ) and were significantly different from the intermediate sites (200 W and 200 E), which had higher median values (7.87,  $n=26$  and 7.96,  $n=26$  for 200 E and 200 W, respectively) and comparable variability (IQ ranges 7.75 - 8.04 and 7.73 - 8.03 for 200 E and 200 W, respectively). At intermediate sites pH sometimes exceeded 8.0, whereas at SEEP the measured pH never reached 8.0. The reference sites had significantly higher pH values (median values of 8.11,  $n=21$  and 8.12,  $n=19$  for REF A and REF B, respectively) and lower variability (Figure 2.3).

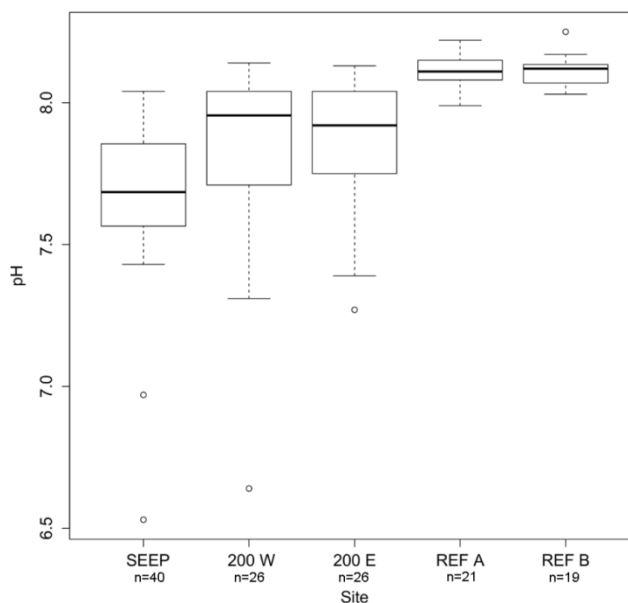


Figure 2.3. Variability in pH at the five study sites off Methana between September 2011 and September 2013. Horizontal line = median, vertical boxes = 25th and 75th percentiles, whiskers = min/max values if smaller than 1.5 times the inter-quartile range and dots = outliers.

Temperature and salinity varied seasonally and were uniform across sites. The minimum temperature was 14.2°C in February, whereas in summer the temperature could reach 26.8°C; salinity varied from 37.5 to 40.0 ppt. Total alkalinity varied from 2.615 to 2.944 mmol kg<sup>-1</sup> with no seasonal trend (Table 2.4), with slightly lower values and less variability than CO<sub>2</sub> seeps off Vulcano, where total alkalinity varies between 2.78 to 3.17 mmol kg<sup>-1</sup> (Boatta *et al.*, 2013). Seawater pCO<sub>2</sub> had a median value of over 1300 µatm at the SEEP site, almost three times the values calculated for the reference sites. The median saturation state of calcite and aragonite was always > 1, although sites with high and intermediate pCO<sub>2</sub> levels were occasionally under-saturated with respect to both calcite and aragonite (Table 2.4).

Table 2.4. Values of measured (pH and total alkalinity (TA)) and calculated (bicarbonate (HCO<sub>3</sub><sup>-</sup>) and carbonate ions (CO<sub>3</sub><sup>2-</sup>) concentrations, pCO<sub>2</sub>, saturation state of calcite (Ω<sub>Ca</sub>) and aragonite (Ω<sub>Ar</sub>)) carbonate system parameters at the five sites using data from six surveys from September 2011 to September 2013. Sample sizes for pH and total alkalinity are shown below site name.

| Site  |        | pH<br>(NBS) | TA<br>(mmol kg <sup>-1</sup> ) | pCO <sub>2</sub><br>(µatm) | HCO <sub>3</sub> <sup>-</sup><br>(mmol kg <sup>-1</sup> ) | CO <sub>3</sub> <sup>2-</sup><br>(mmol kg <sup>-1</sup> ) | Ω <sub>Ar</sub> | Ω <sub>Ca</sub> |
|---|--------|-------------|--------------------------------|----------------------------|---|---|-----------------|-----------------|
| SEEP<br>(n <sub>pH</sub> =40, n <sub>TA</sub> =23)  | Min    | 6.53        | 2.639                          | 24092                      | 2.771   | 0.006   | 0.09            | 0.13            |
|   | Median | 7.69        | 2.794                          | 1754                       | 2.538   | 0.104   | 1.16            | 2.45            |
|   | Max    | 7.99        | 2.944                          | 691                        | 2.243   | 0.225   | 3.45            | 5.20            |
| 200 W<br>(n <sub>pH</sub> =26, n <sub>TA</sub> =24) | Min    | 6.64        | 2.696                          | 18652                      | 2.773   | 0.007   | 0.11            | 0.17            |
|   | Median | 7.96        | 2.771                          | 872                        | 2.366   | 0.177   | 2.70            | 4.12            |
|   | Max    | 8.14        | 2.941                          | 526                        | 2.138   | 0.271   | 4.18            | 6.29            |
| 200 E<br>(n <sub>pH</sub> =26, n <sub>TA</sub> =22) | Min    | 7.27        | 2.693                          | 4505                       | 2.658   | 0.038   | 0.57            | 0.88            |
|   | Median | 7.88        | 2.739                          | 1042                       | 2.403   | 0.152   | 2.30            | 3.50            |
|   | Max    | 8.13        | 2.836                          | 532                        | 2.114   | 0.263   | 4.05            | 6.10            |
| REF A<br>(n <sub>pH</sub> =21, n <sub>TA</sub> =18) | Min    | 7.99        | 2.640                          | 773                        | 2.261   | 0.183   | 2.84            | 4.30            |
|   | Median | 8.11        | 2.708                          | 550                        | 2.106   | 0.246   | 3.78            | 5.70            |
|   | Max    | 8.22        | 2.769                          | 393                        | 2.049   | 0.269   | 4.04            | 6.18            |
| REF B<br>(n <sub>pH</sub> =19, n <sub>TA</sub> =15) | Min    | 8.03        | 2.615                          | 674                        | 2.254   | 0.185   | 2.81            | 4.30            |
|   | Median | 8.12        | 2.697                          | 539                        | 2.145   | 0.231   | 3.54            | 5.33            |
|   | Max    | 8.25        | 2.858                          | 362                        | 2.024   | 0.280   | 4.23            | 6.46            |

### 2.3.2 Seawater nutrient concentrations

Nutrient concentrations were similar to background levels in the Saronikos Gulf (Frigilos, 1991) except for silicate, which was mostly higher than the background value of 1.22  $\mu\text{M}$  even at one of the reference sites (Table 2.5). When values were < LOQ. (Limit Of Quantification) they were substituted with LOQ/2; LOQ. = 0.126  $\mu\text{M}$  for  $\text{NO}_2^- + \text{NO}_3^-$  and 0.102  $\mu\text{M}$  for  $\text{NH}_4^+$ . Statistically significant differences between sites were only detected for nitrite and silicate. Nitrite, however, had a very small range, varying from  $0.040 \pm 0.005 \mu\text{M}$  in REF B to  $0.054 \pm 0.002 \mu\text{M}$  in 200 E, and these were the only two sites that were significantly different. Silicate had a wider range (from  $1.180 \pm 0.269 \mu\text{M}$  in REF B to  $6.371 \pm 1.841 \mu\text{M}$  in 200 W); only site 200 W was significantly different from the reference sites according to pairwise comparisons. No significant differences and relatively uniform values were measured for phosphate, whereas nitrate and ammonium showed higher values at 200 E, although these differences were not significant, possibly due to high within-site variability.

Table 2.5. Average seawater nutrient concentrations ( $\pm$  SD, n=3) at Methana in June 2013. For the five sites, nitrite ( $\text{NO}_2^-$ ), nitrate ( $\text{NO}_3^-$ ), ammonium ( $\text{NH}_4^+$ ), phosphate ( $\text{PO}_4^{3-}$ ) and silicate ( $\text{SiO}_4^{4-}$ ) concentrations are shown. Background values (Bgd) for the Aegean Sea from Friligos (1991). Different letters indicate significant differences according to post-hoc pairwise comparisons; n.d. = not determined.

|                                       | SEEP                    | 200 W                   | 200 E               | REF A                   | REF B               | Bgd  |
|---------------------------------------|-------------------------|-------------------------|---------------------|-------------------------|---------------------|------|
| $\text{NO}_3^-$ ( $\mu\text{M}$ )     | $0.070 \pm 0.062$       | $0.094 \pm 0.069$       | $0.559 \pm 0.297$   | $0.054 \pm 0.054$       | $0.085 \pm 0.045$   | 0.42 |
| $\text{NO}_2^-$ ( $\mu\text{M}$ )     | $0.054 \pm 0.003^{a,b}$ | $0.044 \pm 0.005^{a,b}$ | $0.059 \pm 0.004^b$ | $0.042 \pm 0.004^{a,b}$ | $0.040 \pm 0.009^a$ | n.d. |
| $\text{NH}_4^+$ ( $\mu\text{M}$ )     | $0.232 \pm 0.172$       | $0.265 \pm 0.189$       | $1.075 \pm 0.318$   | $0.203 \pm 0.189$       | $0.298 \pm 0.091$   | 0.36 |
| $\text{PO}_4^{3-}$ ( $\mu\text{M}$ )  | $0.025 \pm 0.008$       | $0.031 \pm 0.011$       | $0.038 \pm 0.009$   | $0.024 \pm 0.006$       | $0.044 \pm 0$       | 0.12 |
| $\text{SiO}_4^{4-}$ ( $\mu\text{M}$ ) | $4.018 \pm 0.671^{a,b}$ | $6.371 \pm 3.189^a$     | $1.607 \pm 0.288^c$ | $1.883 \pm 0.221^{b,c}$ | $1.180 \pm 0.466^c$ | 1.22 |

### 2.3.3 Free sulphides in seawater

Free sulphides concentrations were below the measurable limit for this method (1  $\mu\text{M}$ ) at all five sites. In contrast, the sample from Loutra thermal baths had a concentration of free sulphides of 35  $\mu\text{M}$ .

### 2.3.4 Heavy metals in sediment

Results in  $\text{mg kg}^{-1}$  of dry sediment (normalised to dry mass) are reported in Table 2.6 for each site and compared to the minimum values reported in a survey of heavy metal concentrations in sediments from the Aegean Sea (Karageorgis *et al.*, 2005). As and Mn results were not considered reliable, as the concentrations determined in the reference material exceeded the confidence interval of the certified values, and are therefore not reported.

Table 2.6. Concentration of analysed elements in  $\text{mg kg}^{-1}$  of dry sediment from three sites with low (SEEP) and intermediate (200 W, 200 E) pH compared to reference values for the Aegean Sea (minimum values reported by Karageorgis *et al.*, 2005; n.m. = not measured); for SEEP, 200 W and 200 E values are expressed as average  $\pm$  standard deviation,  $n=3$ .

| Element | SEEP                | 200 W              | 200 E                | Reference values |
|---------|---------------------|--------------------|----------------------|------------------|
| Al      | 43916 $\pm$ 545     | 41804 $\pm$ 775    | 43166 $\pm$ 739      | 27000            |
| Ca      | 25056 $\pm$ 428     | 23119 $\pm$ 282    | 24463 $\pm$ 380      | 46900            |
| Cd      | 0.07 $\pm$ 0.04     | 0.06 $\pm$ 0.01    | 0.05 $\pm$ 0.01      | n.m.             |
| Co      | 2.85 $\pm$ 0.35     | 3.53 $\pm$ 0.35    | 2.24 $\pm$ 0.16      | 10               |
| Cr      | 11.71 $\pm$ 0.57    | 15.46 $\pm$ 1.00   | 9.37 $\pm$ 0.87      | 39               |
| Cu      | 3.67 $\pm$ 0.32     | 4.41 $\pm$ 0.37    | 3.95 $\pm$ 0.95      | 4                |
| Fe      | 9436 $\pm$ 266      | 17802 $\pm$ 1084   | 8443 $\pm$ 257       | 13700            |
| Li      | 7.39 $\pm$ 1.02     | 7.28 $\pm$ 0.70    | 7.11 $\pm$ 0.43      | n.m.             |
| Mg      | 2444 $\pm$ 153      | 2943 $\pm$ 123     | 2271 $\pm$ 29        | 17900            |
| Ni      | 5.32 $\pm$ 0.81     | 5.89 $\pm$ 0.59    | 3.72 $\pm$ 0.31      | 35               |
| Pb      | 14.99 $\pm$ 3.94    | 16.30 $\pm$ 2.56   | 10.76 $\pm$ 5.08     | 17               |
| S       | 796.45 $\pm$ 100.16 | 561.06 $\pm$ 49.52 | 1746.45 $\pm$ 247.45 | n.m.             |
| Zn      | 13.31 $\pm$ 0.52    | 20.43 $\pm$ 0.78   | 12.43 $\pm$ 0.87     | 33               |

Average values of the two ecological risk indicators (SQG-Q\_PEL and SQG-Q\_ERM) are shown in Figure 2.4; all three sites are unimpacted according to

both indexes, except for the site 200 W, which is classified as impacted according to the SQG-Q\_PEL index.

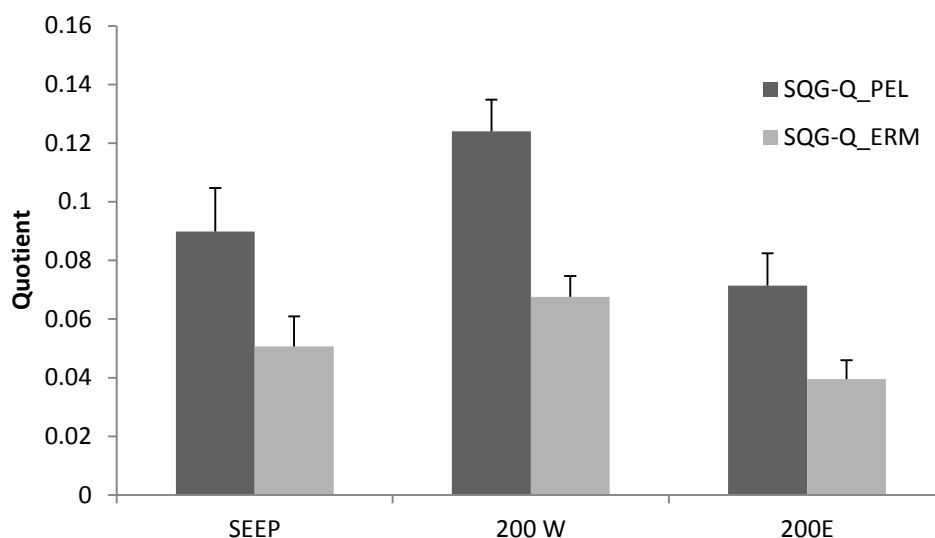


Figure 2.4. Mean ( $\pm$  SD) Sediment Quality Guidelines-quotient (SQG-Q) calculated with PEL and ERM for each site. If the index value is below 0.1, biological effects of heavy metals are unlikely. This is the case of the average value for most samples analysed here, except for 200 W, which is considered impacted according to the SQG-Q\_PEL index. N=9 for all sites except SEEP, where n=6.

### 2.3.5 Heavy metals in macroalgae

Log-transformed metal concentrations were significantly different between sites for all elements analysed. Average concentration of elements in *Dictyota* sp. tissues and results of pairwise comparisons test are shown in Table 2.7. There was large spatial variability in metal content, but no specific metal concentration consistently increased with decreasing pH. Elevated concentrations were recorded at station 200 W for aluminium, arsenic and iron, and at REF A for aluminium and zinc.



Table 2.7. *Dictyota* sp. metal content at the five sites. Means ( $\pm$  SD; mg kg<sup>-1</sup> dry mass; n=5) are shown for each metal and site; different letters indicate significant differences according to Tukey HSD test.

| Element | SEEP                                | 200 W                               | 200 E                               | REF A                                 | REF B                               |
|---------|-------------------------------------|-------------------------------------|-------------------------------------|---------------------------------------|-------------------------------------|
| Al      | 66.58 $\pm$<br>66.58 <sup>a</sup>   | 391.84 $\pm$<br>497.99 <sup>b</sup> | 75.01 $\pm$<br>31.78 <sup>a,b</sup> | 314.62 $\pm$<br>243.57 <sup>a,b</sup> | 89.77 $\pm$<br>39.92 <sup>a,b</sup> |
| As      | 15.90 $\pm$<br>2.30 <sup>a</sup>    | 39.02 $\pm$<br>5.06 <sup>d</sup>    | 25.79 $\pm$<br>6.00 <sup>c</sup>    | 18.41 $\pm$<br>2.91 <sup>a,b</sup>    | 22.52 $\pm$<br>0.83 <sup>b,c</sup>  |
| Cd      | 0.014 $\pm$<br>0.004 <sup>a</sup>   | 0.018 $\pm$<br>0.006 <sup>a,b</sup> | 0.034 $\pm$<br>0.013 <sup>b,c</sup> | 0.573 $\pm$<br>0.228 <sup>c</sup>     | 0.067 $\pm$<br>0.035 <sup>d</sup>   |
| Co      | 0.059 $\pm$<br>0.051 <sup>a</sup>   | 0.107 $\pm$<br>0.044 <sup>a</sup>   | 0.096 $\pm$<br>0.029 <sup>a</sup>   | 1.613 $\pm$<br>0.706 <sup>b</sup>     | 0.119 $\pm$<br>0.036 <sup>a</sup>   |
| Cr      | 0.857 $\pm$<br>0.156 <sup>a,b</sup> | 2.526 $\pm$<br>1.179 <sup>c</sup>   | 0.579 $\pm$<br>0.112 <sup>a</sup>   | 1.204 $\pm$<br>0.544 <sup>b</sup>     | 1.093 $\pm$<br>0.489 <sup>a,b</sup> |
| Cu      | 2.069 $\pm$<br>0.510 <sup>a</sup>   | 3.160 $\pm$<br>0.602 <sup>a,b</sup> | 3.435 $\pm$<br>1.273 <sup>a,b</sup> | 7.726 $\pm$<br>3.337 <sup>c</sup>     | 4.771 $\pm$<br>0.678 <sup>b,c</sup> |
| Fe      | 587.1 $\pm$<br>95.7 <sup>b</sup>    | 5659.8 $\pm$<br>1350.3 <sup>a</sup> | 485.5 $\pm$<br>104.6 <sup>b,c</sup> | 316.3 $\pm$<br>197.9 <sup>c,d</sup>   | 146.3 $\pm$<br>72.8 <sup>d</sup>    |
| Ni      | 0.916 $\pm$<br>0.223 <sup>a</sup>   | 1.325 $\pm$<br>0.281 <sup>a</sup>   | 1.338 $\pm$<br>0.231 <sup>a</sup>   | 4.181 $\pm$<br>1.292 <sup>b</sup>     | 2.554 $\pm$<br>0.596 <sup>b</sup>   |
| Pb      | 2.704 $\pm$<br>0.480 <sup>a</sup>   | 17.605 $\pm$<br>21.164 <sup>b</sup> | 2.378 $\pm$<br>0.616 <sup>a</sup>   | 25.979 $\pm$<br>26.174 <sup>b</sup>   | 10.820 $\pm$<br>12.842 <sup>b</sup> |
| Zn      | 10.95 $\pm$<br>11.73 <sup>a</sup>   | 11.70 $\pm$<br>1.19 <sup>a</sup>    | 8.22 $\pm$<br>1.85 <sup>a</sup>     | 42.02 $\pm$<br>20.75 <sup>b</sup>     | 14.68 $\pm$<br>1.33 <sup>a,b</sup>  |

Values higher than ranges reported in the literature for seaweed tissues from unpolluted sites (Table 2.8) were found for aluminium, arsenic and iron at 200W and for aluminium and zinc in REF A.

Table 2.8. Comparison of metal concentration (mg kg<sup>-1</sup> dry mass) in *Dictyota* spp. measured in this study with values found in the literature for unpolluted sites (n.d. = not determined; b.d.l. = below detection limit).

| Element | This study<br>(means range) | Abdallah <i>et al.</i> , 2005<br>(mean $\pm$ SD, n= 3) | McDermid and Stuercke, 2003<br>(range) | Raman <i>et al.</i> , 2013<br>(mean $\pm$ S.D., n=3) | Maher and Clarke, 1984 |
|---------|-----------------------------|--|--|--|------------------------|
| Al      | 66 – 391                    | n.d.   | n.d.                                   | n.d.   | n.d.                   |
| As      | 15 – 39                     | n.d.   | n.d.                                   | n.d.   | 26.3                   |
| Cd      | 0.014 – 0.573               | 0.98 $\pm$ 0.3   | n.d.                                   | 3.9 $\pm$ 0.3  | n.d.                   |
| Co      | 0.059 – 1.613               | 4.3 $\pm$ 1.2  | n.d.                                   | 5.5 $\pm$ 0.2  | n.d.                   |
| Cr      | 0.579 – 2.526               | 1.1 $\pm$ 0.3  | n.d.                                   | b.d.l.   | n.d.                   |
| Cu      | 2 – 8                       | 1.3 $\pm$ 0.4  | 5                                      | 6.4 $\pm$ 0.3  | n.d.                   |
| Fe      | 316 – 5659                  | n.d.   | 438 - 608                              | 504 $\pm$ 12.4                                       | n.d.                   |
| Ni      | 0.916 – 4.181               | 2.2 $\pm$ 0.6  | n.d.                                   | 27 $\pm$ 0.4   | n.d.                   |
| Pb      | 2 – 25                      | 19.2 $\pm$ 5.5   | n.d.                                   | 28.5 $\pm$ 3.5                                       | n.d.                   |
| Zn      | 8 – 42                      | 4.9 $\pm$ 1.2  | 13 - 16                                | 11.7 $\pm$ 0.3                                       | n.d.                   |

### 2.3.6 Wave exposure

Values of modified effective fetch and maximum fetch for the three sites examined are shown in Table 2.9. All sites are classified as semi-exposed with regards to wave exposure.

Table 2.9. Table showing modified effective fetch (in km) and maximum fetch (in km) for the three sites examined. Fetch values were not calculated for the sites 200 W and 200 E because they were extremely close and with a very similar orientation to SEEP. All sites are considered semi-exposed according to the classification in table 2.3.

| Site  | Modified effective fetch (km) | Maximum fetch (km) |
|-------|-------------------------------|--------------------|
| SEEP  | 11.17                         | 51.37              |
| REF A | 18.91                         | 130.42             |
| REF B | 15.81                         | 53.12              |

## 2.4 Discussion

Seeps off northern Methana had a median pH value (7.69) similar to that predicted for 2100 according to the IPCC “business as usual” scenario (Caldeira and Wickett, 2005), whereas the reference sites had median values above 8. The seeps had no confounding gradients in temperature, salinity, total alkalinity, hydrogen sulphide or wave exposure. The low pH area off Methana had pCO<sub>2</sub> levels comparable to those reported at other ocean acidification analogues (Hall-Spencer *et al.*, 2008; Kroeker *et al.*, 2011; Fabricius *et al.*, 2011; Kerrison *et al.*, 2011; Boatta *et al.*, 2013), making it suitable to assess community responses to increased pCO<sub>2</sub>.

Enrichment in silicate, which was significantly different from reference values in one of the intermediate sites, is likely due to water-rock interactions common in

hydrothermal environments (D'Alessandro *et al.*, 2008). However, it is unlikely that silicate is limiting in the Aegean Sea; for instance, Si becomes limiting to diatoms when the N:Si ratio in seawater is higher than two (Gilpin *et al.*, 2004), whereas the background ratio for the Aegean Sea is 0.64 (Friligos, 1991). Significant differences in nitrite concentrations among sites are unlikely to explain the community changes either, as their range is very small (0.040 – 0.059  $\mu\text{M}$ ). Mediterranean organisms are normally not limited by silicate or inorganic nitrogen, but by phosphate (Zohary and Robarts, 1998), for which no confounding gradient was found.

No free sulphides were detected near the seeps, although they were present at the Loutra thermal baths, over 10 km from the study site. Hydrogen sulphide is toxic for cellular respiration, and it is often emitted from Mediterranean volcanic vents (Dando *et al.*, 1999; Caramanna *et al.*, 2011). However, sulphides are extremely reactive and oxidise quickly to sulphates in oxygenated waters. It is therefore common to find very low or undetectable sulphide concentrations just a few meters away from volcanic seeps. For instance, at Vulcano sulphides become undetectable at 30 m from the main vents, even though hydrogen sulphide gas has a concentration of 400 ppm at the main bubbling site (Boatta *et al.*, 2013).

No enrichment in sediment heavy metal in the low and intermediate pH sites was detected, even though sediment enriched in various elements have been reported from a nearby area (Hübner *et al.*, 2004). In addition, analysis of *Dictyota* sp. shows that no metal consistently increased in concentration as  $\text{pCO}_2$  increased. Brown algae are considered a good indicator of bioavailable metals since they greatly bioaccumulate metals, often proportionally to metals concentration in surrounding seawater (Phillips, 1990). Values higher than

ranges reported in the literature were found for aluminium, arsenic and iron at 200 W and for aluminium and zinc in REF A (Table 2.8). Aluminium variability is likely to be related to local mineralogy (Karageorgis *et al.*, 2005), while enrichment in the other elements has previously been linked to hydrothermal activity (Hübner *et al.*, 2004). Metal bioaccumulation is a common occurrence at shallow and deep hydrothermal vents (Tarasov *et al.*, 2005; Couto *et al.*, 2010), but at Methana metal enrichment did not seem to have major effects at the community and species level. The intermediate and reference sites enriched in some elements (200 W and REF A) were not significantly different from the other intermediate and reference sites (200 E and REF B) with regards to key species percent cover and overall community structure (see Chapter 3).

As with other carbon dioxide seeps used as natural analogues for ocean acidification, Methana has some limitations. Motile taxa such as fish are able to move in and out of high CO<sub>2</sub> areas (Riebesell, 2008) and pelagic larvae can come from unaffected populations (Cigliano *et al.*, 2010). Moreover, carbonate chemistry is much more variable near the seeps than in reference conditions, as changes in current direction and intensity influence the dispersal of the dissolved gas emissions. Compared to other volcanic seeps, at Methana seawater pCO<sub>2</sub> is high and variable on a greater scale (>15 vs <0.3 km of shoreline; Hall-Spencer *et al.*, 2008; Fabricius *et al.*, 2011; Boatta *et al.*, 2013). Thus, Methana might offer an opportunity to study ecological processes such as recruitment in a high CO<sub>2</sub> area probably less influenced by unaffected populations than smaller sites.

The need to translate results from laboratory experiments to more realistic systems has led to several areas with naturally high pCO<sub>2</sub> to be used to infer biological community responses to ocean acidification. Examples include

estuaries acidified by acid sulphate soils (Amaral *et al.*, 2011), groundwater submarine springs (Crook *et al.*, 2012), upwelling regions (Manzello, 2010; Thomsen *et al.*, 2010; Mayol *et al.*, 2012) and rockpools with different carbonate chemistry (Moulin *et al.*, 2011; Evans *et al.*, 2013). None of the above are perfect ocean acidification analogues, as they can have confounding gradients in salinity and alkalinity (groundwater springs) or in temperature and nutrients (upwelling areas). In addition, low pH recorded in groundwater springs and acidified estuaries is not always caused by increased carbon dioxide concentrations, so only the effects of low pH on biological communities can be tested. However, studies from low pH/high CO<sub>2</sub> sites mostly report decreased abundance and diversity of calcifying organisms, in accord with findings from CO<sub>2</sub> seeps and laboratory experiments (Hall-Spencer *et al.*, 2008; Kroeker *et al.*, 2013a; Fabricius *et al.*, 2014). General patterns of community responses to ocean acidification can then be detected using areas with naturally low pH, even though confounding factors should always be taken into account.

Overall, our data show that the examined seeps off Methana offer an opportunity to study the effects of high pCO<sub>2</sub> levels on natural benthic communities in an oligotrophic environment. The general limitations of using CO<sub>2</sub> seeps should, however, be taken into account. These seeps could also be used to study how ecological processes are influenced by carbon dioxide on a scale of kilometres, not tens of meters as the other seeps currently used as ocean acidification analogues.

## Chapter 3

### Changes in subtidal macroalgal communities along pCO<sub>2</sub> gradients at Mediterranean volcanic seeps

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## Abstract

Shifts in macroalgal communities have been detected along pCO<sub>2</sub> gradients at volcanic seeps in a few temperate (Italy) and tropical (Papua New Guinea) settings. However, replication of these observations is needed to expand our ability to predict how marine ecosystems will change due to ocean acidification. The present study determined macroalgal diversity and abundance along pCO<sub>2</sub> gradients caused by volcanic seeps off Methana (Greece) and Vulcano (Italy) using visual census and destructive sampling methods, respectively. At Methana, *Cystoseira corniculata* was dominant in autumn and *Sargassum vulgare* C.Agardh was dominant in spring near the seeps. The articulated coralline alga *Jania rubens* had significantly higher cover at reference sites, but only in autumn. Diversity decreased with increasing CO<sub>2</sub> regardless of sampling season. At Vulcano, the main habitat-forming algal species changed as CO<sub>2</sub> level increased: at the reference site *Cystoseira* spp. and *Dictyopteris polypodioides* (A.P.De Candolle) J.V.Lamouroux were dominant, at elevated pCO<sub>2</sub> levels *Sargassum vulgare* greatly increased in abundance replacing *D. polypodioides*. These data are consistent with results from laboratory experiments and observations at other Mediterranean CO<sub>2</sub> seep sites in that benthic communities decreased in calcifying algal cover with increasing pCO<sub>2</sub>. This chapter demonstrates that natural pCO<sub>2</sub> gradients can help us envisage how benthic communities will be affected by ocean acidification in a range of environmental conditions, and that benthic community responses to ocean acidification can be strongly affected by season.

### 3.1 Introduction

Studies on single species can be very useful for formulating hypotheses about how biological communities may respond to ocean acidification. However, work on global warming demonstrates that most temperature-associated causes of severe population decline originate not from direct physiological responses to heat, but result from modified species interactions (Cahill *et al.*, 2013). Similar trends arise in lake acidification (Locke and Sprules, 2000) and it is anticipated that consequences of ocean acidification will be similar (Gaylord *et al.*, 2014). Responses of seaweed species to increased carbon dioxide are poorly known (Harley *et al.*, 2012; Koch *et al.*, 2013), with laboratory experiments to date concentrated on calcifying red and green algae (Gao *et al.*, 1993; Büdenbender *et al.*, 2011; Price *et al.*, 2011). So far, evidence indicates that calcifying algae will be negatively affected by ocean acidification while some fleshy algae may thrive (Kroeker *et al.*, 2013a; Brodie *et al.*, 2014). There is very little information on brown seaweed responses to elevated pCO<sub>2</sub> despite the fact that they are key species in temperate rocky habitats worldwide (Steneck *et al.*, 2002). In addition, a species can respond differently to ocean acidification in single species experiments and in natural communities because of inter-specific interaction; for instance, crustose coralline algae may cope with elevated CO<sub>2</sub>, but be outcompeted by fleshy algae (Kroeker *et al.*, 2013c).

Our limited ability to predict community responses of macroalgal communities to ocean acidification worldwide and the few experiments performed on Mediterranean species add value to studies examining community responses using CO<sub>2</sub> seeps in the Mediterranean Sea. Results from surveys at seeps off Ischia and Vulcano (both in Italy) show how increased carbon dioxide is likely to cause shifts in macroalgal communities. As CO<sub>2</sub> increases at these sites,



coralline algae are replaced by canopy-forming brown algae such as *Sargassum vulgare* in the shallow subtidal off Ischia (Porzio *et al.*, 2011) or brown foliose algae such as *Dictyota* spp. and non-calcified *Padina* sp. off Vulcano (Johnson *et al.*, 2012; Graziano *et al.*, unpublished data). This response to increased CO<sub>2</sub> differs from shifts towards opportunistic green macroalgal species such as *Ulva* spp. or mat-forming algae reported in stressed marine benthic ecosystems, such as those exposed to eutrophication (Airoldi and Beck, 2007; Connell *et al.*, 2008), where decreased floral complexity can have detrimental effects on local biodiversity (Schermer *et al.*, 2013) and indirectly affect the abundance of many commercial species (Harley *et al.*, 2012). On the other hand, increased abundance of brown perennial algae near volcanic CO<sub>2</sub> seeps suggests that carbon dioxide may be a useful resource that can benefit perennial habitat-forming algae (Porzio *et al.*, 2011; Connell *et al.*, 2013).

This study aims to assess changes in macroalgal communities along pCO<sub>2</sub> gradients off Greece and Italy. I characterised benthic communities at a shallow subtidal site in the Eastern Mediterranean Sea (Methana, Greece) in spring and autumn as well as those off Vulcano (Italy) at a depth of 3-5 metres in spring. I was mindful of the fact that responses to increased carbon dioxide in these two environments could be substantially different from those previously recorded, since the Aegean Sea has lower average nutrient concentrations than those in the Tyrrhenian Sea (Moutin and Raimbault, 2002). Food limitation has been shown to exacerbate the negative influence of ocean acidification on benthic invertebrates (Melzner *et al.*, 2011; Rodolfo-Metalpa *et al.*, 2011), so nutrient limitation may act in combination with ocean acidification to negatively affect seaweed communities. At ambient levels of CO<sub>2</sub> around Methana and Vulcano,

subtidal brown algae such as *Cystoseira* spp. and *Dictyopteris polypodioides* have the highest biomass (Pérès and Picard, 1964). These subtidal species are expected to be less sensitive to ocean acidification than calcifying algae (Kroeker *et al.*, 2013a), but they could be more sensitive to ocean acidification than intertidal brown algae. As subtidal habitats are more stable than intertidal ones, subtidal organisms are generally thought to be more vulnerable to environmental changes (Lobban and Harrison, 1994).

In addition, there are very few studies dealing with the seasonal patterns of benthic community changes along pCO<sub>2</sub> gradients, even though temperate coastal waters vary greatly among seasons (Coma *et al.*, 2000). These ecosystems undergo large yearly changes in light and temperature regimes, which indirectly influence other factors important for biological communities, such as nutrient levels (Pingree *et al.*, 1976). In the Mediterranean Sea, these three factors strongly influence macroalgal communities: macroalgal biomass peaks in late spring, and community composition changes among seasons (Sala and Boudouresque, 1997). Specifically, many mat-forming algae disappear and most erect algae decrease in cover during the cold season (Piazzi *et al.*, 2004).

## **3.2 Methods**

### **3.2.1 Methana experimental design and data analysis**

The first part of the work for this thesis chapter was conducted along a pCO<sub>2</sub> gradient off Methana (Greece). Five sites at three pCO<sub>2</sub> levels were used: SEEP (high CO<sub>2</sub> level), 200 W and 200 E (intermediate CO<sub>2</sub> level), REF A and REF B (reference CO<sub>2</sub> level); for a detailed description of study sites, see Chapter 2. Benthic community composition was assessed in May and

September 2012 using visual census (detailed sampling dates and sample sizes are reported in Table 1.1B): samples were collected between 0.7 and 1.0 m below mean sea level using 20x20 cm quadrats on horizontal and sub-horizontal rocky substrata (Fraschetti *et al.*, 2005). A frame with 25 4x4 cm squares was used to assess percentage cover (C%) and number of taxa (S). Percentage cover of algae and sessile invertebrates was determined by assigning each taxon a score ranging from 0 to 4 within each square and summing the 25 estimates (Dethier *et al.*, 1993). Final values were expressed as percentages. Taxa were identified to the lowest possible level, usually species, except for turf algae; this functional group was defined as sparse to thick mats of small and juvenile algae less than 2 cm high (Steneck and Dethier, 1994). Seven replicate quadrats randomly chosen but placed at least 4-5 m from each other were assessed for every site in May 2012 and six replicates were collected in September 2012. The selected sample size represented the maximum number of samples that could be randomly collected from sub-horizontal surfaces at the smallest site (SEEP, ~20 m of shoreline) at the selected depth (0.7-1.0 m below sea level). Samples were collected in May, when Mediterranean seaweeds reach their biomass peak (Ballesteros, 1984) and September, when Greek shallow benthic communities have been exposed for over three months to temperatures > 25°C (Maria Salomidi, personal communication).

Differences in community structure and composition were assessed using Permutational Multivariate Analysis of Variance (PERMANOVA, (Anderson *et al.*, 2003); PRIMER 6 and PERMANOVA + package (Clarke and Gorley, 2006)). The analysis had two fixed factors: season (two levels) and site (five levels). The analyses were performed on Bray-Curtis measures of square root

transformed data, using 9999 permutations of residuals under a reduced model. Pair-wise comparisons were then performed for significant factors with more than two levels. Since site was a significant factor in the PERMANOVA, its levels were compared using a SIMPER analysis to identify the taxa driving dissimilarities.

Macroalgal cover data were used to calculate Shannon diversity (Shannon and Weaver, 1949) and Pielou's evenness (Pielou, 1966) for each sample. The two indices were analysed using an ANOVA followed by a Tukey HSD test for multiple comparisons. After testing for normality and homoscedasticity, canopy-forming and calcifying algae arcsin-transformed percent cover was analysed using a two-way ANOVA with site and functional group as fixed factors; seasons were tested separately. The site\*species interaction was then decomposed to obtain multiple comparisons among sites for each season separately. The same analysis was then performed for selected single species. All univariate analyses were performed using SPSS v19 (IBM, USA).

### **3.2.2 Vulcano study site**

The second part of the work for this thesis chapter was conducted along a pCO<sub>2</sub> gradient off the island of Vulcano in the Eolian archipelago (Italy). This island is an active volcano formed in the collision of the Eurasian and Asian plates. The gradient is caused by submarine volcanic seeps emitting over 97% CO<sub>2</sub> (Inguaggiato *et al.*, 2012; Boatta *et al.*, 2013), which lower seawater pH to 5.5-5.6 at the main bubbling site. The hydrothermal fluids also contain hydrogen sulphide (Italiano *et al.*, 1984; Capaccioni *et al.*, 2001), which is potentially toxic to cellular respiration. However, hydrogen sulphide rapidly oxidises to sulphate; sulphide concentrations are in fact very low (< 50 µMol kg<sup>-1</sup>) at > 20 m from the main seeps as water is well-oxygenated all over the bay (Boatta *et al.*, 2013). I

chose an area for this study that was a ~200 m stretch of rocky shore at >30 m from the main seeps, where pH ranges from 7.55 to 8.2 (Boatta *et al.*, 2013). Along this gradient, the three sites (pH  $\pm$  S.D.) shown in Figure 3.1 were selected, one with high (High CO<sub>2</sub>; pH 7.57  $\pm$  0.23, n = 19), one with intermediate (Mid CO<sub>2</sub>; pH 7.94  $\pm$  0.16, n = 18) and one with reference pCO<sub>2</sub> (REF A; pH 8.14  $\pm$  0.05, n = 11); pH data are from monitoring conducted in September 2009 and April 2010 from Graziano *et al.* (unpublished data).

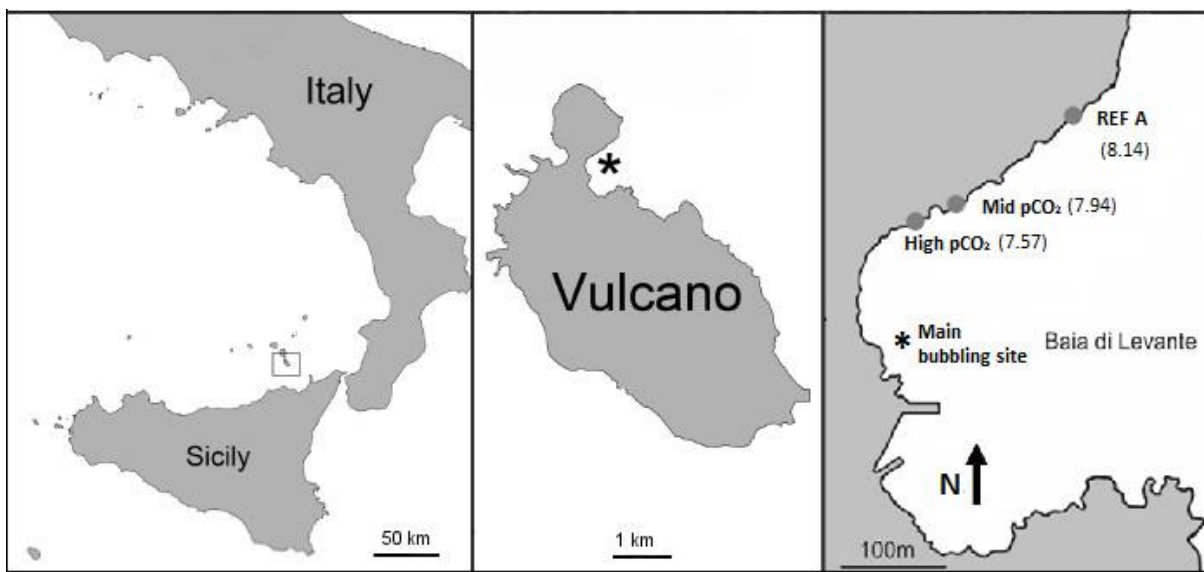


Figure 3.1. Vulcano Island (Sicily, Southern Italy) and study sites; the asterisk marks the main bubbling area, whereas grey circles indicate the three sampling sites and their average pH, with decreasing pCO<sub>2</sub> moving away from the bubbling area.

Geochemical surveys in Levante bay in 2011 have shown that these seeps do not influence the main seawater elements and data in Table 3.1A demonstrate that temperature, salinity and total alkalinity do not change significantly among the sites used for this chapter (Boatta *et al.*, 2013). Nutrients were also measured (Table 3.1B); nitrite and silicate concentrations were significantly different among sites, while nitrate levels were relatively stable along the CO<sub>2</sub> gradient and phosphate levels remained below detection limit (~10 nmol L<sup>-1</sup>) at

all sites (Johnson, 2012). The increase in nitrates was very small (0.01  $\mu\text{M}$ ), and silicates are not believed to be limiting for marine organisms at the reference site, as phosphates are the limiting nutrient in Mediterranean waters (Zohary and Robarts, 1998).

Table 3.1. (A) Mean ( $\pm\text{SD}$ ) seawater temperature (T), salinity (S) and total alkalinity (TA) recorded from September 2009 to July 2011 at three sites used for this chapter (data from Boatta *et al.*, 2013). (B) Mean ( $\pm\text{SD}$ ) dissolved nutrient concentrations at three studied sites at Vulcano (n = 5-6). Phosphate was also determined but at all stations was below the detection limit of the AutoAnalyser, i.e.  $\sim 10\text{ nmol L}^{-1}$  (data from Johnson, 2012).

| A) | Site               | T ( $^{\circ}\text{C}$ ) | S (‰)            | n  | TA ( $\mu\text{Eq kg}^{-1}$ ) | n |
|----|--------------------|--------------------------|------------------|----|-------------------------------|---|
|    | High $\text{CO}_2$ | 21.71 $\pm$ 4.93         | 37.49 $\pm$ 0.64 | 41 | 2524.7 $\pm$ 0.6              | 9 |
|    | Mid $\text{CO}_2$  | 21.67 $\pm$ 4.93         | 37.59 $\pm$ 0.58 | 41 | 2520.5 $\pm$ 6.6              | 9 |
|    | REF A              | 21.66 $\pm$ 4.22         | 37.45 $\pm$ 0.66 | 22 | 2532.5 $\pm$ 20.9             | 7 |

| B) | Site               | Nitrite ( $\mu\text{M}$ ) | Nitrate ( $\mu\text{M}$ ) | Silicate ( $\mu\text{M}$ ) |
|----|--------------------|---------------------------|---------------------------|----------------------------|
|    | High $\text{CO}_2$ | 0.02 $\pm$ 0.002          | 0.33 $\pm$ 0.22           | 19.39 $\pm$ 2.77           |
|    | Mid $\text{CO}_2$  | 0.02 $\pm$ 0.007          | 0.16 $\pm$ 0.13           | 15.12 $\pm$ 5.48           |
|    | REF A              | 0.01 $\pm$ 0.002          | 0.24 $\pm$ 0.11           | 3.43 $\pm$ 0.11            |

As for minor seawater elements, there was a marked increase in iron concentration, which reached values up to three orders of magnitude higher than ambient concentrations reported for the Mediterranean Sea (Sarhou and Jeandel, 2001; Figure 3.2). Iron is an essential micronutrient for marine algae and is a limiting factor for their growth in many areas in the world ocean (Geider and La Roche, 1994). In the Mediterranean Sea, however, iron is only limiting in special circumstances (Bennet and Guien, 2006) due to the very low phosphate concentrations in the region (Zohary and Robarts, 1998). The minimum iron value measured off Vulcano during a recent geochemical survey was 57 nMol/kg (Boatta *et al.*, 2013), much higher than phosphate concentrations in the area ( $< 10\text{ nMol/kg}$ ; Johnson, 2012). According to the modified Redfield ratio

(Martin *et al.*, 1990), iron is limiting when its concentration is lower than 10% of that of phosphate, which is not the case in Vulcano.

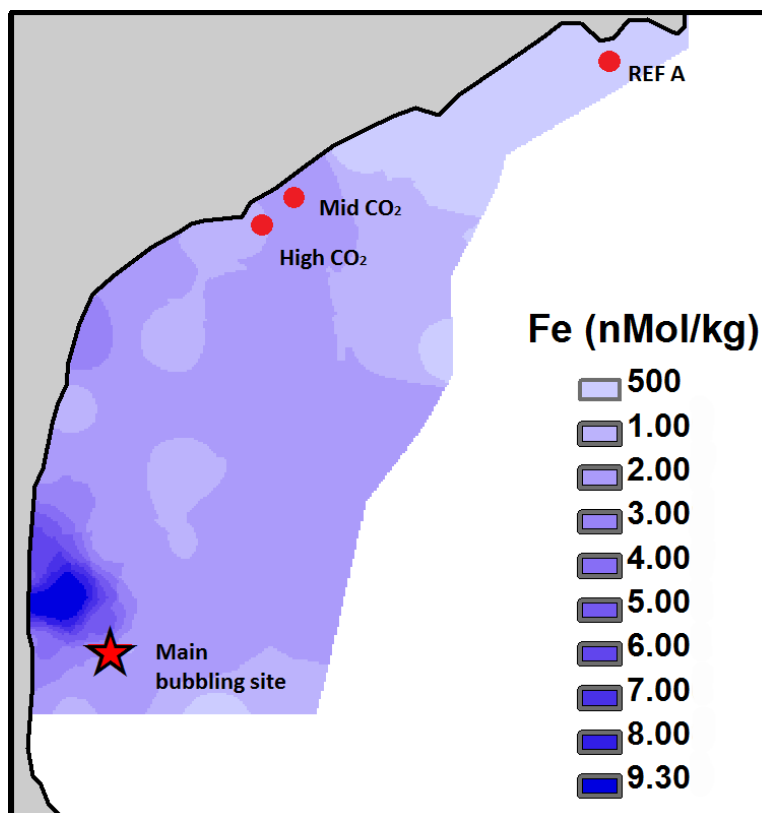


Figure 3.2. Distribution map adapted from a recent geochemical survey (Boatta *et al.*, 2013) in the Baia di Levante showing the iron concentrations measured in the area in April 2011. The sites used for this thesis have been superimposed (red dots); the red star indicates the main bubbling site. Data for these maps were collected from around 70 sampling points within the bay.

Elevated heavy metal concentrations are also a common feature of volcanic areas (Tarasov *et al.*, 2005). A recent geological survey of Baia di Levante (Figure 3.3) revealed that parts of the bay have sediment of poor or bad quality according to the Marine Sediment Pollution Index (MSPi; Shin and Lam, 2001). The sites chosen for the present study have average or good sediment condition, which only have a moderate potential for causing adverse biological effects according to the Sediment Quality Guideline Quotient (SQG-Q, for its

definition see Chapter 2; Vizzini *et al.*, 2013). The change in pCO<sub>2</sub> values caused by the seeps is therefore the most likely driver of any biological change observed at the study sites.

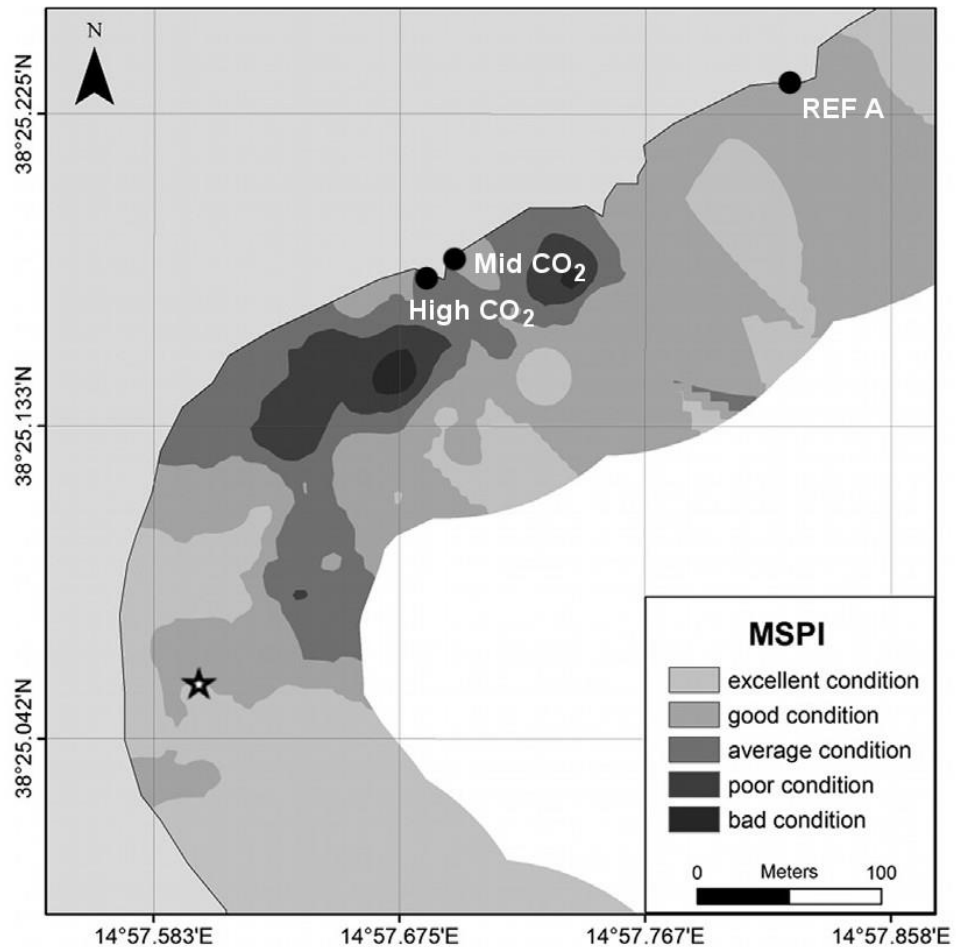


Figure 3.3. Results from a recent geochemical survey in the Baia di Levante showing the Marine Sediment Pollution Index (MSPI) from sediment collected in autumn 2011. The sites used for this thesis have been superimposed (black dots); the star indicates the main bubbling site. Data for these maps were collected from 50 sampling points within the bay (Vizzini *et al.*, 2013).

### 3.2.3 Vulcano experimental design and data analysis

Macroalgae were collected by scraping 20 x 20 cm squares of rock with hammer and chisel following a method developed for this type of Mediterranean shore (Ballesteros, 1986). Four replicates were collected in three sites in May



2010 by Mariagrazia Graziano and Kristy Kroeker, immediately frozen and transported to the laboratory (detailed sampling dates and sample sizes are reported in Table 1.1B). Samples were then sorted and identified to the lowest possible taxonomic level, mostly to the species level, except for turf algae; this functional group was defined as sparse to thick mats of small and juvenile algae less than 2 cm high (Steneck and Dethier, 1994). For biomass determination, dry biomass of each taxon was obtained drying the identified samples in an oven at 60°C for 24 hours. The obtained data was analysed using the same procedure outlined above for Methana, but using an experimental design with one fixed factor with three levels (“Site”) and log-transforming the biomass data for functional groups and single species analyses.

### **3.3 Results**

#### **3.3.1 Methana benthic community**

Overall, 18 macroalgal taxa and three invertebrate taxa (two sponges and one hydrozoan) were recorded. Benthic communities significantly differed among sites and seasons (Table 3.2A), with a significant interaction between the two factors (pseudo- $F_{4,55}=1.754$ ,  $p(\text{perm})=0.0457$ ). In spring the high  $p\text{CO}_2$  site was significantly different from the reference sites, while the intermediate  $p\text{CO}_2$  sites were not significantly different from any of them. In autumn, the high  $p\text{CO}_2$  site was significantly different from all other sites (Table 3.2B). Site had a significant effect on diversity ( $p = 0.049$ , Table 3.3) with a clear decrease as  $\text{CO}_2$  increased ( $0.94 \pm 0.10$ ,  $n=26$  to  $0.55 \pm 0.08$ ,  $n=13$ ; mean  $\pm$  SE).

Table 3.2. (A) PERMANOVA analysis on square-root transformed percentage cover of Methana benthic communities. The first table shows main factors and their interaction and degrees of freedom (df), sum of squares (SS), pseudo-F, permutational p and unique permutations for each of them. Season, site and their interaction all have a significant effect. (B) Results from pair-wise comparisons between sites for each season (different letters represent significantly different groups).

| (A) | Source        | df | SS       | Pseudo-F | p (perm)      | Unique perms |
|-----|---------------|----|----------|----------|---------------|--------------|
|     | Season        | 1  | 31069    | 19.234   | <b>0.0001</b> | 9949         |
|     | Site          | 4  | 21820    | 3.377    | <b>0.0001</b> | 9918         |
|     | Season x Site | 4  | 11330    | 1.754    | <b>0.0457</b> | 9916         |
|     | Residual      | 55 | 88840    |          |               |              |
|     | Total         | 64 | 1.5273E5 |          |               |              |

| (B) | Season | Sites             |                      |                      |                                       |
|-----|--------|-------------------|----------------------|----------------------|---------------------------------------|
|     | Spring | SEEP <sup>a</sup> | 200 W <sup>a,b</sup> | 200 E <sup>a,b</sup> | REF A <sup>b</sup> REF B <sup>b</sup> |
|     | Autumn | SEEP <sup>a</sup> | 200 W <sup>b</sup>   | 200 E <sup>b</sup>   | REF A <sup>b</sup> REF B <sup>b</sup> |

Shannon diversity (H') and Pielou's evenness (J') of benthic communities off Methana were pooled among seasons and sites as no significant differences were detected between seasons and between sites with the same CO<sub>2</sub> level (Figure 3.4). Although the factor 'site' had a significant effect on Shannon diversity (Table 3.3), no significant differences were detected by the Tukey test. However, both indices show a clear decreasing trend in the diversity of the rocky shore sessile communities from reference to high CO<sub>2</sub> levels, with Shannon's diversity index almost halving (0.94 ± 0.40 to 0.55 ± 0.37; mean ± SD).

Table 3.3. ANOVA results for (A) Shannon diversity ( $H'$ ) and (B) Pielou's evenness ( $J'$ ) of Methana benthic communities. The Tables show main factors and their interactions and sum of squares (SS), degrees of freedom (df), Mean Squares (MS), F-ratios (F) and p values. Significant p values ( $< 0.05$ ) are highlighted. Results from pairwise comparisons among sites were never significant and are not reported.

| A) Source     | Type III SS | df | MS    | F     | p            |
|---------------|-------------|----|-------|-------|--------------|
| Site          | 1.438       | 4  | 0.359 | 2.557 | <b>0.049</b> |
| Season        | 0.261       | 1  | 0.261 | 1.859 | 0.178        |
| Site x Season | 0.232       | 4  | 0.058 | 0.413 | 0.798        |
| Error         | 7.731       | 55 | 0.141 |       |              |
| Total         | 9.664       | 64 |       |       |              |

| B) Source     | Type III SS | df | MS    | F     | p     |
|---------------|-------------|----|-------|-------|-------|
| Site          | 0.282       | 4  | 0.071 | 1.285 | 0.287 |
| Season        | 0.166       | 1  | 0.166 | 3.022 | 0.088 |
| Site x Season | 0.119       | 4  | 0.030 | 0.539 | 0.708 |
| Error         | 3.022       | 55 | 0.055 |       |       |
| Total         | 3.568       | 64 |       |       |       |

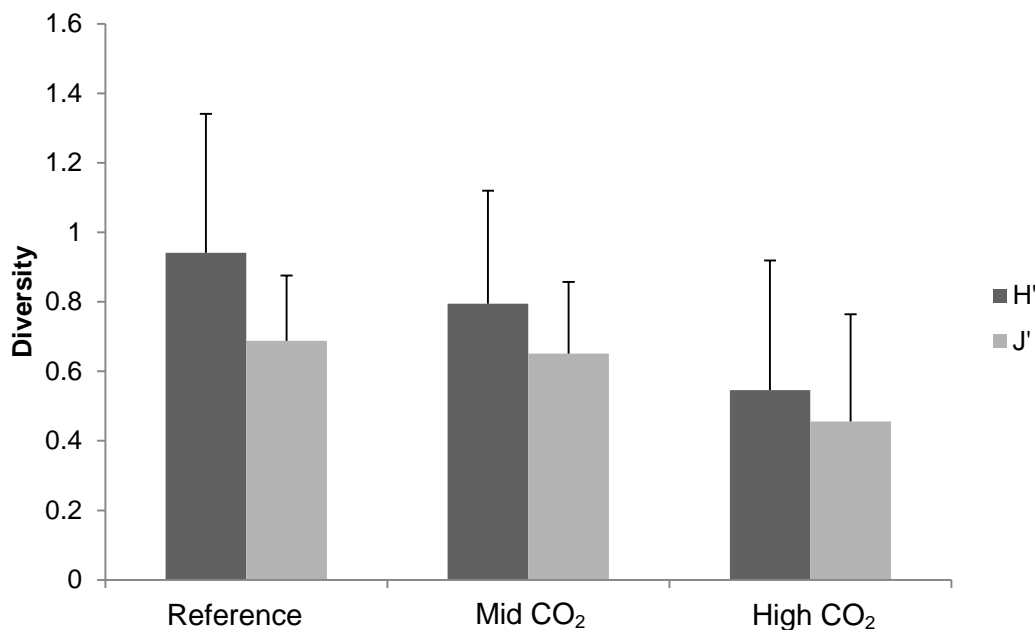


Figure 3.4. Mean ( $\pm$  SD) Shannon diversity ( $H'$ ) and Pielou's evenness ( $J'$ ) at high ( $n=13$ ), intermediate ( $n=26$ ) and reference ( $n=26$ ) CO<sub>2</sub> at Methana rocky shores at depths between 0.7 and 1.0 m pooling sites and season.

The SIMPER analysis among sites shows which taxa contributed most to the detected differences (Table 3.4). The main drivers of differences between groups were canopy-forming algae such as *Cystoseira corniculata* and *Sargassum vulgare* and calcareous algae such as coralline crustose algae (CCA), the articulated coralline alga *Jania rubens* and the calcified brown alga *Padina pavonica*.

Table 3.4. SIMPER analysis of Methana benthic communities showing the average dissimilarities between each pair of sites and which species contributed up to 90% of the dissimilarity. For each taxon, the average abundance in the two groups that are being compared, their average dissimilarity, the dissimilarity to standard deviation ration and the taxon contribution and cumulative contribution are shown. CCA stands for coralline crustose algae.

| <b>Groups SEEP &amp; 200 E; Average dissimilarity = 65.40</b> |          |          |         |         |          |       |
|---|----------|----------|---------|---------|----------|-------|
| Taxon   | SEEP     | 200 E    | Av.Diss | Diss/SD | Contrib% | Cum.% |
|   | Av.Abund | Av.Abund |         |         |          |       |
| <i>Cystoseira corniculata</i>                                 | 5.27     | 6.76     | 14.85   | 1.28    | 22.71    | 22.71 |
| CCA   | 0.11     | 2.86     | 8.80    | 0.88    | 13.45    | 36.16 |
| <i>Sargassum vulgare</i>                                      | 2.58     | 0.85     | 8.46    | 0.74    | 12.93    | 49.09 |
| <i>Jania rubens</i>   | 0.00     | 2.63     | 7.70    | 0.78    | 11.77    | 60.85 |
| <i>Dictyota</i> sp.   | 1.16     | 1.59     | 6.14    | 0.91    | 9.38     | 70.24 |
| <i>Sargassum</i> sp.  | 1.50     | 0.00     | 4.28    | 0.45    | 6.54     | 76.78 |
| <i>Padina pavonica</i> (not calcified)                        | 1.12     | 0.27     | 3.93    | 0.51    | 6.02     | 82.79 |
| Bare substratum   | 1.08     | 0.00     | 3.16    | 0.60    | 4.83     | 87.62 |
| <i>Falkenbergia</i> sp.                                       | 0.46     | 0.00     | 1.38    | 0.51    | 2.10     | 89.73 |
| <i>Cladophora</i> sp.   | 0.50     | 0.00     | 1.36    | 0.39    | 2.08     | 91.80 |

| <b>Groups SEEP &amp; 200 W; Average dissimilarity = 63.83</b> |          |          |         |         |           |       |
|---|----------|----------|---------|---------|-----------|-------|
| Taxon   | SEEP     | 200 W    | Av.Diss | Diss/SD | Contrib % | Cum.% |
|   | Av.Abund | Av.Abund |         |         |           |       |
| <i>Cystoseira corniculata</i>                                 | 5.27     | 6.52     | 14.63   | 1.30    | 22.92     | 22.92 |
| <i>Sargassum vulgare</i>                                      | 2.58     | 0.59     | 8.96    | 0.70    | 14.04     | 36.96 |
| <i>Cladostephus spongiosus</i>                                | 0.36     | 1.95     | 6.54    | 0.85    | 10.24     | 47.20 |
| <i>Dictyota</i> sp.   | 1.16     | 1.56     | 6.18    | 0.85    | 9.68      | 56.88 |
| <i>Jania rubens</i>   | 0.00     | 1.90     | 5.46    | 0.65    | 8.56      | 65.44 |
| CCA   | 0.11     | 1.57     | 4.81    | 0.63    | 7.53      | 72.97 |
| <i>Sargassum</i> sp.  | 1.50     | 0.55     | 4.27    | 0.52    | 6.69      | 79.65 |

|  |      |      |      |      |      |       |
|--|------|------|------|------|------|-------|
| <i>Padina pavonica</i> (not calcified) | 1.12 | 0.26 | 3.96 | 0.51 | 6.21 | 85.86 |
| Bare substratum                        | 1.08 | 0.64 | 2.89 | 0.58 | 4.53 | 90.39 |

**Groups 200 E & 200 W; Average dissimilarity = 43.27**

| Taxon                                  | 200 E    | 200 W    | Av.Diss | Diss/SD | Contrib% | Cum.% |
|--|----------|----------|---------|---------|----------|-------|
|  | Av.Abund | Av.Abund |         |         |          |       |
| <i>Cystoseira corniculata</i>          | 6.76     | 6.52     | 9.58    | 1.11    | 22.14    | 22.14 |
| CCA                                    | 2.86     | 1.57     | 8.68    | 0.92    | 20.06    | 42.21 |
| <i>Cladostephus spongiosus</i>         | 0.00     | 1.95     | 5.86    | 0.82    | 13.54    | 55.74 |
| <i>Jania rubens</i>                    | 2.63     | 1.90     | 3.41    | 0.60    | 7.87     | 63.61 |
| <i>Dictyota</i> sp.                    | 1.59     | 1.56     | 3.11    | 0.66    | 7.18     | 70.80 |
| <i>Sargassum vulgare</i>               | 0.85     | 0.59     | 2.81    | 0.74    | 6.50     | 77.30 |
| Bare substratum                        | 0.00     | 0.64     | 1.62    | 0.40    | 3.75     | 81.05 |
| <i>Padina pavonica</i> (not calcified) | 0.27     | 0.26     | 1.54    | 0.40    | 3.56     | 84.61 |
| <i>Halopteris scoparia</i>             | 0.15     | 0.42     | 1.54    | 0.39    | 3.56     | 88.17 |
| <i>Sargassum</i> sp.                   | 0.00     | 0.55     | 1.44    | 0.49    | 3.33     | 91.49 |

**Groups SEEP & REF A; Average dissimilarity = 74.58**

| Taxon                                  | SEEP     | REF A    | Av.Diss | Diss/SD | Contrib% | Cum.% |
|--|----------|----------|---------|---------|----------|-------|
|  | Av.Abund | Av.Abund |         |         |          |       |
| <i>Cystoseira corniculata</i>          | 5.27     | 5.01     | 15.26   | 1.36    | 20.46    | 20.46 |
| <i>Jania rubens</i>                    | 0.00     | 3.77     | 11.28   | 0.94    | 15.13    | 35.59 |
| <i>Sargassum vulgare</i>               | 2.58     | 0.34     | 8.76    | 0.70    | 11.75    | 47.34 |
| Bare substratum                        | 1.08     | 1.71     | 6.83    | 1.29    | 9.15     | 56.50 |
| <i>Dictyota</i> sp.                    | 1.16     | 1.98     | 6.49    | 0.76    | 8.70     | 65.20 |
| CCA                                    | 0.11     | 1.83     | 5.13    | 0.87    | 6.87     | 72.07 |
| <i>Sargassum</i> sp.                   | 1.50     | 0.00     | 4.28    | 0.45    | 5.74     | 77.81 |
| <i>Padina pavonica</i> (not calcified) | 1.12     | 0.00     | 3.27    | 0.44    | 4.38     | 82.19 |
| <i>Cystoseira amentacea</i>            | 0.00     | 0.93     | 2.93    | 0.53    | 3.93     | 86.12 |
| <i>Padina pavonica</i> (calcified)     | 0.00     | 0.75     | 2.44    | 0.52    | 3.27     | 89.40 |
| <i>Halopteris scoparia</i>             | 0.00     | 0.66     | 2.03    | 0.40    | 2.73     | 92.12 |

**Groups 200 E & REF A; Average dissimilarity = 46.56**

| Taxon                              | 200 E    | REF A    | Av.Diss | Diss/SD | Contrib% | Cum.% |
|------------------------------------|----------|----------|---------|---------|----------|-------|
|                                    | Av.Abund | Av.Abund |         |         |          |       |
| <i>Cystoseira corniculata</i>      | 6.76     | 5.01     | 11.28   | 1.21    | 24.22    | 24.22 |
| CCA                                | 2.86     | 1.83     | 6.48    | 0.78    | 13.93    | 38.15 |
| Bare substratum                    | 0.00     | 1.71     | 5.02    | 0.99    | 10.77    | 48.92 |
| <i>Dictyota</i> sp.                | 1.59     | 1.98     | 4.84    | 0.84    | 10.39    | 59.31 |
| <i>Jania rubens</i>                | 2.63     | 3.77     | 4.39    | 0.86    | 9.43     | 68.74 |
| <i>Sargassum vulgare</i>           | 0.85     | 0.34     | 3.15    | 0.77    | 6.77     | 75.50 |
| <i>Cystoseira amentacea</i>        | 0.00     | 0.93     | 2.81    | 0.53    | 6.04     | 81.55 |
| <i>Padina pavonica</i> (calcified) | 0.00     | 0.75     | 2.34    | 0.52    | 5.04     | 86.58 |
| <i>Halopteris scoparia</i>         | 0.15     | 0.66     | 2.30    | 0.48    | 4.95     | 91.53 |

**Groups 200 W & REF A; Average dissimilarity = 53.21**

| Taxon                                 | 200 W    | REF A    | Av.Diss | Diss/SD | Contrib% | Cum.% |
|---------------------------------------|----------|----------|---------|---------|----------|-------|
|                                       | Av.Abund | Av.Abund |         |         |          |       |
| <i>Cystoseira corniculata</i>         | 6.52     | 5.01     | 10.33   | 1.20    | 19.42    | 19.42 |
| CCA                                   | 1.57     | 1.83     | 6.12    | 0.92    | 11.50    | 30.92 |
| Bare substratum                       | 0.64     | 1.71     | 5.85    | 1.14    | 10.99    | 41.91 |
| <i>Jania rubens</i>                   | 1.90     | 3.77     | 5.74    | 0.80    | 10.78    | 52.69 |
| <i>Cladostephus spongiosus</i>        | 1.95     | 0.00     | 5.66    | 0.81    | 10.64    | 63.33 |
| <i>Dictyota</i> sp.                   | 1.56     | 1.98     | 4.96    | 0.78    | 9.32     | 72.65 |
| <i>Cystoseira amentacea</i>           | 0.00     | 0.93     | 2.82    | 0.53    | 5.31     | 77.96 |
| <i>Halopteris scoparia</i>            | 0.42     | 0.66     | 2.56    | 0.48    | 4.82     | 82.77 |
| <i>Sargassum vulgare</i>              | 0.59     | 0.34     | 2.40    | 0.58    | 4.51     | 87.29 |
| <i>Padina pavonica</i><br>(calcified) | 0.00     | 0.75     | 2.35    | 0.52    | 4.42     | 91.71 |

**Groups SEEP & REF B; Average dissimilarity = 72.33**

| Taxon                                     | SEEP     | REF B    | Av.Diss | Diss/SD | Contrib% | Cum.% |
|---|----------|----------|---------|---------|----------|-------|
|   | Av.Abund | Av.Abund |         |         |          |       |
| <i>Cystoseira corniculata</i>             | 5.27     | 4.64     | 15.07   | 1.30    | 20.84    | 20.84 |
| <i>Sargassum vulgare</i>                  | 2.58     | 0.60     | 8.90    | 0.71    | 12.31    | 33.15 |
| <i>Sargassum</i> sp.                      | 1.50     | 1.86     | 7.36    | 0.81    | 10.18    | 43.33 |
| CCA                                       | 0.11     | 2.44     | 6.80    | 1.15    | 9.41     | 52.73 |
| <i>Jania rubens</i>                       | 0.00     | 2.09     | 5.96    | 0.79    | 8.24     | 60.97 |
| <i>Dictyota</i> sp.                       | 1.16     | 1.61     | 5.76    | 0.76    | 7.97     | 68.94 |
| Bare substratum                           | 1.08     | 1.46     | 4.65    | 0.88    | 6.42     | 75.36 |
| <i>Padina pavonica</i><br>(calcified)     | 0.00     | 1.18     | 3.66    | 0.87    | 5.06     | 80.42 |
| <i>Padina pavonica</i> (not<br>calcified) | 1.12     | 0.00     | 3.26    | 0.44    | 4.51     | 84.92 |
| <i>Halopteris scoparia</i>                | 0.00     | 0.97     | 2.98    | 0.43    | 4.12     | 89.04 |
| <i>Falkenbergia</i> sp.                   | 0.46     | 0.00     | 1.32    | 0.51    | 1.82     | 90.86 |

**Groups 200 E & REF B; Average dissimilarity = 53.38**

| Taxon                                     | 200 E    | REF B    | Av.Diss | Diss/SD | Contrib% | Cum.% |
|---|----------|----------|---------|---------|----------|-------|
|   | Av.Abund | Av.Abund |         |         |          |       |
| <i>Cystoseira corniculata</i>             | 6.76     | 4.64     | 12.03   | 1.18    | 22.55    | 22.55 |
| CCA                                       | 2.86     | 2.44     | 7.52    | 1.06    | 14.08    | 36.62 |
| <i>Sargassum</i> sp.                      | 0.00     | 1.86     | 5.37    | 0.69    | 10.06    | 46.69 |
| <i>Jania rubens</i>                       | 2.63     | 2.09     | 4.08    | 0.76    | 7.64     | 54.33 |
| Bare substratum                           | 0.00     | 1.46     | 4.00    | 0.70    | 7.49     | 61.81 |
| <i>Sargassum vulgare</i>                  | 0.85     | 0.60     | 3.96    | 0.68    | 7.42     | 69.23 |
| <i>Dictyota</i> sp.                       | 1.59     | 1.61     | 3.96    | 0.79    | 7.41     | 76.64 |
| <i>Padina pavonica</i><br>(calcified)     | 0.00     | 1.18     | 3.50    | 0.87    | 6.56     | 83.20 |
| <i>Halopteris scoparia</i>                | 0.15     | 0.97     | 3.12    | 0.49    | 5.84     | 89.04 |
| <i>Padina pavonica</i> (not<br>calcified) | 0.27     | 0.00     | 0.83    | 0.30    | 1.55     | 90.59 |

**Groups 200 W & REF B; Average dissimilarity = 57.06**

| Taxon                                 | 200 W    | REF B    |         | Diss/SD | Contrib% | Cum.% |
|---------------------------------------|----------|----------|---------|---------|----------|-------|
|                                       | Av.Abund | Av.Abund | Av.Diss |         |          |       |
| <i>Cystoseira corniculata</i>         | 6.52     | 4.64     | 11.48   | 1.23    | 20.13    | 20.13 |
| CCA                                   | 1.57     | 2.44     | 6.89    | 1.09    | 12.07    | 32.20 |
| <i>Cladostephus spongiosus</i>        | 1.95     | 0.00     | 5.55    | 0.82    | 9.73     | 41.93 |
| <i>Sargassum</i> sp.                  | 0.55     | 1.86     | 5.48    | 0.80    | 9.60     | 51.54 |
| Bare substratum                       | 0.64     | 1.46     | 4.21    | 0.78    | 7.38     | 58.92 |
| <i>Dictyota</i> sp.                   | 1.56     | 1.61     | 4.17    | 0.76    | 7.32     | 66.24 |
| <i>Jania rubens</i>                   | 1.90     | 2.09     | 4.04    | 0.75    | 7.09     | 73.32 |
| <i>Halopteris scoparia</i>            | 0.42     | 0.97     | 3.83    | 0.54    | 6.71     | 80.03 |
| <i>Padina pavonica</i><br>(calcified) | 0.00     | 1.18     | 3.49    | 0.87    | 6.12     | 86.15 |
| <i>Sargassum vulgare</i>              | 0.59     | 0.60     | 3.22    | 0.52    | 5.64     | 91.79 |

**Groups REF A & REF B; Average dissimilarity = 54.82**

| Taxon                                 | REF A    | REF B    |         | Diss/SD | Contrib% | Cum.% |
|---------------------------------------|----------|----------|---------|---------|----------|-------|
|                                       | Av.Abund | Av.Abund | Av.Diss |         |          |       |
| <i>Cystoseira corniculata</i>         | 5.01     | 4.64     | 10.88   | 1.19    | 19.84    | 19.84 |
| <i>Jania rubens</i>                   | 3.77     | 2.09     | 6.05    | 0.89    | 11.03    | 30.87 |
| Bare substratum                       | 1.71     | 1.46     | 6.03    | 1.13    | 10.99    | 41.86 |
| <i>Sargassum</i> sp.                  | 0.00     | 1.86     | 5.24    | 0.68    | 9.55     | 51.41 |
| <i>Dictyota</i> sp.                   | 1.98     | 1.61     | 4.95    | 0.75    | 9.03     | 60.44 |
| CCA                                   | 1.83     | 2.44     | 4.91    | 1.23    | 8.95     | 69.39 |
| <i>Halopteris scoparia</i>            | 0.66     | 0.97     | 4.17    | 0.61    | 7.60     | 76.99 |
| <i>Padina pavonica</i><br>(calcified) | 0.75     | 1.18     | 3.78    | 0.93    | 6.89     | 83.88 |
| <i>Cystoseira amentacea</i>           | 0.93     | 0.00     | 2.65    | 0.53    | 4.84     | 88.71 |
| <i>Sargassum vulgare</i>              | 0.34     | 0.60     | 2.58    | 0.40    | 4.70     | 93.42 |

Taxa driving community differences among sites (Table 3.4) were grouped into two categories; canopy-forming algae (*Cystoseira corniculata*, *Cystoseira amentacea* (C.Agardh) Bory de Saint-Vincent, *Sargassum vulgare* and *Cladostephus spongiosus* (Hudson) C.Agardh) and calcifying algae (CCA, *Jania rubens*, *Corallina* sp., *Amphiroa* sp. and *Padina pavonica*). The two categories are shown for May (Figure 3.5A) and September (Figure 3.5B). As no significant differences were found within intermediate and reference sites,  $p\text{CO}_2$  levels were pooled for clarity. Both categories showed very strong seasonal patterns: no differences in canopy-forming algal cover were detected

in May, but in September the high  $p\text{CO}_2$  site had higher canopy cover than the reference sites. Likewise, calcifying algae showed no significant difference among  $p\text{CO}_2$  levels in spring, but in autumn the high  $p\text{CO}_2$  site had a significantly lower cover of calcareous algae compared to intermediate and control  $p\text{CO}_2$  levels.

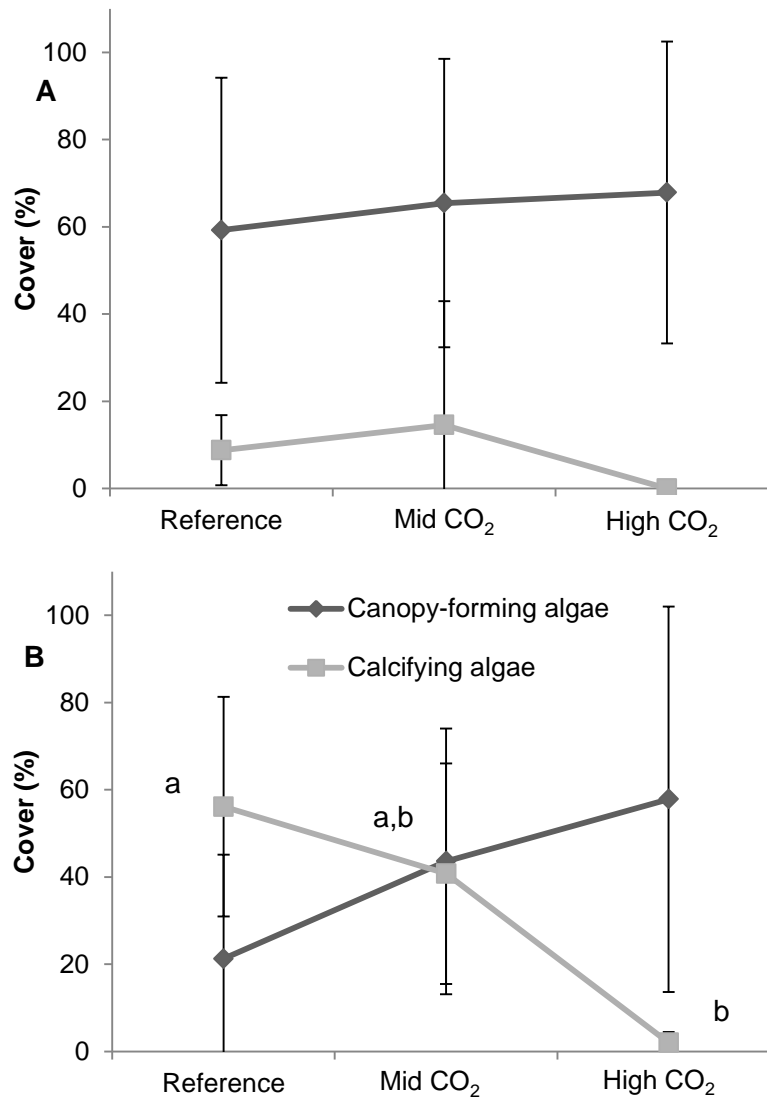


Figure 3.5. Mean percentage cover ( $\pm$  SD) of canopy-forming algae (black) and calcifying algae (grey) in May (a) and September (b) at high ( $n=6$ ), intermediate ( $n=14$ ) and reference ( $n=14$ ) CO<sub>2</sub> conditions off Methana. Different letters indicate significant differences between groups.



The species forming these two categories changed along the  $p\text{CO}_2$  gradient depending on the season, and the main canopy-forming and calcareous species covers are shown for May and September in Figure 3.7A and 3.7B, respectively. As no significant differences were found within intermediate and reference sites,  $p\text{CO}_2$  levels were pooled for clarity. In spring, *S. vulgare* was more abundant at the high  $p\text{CO}_2$  site, but it was almost absent from all sites in autumn. In contrast, *C. corniculata* cover significantly increased in the high  $p\text{CO}_2$  site from spring to autumn, while the opposite was true for the intermediate and reference sites, where *C. corniculata* cover decreased from spring to autumn. As for the coralline algae, CCAs recruited earlier than *J. rubens* and reached their maximum cover in spring at the intermediate sites, while in the reference sites their cover increased from spring to autumn. The articulate coralline alga *J. rubens* had extremely low abundances at all sites in spring, while in autumn its percent cover decreased with increasing  $p\text{CO}_2$  levels (Figure 3.6).

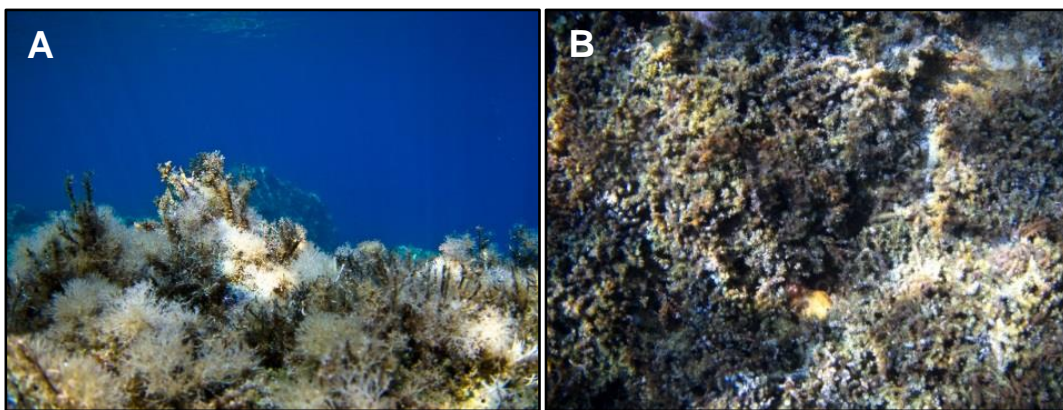


Figure 3.6. Typical appearance of macroalgal communities off Methana in autumn at (A) reference sites, with high cover of the articulated coralline alga *J. rubens*, and (B) near the  $\text{CO}_2$  seeps, where *C. corniculata* is dominant (photos by Maria Salomidi).

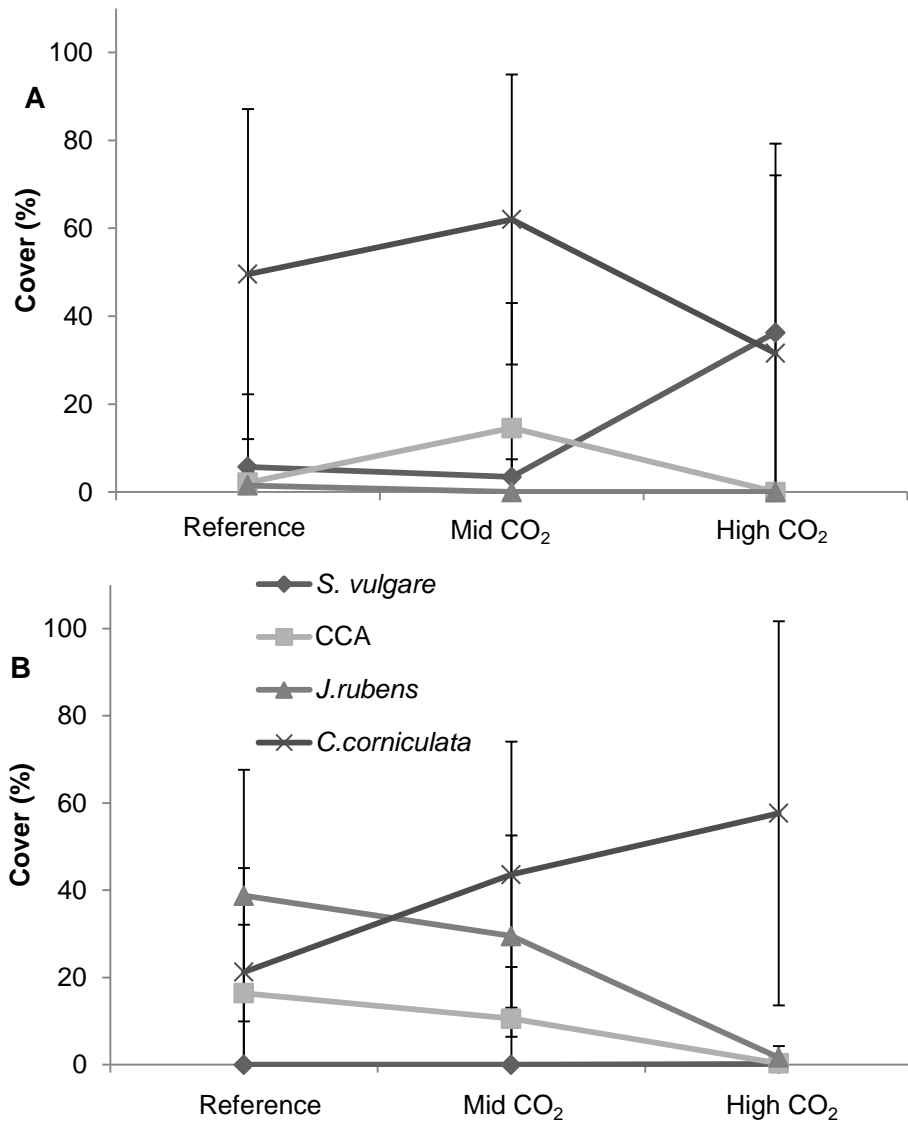


Figure 3.7. Mean percentage cover ( $\pm$  SD) of dominant macroalgal species in May (A) and September (B) at high ( $n=6$ ), intermediate ( $n=14$ ) and reference ( $n=14$ ) levels of CO<sub>2</sub> in Methana. Different letters and numbers indicate significant differences between groups.

### 3.3.2 Vulcano macroalgal communities

At Vulcano, 32 macroalgal taxa were recorded, five of which were calcifying algae. Results from PERMANOVA analysis on square-root transformed data (Table 3.5a) show that macroalgal communities were significantly different among sites (pseudo- $F_{2,9}=2.702$ ,  $p(\text{perm})=0.0005$ ). Pair-wise comparisons (Table 3.5b) show that there was a significant difference between the

communities at the High CO<sub>2</sub> and reference sites ( $t_{2,6}=1.793$ ,  $p(\text{MC})=0.045$ ), whereas the Mid CO<sub>2</sub> site was intermediate between the other two.

Table 3.5. (A) PERMANOVA analysis on square-root transformed biomass of Vulcano benthic communities. The Table shows degrees of freedom (df), sum of squares (SS), pseudo-F, permutational p and unique permutations for the factor “pCO<sub>2</sub> level”. (B) Since “Site” had a significant effect ( $p<0.05$ ), pair-wise comparisons between CO<sub>2</sub> levels were carried out and are shown in the lower part of the table. Since the number of possible permutations was low (<100), Monte Carlo p ( $p(\text{MC})$ ) was used as the most reliable p value, and shows that the t-values of High CO<sub>2</sub> and reference site were significantly different.

| (A) | Source   | df | SS     | MS     | Pseudo-F | p (perm)     | Unique perms |
|-----|----------|----|--------|--------|----------|--------------|--------------|
|     | Site     | 2  | 6950.7 | 3475.4 | 2.7017   | <b>0.005</b> | 4732         |
|     | Residual | 9  | 11577  | 1286.4 |          |              |              |
|     | Total    | 11 | 18528  |        |          |              |              |

| (B) | Groups                                     | t      | p (perm)      | Unique perms | p (MC)        |
|-----|--|--------|---------------|--------------|---------------|
|     | High CO <sub>2</sub> , Mid CO <sub>2</sub> | 1.5833 | 0.0277        | 35           | 0.0692        |
|     | High CO <sub>2</sub> , REF A               | 1.7925 | <b>0.0291</b> | 35           | <b>0.0450</b> |
|     | Mid CO <sub>2</sub> , REF A                | 1.5589 | 0.0259        | 35           | 0.0912        |

Shannon diversity (H') and Pielou's evenness (J') were not significantly different among sites (Table 3.6), and Figure 3.8 shows that no specific trend was detectable.

Table 3.6. ANOVA results for (A) Shannon diversity ( $H'$ ) and (B) Pielou's evenness ( $J'$ ) of Vulcano macroalgal communities. The Tables show main factors and their interactions and sum of squares (SS), degrees of freedom (df), Mean Squares (MS), F-ratios (F) and p values.

| (A) | Source | Type III SS | df | MS    | F     | p     |
|-----|--------|-------------|----|-------|-------|-------|
|     | Site   | 0.657       | 2  | 0.328 | 0.911 | 0.436 |
|     | Error  | 3.245       | 9  | 0.361 |       |       |
|     | Total  | 3.902       | 11 |       |       |       |
| (B) | Source | Type III SS | df | MS    | F     | p     |
|     | Site   | 0.087       | 2  | 0.043 | 0.963 | 0.418 |
|     | Error  | 0.405       | 9  | 0.045 |       |       |
|     | Total  | 0.491       | 11 |       |       |       |

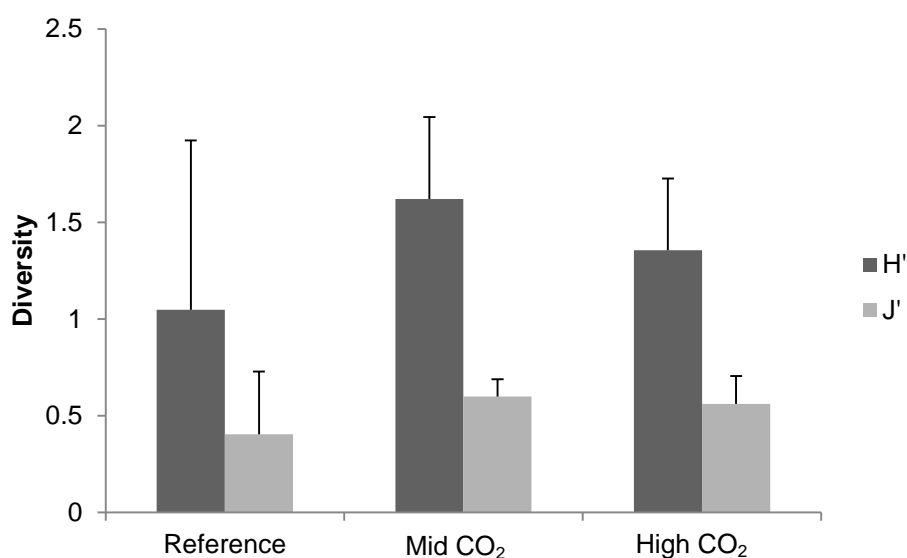


Figure 3.8. Mean ( $\pm$  SD,  $n = 4$ ) Shannon diversity ( $H'$ ) and Pielou's evenness ( $J'$ ) at high, intermediate and reference  $pCO_2$  at Vulcano.

SIMPER analysis shows which taxa contribute most to the detected differences among sites (Table 3.7). Dissimilarity levels are highest between the high  $CO_2$  and reference sites (60.20), followed by the dissimilarity between reference and intermediate sites (59.77); intermediate and high  $pCO_2$  sites were the most similar groups (average dissimilarity = 58.03). These results are consistent with

the pair-wise comparison of macroalgal communities (see Table 3.5b). The main drivers of differences between groups were canopy-forming algae such as *Cystoseira* sp., *Sargassum vulgare* and *Dictyopteris polypodioides*, calcifying algae such as crustose coralline algae (CCA) and the calcified brown alga *Padina pavonica*, green algae such as *Flabellia petiolata* (Turra) Nizamuddin, *Caulerpa prolifera* (Forsskål) J.V.Lamouroux and *Caulerpa racemosa* (Forsskål) J.Agardh and turf algae.

Table 3.7. SIMPER analysis of Vulcano benthic communities showing average dissimilarities between each pair of pCO<sub>2</sub> levels and which species contributed to the dissimilarity by up to 90%. For each taxon, the average abundance at the two groups that are being compared, their average dissimilarity, the dissimilarity to standard deviation ratio and the taxon contribution and cumulative contribution are shown.

| <b>Groups High CO<sub>2</sub> &amp; Mid CO<sub>2</sub>; Average dissimilarity = 58.03</b> |                            |                           |                |                |               |             |
|---|----------------------------|---------------------------|----------------|----------------|---------------|-------------|
|   | <b>High CO<sub>2</sub></b> | <b>Mid CO<sub>2</sub></b> |                |                |               |             |
| <b>Taxa</b>   | <b>Av.Ab</b>               | <b>Av.Ab</b>              | <b>Av.Diss</b> | <b>Diss/SD</b> | <b>Contr%</b> | <b>Cum%</b> |
| <i>Cystoseira</i> sp.   | 4.39                       | 1.91                      | 12.11          | 1.57           | 20.87         | 20.87       |
| <i>Flabellia petiolata</i>  | 2.45                       | 0.61                      | 7.62           | 1.74           | 13.13         | 34.00       |
| <i>Sargassum</i> sp.  | 1.29                       | 0.00                      | 5.03           | 0.95           | 8.66          | 42.66       |
| <i>Caulerpa prolifera</i>   | 1.40                       | 0.40                      | 4.86           | 1.37           | 8.37          | 51.04       |
| CCA   | 0.38                       | 1.05                      | 4.01           | 1.17           | 6.91          | 57.94       |
| <i>Peyssonnelia</i> sp.   | 0.06                       | 0.74                      | 3.08           | 0.70           | 5.31          | 63.25       |
| Turf algae  | 2.05                       | 1.61                      | 2.96           | 1.70           | 5.10          | 68.36       |
| <i>Cystoseira</i> with<br><i>Peyssonnelia</i><br>epiphyte                                 | 0.00                       | 0.53                      | 2.27           | 0.54           | 3.92          | 72.28       |
| <i>Dictyopteris</i><br><i>polypodioides</i>   | 0.00                       | 0.55                      | 2.21           | 1.51           | 3.81          | 76.09       |
| <i>Dictyota</i> sp.   | 0.80                       | 0.87                      | 2.01           | 1.32           | 3.47          | 79.56       |
| <i>Caulerpa racemosa</i>  | 0.07                       | 0.49                      | 1.86           | 1.14           | 3.20          | 82.76       |
| <i>Taonia atomaria</i>  | 0.34                       | 0.15                      | 1.75           | 0.86           | 3.02          | 85.78       |
| <i>Padina pavonica</i>  | 0.00                       | 0.29                      | 1.25           | 2.72           | 2.16          | 87.94       |
| <i>Halopteris scoparia</i>  | 0.00                       | 0.31                      | 1.25           | 0.54           | 2.15          | 90.08       |
| <b>Groups High CO<sub>2</sub> &amp; REF A; Average dissimilarity = 60.20</b>              |                            |                           |                |                |               |             |
|   | <b>High CO<sub>2</sub></b> | <b>REF A</b>              |                |                |               |             |
| <b>Taxa</b>   | <b>Av.Ab</b>               | <b>Av.Ab</b>              | <b>Av.Diss</b> | <b>Diss/SD</b> | <b>Contr%</b> | <b>Cum%</b> |
| <i>Cystoseira</i> sp.   | 4.39                       | 4.77                      | 12.64          | 1.08           | 21.00         | 21.00       |
| <i>Flabellia petiolata</i>  | 2.45                       | 0.37                      | 7.77           | 2.03           | 12.9          | 33.91       |

|   |      |      |      |      |      |       |
|---|------|------|------|------|------|-------|
| <i>Caulerpa prolifera</i>                           | 1.40 | 0.00 | 5.97 | 2.24 | 9.92 | 43.83 |
| Turf algae  | 2.05 | 0.80 | 5.04 | 1.54 | 8.37 | 52.20 |
| <i>Sargassum</i> sp.                                | 1.29 | 0.41 | 4.91 | 1.09 | 8.16 | 60.35 |
| <i>Dictyopteris polypodioides</i>                   | 0.00 | 1.17 | 4.50 | 0.92 | 7.47 | 67.82 |
| <i>Halopteris scoparia</i>                          | 0.00 | 0.73 | 2.80 | 0.90 | 4.66 | 72.48 |
| CCA   | 0.38 | 0.75 | 2.77 | 1.12 | 4.60 | 77.08 |
| Articulated coralline                               | 0.00 | 0.56 | 2.32 | 2.04 | 3.85 | 80.93 |
| <i>Taonia atomaria</i>                              | 0.34 | 0.25 | 1.82 | 0.97 | 3.02 | 83.95 |
| <i>Dictyota</i> sp.                                 | 0.80 | 0.78 | 1.80 | 1.22 | 2.99 | 86.94 |
| <i>Dictyota fasciola</i>                            | 0.00 | 0.36 | 1.33 | 0.75 | 2.21 | 89.15 |
| <i>Cystoseira</i> with <i>Peyssonnelia</i> epiphyte | 0.00 | 0.28 | 1.03 | 0.54 | 1.72 | 90.86 |

Groups Mid CO<sub>2</sub> & REF A; Average dissimilarity = 59.77

| Taxa  | Mid CO <sub>2</sub> | REF A | Av.Diss | Diss/SD | Contr% | Cum%  |
|---|---------------------|-------|---------|---------|--------|-------|
|   | Av.Ab               | Av.Ab |         |         |        |       |
| <i>Cystoseira</i> sp.                               | 1.91                | 4.77  | 13.93   | 1.19    | 23.31  | 23.31 |
| <i>Dictyopteris polypodioides</i>                   | 0.55                | 1.17  | 5.19    | 1.44    | 8.68   | 31.99 |
| Turf algae  | 1.61                | 0.80  | 4.07    | 1.26    | 6.82   | 38.80 |
| CCA   | 1.05                | 0.75  | 3.77    | 1.28    | 6.31   | 45.11 |
| <i>Halopteris scoparia</i>                          | 0.31                | 0.73  | 3.27    | 1.00    | 5.47   | 50.58 |
| <i>Peyssonnelia</i> sp.                             | 0.74                | 0.00  | 3.24    | 0.70    | 5.43   | 56.01 |
| <i>Cystoseira</i> with <i>Peyssonnelia</i> epiphyte | 0.53                | 0.28  | 3.00    | 0.74    | 5.02   | 61.03 |
| <i>Dictyota</i> sp.                                 | 0.87                | 0.78  | 2.72    | 1.23    | 4.55   | 65.58 |
| <i>Flabellia petiolata</i>                          | 0.61                | 0.37  | 2.67    | 1.25    | 4.47   | 70.05 |
| Articulated coralline                               | 0.00                | 0.56  | 2.62    | 2.28    | 4.38   | 74.42 |
| <i>Caulerpa racemosa</i>                            | 0.49                | 0.00  | 2.03    | 1.04    | 3.40   | 77.83 |
| <i>Sargassum</i> sp.                                | 0.00                | 0.41  | 1.73    | 0.59    | 2.89   | 80.72 |
| <i>Caulerpa prolifera</i>                           | 0.4                 | 0.00  | 1.70    | 0.86    | 2.84   | 83.56 |
| <i>Dictyota fasciola</i>                            | 0.00                | 0.36  | 1.49    | 0.76    | 2.49   | 86.05 |
| <i>Padina pavonica</i>                              | 0.29                | 0.02  | 1.25    | 3.17    | 2.09   | 88.14 |
| <i>Taonia atomaria</i>                              | 0.15                | 0.25  | 1.15    | 1.27    | 1.93   | 90.07 |

Taxa driving differences among sites were grouped in four categories, canopy-forming algae, calcifying algae, non-calcifying green algae and turf algae. The canopy-forming algae category consisted of the sum of the biomass of *Cystoseira* sp., *Dictyopteris polypodioides* and *Sargassum vulgare*; the calcifying algae group included CCA, articulate coralline algae, *Padina*

*pavonica*, *Peyssonnelia* sp. and *Acetabularia acetabulum* P.C.Silva. Non-calcifying green algae consisted of *Flabellia petiolata*, *Caulerpa prolifera* and *Caulerpa racemosa*, while turf algae were already grouped in one category. Biomass of these categories is shown below (Figure 3.9), and the pattern is consistent with that of Methana in spring. Canopy-forming algae did not show a clear pattern and calcifying algae had the highest biomass at the intermediate site, although this difference was not significant (see Figure 3.5a). Non-calcifying green algae had a significant increase as pCO<sub>2</sub> increased, and turf algae biomass was significantly higher in the high pCO<sub>2</sub> site than in the control, with the Mid CO<sub>2</sub> site having intermediate values between the other two sites.

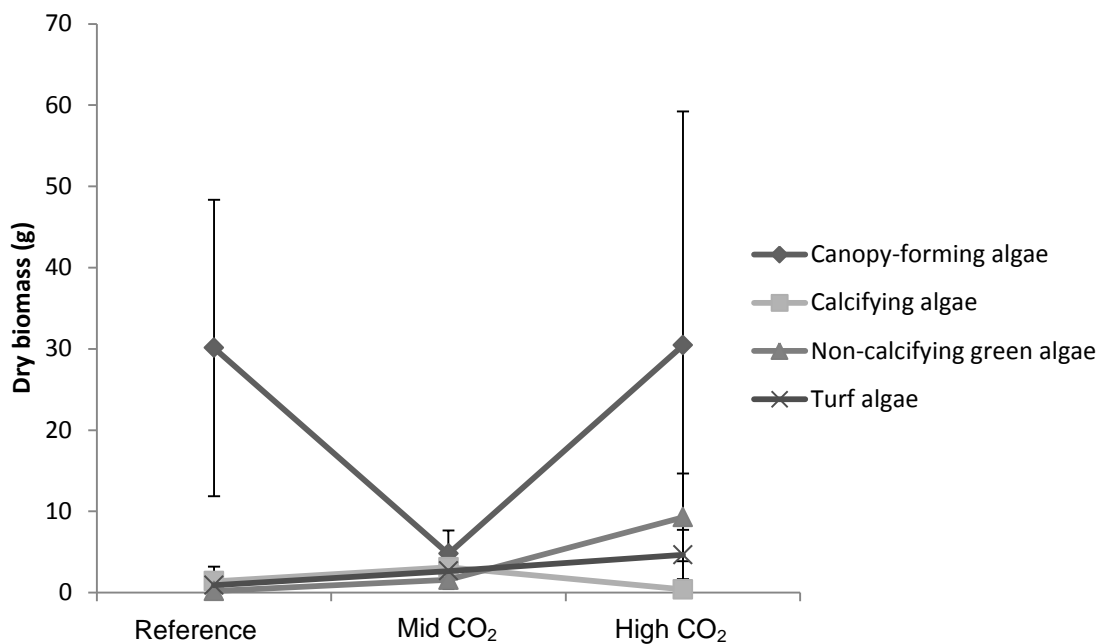


Figure 3.9. Mean ( $\pm$  SD, n=4) biomass of canopy-forming algae, calcifying algae, non-calcifying green algae and turf algae at sites with high, mid and reference pCO<sub>2</sub> at Vulcano.

The most representative canopy-forming and non-calcifying green algae biomass is shown below (Figure 3.10). The two canopy-forming algae *S. vulgare* and *D. polypodioides* showed opposite trends, with the first substituting the latter as pCO<sub>2</sub> increased. On the other hand, the two most abundant non-

calcifying green algae (*F. petiolata* and *C. prolifera*) had a significant increase in biomass at the high CO<sub>2</sub> site compared to the reference site.

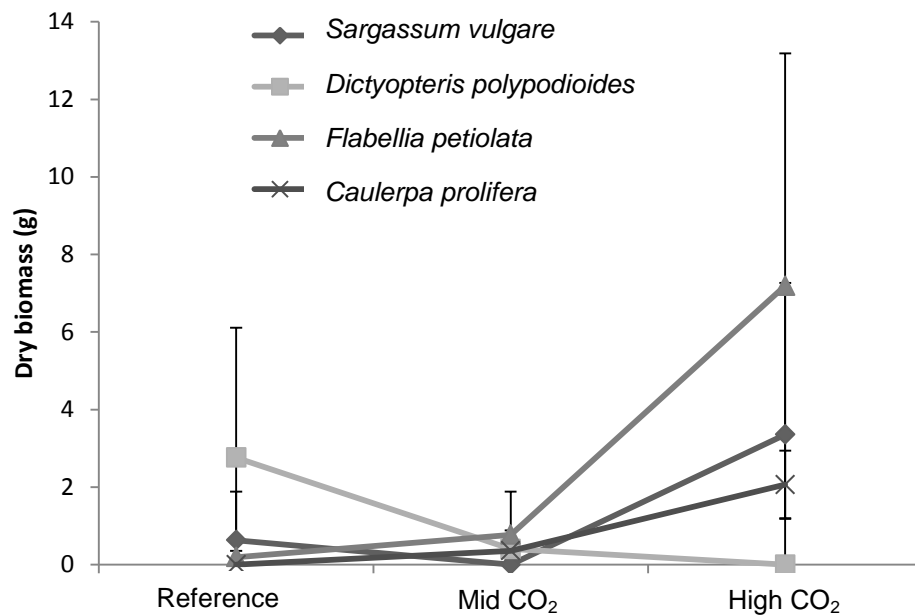


Figure 3.10. Mean ( $\pm$  SD, n=4) biomass of *Sargassum vulgare*, *Dictyopteris polypodioides*, *Flabellia petiolata* and *Caulerpa prolifera* at sites with high, mid and reference pCO<sub>2</sub> at Vulcano.

### 3.4 Discussion

These results show that increased seawater pCO<sub>2</sub> can have profound effects on macroalgal communities in oligotrophic conditions and that sampling season strongly affects the response of benthic communities to ocean acidification. At Methana, coralline algal cover decreased while canopy-forming algae became more abundant as pCO<sub>2</sub> increased, but the difference was only statistically significant in autumn. Macroalgal communities off Methana had year-round decreased diversity, especially of calcifying species, as carbon dioxide increased, in line with results from surveys at other CO<sub>2</sub> seeps (Porzio *et al.*, 2011; Fabricius *et al.*, 2011,2014) and from laboratory experiments (Hale *et al.*, 2011; Kroeker *et al.*, 2013a).



The seasonal effects of ocean acidification on macroalgal communities have not been detected until now since most field studies have been carried out in one season, while laboratory and mesocosm experiments rarely last long enough to incorporate seasonality effects. Godbold and Solan (2013) found that seasonality greatly affected invertebrate responses to both ocean acidification and increased temperature. A recent study showed that in tropical seagrass epiphyte communities fleshy algae substituted coralline algae as CO<sub>2</sub> increased, and that this pattern was more pronounced in winter (Campbell and Fourqurean, 2014). One of the very few long-term studies of algal physiological responses to ocean acidification showed that coralline algae respond differently to increased pCO<sub>2</sub> depending on season: net calcification was negatively affected by the interaction of increased pCO<sub>2</sub> and temperature in summer, but not in the other seasons (Martin *et al.*, 2013).

At Vulcano, where samples were only collected in spring, results are consistent with Methana; calcifying algal biomass decreased with increasing pCO<sub>2</sub> whereas biomass of canopy-forming algae did not show any specific trend, although the dominant canopy-forming species shifted with increasing CO<sub>2</sub>. Here, more taxa were detected because of the different method used, revealing that less conspicuous species such as non-calcifying green algae (e.g. *Flabellia petiolata*) and turf algae increased with increasing seawater pCO<sub>2</sub>.

Non-calcifying green algal biomass increased at elevated pCO<sub>2</sub>; this trend was particularly evident for *Flabellia petiolata* and *Caulerpa prolifera*. Increased *F. petiolata* abundance at intermediate pCO<sub>2</sub> levels was also reported from Ischia, where this species becomes dominant when pH drops to about 7.8 (Porzio *et al.*, 2011); this is consistent with findings from Vulcano, where average pH at the high CO<sub>2</sub> site is about 7.6 (Boatta *et al.*, 2013). The reason why *F. petiolata*

benefits from increased CO<sub>2</sub> is not currently known, but previous studies have shown that this species is unlikely to use carbon concentrating mechanisms (CCMs; Raven *et al.*, 2002; Mercado *et al.*, 2009). It is therefore possible that increased CO<sub>2</sub> levels give this species a competitive advantage as they would have more substratum available for photosynthesis (Cornwall *et al.*, 2012; Koch *et al.*, 2013).

The increase in fleshy algae with increasing pCO<sub>2</sub> recorded in Vulcano is consistent with previous results from volcanic seeps (Porzio *et al.*, 2011; Graziano *et al.*, unpublished data). Shifts towards fleshy macroalgae in response to ocean acidification have also been reported in laboratory and mesocosm studies, where small fleshy algae increase their biomass and percent cover by outcompeting coralline crusts as CO<sub>2</sub> goes up (Connell and Russell, 2010). Since these algae can have a negative effect on kelp recruitment, ocean acidification has the potential to cause dramatic phase shifts in temperate habitats (Connell and Russell 2010), although nutrient and light levels will determine their significance (Russell *et al.*, 2009; Russell *et al.*, 2011). In contrast, at Methana turf-forming algae cover did not increase as CO<sub>2</sub> increased. Another shallow subtidal survey off Italian CO<sub>2</sub> seeps (Porzio *et al.*, 2011) detected a decrease in turf-forming algal biomass at pCO<sub>2</sub> levels of about 1000 ppm. This shows that shifts to turf-forming algae do not necessarily happen at intermediate pCO<sub>2</sub> levels, especially if not associated with increased nutrient levels (Connell and Russell, 2010) or other disturbances disrupting fucoid algal cover (Falkenberg *et al.*, 2012).

Decreased abundance of calcifying algae at high CO<sub>2</sub> sites off Vulcano and Methana is consistent with previous results from volcanic seeps off Ischia, in Italy (Porzio *et al.*, 2011). Here, the articulated coralline *Jania rubens* was one

of the dominant species at reference CO<sub>2</sub> levels, but it was absent at elevated CO<sub>2</sub>. At Methana, this species was not completely absent from the high CO<sub>2</sub> site, possibly because here the average pH was higher than in Ischia (see Chapter 2). Cover of crustose coralline algae (CCA) decreased with increasing pCO<sub>2</sub> as well both in Italy and in Greece, confirming that calcifying algae are likely to be threatened by ocean acidification (Gao *et al.*, 1993; Anthony *et al.*, 2008; Kuffner *et al.*, 2008; Martin *et al.*, 2008; Ries *et al.*, 2009; Martin and Gattuso, 2009; Gao and Zheng, 2010), especially those species living near their thermal limit (Koch *et al.*, 2013). CCA producing Mg-calcite appear to be extremely sensitive to ocean acidification, whereas those species containing dolomite-rich calcium carbonate seem more resistant to dissolution in high CO<sub>2</sub> conditions (Nash *et al.*, 2012). Intermediate pCO<sub>2</sub> levels seem to increase CCA abundance in spring both off Vulcano and Methana, possibly because carbon fertilisation could enhance calcification. This pattern has been observed in some laboratory studies (Ries *et al.*, 2009; Hofmann *et al.*, 2012) when pCO<sub>2</sub> is below 1000 µatm. However, calcifying algae appear unable to cope with the high energetic demands of calcification when pCO<sub>2</sub> reaches levels above 1000 µatm (Bradassi *et al.*, 2013). Recent studies found that CCA are more sensitive to rates, not magnitude, of ocean acidification (Kamenos *et al.*, 2013) and that fluctuating pH reduces growth in an articulated coralline alga (Cornwall *et al.*, 2013): high variability in pCO<sub>2</sub> at the seeps could therefore lead to an over-estimation of its negative effects on coralline algae.

The increase in canopy-forming algal cover at high CO<sub>2</sub> was mostly caused by an increased abundance of *Sargassum vulgare* in spring along both pCO<sub>2</sub> gradients and of *Cystoseira corniculata* in autumn at Methana. *Sargassum vulgare* was more abundant at high CO<sub>2</sub> also at volcanic seeps off Ischia

(Porzio *et al.*, 2011). However, this species was absent at Methana in autumn because of its pronounced seasonal cycle (Belegratis *et al.*, 1999). As for *C. corniculata*, it is likely that the higher autumnal cover at the elevated pCO<sub>2</sub> site was due to the absence of *S. vulgare* and *J. rubens*. In fact, the genus *Sargassum* can be advantaged over *Cystoseira* when competing for space (Engelen *et al.*, 2008), while *J. rubens* is an epiphyte that can overgrow canopy-forming algae and become dominant in autumn (Belegratis *et al.*, 1999). Physiological responses of *J. rubens* to high pCO<sub>2</sub> are likely to be the main determinant of its decrease in cover, but enhanced defensive compound production by *C. corniculata* cannot be excluded. It has in fact been shown that some fucoid algae are carbon limited, and elevated CO<sub>2</sub> can cause a sharp increase in their defensive compound contents (Swanson and Fox, 2007).

At Vulcano, *Dictyopteris polypodioides* biomass sharply decreased as pCO<sub>2</sub> increased. This is surprising, as *Dictyota* sp. did not seem to be affected by ocean acidification at Vulcano and actually increase its biomass with increasing CO<sub>2</sub> at Ischia (Porzio *et al.*, 2011). This pattern could be either explained by differences in these species' physiologies or by their different palatability to herbivores, as abundances of their main consumer, the sea urchin *Paracentrotus lividus*, decrease with increasing pCO<sub>2</sub> at Vulcano (Johnson *et al.*, 2012; Calosi *et al.*, 2013a). Since no studies on this species' physiological responses to ocean acidification have been conducted so far, there is no information available on the underlying physiological mechanisms. This is a further proof that biological response to ocean acidification can greatly vary even within families (Miller *et al.*, 2009; Kroeker *et al.*, 2011), and that some non-calcifying species can be as sensitive to increased pCO<sub>2</sub> as calcifiers.

Overall, this study shows that phase shifts in benthic communities as seawater pCO<sub>2</sub> increases are likely to be consistent between Western and Eastern Mediterranean Sea and between intertidal and shallow subtidal (2-3 metres depth) habitats. Loss of diversity and reduced abundance of ecologically important calcifying algae at elevated carbon dioxide levels found in this study add to a growing body of evidence showing that ocean acidification is likely to alter community composition (Hall-Spencer *et al.*, 2008; Fabricius *et al.*, 2011; Kroeker *et al.*, 2011; Porzio *et al.*, 2011; Hofmann *et al.*, 2012; Brodie *et al.*, 2014). Changes in benthic community structure have potential profound effects on biological processes such as food web dynamics, nutrient cycling and primary productivity (Tilman, 1999), thus affecting ecosystem functioning.

## **Chapter 4**

# **Canopy algal epifauna changes at elevated pCO<sub>2</sub> at two Mediterranean volcanic seeps**

## Abstract

Only a few studies have dealt with epifaunal community responses to ocean acidification, and they have not reported consistent results. As for canopy-forming algal epifauna, there is virtually no information on their responses to elevated pCO<sub>2</sub>. This chapter investigates how epifauna of the main canopy-forming macroalgae at volcanic seeps in Italy and Greece changed with increasing pCO<sub>2</sub>. Rocky shores at both sites were dominated by furoid algae; at Vulcano (Italy) there was a change from *Dictyopteris polypodioides* to *Sargassum vulgare* with increasing pCO<sub>2</sub>, while the genus *Cystoseira* remained abundant at all sites, but there was a shift in species. In contrast, *Cystoseira corniculata* was the main canopy-forming alga at volcanic seeps off Methana (Greece) and at nearby reference sites. Canopy-forming algal samples were collected at Methana (*C. corniculata*) and Vulcano (*Cystoseira* spp. and *S. vulgare*) to examine their epifaunal communities. The hypotheses tested were: (i) abundance and diversity of calcifiers will decrease as CO<sub>2</sub> increases; (ii) the magnitude of change in epifaunal communities will differ depending on the macroalgal species they inhabit. At both sites fauna was collected in spring, from 20 x 20 cm quadrats off Methana and by collecting individual thalli of furoid algae off Vulcano. Although macroalgal morphology and mobile epifauna changed significantly with increasing pCO<sub>2</sub>, sessile epiphyte communities did not show consistent changes among pCO<sub>2</sub> levels. The lack of a clear CO<sub>2</sub> effect on epiphytes could be due to the ability of canopy-forming algae to locally raise pH due to photosynthesis; epifauna may still be affected by changes in pCO<sub>2</sub> because it is more mobile than epiphytes and therefore often leaves the macroalgal boundary layer. The abundance of calcifying organisms was strongly affected by increasing pCO<sub>2</sub>, whereas non-calcified taxa such as many

polychaetes were more abundant at high CO<sub>2</sub>, probably because of reduced competition for space and resources. However, at Vulcano the structure of epifaunal communities inhabiting *S. vulgare* did not change significantly with pCO<sub>2</sub>, unlike epifaunal communities living on *Cystoseira* spp. at Vulcano and *C. corniculata* at Methana. Thus, canopy-forming macroalgae and their associated communities are expected to change as seawater carbon dioxide levels increase, but the magnitude of change is expected to differ depending on the macroalgal host.

#### **4.1 Introduction**

Although research on biological responses to ocean acidification is in its infancy, there is evidence that an increase in seawater pCO<sub>2</sub> often has strong negative effects on calcifying organisms (Kroeker *et al.*, 2013a). Evidence from volcanic CO<sub>2</sub> seeps used as ocean acidification analogues supports this conclusion (e.g. Hall-Spencer *et al.*, 2008; Cigliano *et al.*, 2010; Fabricius *et al.*, 2011; Kroeker *et al.*, 2011; Inoue *et al.*, 2013), although abundant food supplies can help animals to cope with increased pCO<sub>2</sub>: for example, mussels and barnacles can remain dominant in eutrophic conditions despite being exposed to high CO<sub>2</sub> levels in Kiel fjord (Thomsen *et al.*, 2010). Community responses do not always reflect single species responses that would be predicted from laboratory-based physiological tests because of biological interactions. For instance, in a mesocosm study of communities from artificial substrata mimicking mat-forming algae Hale *et al.* (2011) reported an unexpected increase in nematode abundance at high CO<sub>2</sub> because of reduced competition for space with taxa sensitive to hypercapnia, such as molluscs. In a field study of turf-associated fauna along pCO<sub>2</sub> gradients in the Mediterranean Sea, Kroeker *et al.* (2011) found that small crustaceans such as amphipods and



tanaisids, not nematodes, increased in abundance at low pH. Crustaceans in the mesocosm study (Hale *et al.*, 2011) decreased with increasing pCO<sub>2</sub>. There are therefore great uncertainties in predicting the responses of benthic communities to ocean acidification, although both studies report a strong decrease in calcifying organisms' abundance and diversity at increased pCO<sub>2</sub> (Hale *et al.* 2011; Kroeker *et al.*, 2011).

Marine macroalgae are dominant on temperate rocky reefs worldwide (Steneck *et al.*, 2002) and are considered ecosystem engineers because they add structural complexity to the substratum. Invertebrate communities associated with macroalgae have higher species richness and diversity than unvegetated substrata (Dean and Connell, 1987). Epifaunal abundance and diversity can be influenced by hydrodynamics and sedimentation rate (Sánchez-Moyano *et al.*, 2000), and is usually correlated with the complexity of their macroalgal habitat. For instance, densely branched Mediterranean macroalgae host more diverse invertebrate communities because of reduced predation risk and hydrodynamism (Chemello and Milazzo, 2002). Differences in epifaunal communities can also be determined by other seaweed characteristics, such as the presence of defensive compounds (Hay *et al.*, 1987; Jormalainen *et al.*, 2001). Macroalgae and seagrasses raise pH near their fronds through photosynthesis; this process controls calcification rates of their coralline algal epiphytes (Semesi *et al.*, 2009) and has been proven to reduce the negative effects of ocean acidification on some macroalgae, especially if water movement is slow (Cornwall *et al.*, 2014). Invertebrates living on macroalgae might be exposed to smaller changes in ambient pCO<sub>2</sub> compared to animals living in the water column or on bare substrata, and community changes may therefore be less dramatic.

At volcanic seeps off Vulcano (Italy), dominant macroalgae change along a pCO<sub>2</sub> gradient: *Cystoseira* spp. and *Dictyopteris polypodioides* are abundant at reference sites, whereas *Sargassum vulgare* becomes extremely abundant at high pCO<sub>2</sub> (see Chapter 3). On the other hand, no such change was recorded at volcanic seeps off Methana (Greece), where *Cystoseira corniculata* remains the dominant canopy-forming species, even though *Sargassum vulgare* cover increases with pCO<sub>2</sub> (see Chapter 3). Macroalgal communities associated with *Sargassum muticum* have been proven to cope better with ocean acidification than those associated with *Cystoseira tamariscifolia* (Olabarria *et al.*, 2013), but responses of canopy-forming algae epifauna to ocean acidification have not been studied yet.

Based on previous evidence, I expect that canopy-forming algae will not be negatively affected as the oceans acidify (Kroeker *et al.*, 2013a). However, diversity of biological communities can still decrease as pCO<sub>2</sub> increases even if their habitat resists ocean acidification (Martin *et al.*, 2008; Fabricius *et al.*, 2014). Epifaunal communities are the main constituent of diets for seagrass-associated fish, and are therefore an important link to higher trophic level organisms, such as juvenile fish (Yamada *et al.*, 2010). The aim of this study was to assess changes in epifauna of the main canopy-forming algal species along two pCO<sub>2</sub> gradients at volcanic seeps off Vulcano (Italy) and Methana (Greece) given their importance for coastal ecosystem functioning and fisheries. The hypotheses tested are; (i) invertebrate diversity and abundance will decrease with increasing CO<sub>2</sub>, and (ii) the manner of these changes in diversity and abundance will depend on the host macroalgal species.

## 4.2 Methods

### 4.2.1 Methana

In May 2012, samples were collected by scraping 20 x 20 cm quadrats of *C. corniculata* (Figure 4.2A) growing on horizontal or sub-horizontal rock using a hammer and chisel while covering the area with a 200 µm mesh size nylon net to avoid loss of vagile fauna. Three samples were collected from each of the five sites described in Chapter 2 (SEEP, 200 W, 200 E, REF A and REF B); detailed sampling dates and sample sizes are reported in Table 1.1C. Samples were fixed in 4% buffered formaldehyde for approximately 48 h, transferred to 70% IMS (Industrial Methylated Spirit) and stored until analysis. Samples were then sorted, separating *C. corniculata* from its epiphytic algae, which were assigned to functional groups, and from its epifauna. *Cystoseira corniculata* and its epiphytes were then dried at 50°C for 72 h and weighed ( $\pm 1$  mg accuracy) to obtain dry mass. Over 29000 individual invertebrates were sorted under a stereoscope and identified to the lowest possible taxonomic level, hereafter termed the operational taxonomic unit (OTU). Amphipods were identified using keys from Bellan-Santini *et al.* (1982, 1989, 1993, 1998), molluscs were identified using the key from Doneddu and Trainito (2005) and taxonomic expertise by Prof. Renato Chemello (University of Palermo, Italy), and all other taxa were identified using the guide from Riedl (1991). The invertebrates collected included foraminiferans, sipunculids, molluscs (bivalves and gastropods), polychaetes (including serpulid worms), crustaceans (amphipods, decapods, isopods, tanaids and copepods) and echinoderms.

### 4.2.2 Vulcano

In June 2013, samples were collected by placing nets (200 µm mesh size) over 15 individual thalli per site of *Cystoseira* spp. (Figure 4.2B) and on 10 thalli per site of *Sargassum vulgare* (Figure 4.2C) and delicately detaching the thallus from the rock with a chisel. Two sites shown in Figure 4.1 were used, one with high pCO<sub>2</sub> (1200 ppm) and one with lower pCO<sub>2</sub> (600 ppm); detailed sampling dates and sample sizes are reported in Table 1.1C. Samples were sieved and transferred to 70% Industrial Methylated Spirit (IMS) for storage. Over 14000 individual invertebrates were sorted under a stereoscope and identified to the lowest possible taxonomic level, hereafter termed the operational taxonomic unit (OTU). Amphipods were identified using keys from Bellan-Santini *et al.* (1982, 1989, 1993, 1998), molluscs were identified using the key from Doneddu and Trainito (2005) and taxonomic expertise by Prof. Renato Chemello (University of Palermo, Italy), and all other taxa were identified using the guide from Riedl (1991). The invertebrates collected included molluscs (bivalves and gastropods), polychaetes, crustaceans (amphipods, decapods, isopods and tanaids) and echinoderms.

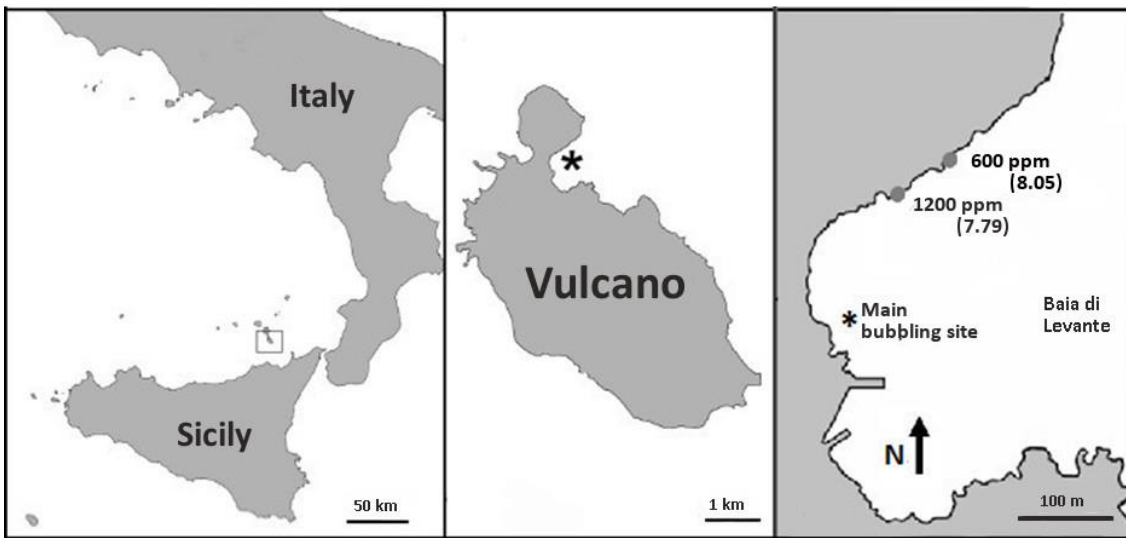


Figure 4.1. Location of Vulcano Island (Sicily, Southern Italy) and of the study area. Asterisk marks the main venting site, grey circles show two experimental sites, with decreasing  $p\text{CO}_2$  moving away from the bubbling site. Average pH from environmental monitoring performed in 2012 (data reported in Chapter 5).

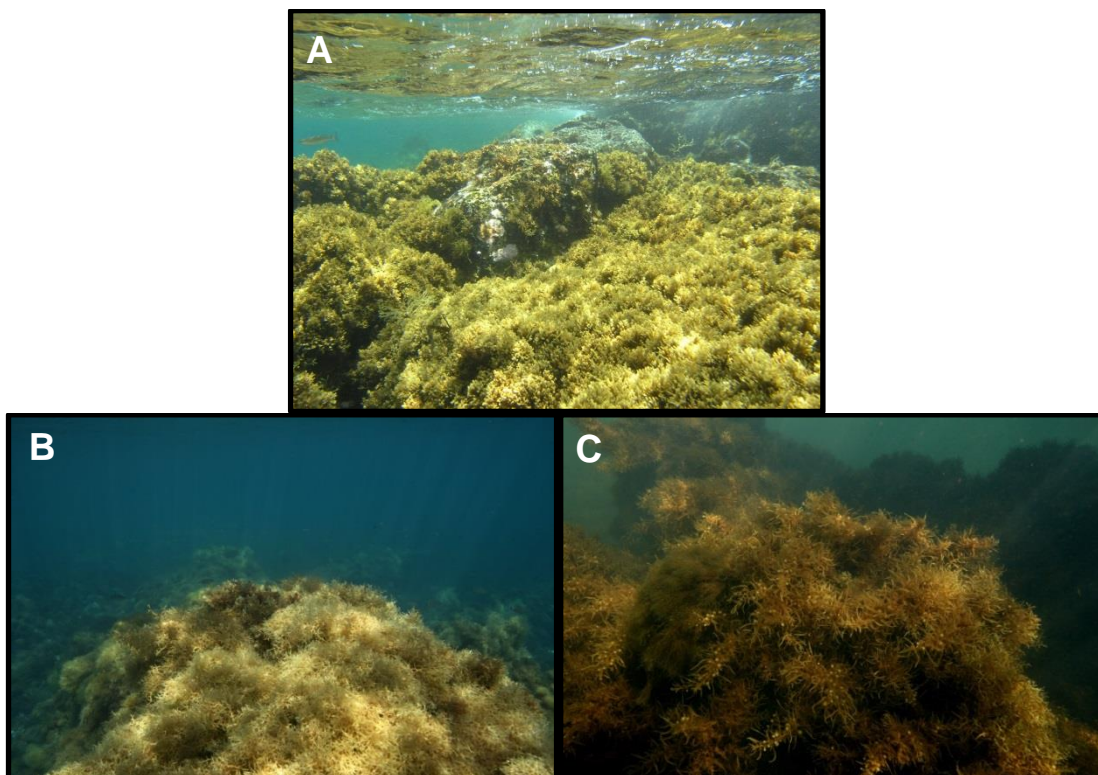


Figure 4.2. Macroalgal species sampled for epifauna at Methana in May 2012 (*Cystoseira corniculata*; A) and at Vulcano in June 2013 (*Cystoseira* spp. and *Sargassum vulgare*; B and C).

Several algal morphological parameters that could influence invertebrate density were measured for each thallus; parameters were selected based on Chemello and Milazzo (2002). Macroalgal biomass was measured with a balance ( $\pm 0.001$  g accuracy) after it was blot-dried (fresh mass) and dried in an oven for 72 h at 50°C (dry mass). The other morphological parameters used were:

- Axis length: length of main axis measured in mm, measured by spreading the macroalgal thalli on graph paper;
- Frond density: number of primary branches on longest frond/axis length;
- Branching arrangement: total tip number/frond density;
- Canopy volume: maximum height (mm) x maximum length (mm) x maximum width (mm), measured by spreading the macroalgal thalli on graph paper;
- Volume: ml of water displaced, measured in a graduated cylinder;
- Interstitial volume: canopy volume - volume;
- Index of compactness: canopy volume/volume;
- Order of branching: counted from the distal branch to the stem. The final branches were classed first order, and whenever two branches of the same order joined, the order of the resultant branch was increased by one;
- Fractal dimension: calculated from black and white photos using the box counting method with the Fractalyse 2.4 software (CNRS, France).

#### **4.2.3 Statistical analyses**

ANOVA was used to analyse the biomass of *C. corniculata* samples from Methana (fixed factor: site) and morphological parameters influencing epifauna

on algal thalli from Vulcano (fixed factors: site and species) after checking they complied with the normality and variance homogeneity requirements of ANOVA. These analyses were performed using SPSS v19 (IBM, USA).

The structure and composition of epiphytic communities from Methana samples and patterns in morphological parameters in samples from Vulcano were tested using a PERMANOVA on square-root (Methana) and normalised (Vulcano) data, with the same experimental designs outlined above. Type III sums of squares with 9,999 unrestricted permutations of the raw data were used for Methana data to account for small sample sizes, whereas Vulcano morphological data were analysed using 9,999 permutations of residuals under a reduced model. Pairwise tests were performed when a factor with more than two levels was significant.

The same procedure was used to analyse epifaunal community data, but a BIO-ENV analysis (Clarke and Ainsworth, 1993) was first used to determine the best combination of variables (epiphyte community and *C. corniculata* biomass for Methana samples, morphological parameters for Vulcano samples) to use as covariates for PERMANOVA (i.e. the combination of variables that explained the most epifaunal variation); when covariates were used, type I sums of squares were used. Epifaunal diversity (Vulcano) and changes in abundance of individual broad taxonomic groups (Vulcano and Methana) were also analysed using the experimental design described above. Where appropriate and meaningful, nMDS plots were used to visually inspect similarities among samples. All analyses above were performed using PRIMER 6 with PERMANOVA+ extension (Plymouth Routines In Multivariate Ecological Research, version 6).

## 4.3 Results

### 4.3.1 Methana

Dry mass of *C. corniculata* growing in 20 x 20 cm quadrats off Methana showed significant differences among study sites (Table 4.1). Pairwise comparisons among sites then showed no consistent differences between different pH levels, although average dry mass of *C. corniculata* decreased from the seep site (mean  $\pm$ SE, n=3: 78.967  $\pm$  12.782 g) to the reference sites (mean  $\pm$ SE, n=6: 44.700  $\pm$  8.996 g), as shown in Figure 4.3.

Table 4.1. ANOVA on biomass of *C. corniculata* from 20 x 20 cm quadrats in May 2012. The Table shows the main factor, sum of squares (SS), degrees of freedom (df), Mean Squares (MS), F-ratios (F) and p values. The p values < 0.05 are highlighted. The lower part of the Table shows subsets detected by post-hoc pairwise comparisons, with different letters representing significantly different groups.

| Source         | Type III SS       | df                   | MS                 | F                    | p                  |
|----------------|-------------------|----------------------|--------------------|----------------------|--------------------|
| Site           | 5163.609          | 4                    | 1290.902           | 5.333                | <b>0.015</b>       |
| Error          | 2420.427          | 10                   | 242.043            |                      |                    |
| Total          | 7584.036          | 14                   |                    |                      |                    |
| <b>Subsets</b> | SEEP <sup>a</sup> | 200 W <sup>a,b</sup> | 200 E <sup>a</sup> | REF A <sup>a,b</sup> | REF B <sup>b</sup> |



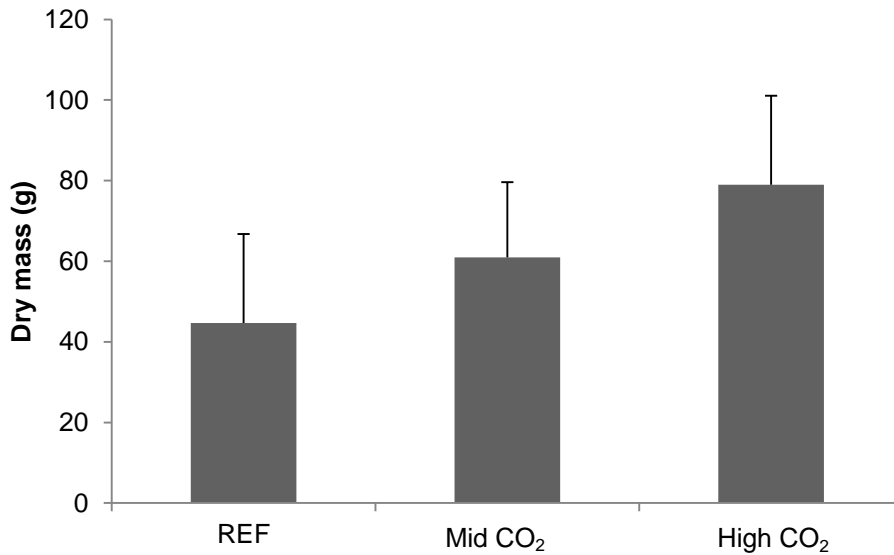


Figure 4.3. Mean ( $\pm$  SD) dry mass of *C. corniculata* from 20 x 20 cm quadrats scraped from rock in May 2012 at reference (REF, n=6), intermediate (n=6) and high CO<sub>2</sub> (n=3) off Methana.

Epiphyte communities on *C. corniculata* also showed significant differences among sites, but no consistent effect of pCO<sub>2</sub> was revealed by pairwise comparisons (Table 4.2).

Table 4.2. PERMANOVA on epiphyte communities of *C. corniculata* from 20 x 20 cm quadrats scraped from rocky substratum in May 2012. The table shows the main factor, degrees of freedom (df), sum of squares (SS), Mean Squares (MS), pseudo F-ratios (Pseudo-F), permutational p and number of unique permutations. Significant p values (< 0.05) are highlighted. The lower part of the table shows subsets detected by post-hoc pairwise comparisons, with different letters representing significantly different groups.

| Source         | df | SS                | MS                   | Pseudo-F             | P(perm)            | Unique perms         |
|----------------|----|-------------------|----------------------|----------------------|--------------------|----------------------|
| Site           | 4  | 9361.90           | 2340.50              | 2.5891               | <b>0.0095</b>      | 9903                 |
| Residual       | 10 | 9039.90           | 903.99               |                      |                    |                      |
| Total          | 14 | 18402.00          |                      |                      |                    |                      |
| <b>Subsets</b> |    | SEEP <sup>a</sup> | 200 W <sup>a,b</sup> | 200 E <sup>a,b</sup> | REF A <sup>b</sup> | REF B <sup>a,b</sup> |

BIO-ENV analysis showed that among the measured covariates, dry biomass of Porifera epiphytes (white species) explained most of the variability between samples. Spearman correlation coefficient for this analysis was 0.101, meaning that white Porifera epiphytes explained 10.1% of the variability of invertebrate abundance among samples. PERMANOVA of the invertebrate data using white Porifera epiphytes as a covariate showed a significant effect of site (Table 4.3). Pairwise comparisons showed that invertebrate communities at SEEP were significantly different from those at reference sites, while sites with intermediate pCO<sub>2</sub> were not significantly different from either SEEP or the closest reference site (REF A).

Table 4.3. PERMANOVA on epifaunal communities of *C. corniculata* from 20 x 20 cm quadrats scraped from rocky substratum in May 2012 using biomass of epiphytic Porifera (white) as covariate. The Table shows the main factors and their interaction, degrees of freedom (df), sum of squares (SS), Mean Squares (MS), pseudo F-ratios (Pseudo-F), permutational p and number of unique permutations. Significant p values (< 0.05) are highlighted. The lower part of the Table shows subsets detected by post-hoc pairwise comparisons, with different letters representing significantly different groups.

| Source          | df | SS                | MS                   | Pseudo-F             | P(perm)              | Unique perms       |
|-----------------|----|-------------------|----------------------|----------------------|----------------------|--------------------|
| Porifera        | 1  | 946.61            | 946.61               | 2.5386               | <b>0.0283</b>        | 9951               |
| Site            | 4  | 8207.80           | 2052.00              | 5.5030               | <b>0.0001</b>        | 9930               |
| Porifera x Site | 3  | 726.85            | 242.28               | 0.6498               | 0.8743               | 9918               |
| Residual        | 6  | 2237.30           | 372.88               |                      |                      |                    |
| Total           | 14 | 12119.00          |                      |                      |                      |                    |
| <b>Subsets</b>  |    | SEEP <sup>a</sup> | 200 W <sup>a,b</sup> | 200 E <sup>a,b</sup> | REF A <sup>b,c</sup> | REF B <sup>c</sup> |

To further explore general patterns of changes in invertebrate communities along the Methana pCO<sub>2</sub> gradient, the community analysis was repeated grouping OTUs into general categories. BIO-ENV analysis of these data showed that among the measured covariates, dry biomass of Porifera epiphytes

(white species) and of Porifera epiphytes (yellow species) explained most of the variability between samples. Spearman correlation coefficient for this analysis was 0.122, meaning that white and yellow Porifera epiphytes explained 12.2% of the variability in invertebrate abundance among samples. However, PERMANOVA of the invertebrate data using the two categories above as covariates showed that they did not have a significant effect. PERMANOVA was then repeated without covariates, and a significant effect of site was detectable (Table 4.4). Pairwise comparisons showed that invertebrate communities at SEEP were significantly different from those at reference sites. Sites with intermediate pCO<sub>2</sub> levels were not significantly different from each other, but 200 W was also not significantly different from SEEP.

Table 4.4. PERMANOVA on invertebrate communities of *C. corniculata* from 20 x 20 cm quadrats in May 2012 grouping taxa into broad taxonomic groups. The Table shows the main factor, degrees of freedom (df), sum of squares (SS), Mean Squares (MS), pseudo F-ratios (Pseudo-F), permutational p and number of unique permutations. Significant p values (< 0.05) are highlighted. The lower part of the Table shows subsets detected by post-hoc pairwise comparisons, with different letters representing significantly different groups.

| <b>Source</b>  | <b>df</b> | <b>SS</b>         | <b>MS</b>            | <b>Pseudo-F</b>    | <b>P(perm)</b>     | <b>Unique perms</b> |
|----------------|-----------|-------------------|----------------------|--------------------|--------------------|---------------------|
| Site           | 4         | 4742.7            | 1185.70              | 8.0678             | <b>0.0001</b>      | 9918                |
| Residual       | 10        | 1469.6            | 146.96               |                    |                    |                     |
| Total          | 14        | 6212.4            |                      |                    |                    |                     |
| <b>Subsets</b> |           | SEEP <sup>a</sup> | 200 W <sup>a,b</sup> | 200 E <sup>b</sup> | REF A <sup>c</sup> | REF B <sup>c</sup>  |

A MDS plot of these data (Figure 4.4) is consistent with pairwise comparisons; it shows that reference sites were clearly different from sites with intermediate and high pCO<sub>2</sub> levels, while the latter were only loosely separated.

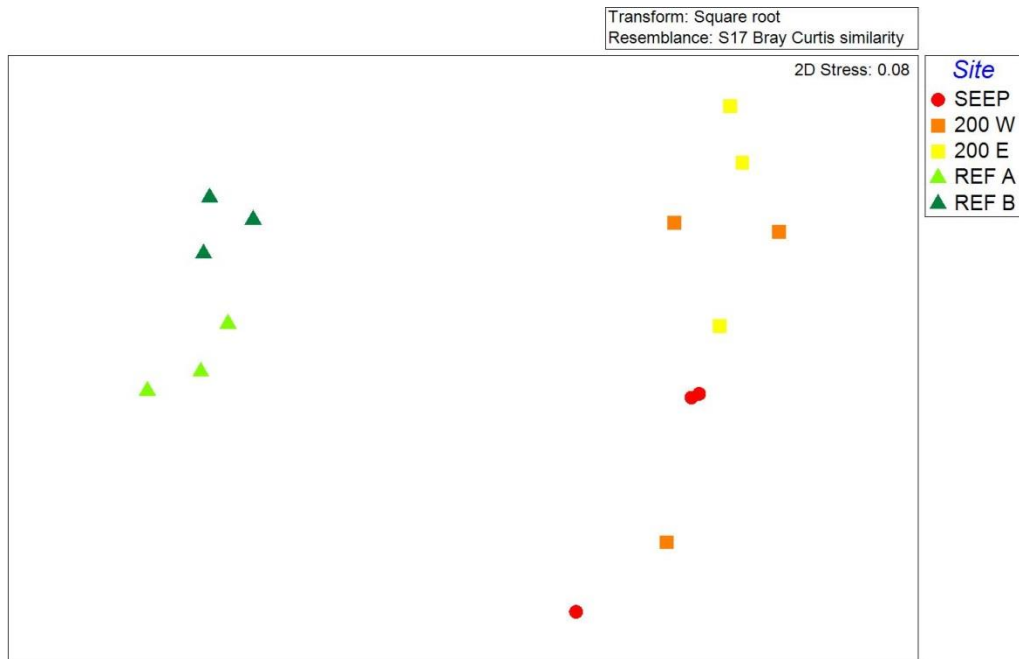


Figure 4.4: MDS plot of invertebrate assemblages on *C. corniculata* thalli collected from 20 x 20 cm quadrats at SEEPS, 200 W, 200 E, REF A and REF B at Methana in May 2012.

Abundances of invertebrate categories that showed significant responses to increased  $p\text{CO}_2$  are reported in Figure 4.5. The most abundant invertebrates were amphipods, polychaetes and foraminifera (Figure 4.5A), while bivalves, ophiuroids, sipuncula, gastropods and serpulids were present in lower abundances (Figure 4.5B). In general, heavily calcified taxa (foraminifera, bivalves, gastropods and serpulids) showed decreased abundances with increasing  $p\text{CO}_2$ , while others (amphipods, polychaetes, ophiuroids) had a parabolic pattern (with highest abundances at intermediate  $\text{CO}_2$ ) whilst sipunculids showed no clear trend in relation to carbon dioxide levels.

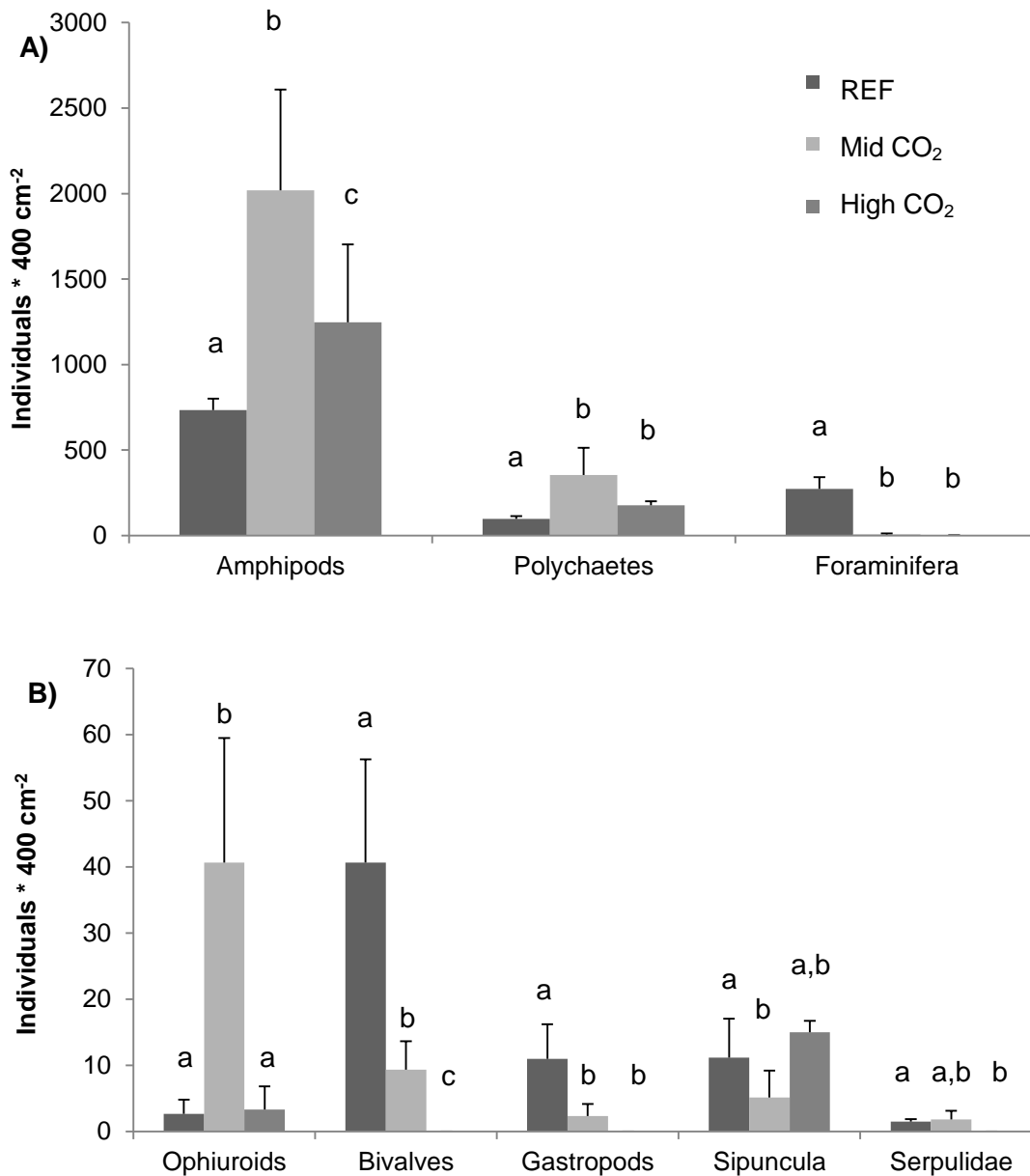


Figure 4.5. Mean ( $\pm$ SD,  $n=3$ ) number of individuals for each of the main taxonomic groups of invertebrates found on *C. corniculata* from 20 x 20 cm quadrats in May 2012 at reference (REF,  $n=6$ ), intermediate ( $n=6$ ) and high CO<sub>2</sub> levels ( $n=3$ ). The groups are divided depending whether they could have more (A) or less (B) than 100 individuals per sample. Different letters represent significantly different groups according to pairwise comparisons.

### 4.3.2 Vulcano

Normalised morphological parameters of macroalgae were significantly different between sites, and changed differently in *Cystoseira* spp. and *S. vulgare* (Table 4.5). All combinations of site and species were significantly different in pairwise comparisons (Table 4.5). The MDS plot clearly shows that while all combinations of site and species were different from each other, *Cystoseira* spp. samples were more tightly grouped than those of *S. vulgare*, which were separated into two very distinct groups (600 and 1200; Figure 4.6).

Table 4.5. PERMANOVA on morphology of *Cystoseira* spp. and *S. vulgare* thalli collected at Vulcano in June 2013. The table shows the main factors and their interaction, degrees of freedom (df), sum of squares (SS), Mean Squares (MS), pseudo F-ratios (Pseudo-F), permutational p and number of unique permutations. Significant p values (< 0.05) are highlighted. The lower part of the table shows subsets detected by post-hoc pairwise comparisons, with different letters representing significantly different groups.

| Source               | df                 | SS                  | MS                 | Pseudo-F            | p(perm)       | Unique perms |
|----------------------|--------------------|---------------------|--------------------|---------------------|---------------|--------------|
| Site                 | 1                  | 32.662              | 32.662             | 5.9684              | <b>0.0003</b> | 9935         |
| Species              | 1                  | 45.812              | 45.812             | 8.3715              | <b>0.0001</b> | 9942         |
| Site x Species       | 1                  | 114.23              | 114.23             | 20.874              | <b>0.0001</b> | 9940         |
| Residual             | 44                 | 240.79              | 5.4724             |                     |               |              |
| Total                | 47                 | 415.99              |                    |                     |               |              |
| Pairwise comparisons | 600 S <sup>a</sup> | 1200 S <sup>b</sup> | 600 C <sup>c</sup> | 1200 C <sup>d</sup> |               |              |

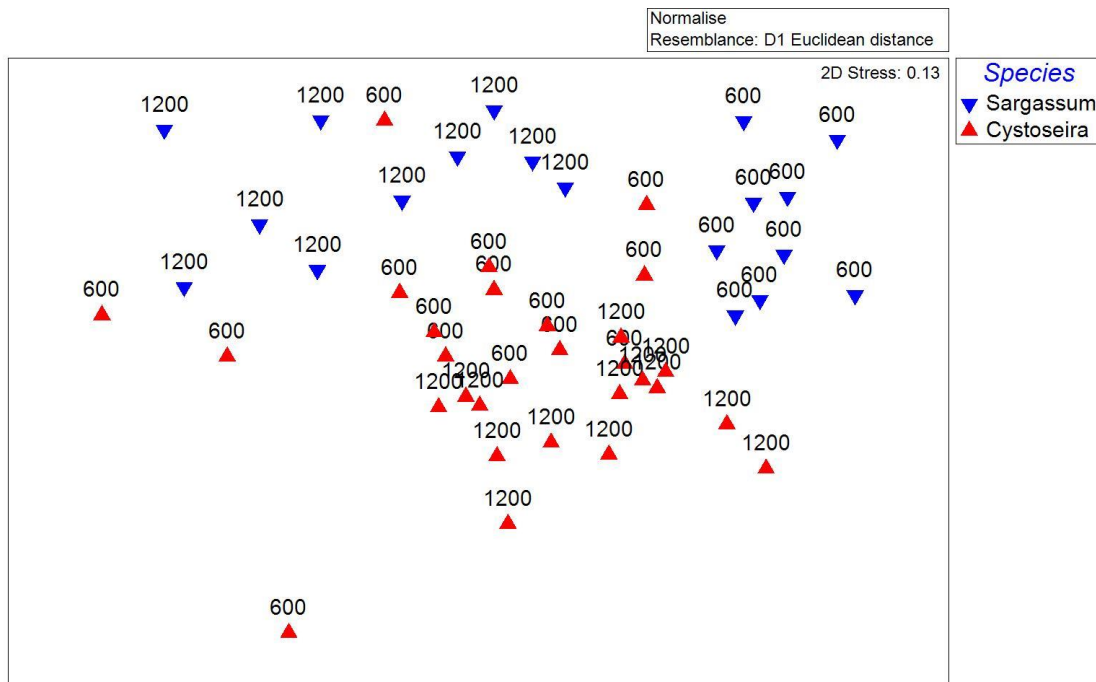


Figure 4.6: MDS plot of macroalgal morphology of *Cystoseira* spp. and *S. vulgare* thalli collected at Vulcano in June 2013 at sites with 600 and 1200 ppm average seawater pCO<sub>2</sub>.

The morphological variables best explaining epifaunal patterns were axis length, order of branching and frond density; these three parameters explained 29.3% of the epifauna variability according to the BIO-ENV analysis. ANOVAs of the parameters above showed that axis length of *Cystoseira* spp. and *S. vulgare* exhibited opposite patterns. Main axes of *S. vulgare* were longer at elevated CO<sub>2</sub>, increasing from under 150 mm to over 400 m, whereas those of *Cystoseira* spp. were shorter at the high CO<sub>2</sub> site (Figure 4.7A). Order of branching was mostly similar for all sites and species, but *S. vulgare* at the low pCO<sub>2</sub> site had a much simpler structure (Figure 4.7B). On the other hand, frond density was not significantly different between sites, but was significantly higher in *Cystoseira* spp. than in *S. vulgare* (Figure 4.7C).

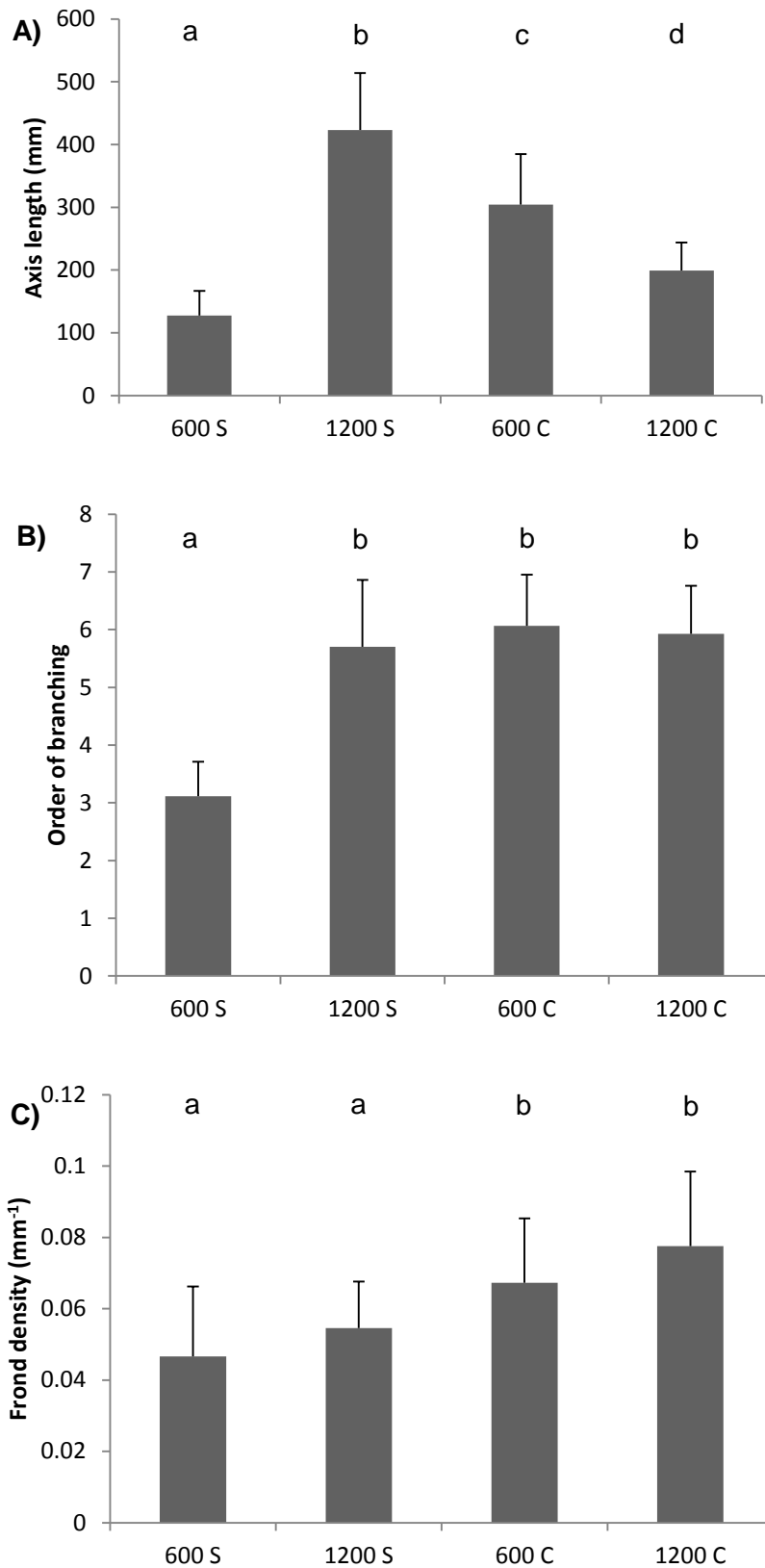


Figure 4.7: Mean ( $\pm$  SD,  $n=9-15$ ) axis length (A), order of branching (B) and frond density (C) of *Cystoseira* spp. and *S. vulgare* thalli collected at Vulcano in June 2013 at sites with 600 and 1200 ppm average seawater  $pCO_2$ . Different letters represent significantly different groups identified by pairwise comparisons.



As for the invertebrate community analysis, no significant interactions between factors and covariates were detected; those interactions were therefore removed from the analysis. The results show that after taking into account the three covariates, invertebrate communities were significantly different between sites and between species, but the two factors did not interact (Table 4.6). Pairwise comparisons revealed that while epifauna of *Cystoseira* spp. was significantly different between sites, epifauna of *S. vulgare* did not change significantly as pCO<sub>2</sub> increased, and its community structure was not significantly different from that of *Cystoseira* spp. (Table 4.6). As analysis of broad taxonomic categories gave the same results, they are not reported here.

Table 4.6. PERMANOVA on invertebrate communities of *Cystoseira* spp. and *S. vulgare* thalli collected at Vulcano in June 2013 using axis length, order of branching and frond density as covariates. The table shows the main factors and their interaction, degrees of freedom (df), sum of squares (SS), Mean Squares (MS), pseudo F-ratios (Pseudo-F), permutational p and number of unique permutations. Significant p values (< 0.05) are highlighted. The lower part of the table shows subsets detected by post-hoc pairwise comparisons, with different letters representing significantly different groups.

| Source               | df                   | SS                    | MS                 | Pseudo-F            | p(perm)       | Unique perms |
|----------------------|----------------------|-----------------------|--------------------|---------------------|---------------|--------------|
| Axis length          | 1                    | 5313.9                | 5313.9             | 6.1971              | <b>0.0001</b> | 9926         |
| Order of branching   | 1                    | 4385.8                | 4385.8             | 5.1148              | <b>0.0001</b> | 9925         |
| Frond density        | 1                    | 2865.6                | 2865.6             | 3.3419              | <b>0.0003</b> | 9935         |
| Site                 | 1                    | 7833.2                | 7833.2             | 9.1352              | <b>0.0001</b> | 9926         |
| Species              | 1                    | 4895.1                | 4895.1             | 5.7088              | <b>0.0002</b> | 9934         |
| Site x Species       | 1                    | 1245.6                | 1245.6             | 1.4527              | 0.1366        | 9924         |
| Residual             | 41                   | 35156                 | 857.47             |                     |               |              |
| Total                | 47                   | 61696                 |                    |                     |               |              |
| Pairwise comparisons | 600 S <sup>a,b</sup> | 1200 S <sup>a,b</sup> | 600 C <sup>a</sup> | 1200 C <sup>b</sup> |               |              |

Epifaunal diversity was not significantly affected by any morphological parameters, so covariates were removed from the analysis. Similarly to the epifaunal community structure, Shannon diversity was significantly affected by site and species, but the two factors did not interact (Table 4.7). Although epifauna of both macroalgae had lower diversity at elevated CO<sub>2</sub>, the difference was clearer in *Cystoseira* spp. (Figure 4.8).

Table 4.7. ANOVA on epifaunal diversity of *Cystoseira* spp. and *S. vulgare* thalli collected at Vulcano in June 2013. The table shows the main factors and their interaction, degrees of freedom (df), sum of squares (SS), Mean Squares (MS), pseudo F-ratios (Pseudo-F), permutational p and number of unique permutations. Significant p values (< 0.05) are highlighted. The lower part of the table shows subsets detected by post-hoc pairwise comparisons, with different letters representing significantly different groups.

| Source               | df                   | SS                  | MS                 | Pseudo-F            | p(perm)       | Unique perms |
|----------------------|----------------------|---------------------|--------------------|---------------------|---------------|--------------|
| Site                 | 1                    | 0.67246             | 0.6724<br>6        | 6.9354              | <b>0.0113</b> | 9824         |
| Species              | 1                    | 0.39158             | 0.3915<br>8        | 4.0385              | <b>0.0485</b> | 9830         |
| Site x Species       | 1                    | 0.10733             | 0.1073<br>3        | 1.1069              | 0.3002        | 9835         |
| Residual             | 44                   | 4.2662              | 0.097              |                     |               |              |
| Total                | 47                   | 5.5516              |                    |                     |               |              |
| Pairwise comparisons | 600 S <sup>a,b</sup> | 1200 S <sup>b</sup> | 600 C <sup>a</sup> | 1200 C <sup>c</sup> |               |              |

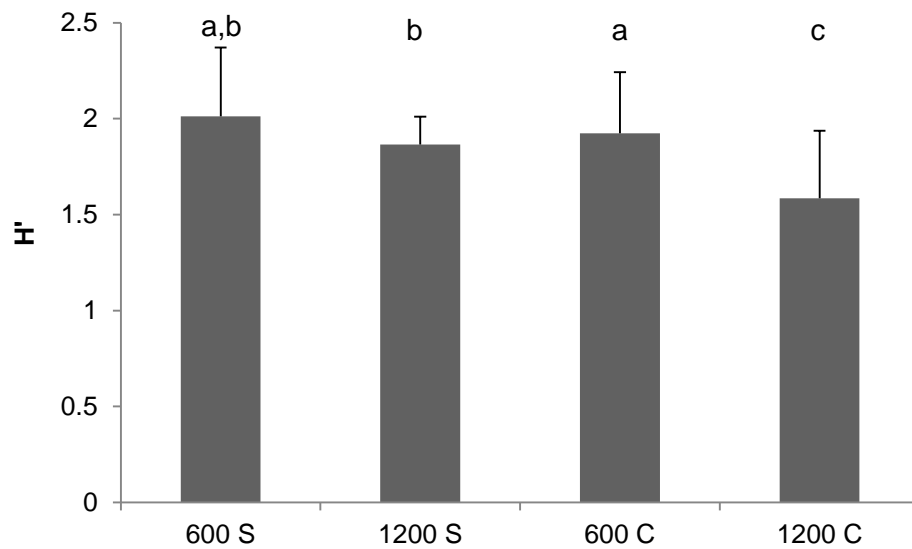


Figure 4.8: Mean ( $\pm$  SD, n=9-15) diversity of epifaunal communities on *Cystoseira* spp. and *S. vulgare* thalli collected at Vulcano in June 2013 at sites with 600 and 1200 ppm average seawater pCO<sub>2</sub>. Different letters represent significantly different groups identified by pairwise comparisons.

Graphs of the number of individuals of the most abundant taxa are shown in Figure 4.9. Values are reported as individuals per ml of water displaced by the macroalga, as this was the morphological parameter that seemed to influence invertebrate abundance the most (BIO-ENV analyses on single taxa, results not shown). Amphipods and tanaids showed a clear decrease with increasing pCO<sub>2</sub> on *S. vulgare*, but a trend towards increasing abundance on *Cystoseira* spp.. Gastropods decreased at the high CO<sub>2</sub> site in both macroalgal species, whereas polychaetes and isopods became more abundant as CO<sub>2</sub> increased.

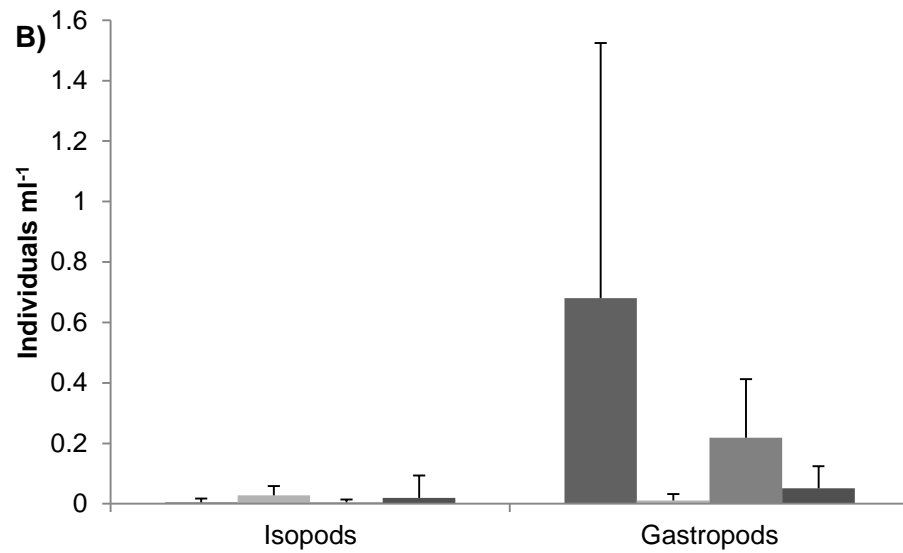
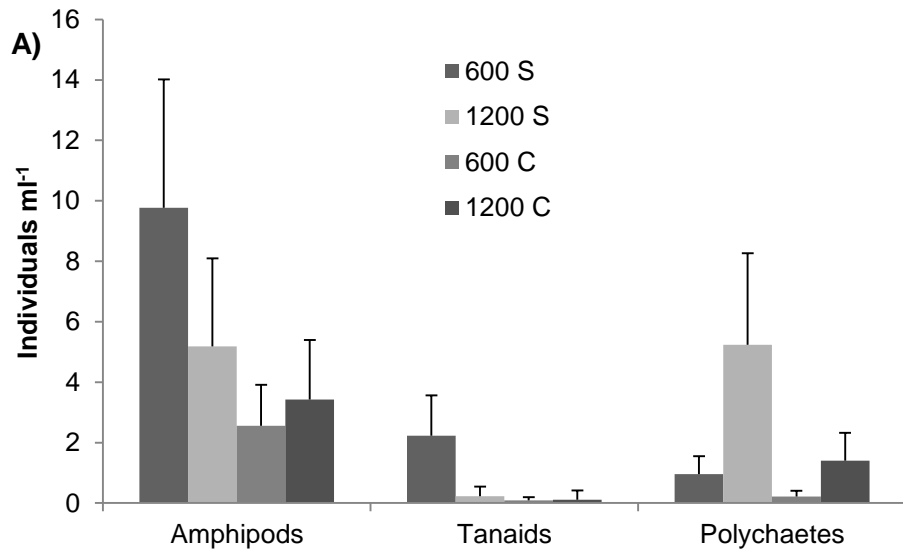


Figure 4.9. Mean ( $\pm$ SD, n=9-15) number of individuals for each of the main taxonomic groups of invertebrates per ml of water displaced by thalli of *Cystoseira* spp. and *S. vulgare* collected at Vulcano in June 2013 at sites with 600 and 1200 ppm average seawater pCO<sub>2</sub>. The groups are divided depending whether they had more (A) or less (B) than 1 individual per ml of water displaced.

## 4.4 Discussion

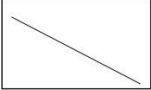

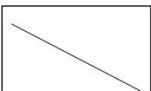
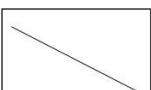
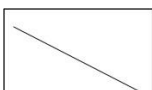
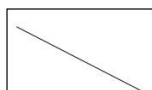
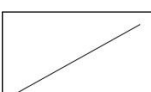
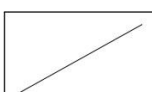
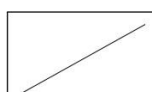




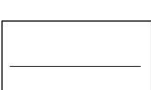




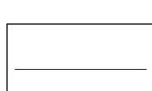

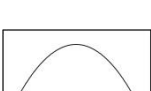
This is the first study to assess how epifauna of different canopy-forming algae responds to elevated CO<sub>2</sub> levels. Epifauna of macroalgae and seagrasses represents the main food source for many commercial fish, especially for juvenile individuals (Yamada *et al.*, 2010); changes in their abundance and specific composition could therefore have dramatic consequences for coastal ecosystems and fisheries. Epifauna of *Cystoseira* spp. collected at two CO<sub>2</sub> seeps was significantly affected by elevated carbon dioxide levels, but epifauna of *Sargassum vulgare* was not affected by changes in pCO<sub>2</sub>. This has implications for ecosystem functioning, as epifauna can contribute 70-98% of secondary productivity on temperate rocky reefs (Taylor, 1998).

Community structure of *Cystoseira* spp. epifauna significantly changed with increasing pCO<sub>2</sub> at both sites, but at Vulcano *S. vulgare* epifauna did not change significantly with pCO<sub>2</sub> levels. The changes in epifaunal communities are in accord with previous studies on epifaunal responses to ocean acidification, which reported significant changes in community structure (Hale *et al.*, 2011; Kroeker *et al.*, 2011). A pH < 7.2 can cause communities to shift from net calcification to net dissolution, although total invertebrate biomass does not change with pCO<sub>2</sub> (Kroeker *et al.*, 2011; Christen *et al.*, 2013). At Vulcano, epifaunal diversity significantly decreased at the high CO<sub>2</sub> site on *Cystoseira* spp. thalli, but epifauna of *S. vulgare* did not show significant changes in community structure and diversity between sites. Although invertebrate communities often decrease in diversity as CO<sub>2</sub> increases (Cigliano *et al.*, 2010; Hale *et al.*, 2011; Kroeker *et al.*, 2011), *Cystoseira*-associated macroalgal communities are more sensitive to ocean acidification compared to those associated with *Sargassum muticum* (Olabarria *et al.*, 2013). In addition, the

increase in *S. vulgare* structural complexity at the high CO<sub>2</sub> site might have helped maintain epifaunal diversity, as morphological complexity of macroalgal hosts has been linked with higher diversity and abundance of their associated epifauna (Chemello and Milazzo, 2002; Bates, 2009).

Heavily calcified taxa decreased in abundance with increasing pCO<sub>2</sub>, both at Methana and Vulcano (results summarised in Table 4.8). The only taxa that increased in abundance at elevated pCO<sub>2</sub> were polychaetes and amphipods, although the former showed a clearer increase. Compensatory increase in abundance of CO<sub>2</sub>-resistant taxa in ocean acidification conditions has been reported by Hale *et al.* (2011) and Kroeker *et al.* (2011) for nematodes and crustaceans, respectively. These results show that different taxa can be advantaged as CO<sub>2</sub> increases depending on system characteristics; specifically, different taxa can be advantaged by decreased predation rates or reduced competition for space and resources (Micheli *et al.*, 1999). Taxa producing carbonate structures are negatively affected by elevated CO<sub>2</sub> (Kroeker *et al.*, 2013a), whereas crustaceans experience less severe effects because their chitinous skeletons are less prone to dissolution than calcium carbonate structures (Whiteley, 2011). At both study sites, crustaceans indeed showed small or unclear changes in abundance at different CO<sub>2</sub> levels. At Vulcano, changes in epifaunal densities with increased CO<sub>2</sub> were more marked in *S. vulgare*, possibly because its increase in thallus length and complexity at the high CO<sub>2</sub> site increased available habitat for epifauna, therefore masking direct effects of carbon dioxide on their abundance.

Table 4.8. Response to increasing CO<sub>2</sub> of the main epifaunal taxa living on *Cystoseira corniculata* (Methana) or *Cystoseira* spp. and *Sargassum vulgare* (Vulcano); symbols indicate increase (↗), decrease (↘) or no change (—) with elevated CO<sub>2</sub>; n.f. = not found.

| Taxon        | Methana   | Vulcano   |   |
|--------------|---|---|---|
|              | <i>Cystoseira corniculata</i>   | <i>Cystoseira</i> spp.  | <i>Sargassum vulgare</i>  |
| Foraminifera |    | n.f.  | n.f.  |
| Sipunculida  |    | n.f.  | n.f.  |
| Bivalves     |   | n.f.  | n.f.  |
| Gastropods   |  |  |  |
| Polychaetes  |  |  |  |
| Serpulids    |  | n.f.  | n.f.  |
| Amphipods    |  |  |  |
| Isopods      |  |  |  |
| Tanaids      |  |  |  |
| Copepods     |  | n.f.  | n.f.  |
| Ophiuroids   |  | n.f.  | n.f.  |

At Methana, biomass of *C. corniculata* and of its epiphytes did not change consistently among pCO<sub>2</sub> levels, even though there were clear differences among sites. *C. corniculata* biomass increased, albeit not significantly, at elevated CO<sub>2</sub>. This is consistent with a recent meta-analysis showing that some fleshy algae exhibit faster growth rates at elevated pCO<sub>2</sub> levels (Kroeker *et al.*, 2013a). The lack of significant effects of pCO<sub>2</sub> on *C. corniculata* epiphytes is surprising, as it contrasts with the clear decrease in calcifying epiphytes off Vulcano as CO<sub>2</sub> increased (Papworth, 2012), for macroalgal communities (Chapter 3) and epifauna (this chapter). Other factors may be influencing epiphyte communities and masking direct effects of CO<sub>2</sub>; for instance, epiphytes are often controlled by grazers such as amphipods or gastropods (Fong *et al.*, 2000; Whalen *et al.*, 2012), whose abundances varied among sites. Another possibility is that *C. corniculata* photosynthesis raises pH near its fronds (Hendriks *et al.*, 2014; Cornwall *et al.*, 2014). This may have reduced the impacts of ocean acidification on epiphytes, but not on mobile epifauna, which often swim in and out of macroalgal fronds (Edgar, 1992).

At Vulcano, macroalgal morphology clearly changed at elevated pCO<sub>2</sub>. *Sargassum vulgare* was competitively advantaged at high CO<sub>2</sub>, as it increased in length and complexity (i.e. order of branching) at the 1200 ppm site. In contrast, *Cystoseira* spp. decreased in length at elevated CO<sub>2</sub>, but their frond densities were higher than that of *S. vulgare* at both sites. While *S. vulgare* was the only species of the genus *Sargassum* present at both study sites, several *Cystoseira* species were present off Vulcano. Specifically, *C. compressa* was very abundant at the 1200 ppm site and *C. humilis* was very abundant at the 600 ppm site. Part of the morphological variability of *Cystoseira* spp. could



therefore be attributed to changes in species composition rather than direct effects of pCO<sub>2</sub> on one species' morphology. Other factors, chiefly wave exposure and light, have long been known to influence macroalgal morphology (Hurd, 2000; Monro and Poore, 2005), but neither changes between the two study sites (Johnson *et al.*, 2013). On the other hand, seasonal changes only have a minor influence on most macroalgal morphological parameters (Wernberg and Vanderklift, 2010), so results from this study are unlikely to be influenced by sampling season. *Sargassum vulgare* cover increased with increasing pCO<sub>2</sub> at other Mediterranean seeps (Porzio *et al.*, 2011; Chapter 3), but this is the first evidence that pCO<sub>2</sub> levels can influence fleshy algal morphology as well.

Magnitude of change in communities associated with canopy-forming algae also depends on the type of community, as macroalgal epiphytes did not change among pCO<sub>2</sub> levels, whereas epifaunal communities did. At Vulcano, epifauna of *Cystoseira* spp. and *S. vulgare* were not significantly different within pCO<sub>2</sub> levels. Epifauna of macroalgal species belonging to the same functional group are indeed not likely to be significantly different (Bates and DeWreede, 2007). However, *S. vulgare* epifauna changed between sites less than that of *Cystoseira* spp., hinting at host-specific patterns of epifaunal change with increasing CO<sub>2</sub>. In addition, the concurrent increase in *S. vulgare* abundance at elevated pCO<sub>2</sub> will amplify changes in epifaunal communities, with knock-on effects on ecosystem functioning (Taylor, 1998).

## Chapter 5

### Effect of herbivores on benthic communities at different pCO<sub>2</sub> levels

Parts of this chapter are currently under review as:

**C. Baggini**, Y. Issaris, M. Salomidi, J.M. Hall-Spencer (2014). Herbivore diversity improves benthic community resilience to ocean acidification. *Journal of Experimental Marine Biology and Ecology* (accepted pending revisions).

## Abstract

Marine volcanic seeps exhibit profound changes in benthic communities along gradients of increasing  $p\text{CO}_2$  on intertidal and subtidal rocky shores. As grazing by fish, sea urchins and gastropods can also structure benthic communities, decreased herbivore densities due to intolerance to acidified conditions may interact with direct  $\text{CO}_2$  effects to determine benthic community structure in a high  $\text{CO}_2$  world. Here, two exclusion experiments were used to test effects of herbivory in benthic communities along  $p\text{CO}_2$  gradients. Limpets were excluded on intertidal shores at volcanic seeps off Vulcano (Italy) to examine their role in changes from coralline to fleshy algal assemblages. At volcanic seeps off Methana (Greece), herbivore exclusions were used to test whether herbivores affect subtidal algal recruitment differently as carbon dioxide levels increase. Off Vulcano, spatial heterogeneity and seasonality of benthic intertidal communities at a reference site was much higher than at a high  $\text{CO}_2$  site. Limpets had weak effects on benthic communities at ambient  $\text{CO}_2$  levels, and no effect at the high  $\text{CO}_2$  site. Limpet abundances significantly decreased as  $p\text{CO}_2$  levels increased, but higher limpet grazing rates at elevated  $\text{CO}_2$  were not sufficient to maintain top-down control on benthic communities. Conversely, sea urchins and herbivorous fish dramatically reduced macroalgal biomass at Methana. This effect was not hampered by increased  $p\text{CO}_2$  despite lower sea urchin densities near the  $\text{CO}_2$  seeps, probably because fish grazing increased. In summary, we found that as long as herbivore fish are present, carbon dioxide levels up to about  $2000 \mu\text{atm}$  are unlikely to significantly reduce the importance of herbivory in structuring Mediterranean benthic communities, even when herbivores strongly control benthic communities. A shift from sea urchin to fish as main

grazers highlights that ocean acidification may cause complex responses at the community level.

## 5.1 Introduction

Ocean acidification is expected to have profound effects on marine ecosystems worldwide (Kroeker *et al.*, 2013a). Studies at volcanic seeps have shown that increased seawater pCO<sub>2</sub> causes changes in benthic macroalgal and invertebrate communities (Kroeker *et al.*, 2011; Porzio *et al.*, 2011). These changes could be caused by physiological effects of CO<sub>2</sub> on macroalgae, altered competitive interactions (Kroeker *et al.*, 2013c), changes in chemical plant defences (Arnold *et al.*, 2012), or a combination of the above. In addition, grazers may have a determining role in the observed community changes given the strong role of herbivory in marine ecosystems (Poore *et al.*, 2012). Some herbivores, such as amphipods, become more abundant as CO<sub>2</sub> increase at volcanic seeps (Cigliano *et al.*, 2010; Kroeker *et al.*, 2011; Suaria *et al.*, unpublished data). Conversely, key grazers such as limpets and sea urchins decrease in abundance with increased CO<sub>2</sub> (Hall-Spencer *et al.*, 2008; Johnson *et al.*, 2012; Calosi *et al.*, 2013a; Graziano *et al.*, unpublished data), but their contribution to community changes along pCO<sub>2</sub> gradients has not previously been tested experimentally.

Coastal environments have low functional redundancy, even when diversity is relatively high (Micheli *et al.*, 2014). Decrease of limpet and sea urchin densities as seawater CO<sub>2</sub> increases thus leave marine ecosystem vulnerable to phase shifts, especially in the absence of herbivorous fish (Hughes, 1994). Numerous dramatic changes to benthic communities due to reduction in grazing rates have

been reported; for instance, tropical coral reefs can be overgrown by macroalgae if grazing pressure is removed (Hughes *et al.*, 2007).

Limpets of the genus *Patella* are abundant grazers in intertidal Mediterranean shores. Three species are particularly common: *P. aspera*, *P. rustica* and *P. caerulea* (Figure 5.1a). They can greatly influence benthic communities, although their influence varies in space and time (Benedetti-Cecchi *et al.*, 2000) and the algal functional groups affected change depending on several factors. For instance, Benedetti-Cecchi *et al.* (1996) showed how limpets strongly affect coarsely branched and coralline algal abundance, but not filamentous algae, whereas the opposite is true for a study performed in the same area, but on artificial structures (Bulleri *et al.*, 2000).

Limpets are negatively affected by increasing CO<sub>2</sub> levels and the consequent decrease in seawater calcium carbonate saturation; their densities decrease with increasing pCO<sub>2</sub> at seeps off Ischia and Vulcano (Hall-Spencer *et al.*, 2008; Graziano *et al.*, unpublished data). They can survive elevated pCO<sub>2</sub> conditions and up-regulate their calcification rates to counter increased shell dissolution rates (Rodolfo-Metalpa *et al.*, 2011). Some gastropod herbivores increase their feeding rates when pCO<sub>2</sub> increases (Falkenberg *et al.*, 2013b), possibly to sustain the higher energetic cost of calcification. The increase in benthic microalgal chlorophyll concentration recorded at volcanic seeps as CO<sub>2</sub> increases (Johnson *et al.*, 2013) could therefore give limpets a significant advantage in coping with high CO<sub>2</sub> conditions through increased food availability. There is thus a possibility that limpets will still affect benthic communities in a high CO<sub>2</sub> ocean, even with decreased densities, by means of increased feeding rates.

In Mediterranean subtidal environments, high densities of the sea urchins *Paracentrotus lividus* and *Arbacia lixula* can cause a shift from photophilic algal assemblages to “barren grounds”, impoverished assemblages dominated by encrusting algae (Sala *et al.*, 1998; Figure 5.1b). Sea urchin grazing can cause a shift to barren grounds in temperate rocky reefs worldwide, which are considered an alternative stable state to kelp beds (Filbee-Dexter and Scheibling, 2014). Once established, barren grounds can be maintained by relatively low sea urchin densities (Chiantore *et al.*, 2008), although these can change back to macroalgal beds if the biomass of carnivorous fish exceeds a critical threshold (Guidetti and Sala, 2007). Herbivorous fish are normally thought to exert weaker top-down control on temperate benthic communities compared to sea urchins (Floeter *et al.*, 2005). However, in the warm-temperate Mediterranean Sea herbivorous fish can limit the distribution of some macroalgal species (Vergés *et al.*, 2009). The main herbivorous fish in the Mediterranean Sea are the sparid *Sarpa salpa* (Figure 5.1c) and the scarid *Sparisoma cretense*, as well as the lessepsian migrants *Siganus luridus* and *Siganus rivulatus*, which can account for over 90% of herbivorous fish biomass in Greek coastal waters (Kalogirou *et al.*, 2012). Increased temperatures in the Mediterranean Sea are helping *Siganus* spp. to expand their range; these fish are therefore causing and maintain barren grounds in the Eastern Mediterranean (Sala *et al.*, 2011; Vergés *et al.*, 2014).

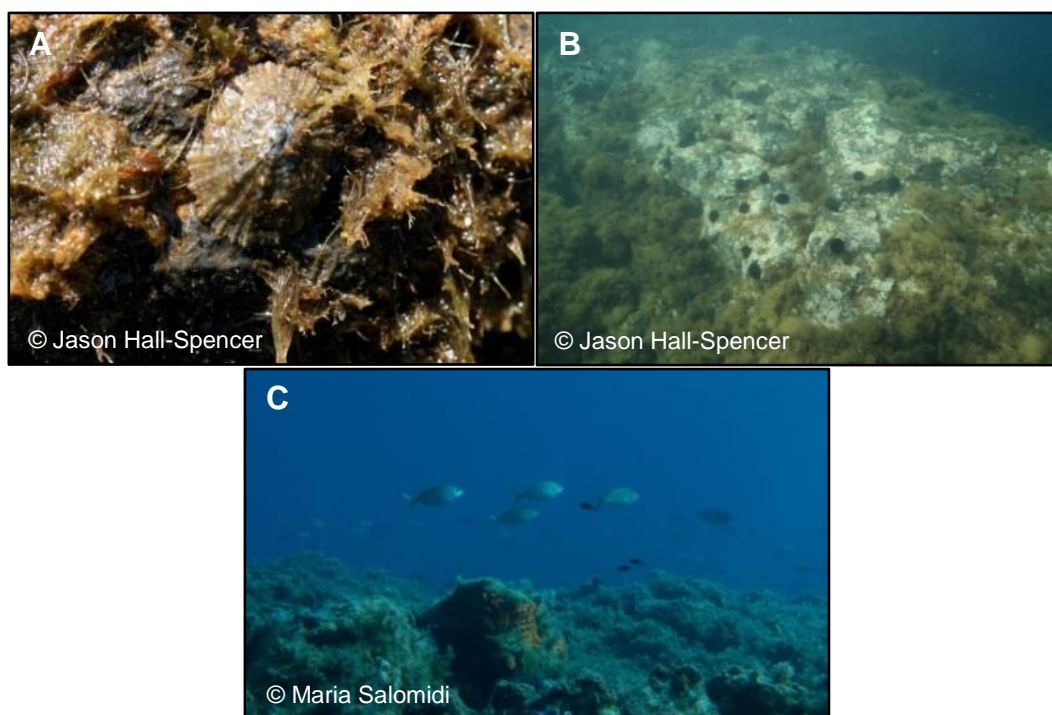


Figure 5.1. Main grazers on Mediterranean intertidal and subtidal shores: (A) Limpet on intertidal shore off Vulcano (Italy); (B) sea urchins reducing macroalgal biomass at shores off Vulcano (Italy); (C) herbivorous fish (*Sarpa salpa*) near CO<sub>2</sub> seeps off Methana (Greece).

Ocean acidification has a detrimental effect on the physiology of many sea urchin species (Dupont *et al.*, 2010) and their densities often decrease as seawater pCO<sub>2</sub> increases (Johnson *et al.*, 2012; Calosi *et al.*, 2013a). On the other hand, many adult fish seem to tolerate carbon dioxide levels predicted for the end of this century (Melzner *et al.*, 2009). Despite this, near-future levels of CO<sub>2</sub> can have profound effects on fish behaviour and sensory functions, particularly at larval and juvenile stages, making many fish species less alert to predators even after prolonged exposure at CO<sub>2</sub> seeps (McCormick *et al.*, 2013; Munday *et al.*, 2014). However, the structure of fish communities seems to be more affected by indirect effects on habitat complexity of ocean acidification than by observed direct effects of elevated pCO<sub>2</sub> on the behaviour of chronically exposed fish (Munday *et al.*, 2014). Being able to move in and out a pCO<sub>2</sub>

gradient adult herbivorous fish could be advantaged by high CO<sub>2</sub> conditions because of increased food availability following decreased competition with sea urchins (Johnson *et al.*, 2012) and decreased plant chemical defences (Arnold *et al.*, 2012)

Our understanding of algal community change due to elevated CO<sub>2</sub> has evolved through a series of studies at volcanic seeps. Initial work led researchers to conclude that a shift from coralline algae dominated to fleshy algal communities was driven by dissolution effects on calcified algae (Hall-Spencer *et al.*, 2008; Martin *et al.*, 2008; Porzio *et al.*, 2011). Subsequent work investigating macroalgal succession indicated that certain coralline algae were able to withstand dissolution at CO<sub>2</sub> levels predicted for the end of this century, but fleshy algae were able to outcompete them at high CO<sub>2</sub> (Kroeker *et al.*, 2013c). In a comparison of a tropical and a temperate CO<sub>2</sub> seep system, Johnson *et al.* (2012) found that *Padina* spp. cover was higher at elevated CO<sub>2</sub> levels despite lower calcium carbonate content of thalli at the high CO<sub>2</sub> site. They postulated that this was possible since sea urchins, their main grazers, were unable to tolerate high CO<sub>2</sub> conditions (see Calosi *et al.*, 2013a; Bray *et al.*, 2014). More recent work demonstrates that most evidence of community changes does not originate from direct physiological responses of species to ocean acidification, but from indirect ocean acidification effects on habitat changes or trophic interactions (Alsterberg *et al.*, 2013; Fabricius *et al.*, 2014; Munday *et al.*, 2014; Gaylord *et al.*, 2014). Volcanic CO<sub>2</sub> seeps can be used to disentangle the direct and indirect effects of ocean acidification on marine benthic communities. Here we formally test these effects of ocean acidification in experiments along natural pCO<sub>2</sub> gradients with and without grazers present on rocky Mediterranean shores. Specifically, two separate exclusion experiments were used to test



effects of herbivory in benthic communities along pCO<sub>2</sub> gradients. Limpets were excluded on intertidal shores at volcanic seeps off Vulcano (Italy) to examine their role in changes from coralline to fleshy algal assemblages. At volcanic seeps off Methana (Greece), herbivore exclusions were used to test whether herbivores differently affect subtidal algal recruitment as carbon dioxide levels increase.

## **5.2 Methods**

### **5.2.1 Vulcano**

#### **5.2.1.1 Study site**

The aim of this experiment was to examine the role of limpet grazing in driving changes from coralline to fleshy algal assemblages as CO<sub>2</sub> levels increase, and it was conducted in an area off Vulcano Island described in Chapter 3. Along this gradient, the two sites shown in Figure 5.2 were selected, one with average pH<sub>NBS</sub> of about 7.8 (named “1200 ppm”) and one located about 50-60 m farther away from the main seeps (named “600 ppm”), with an average pH of approximately 8.05, slightly lower and more variable than most Mediterranean coastal waters (Boatta *et al.*, 2013). The sites were visited four times during the experiment (start of experiment, i.e. May 2012; July 2012; September 2012; November 2012 - detailed sampling dates and sample sizes are reported in Table 1.1D), and each time pH (NBS scale), temperature and salinity were measured at about 0.5 m depth using a calibrated YSI (556 MPS) pH meter. For pH, means were calculated from hydrogen ion concentrations and then re-converted to pH. The other carbonate chemistry parameters were calculated with CO2Sys (Lewis and Wallace, 1998) using the average total alkalinity value

resulting from monitoring at the site in 2011 ( $2.525 \text{ mMol kg}^{-1}$ , Boatta *et al.*, 2013).

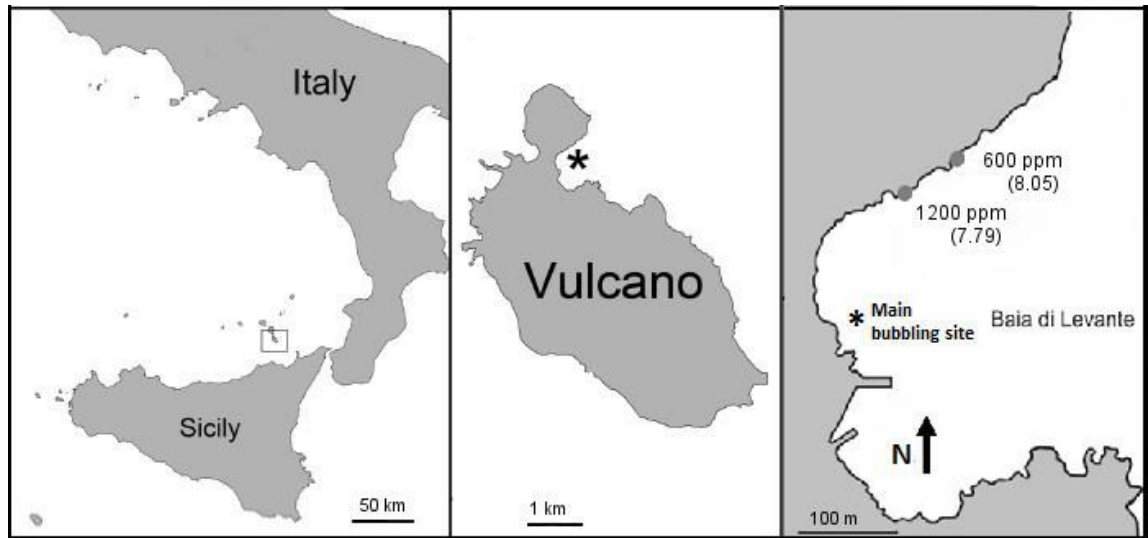


Figure 5.2. Position of Vulcano Island (Sicily, Southern Italy) and of the study area. Asterisk marks the main venting site, grey circles show the two experimental sites, with decreasing  $\text{pCO}_2$  moving away from the bubbling site (modified from Graziano *et al.*, unpublished data). Average pH measured during the experiment shown in parentheses ( $n=6$ ).

### 5.2.1.2 Limpet exclusion

At each site, twelve 15 cm diameter circular plots were selected in the intertidal zone (defined as the area 10 cm above the limit of the canopy-forming algae, *Cystoseira* spp.). All plots were chosen on vertical flat surfaces with similar wave exposure, as limpet grazing is more intense on vertical surfaces (Marco Milazzo, personal communication). Six of these plots were enclosed using 5 cm high copper rings, which are very rarely crossed by limpets (Harley, 2002). The rings were screwed to the substratum and any space between the ring and the rock was filled using epoxy putty. Half rings were attached in the same way to three of the remaining plots to serve as procedural controls, while the three remaining plots were marked with epoxy putty at the corners and were used as controls (Figure 5.3).

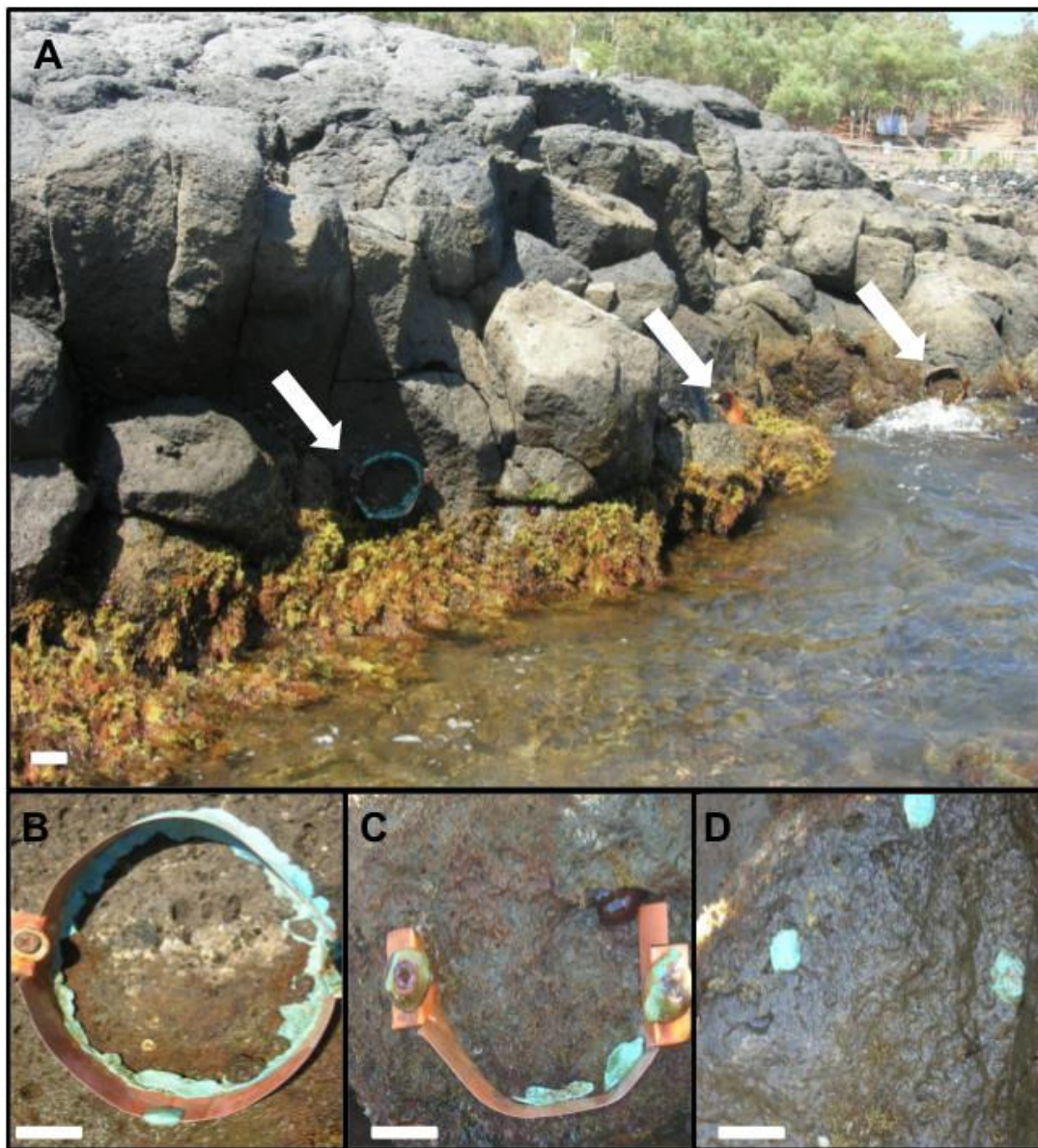


Figure 5.3. Rocky shore on Vulcano showing experimental units (arrows) at the 1200 ppm site during low tide (A). In the lower part of the figure the three treatments are shown: limpet exclosure (15 cm diameter, B), procedural control (C) and control (D); scale bars = 5 cm.

The experiment lasted six months; benthic diversity and abundance in the plots were assessed using visual census approximately every two months (start of experiment, i.e. May 2012; July 2012; September 2012; November 2012). In November 2012, a 10 x 10 cm quadrat in the centre of each plot was denuded of all macroalgae using a hammer and chisel. The samples were preserved in

70% ethanol and were identified to the lowest possible taxonomic level in the laboratory. After identification, algae from each taxon were left to dry at 60°C in the oven for 72h and weighed to obtain dry mass.

#### **5.2.1.3 Limpet abundances and feeding rates**

During each visit, limpet abundance was determined at low tide in nine haphazardly chosen replicate plots per site. The plots were the same size as the experimental plots. Limpets were counted and their shell length measured using a Vernier calliper (accuracy  $\pm 1$  mm). In July, limpet densities were also determined at high tide to determine whether there was a significant difference in limpet densities related to this parameter.

In November 2012, limpet feeding rates were also measured using wax discs (Thompson *et al.*, 1997). Individually numbered 14 mm diameter plastic holders filled with wax were placed in holes drilled in the rocky substratum. In each site, three grids of 16 holes in which wax discs could be placed were drilled, in a 4x4 configuration with 15 cm gaps between each hole. Discs were left for 14 days on the shore and then collected, and in the laboratory number of grazed discs and percentage cover of grazing marks were determined.

#### **5.2.1.4 Statistical analyses**

Benthic species composition and abundance from visual census was tested using a three-factor PERMANOVA. "Site" and "treatment" were considered fixed factors with two (600 and 1200 ppm) and three (exclusion, procedural control and control) levels, respectively, whereas "date" was a random orthogonal factor. A square-root transformation was used to reduce the influence of abundant taxa in the community, a Bray-Curtis dissimilarity matrix was built and

Type III sums of squares with 9,999 unrestricted permutations of the raw data were used to account for small sample sizes. Although the design includes repeated measures on the same plots, sphericity and normality are not necessary for PERMANOVA because the test uses a permutation procedure to generate a distribution for the pseudo-F statistic (analogous to the F statistic in ANOVA). When a limited number of permutations (<100) was available, Monte Carlo p-values were preferred over permutational p-values, which are not reliable in these cases (Anderson *et al.*, 2003). The scraping samples were analysed in the same way, but the experimental design only included the “treatment” and “site” factors. Percent changes in key groups of macroalgae were also analysed using this design.

Variance derived from significant interactions was then decomposed to determine which factor determined the significant interaction, and pairwise tests were performed when necessary. A SIMPER analysis was then used to determine the contribution of each taxon to the average Bray-Curtis dissimilarity between levels of a factor if the PERMANOVA analysis was significant. The same procedure was used to analyse scrapings data, but the design was modified to include only the “site” and “treatment” factors. All analyses above were performed using PRIMER 6 with PERMANOVA+ extension (Plymouth Routines In Multivariate Ecological Research, version 6).

Limpet abundance and length as well as percent cover of marks on wax discs and macroalgal biomass from scrapings were analysed using a two-way ANOVA with “site” and “date” as factors after checking they complied with the normality and variance homogeneity requirements of the analysis. However, no “date” factor was used for the analysis of limpet grazing rates and macroalgal biomass. All the analyses above were performed using SPSS v19.

## 5.2.2 Methana

To assess whether subtidal herbivores differently affect algal recruitment at different carbon dioxide levels, we conducted a second exclusion experiment at Methana CO<sub>2</sub> seeps.

### 5.2.2.1 Herbivore surveys

Off Methana, herbivore densities were determined at a site near the seeps (SEEP) and at a reference site (REF A; see Chapter 2 for sites description - detailed sampling dates and sample sizes are reported in Table 1.1D). Densities of *Paracentrotus lividus* and *Arbacia lixula* were determined separately using transects: individuals present between 1 and 2 m depth were counted by snorkelers along five transects (5 m long and 1 m wide) per site per species in September 2012 and June 2013. Fish community composition and biomass were quantified in September 2013 by Maria Salomidi and Yiannis Issaris using a standard visual census technique (while SCUBA diving) within belt transects of 25 m length and 5 m width placed at 3m depth (three replicates, 125 m<sup>2</sup> surface each). The observer conducting the fish survey moved at constant speed identifying, counting and attributing all individuals to 5 cm size classes within 2.5 m on either side of the 25 m transect line (La Mesa and Vacchi, 1999; Giakoumi *et al.*, 2012). Length estimates of fish from the visual surveys were converted to wet biomass by using the allometric length-biomass conversion:  $B = a L^b$ , where B is biomass in grams and L is total length in cm. The constant parameters a and b corresponding to the closest geographical area were obtained from Morey *et al.* (2003).

### 5.2.2.2 Herbivore exclusion

Four sterile 10 x 10 cm ceramic tiles were attached to rocks using epoxy putty and deployed at the two Methana study sites at ~ 2 m depth by snorkelers as



controls; four tiles per site were enclosed in a 1 cm mesh cage to exclude herbivores, and four additional tiles per site were enclosed in a cage missing one side to act as procedural controls (Figure 5.4). The cages were painted using non-toxic antifouling paint (EP-2000, ePaint, Florida) to prevent epiphytes from growing and shading the tiles. Tiles were deployed in September 2012 and recovered in June 2013, when seaweed biomass reaches its annual peak (Ballesteros, 1984).

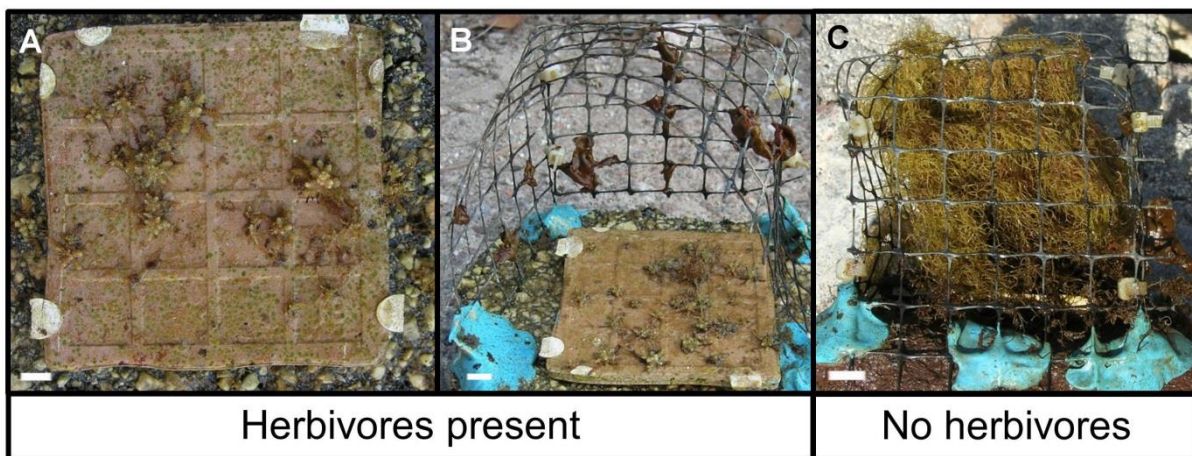


Figure 5.4. Pictures of the three treatments for the herbivore exclusion experiment at Methana taken at the end of the experiment (June 2013): control tile (10 x 10 cm; A), procedural control (B) and herbivore exclusion (C); scale bars = 1 cm.

After recovery, tiles were detached from the rock, put in individual zip-lock bags and stored frozen. In the laboratory, their cover was visually assessed and quantified as percent cover of functional groups. The functional groups used were: fucoid algae (mostly *Cystoseira* sp.), erect brown algae, fleshy brown algae (mostly *Dictyota* sp.), calcifying brown algae (mostly *Padina pavonica*), turf algae (mat-forming algae shorter than 2 cm, mostly *Halopteris scoparia* (Linnaeus) Sauvageau), encrusting black, encrusting green, filamentous green, articulated coralline algae, coralline crustose algae (CCA), serpulid worms, biofilm, bare substratum. The biomass of turf and erect algae was measured by

scraping the algae from the tiles, drying them at 60°C for 72 h and weighing them to obtain dry mass.

### **5.2.2.3 Statistical analyses**

Sea urchin data were analysed with a three-way ANOVA after transforming them (fourth root) to comply with the normality and variance homogeneity requirements of ANOVA. The ANOVA had three fixed factors (species, date and site). Log-transformed biomass of the three recorded herbivorous fish was also analysed using an ANOVA with site and species as fixed factors. All the analyses above were performed using SPSS v19.

Tiles species composition and abundance from visual census was tested using a two-factor PERMANOVA with “site” and “treatment” as fixed factors. A square-root transformation was used to reduce the influence of abundant taxa in the community, a Bray-Curtis dissimilarity matrix was built and Type III sums of squares with 9,999 unrestricted permutations of the raw data were used to account for small sample sizes. Pairwise tests were performed when a factor with more than two levels was significant. A nMDS plot was used to visually inspect the similarities among samples. The same procedure was used to analyse biomass data.

Percent cover or biomass changes in key groups of macroalgae were analysed with a permutational ANOVA using the experimental design described above, but using dissimilarity matrices based on Euclidean distances. Percent cover was used for those functional groups that could not be reliably scraped from the tile (i.e. CCA, encrusting green, encrusting black, biofilm and bare substratum). All analyses above were performed using PRIMER 6 with PERMANOVA+ extension (Plymouth Routines In Multivariate Ecological Research, version 6).



## 5.3 Results

### 5.3.1 Vulcano

#### 5.3.1.1 Environmental parameters

Measured and calculated carbonate chemistry parameters are shown in Table 5.1. Over the experiment duration, pH in the 1200 ppm site was approximately 7.8, more than 0.2 points lower than the 600 ppm site. In contrast, measured temperature and salinity were not significantly different between the two sites. At the elevated CO<sub>2</sub> site, seawater pCO<sub>2</sub> was double than in the reference site, even though seawater was still saturated with respect to both calcite and aragonite.

Table 5.1. Mean ( $\pm$  SD, n=6) pH, temperature (T) and salinity (S) measured during the experiment at Vulcano between May - October 2012 and pCO<sub>2</sub>, bicarbonate ions (HCO<sub>3</sub><sup>-</sup>), carbonate ions (CO<sub>3</sub><sup>2-</sup>), seawater saturation with respect to calcite ( $\Omega_{Ca}$ ) and aragonite ( $\Omega_{Ar}$ ) calculated using CO2Sys.

|                 | <b>pH<sub>NBS</sub></b> | <b>T</b><br>(°C)    | <b>S</b><br>(ppt)   | <b>pCO<sub>2</sub></b><br>( $\mu$ atm) | <b>HCO<sub>3</sub><sup>-</sup></b><br>(mmol<br>kg <sup>-1</sup> ) | <b>CO<sub>3</sub><sup>2-</sup></b><br>(mmol<br>kg <sup>-1</sup> ) | <b><math>\Omega_{Ca}</math></b> | <b><math>\Omega_{Ar}</math></b> |
|-----------------|-------------------------|---------------------|---------------------|--|---|---|---------------------------------|---------------------------------|
| <b>600 ppm</b>  | 8.05<br>$\pm$ 0.04      | 25.03<br>$\pm$ 1.20 | 38.72<br>$\pm$ 0.15 | 602<br>$\pm$ 51                        | 2025<br>$\pm$ 39  | 205<br>$\pm$ 16   | 4.77<br>$\pm$ 0.37              | 3.16<br>$\pm$ 0.25              |
| <b>1200 ppm</b> | 7.79<br>$\pm$ 0.17      | 25.20<br>$\pm$ 2.78 | 38.72<br>$\pm$ 0.39 | 1211<br>$\pm$ 192                      | 2200<br>$\pm$ 53  | 133<br>$\pm$ 22   | 3.10<br>$\pm$ 0.50              | 2.06<br>$\pm$ 0.33              |

#### 5.3.1.2 Limpet exclusion, abundance and feeding rates

Copper rings were highly effective at excluding limpets from experimental plots. No limpets were found in the enclosure plots during subsequent visits, except for July, when 1-2 small limpets (length < 2 mm) had recruited into three enclosure plots at the 600 ppm site, but they were removed and no limpets crossed the copper rings. The visual census data and the scraping data had

similar results, so only the visual census data analysis is reported for simplicity. Results from the PERMANOVA analysis (Table 5.2) show that the experimental treatment had a different effect at the two sites (Site\*Treatment pseudo- $F_{2,68} = 3.997$ ,  $p(\text{perm}) = 0.0086$ ).

Table 5.2. PERMANOVA analyses of square-root transformed percentage benthic cover in the experimental plots for the experiment performed at Vulcano from May to October 2012. The first table shows main factors and their interactions and degrees of freedom (df), sum of squares (SS), pseudo-F, permutational p and unique permutations for each of them. Treatment x Date interaction and Date both have a significant effect ( $p < 0.05$ ). The second table shows pair-wise comparisons between treatments at both sites with no significant differences between the t-values of any of the treatments at the 1200 ppm site, while all comparisons were significant at the 600 ppm site.

| Source                    | df | SS       | Pseudo-F | p(perm)       | Unique perms |
|---------------------------|----|----------|----------|---------------|--------------|
| Site                      | 1  | 11014.0  | 8.3517   | <b>0.0054</b> | 6367         |
| Treatment                 | 2  | 3322.3   | 2.5128   | 0.0926        | 9950         |
| Date                      | 3  | 20601.0  | 5.9490   | <b>0.0001</b> | 9930         |
| Site x Treatment          | 2  | 2337.8   | 3.9974   | <b>0.0086</b> | 9955         |
| Site x Date               | 3  | 3996.6   | 1.1541   | 0.3159        | 9938         |
| Treatment x Date          | 6  | 3827.9   | 0.5527   | 0.9450        | 9905         |
| Site x Treatment x Date** | 5  | 1374.9   | 0.2382   | 0.9989        | 9927         |
| Res                       | 68 | 78493.0  |          |               |              |
| Total                     | 90 | 133570.0 |          |               |              |

\*\* Term has one or more empty cells

| Within level '1200 ppm' of factor 'Site' |         |         |              |
|--|---------|---------|--------------|
| Groups                                   | t       | p(perm) | Unique perms |
| exclosure, proc control                  | 0.81312 | 0.5255  | 1259         |
| exclosure, control                       | 1.2114  | 0.2832  | 4344         |
| proc control, control                    | 1.0702  | 0.3976  | 420          |

| Within level '600 ppm' of factor 'Site' |        |               |              |
|---|--------|---------------|--------------|
| Groups                                  | t      | P(perm)       | Unique perms |
| exclosure, proc control                 | 7.4724 | <b>0.0016</b> | 840          |
| exclosure, control                      | 2.021  | <b>0.0444</b> | 840          |
| proc control, control                   | 4.2419 | <b>0.0019</b> | 840          |

There was also a significant difference between sampling dates (pseudo- $F_{3,68}=5.949$ ,  $p(\text{perm})=0.0001$ ), which was consistent among sites and

treatments. Pair-wise comparisons between treatments in each site obtained by decomposing the variance in the site\*treatment interaction are shown in the lower part of Table 5.2. It is evident that the 600 ppm site had a much higher heterogeneity compared to the elevated CO<sub>2</sub> site because all pairwise comparisons were significant in the former site. However, this means that no conclusion on the overall treatment effect can be drawn.

The SIMPER analysis between sites and among treatment levels at the 600 ppm site showed which taxa contributed the most to the detected differences (Table 5.3). The main drivers of differences between sites were bare rock and brown turf, which together account for almost 40% of the total variability. Both categories increased at the 1200 ppm site, whereas *Padina*, CCA, *Dictyotales* and *Cystoseira* showed the opposite trend. The main drivers of differences among treatments were the dominant categories such as turf algae and bare substratum. Those taxa that changed most among treatment levels such as the calcareous brown alga *Padina pavonica* and the barnacle *Chtamalus stellatus* were also important determinants of the differences between treatments.

Table 5.3. SIMPER analysis showing the average dissimilarities between sites, as well as that among treatments at the 600 ppm site at Vulcano in 2012 pooling dates and which cover group contributes to the dissimilarity up to 90%. For each species, the average abundance in the two groups that are being compared, their average dissimilarity, the dissimilarity to standard deviation ration and the taxon contribution and cumulative contribution are shown.

| <b>Groups 600 ppm and 1200 ppm; Average dissimilarity = 53.94</b> |                 |                 |                |                |                 |              |
|---|-----------------|-----------------|----------------|----------------|-----------------|--------------|
| <b>Taxa</b>   | <b>1200 ppm</b> | <b>600 ppm</b>  | <b>Av.Diss</b> | <b>Diss/SD</b> | <b>Contrib%</b> | <b>Cum.%</b> |
|   | <b>Av.Abund</b> | <b>Av.Abund</b> |                |                |                 |              |
| Bare rock   | 5.22            | 4.14            | 10.81          | 1.30           | 20.04           | 20.04        |
| Brown turf  | 6.26            | 5.10            | 9.56           | 1.25           | 17.72           | 37.77        |
| <i>Padina</i>   | 0.64            | 2.64            | 7.08           | 1.10           | 13.13           | 50.89        |
| CCA   | 0.60            | 2.49            | 6.68           | 1.33           | 12.38           | 63.28        |

|   |                  |                     |                |                |                 |              |
|---|------------------|---------------------|----------------|----------------|-----------------|--------------|
| <i>Dictyotales</i>  | 0.19             | 1.22                | 3.59           | 0.80           | 6.66            | 69.94        |
| <i>Cystoseira</i>   | 0.51             | 0.68                | 2.89           | 0.62           | 5.36            | 75.30        |
| <i>Anadyomene</i>   | 0.53             | 0.55                | 2.38           | 0.80           | 4.40            | 79.70        |
| <i>Chthamalus</i>   | 0.05             | 0.76                | 2.26           | 0.59           | 4.19            | 83.89        |
| <i>Dasycladus</i>   | 0.09             | 0.73                | 2.16           | 0.58           | 4.00            | 87.89        |
| Green turf  | 0.43             | 0.05                | 1.45           | 0.31           | 2.68            | 90.57        |
| <b>Groups exclosure and control; Average dissimilarity = 42.52</b>      |                  |                     |                |                |                 |              |
|   | <b>Exclosure</b> | <b>Control</b>      |                |                |                 |              |
| <b>Taxa</b>   | <b>Av.Abund</b>  | <b>Av.Abund</b>     | <b>Av.Diss</b> | <b>Diss/SD</b> | <b>Contrib%</b> | <b>Cum.%</b> |
| Bare rock   | 4.04             | 4.82                | 9.06           | 1.4            | 21.31           | 21.31        |
| Brown turf  | 5.35             | 4.97                | 6.45           | 1.07           | 15.17           | 36.48        |
| <i>Padina</i>   | 2.73             | 1.32                | 5.87           | 1.24           | 13.81           | 50.29        |
| Dictyotales   | 1.02             | 1.86                | 4.72           | 0.96           | 11.1            | 61.39        |
| CCA   | 2.68             | 3.01                | 3.93           | 1.11           | 9.24            | 70.63        |
| <i>Chthamalus</i>   | 1.28             | 0.19                | 3.04           | 0.74           | 7.14            | 77.77        |
| <i>Cystoseira</i>   | 0.9              | 0.26                | 2.85           | 0.64           | 6.71            | 84.47        |
| <i>Dasycladus</i>   | 0.74             | 0.48                | 2.28           | 0.69           | 5.36            | 89.83        |
| <i>Anadyomene</i>   | 0.55             | 0.29                | 1.34           | 0.64           | 3.16            | 92.99        |
| <b>Groups exclosure and proc control; Average dissimilarity = 45.14</b> |                  |                     |                |                |                 |              |
|   | <b>Exclosure</b> | <b>Proc control</b> |                |                |                 |              |
| <b>Taxa</b>   | <b>Av.Abund</b>  | <b>Av.Abund</b>     | <b>Av.Diss</b> | <b>Diss/SD</b> | <b>Contrib%</b> | <b>Cum.%</b> |
| <i>Padina</i>   | 2.73             | 3.77                | 7.66           | 1.22           | 16.98           | 16.98        |
| Bare rock   | 4.04             | 3.69                | 7.51           | 1.11           | 16.63           | 33.6         |
| Brown turf  | 5.35             | 4.75                | 6.56           | 1.06           | 14.53           | 48.14        |
| CCA   | 2.68             | 1.59                | 5.28           | 1.23           | 11.71           | 59.84        |
| Dictyotales   | 1.02             | 0.97                | 3.17           | 0.89           | 7.03            | 66.87        |
| <i>Chthamalus</i>   | 1.28             | 0.29                | 3.06           | 0.76           | 6.78            | 73.65        |
| <i>Cystoseira</i>   | 0.9              | 0.66                | 2.99           | 0.8            | 6.62            | 80.27        |
| <i>Dasycladus</i>   | 0.74             | 0.94                | 2.97           | 0.75           | 6.58            | 86.84        |
| <i>Anadyomene</i>   | 0.55             | 0.8                 | 1.77           | 0.8            | 3.92            | 90.76        |
| <b>Groups control and proc control; Average dissimilarity = 48.22</b>   |                  |                     |                |                |                 |              |
|   | <b>Control</b>   | <b>Proc control</b> |                |                |                 |              |
| <b>Taxa</b>   | <b>Av.Abund</b>  | <b>Av.Abund</b>     | <b>Av.Diss</b> | <b>Diss/SD</b> | <b>Contrib%</b> | <b>Cum.%</b> |
| Bare rock   | 4.82             | 3.69                | 9.59           | 0.99           | 19.88           | 19.88        |
| <i>Padina</i>   | 1.32             | 3.77                | 9.31           | 1.14           | 19.31           | 39.2         |
| Brown turf  | 4.97             | 4.75                | 7.23           | 0.88           | 15              | 54.19        |
| CCA   | 3.01             | 1.59                | 5.12           | 0.97           | 10.62           | 64.81        |
| Dictyotales   | 1.86             | 0.97                | 4.91           | 0.96           | 10.18           | 74.99        |
| <i>Dasycladus</i>   | 0.48             | 0.94                | 2.93           | 0.64           | 6.07            | 81.06        |
| <i>Cystoseira</i>   | 0.26             | 0.66                | 2.24           | 0.71           | 4.65            | 85.71        |
| <i>Anadyomene</i>   | 0.29             | 0.8                 | 2.04           | 0.85           | 4.24            | 89.95        |
| <i>Chthamalus</i>   | 0.19             | 0.29                | 0.94           | 0.49           | 1.96            | 91.91        |

Some individual species showed patterns related to the experimental treatment (Figure 5.5). The treatments had a significant effect on percent cover of *Padina pavonica* and *Dictyota* sp., but there was no pattern coherent with grazing reduction (i.e. control and procedural control had different values), meaning that these two taxa likely responded to some artefact effect such as changes in light

or water circulation. On the other hand, the barnacle *Chthamalus stellatus* and the red alga *Laurencia* sp. significantly increased their cover when limpets were excluded, the former only at the 600 ppm site and the latter showing a diminished effect at the elevated CO<sub>2</sub> site.

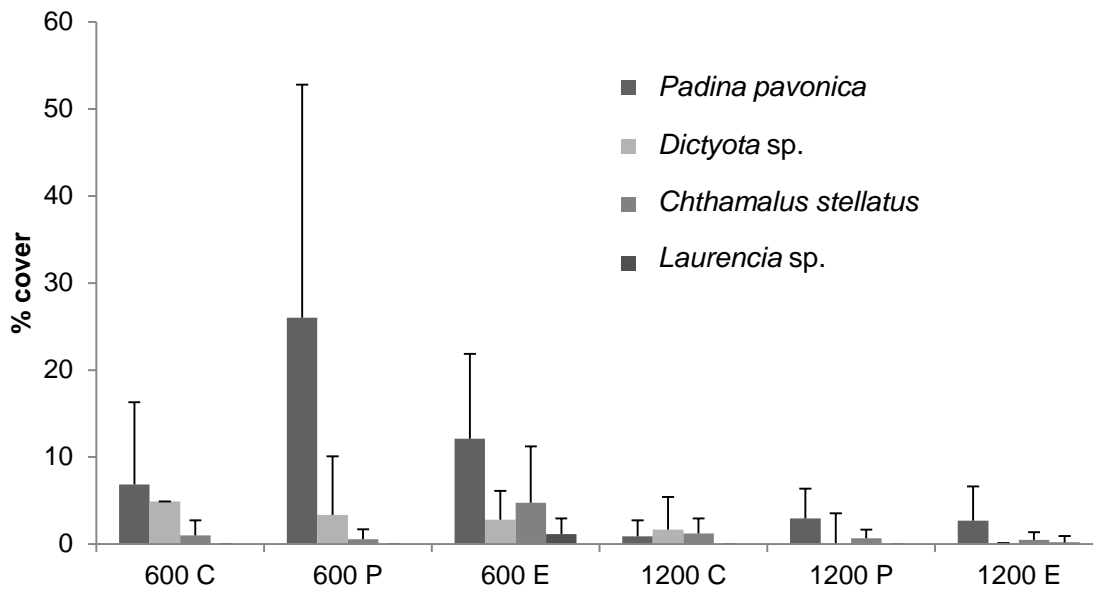


Figure 5.5. Mean percent cover ( $\pm$  SD, n=9-18) of species significantly affected by experimental treatments at the exclusion experiment at Vulcano in 2012 (C is control, P is procedural control, E is exclusion) pooling sampling dates at the 600 ppm and 1200 ppm sites.

Statistical analysis of limpet abundance data shows that both site (pseudo- $F_{1,4}=18.223$ ,  $p(\text{perm})=0.006$ ) and date (pseudo- $F_{4,78}=3.5842$ ,  $p(\text{perm})=0.01$ ) had a significant effect. Pairwise comparisons confirm that at the 1200 ppm site there was no significant seasonal pattern. Conversely, at the 600 ppm site limpet abundances were higher than those in the 1200 ppm site in spring and summer, but in autumn there was a sudden drop in limpet densities, bringing their values close to those of the 1200 ppm site (Figure 5.6). Limpet abundances sampled at high and low tide in July did not differ significantly.

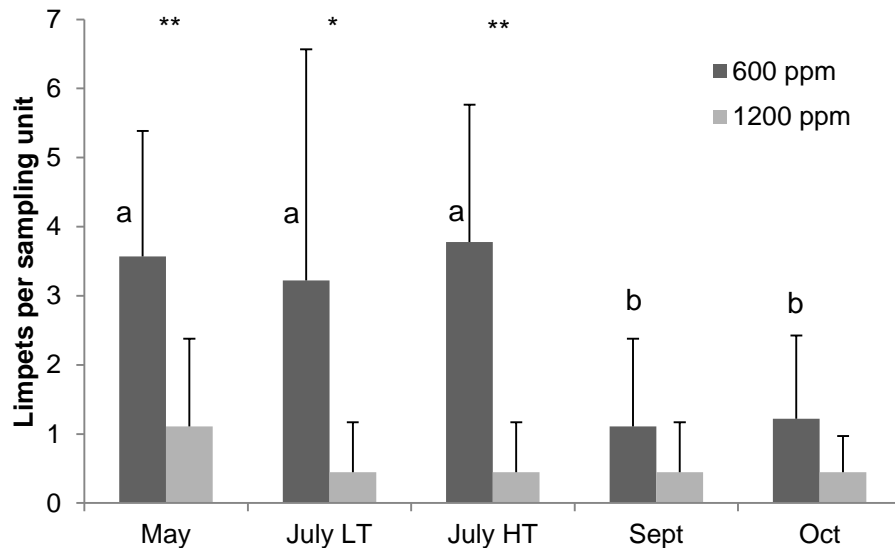


Figure 5.6. Mean ( $\pm$  SD,  $n=9$ ) limpet abundance per sampling unit on the four sampling dates for 600 ppm and 1200 ppm sites at Vulcano during the exclusion experiment (May-October 2012). In July limpet densities were assessed twice, once at low tide (LT) and once at high tide (HT) to determine the variability of limpet abundances within a tidal cycle; all other densities were determined at low tide. Different letters mean that limpet abundances are significantly different among sampling dates. Asterisks show when the two sites are significantly different (\* =  $p < 0.05$ ; \*\* =  $p < 0.01$ ).

As for limpet length measurements, the permutational ANOVA results report only a significant effect of site (pseudo- $F_{1,7}=17.861$ ,  $p(\text{perm})=0.028$ ). Limpets from the 1200 ppm site were bigger than those living in the 600 ppm site, especially in spring and summer. This difference, however, was never significant in pairwise comparisons and was strongly reduced in autumn (Figure 5.7).

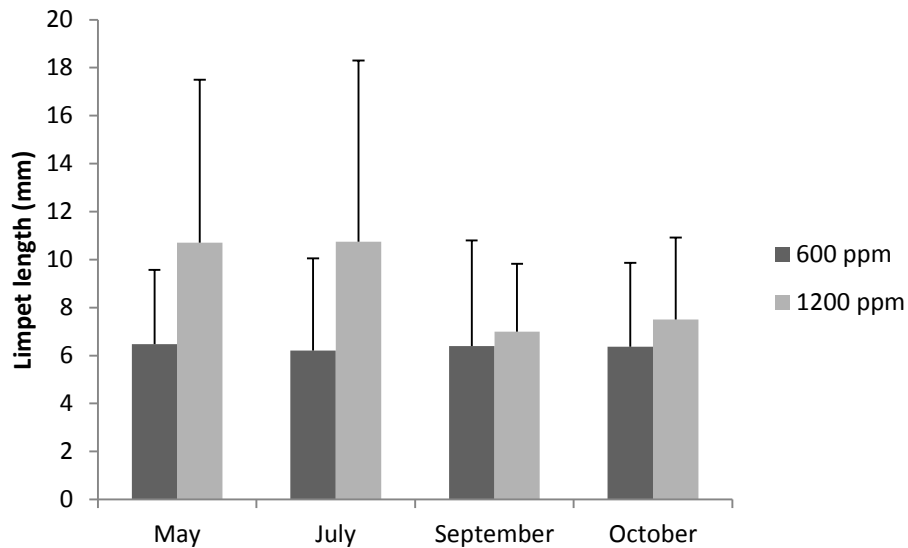


Figure 5.7. Mean ( $\pm$  SD) limpet length in the four sampling dates (May, July, September and October 2012) at the 600 and 1200 ppm sites off Vulcano. Limpets in the 1200 ppm site are slightly longer than those in the 600 ppm site. N=4-29.

After verifying that quadrat and position of disc in the quadrat (high or low) had no significant effect on the percentage of grazed disc, arcsin-transformed data were analysed using a one-way ANOVA. Results from the analysis show that there was no significant difference in grazing rates between sites, even though there was a clear trend for higher grazing rates at the 600 ppm site (Figure 5.8).

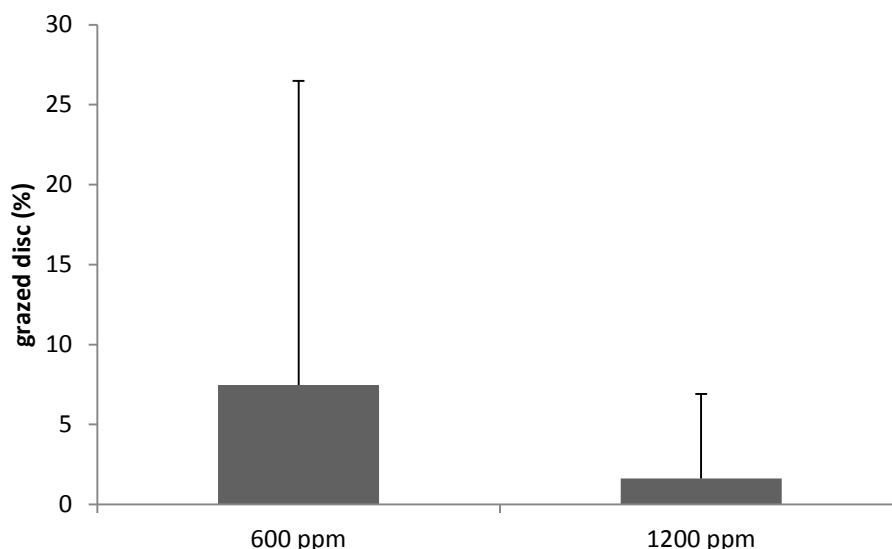


Figure 5.8: Mean ( $\pm$  SD) percentage of wax disc grazed by limpets in the 600 ppm site (n=41) and the 1200 ppm site (n = 34) at Vulcano in November 2012.

### 5.3.2 Methana

#### 5.3.2.1 Environmental parameters

Measured and calculated carbonate chemistry parameters are shown in Table 5.4. The mean pH near the seeps was approximately 7.7, more than 0.3 points lower than the reference site. On the other hand, temperature and salinity were not significantly different between the two sites. At the high CO<sub>2</sub> site, seawater pCO<sub>2</sub> was double that of the reference site, even though on average seawater was still saturated with respect to both calcite and aragonite.

Table 5.4. Mean ( $\pm$  SD, n=11-24) pH, temperature (T) and salinity (S) measured at Methana in September 2012 and June 2013 as well as pCO<sub>2</sub>, bicarbonate ions (HCO<sub>3</sub><sup>-</sup>), carbonate ions (CO<sub>3</sub><sup>2-</sup>), seawater saturation with respect to calcite ( $\Omega_{Ca}$ ) and aragonite ( $\Omega_{Ar}$ ) calculated using CO2Sys.

|              | pH <sub>NBS</sub>  | T<br>(°C)           | S<br>(ppt)          | pCO <sub>2</sub><br>( $\mu$ atm) | HCO <sub>3</sub> <sup>-</sup><br>(mmol<br>kg <sup>-1</sup> ) | CO <sub>3</sub> <sup>2-</sup><br>(mmol<br>kg <sup>-1</sup> ) | $\Omega_{Ca}$      | $\Omega_{Ar}$      |
|--------------|--------------------|---------------------|---------------------|----------------------------------|--|--|--------------------|--------------------|
| <b>SEEP</b>  | 7.70<br>$\pm$ 0.16 | 25.34<br>$\pm$ 0.85 | 38.77<br>$\pm$ 0.93 | 1676.8<br>$\pm$ 643.5            | 2485.4<br>$\pm$ 112.4  | 125.0<br>$\pm$ 46.5  | 2.91<br>$\pm$ 1.06 | 1.93<br>$\pm$ 0.71 |
| <b>REF A</b> | 8.09<br>$\pm$ 0.06 | 25.01<br>$\pm$ 1.05 | 38.94<br>$\pm$ 0.87 | 586.9<br>$\pm$ 106.7             | 2140.5<br>$\pm$ 63.3   | 232.1<br>$\pm$ 25.9  | 5.40<br>$\pm$ 0.59 | 3.57<br>$\pm$ 0.39 |



### 5.3.2.2 Herbivore surveys

Sea urchin densities significantly differed between REF A and SEEP, and the densities of the two species were significantly different as well (Table 5.5). On the other hand, no effect of date was detected, and the lack of significant interactions indicates that both *A. lixula* and *P. lividus* densities changed consistently between sites. As no significant effect of date was detected, sea urchin densities were pooled between dates for easier representation.

Table 5.5. ANOVA on fourth-root transformed sea urchin densities measured at Methana in September 2012 and June 2013. The table shows main factors and their interactions and sum of squares (SS), degrees of freedom (df), Mean Squares (MS), F-ratios (F) and p values. Significant p values (< 0.05) are highlighted.

| Source                | SS     | df | MS    | F      | p                 |
|-----------------------|--------|----|-------|--------|-------------------|
| Site                  | 5.629  | 1  | 5.629 | 17.047 | <b>&lt; 0.001</b> |
| Date                  | 0.704  | 1  | 0.704 | 2.131  | 0.153             |
| Species               | 3.952  | 1  | 3.952 | 11.969 | <b>0.001</b>      |
| Site * Date           | 0.085  | 1  | 0.085 | 0.257  | 0.615             |
| Site * Species        | 0.029  | 1  | 0.029 | 0.089  | 0.767             |
| Date * Species        | 0.487  | 1  | 0.487 | 1.476  | 0.232             |
| Site * Date * Species | 0.068  | 1  | 0.068 | 0.206  | 0.653             |
| Error                 | 11.887 | 36 | 0.330 |        |                   |
| Total                 | 51.118 | 44 |       |        |                   |

Densities of *A. lixula* were consistently higher than those of *P. lividus* (Figure 5.9), with average densities of the former species ranging from 1.9 to 7.5 individuals in a five-metre transect. On the other hand, *P. lividus* densities ranged from 0.2 to 1.6 individuals. There was also a clear reduction in the densities of both species near the seeps, with *P. lividus* being almost absent at the high CO<sub>2</sub> site.

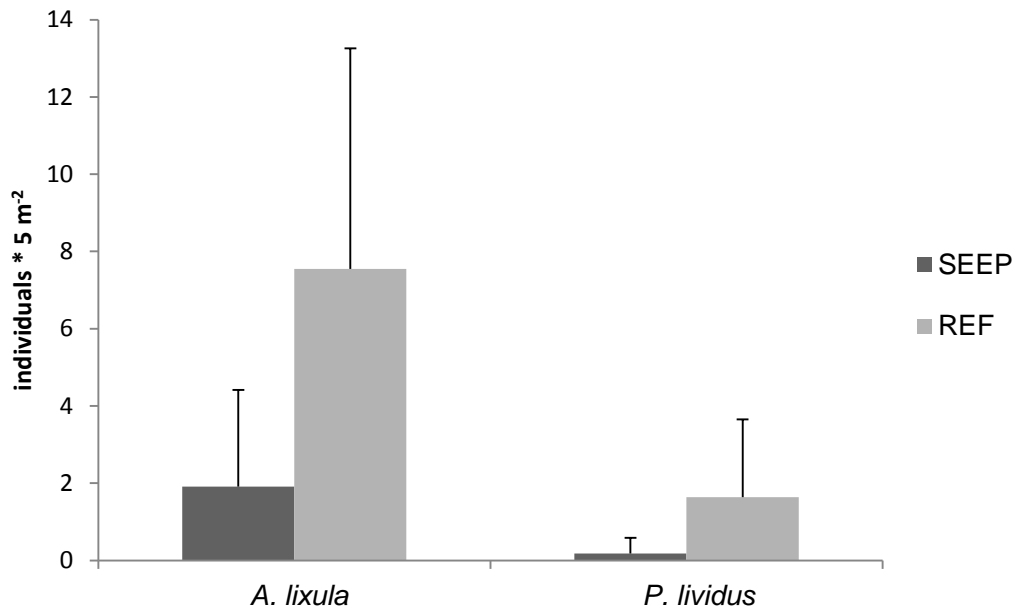


Figure 5.9. Average number ( $\pm$  SD, n=11) of sea urchin individuals along a 5 m transect at Methana study sites (SEEP and REF A) pooling data from September 2012 and June 2013.

Three herbivorous fish species were recorded at the study sites: *Sarpa salpa*, *Siganus luridus* and *Sparisoma cretense*. Results from ANOVA (Table 5.6) show that, just as with sea urchins, both site and species had a significant effect on fish biomass. No significant interactions were found, meaning that changes in all species' biomass between sites followed a similar pattern.

Table 5.6. ANOVA on log-transformed herbivorous fish biomass at Methana in June 2013 showing main factors and their interactions and sum of squares (SS), degrees of freedom (df), Mean Squares (MS), F-ratios (F) and p values. Significant p values (< 0.05) are highlighted.

| Source         | SS      | df | MS     | F     | p            |
|----------------|---------|----|--------|-------|--------------|
| Species        | 52.403  | 2  | 26.201 | 4.291 | <b>0.039</b> |
| Site           | 35.608  | 1  | 35.608 | 5.831 | <b>0.033</b> |
| Species * Site | 17.190  | 2  | 8.595  | 1.408 | 0.282        |
| Error          | 73.279  | 12 | 6.107  |       |              |
| Total          | 363.430 | 18 |        |       |              |

All herbivorous fish species increased in biomass (i.e. total biomass per 25 m transect) near the seeps (Figure 5.10), but the magnitude of the change was very different among species: while *S. cretense* had a low biomass that changed very little between sites, the two other species had very marked changes in biomass between sites. *S. luridus* was present at both sites and its mean biomass increased from 65 to 1565 g from REF A to SEEP. *S. salpa* was absent from REF A, while at SEEP it was the dominant species in terms of biomass ( $2009 \pm 3145$  g).

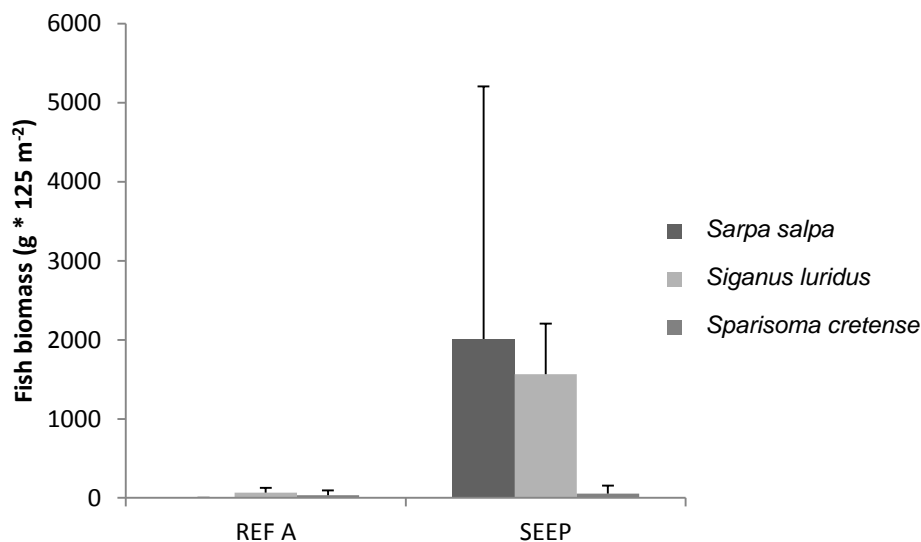


Figure 5.10. Average total biomass ( $\pm$  SD,  $n=3$ ) of herbivorous fish (*Sarpa salpa*, *Siganus luridus* and *Sparisoma cretense*) per 25 m transect at REF A and SEEP in September 2013.

### 5.3.2.3 Herbivore exclusion

PERMANOVA analysis of tiles cover (Table 5.7) shows that both site and treatment had a significant effect on benthic assemblages, but there was no interaction between the two factors. Since the treatment factor was significant, pairwise comparisons were performed among treatment levels to detect which

pairs were significantly different. The results (Table 5.7, lower part) show that exclusions were significantly different from both control and procedural control, which did not differ between each other.

Table 5.7. PERMANOVA on square-root transformed percentage cover of the uncaged and caged tiles deployed at Methana from September 2012 to June 2013. The first table shows main factors and their interactions and degrees of freedom (df), sum of squares (SS), Mean Square (MS), pseudo-F, permutational p and unique permutations for each of them. The second table shows pair-wise comparisons between treatments pooling sites; for each comparison the t value, p value and number of unique permutations are shown.

| Source           | df | SS     | MS     | Pseudo-F | P(perm)       | Unique perms |
|------------------|----|--------|--------|----------|---------------|--------------|
| Site             | 1  | 5380.7 | 5380.7 | 5.3584   | <b>0.0003</b> | 9938         |
| Treatment        | 2  | 11675  | 5837.4 | 5.8133   | <b>0.0001</b> | 9937         |
| Site x Treatment | 2  | 2318.5 | 1159.2 | 1.1544   | 0.3204        | 9945         |
| Residual         | 15 | 15062  | 1004.2 |          |               |              |
| Total            | 20 | 34487  |        |          |               |              |

| Groups                  | t      | P(perm)       | Unique perms |
|-------------------------|--------|---------------|--------------|
| Control, Exclusion      | 2.7397 | <b>0.0001</b> | 9937         |
| Control, Proc control   | 1.2182 | 0.2271        | 9918         |
| Exclusion, Proc control | 2.3722 | <b>0.0009</b> | 9878         |

The MDS plot (Figure 5.11) shows that SEEP and REF A were clearly different for all treatments. Controls and procedural controls were closely grouped whereas exclusion tiles were very different. At the SEEP site, a different group of algae (erect brown algae, fleshy brown algae, calcifying brown algae) was dominant in each exclusion tile, whereas at the reference site there was mostly an increase in calcifying brown algal cover when herbivores were excluded.

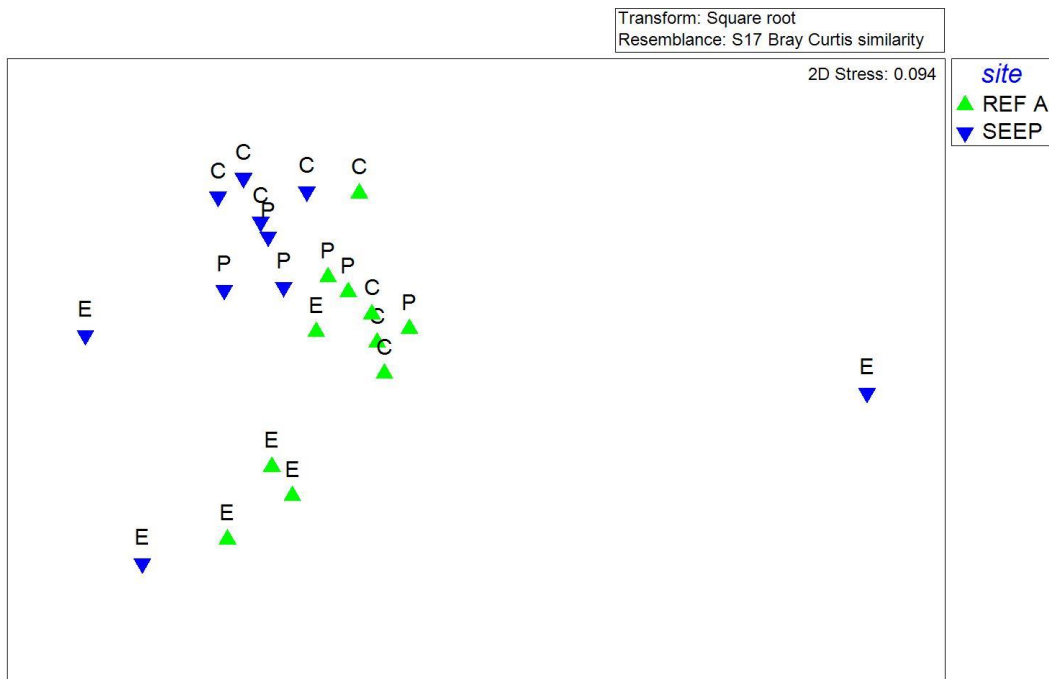


Figure 5.11: MDS plot for the herbivore exclusion experiment performed at Methana from September 2012 to June 2013; green triangles represent tiles placed in REF A, blue triangles are tiles placed in SEEP. Letters above the symbols represent the treatments: C is control, P is procedural control, E is exclusion.

Statistical analysis of the scraping data produced results analogous to the percent cover data at the community level, so only the latter are reported as they are more comprehensive (i.e. they also include encrusting forms). Total biomass significantly differed among treatments (Table 5.8), ranging from about 0.1 g in the controls to approximately 3 g in the exclusions (Figure 5.12). However, at the reference site procedural controls had values intermediate between controls and exclusions.

Table 5.8. PERMANOVA on square-root transformed biomass of macroalgae growing on uncaged and caged tiles deployed at Methana from September 2012 to June 2013. The table shows main factors and their interactions and degrees of freedom (df), sum of squares (SS), Mean Square (MS), pseudo-F, permutational p and unique permutations for each of them.

| Source           | df | SS     | MS     | Pseudo-F | p(perm)       | Unique perms |
|------------------|----|--------|--------|----------|---------------|--------------|
| Site             | 1  | 0.2548 | 0.2548 | 1.5942   | 0.2271        | 9835         |
| Treatment        | 2  | 8.3648 | 4.1824 | 26.166   | <b>0.0002</b> | 9955         |
| Site x Treatment | 2  | 0.3737 | 0.1869 | 1.1691   | 0.334         | 9959         |
| Residual         | 15 | 2.3976 | 0.1598 |          |               |              |
| Total            | 20 | 11.706 |        |          |               |              |

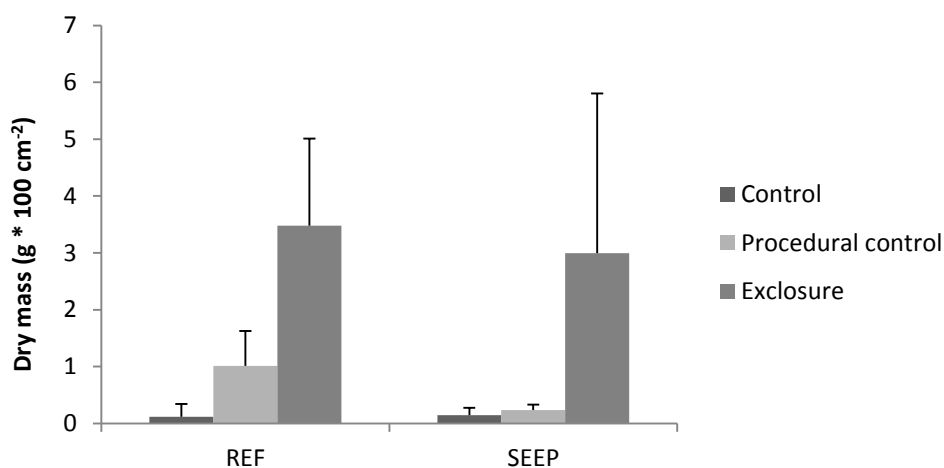


Figure 5.12. Average biomass ( $\pm$  SD) of fleshy and erect algae grown on tiles for all three treatments of the herbivore exclusion experiment conducted at Methana from September 2012 to June 2013;  $n = 3-4$ .

Eight functional groups were significantly different between treatments or sites, four turf or erect and four encrusting forms. Overall, turf and erect algae increased in herbivore exclusions (Figure 5.13A), whereas encrusting forms showed the opposite trend (Figure 5.13B). Biofilm percent cover did not show any clear effect of herbivore exclusion, but it significantly increased at the high CO<sub>2</sub> site. The effect of herbivore exclusion was always clear at SEEP, while at

REF A some functional groups (turf algae, CCA and bare substratum) had biomass or cover values similar between exclusion and procedural control. There were significant differences between sites as well, with turf algae, calcifying brown algae and CCA decreasing as CO<sub>2</sub> increased and fucoid algae, fleshy brown algae, biofilm and bare substratum showing the opposite trend.

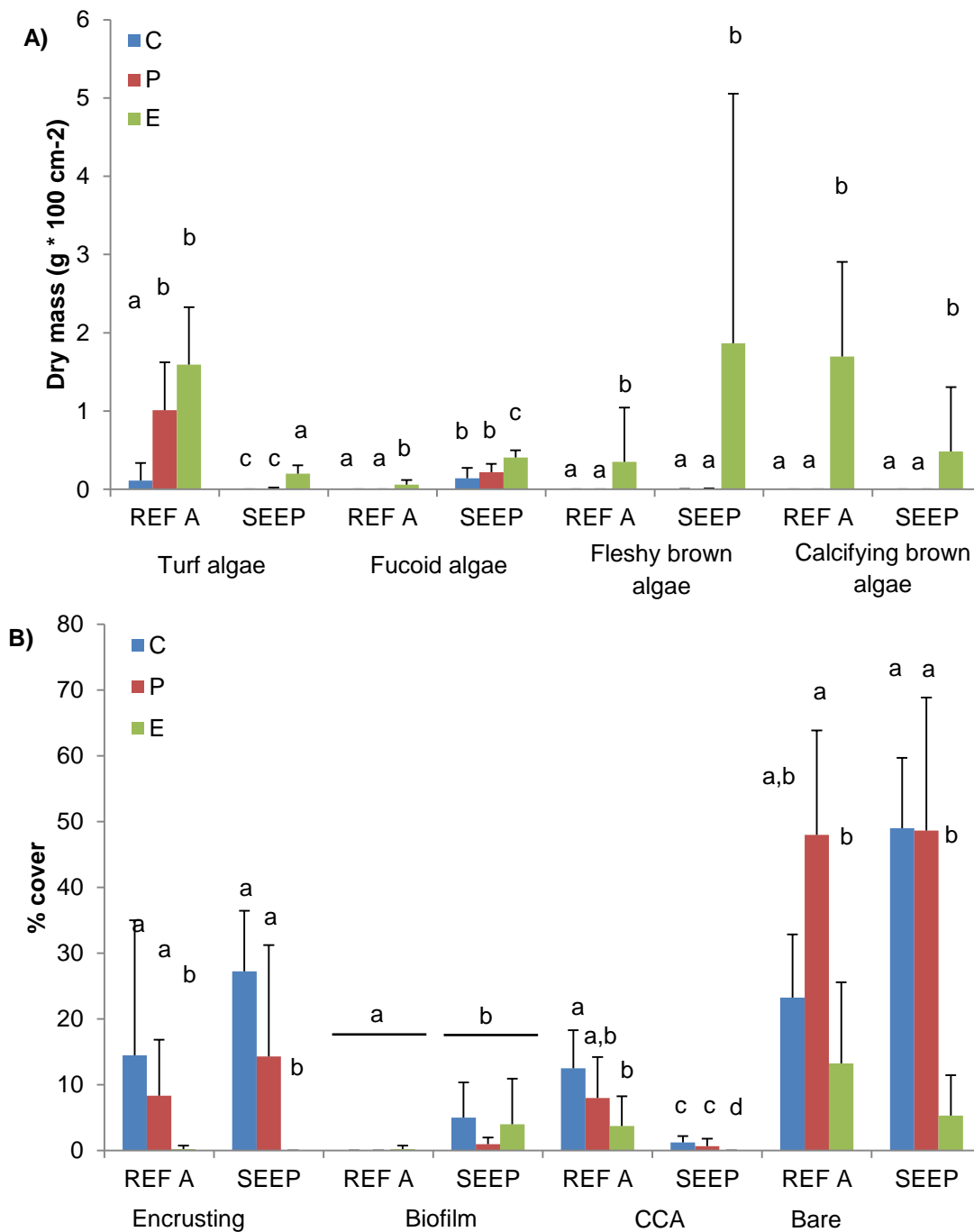


Figure 5.13. Mean ( $\pm$  SD, n=3-4) biomass (A) or percent cover (B) of functional groups that showed significant differences between sites (REF A and SEEP) or treatments (C=controls;

P=procedural controls; E=herbivore exclusions). Different letters indicate significantly different sub-groups within a functional group.

## 5.4 Discussion

The relative role of bottom-up and top-down processes in shaping marine ecosystems has long been a critical issue in marine ecology research. Previous research has shown that relative importance of these two types of processes is highly context-dependant (Burkepile and Hay, 2006). This study shows that in Mediterranean intertidal and subtidal rocky reefs increased pCO<sub>2</sub> (bottom-up) has a significant effect on benthic communities. On the other hand, limpet herbivory (top-down) only had a weak influence on benthic communities at an intertidal rocky shore off Vulcano (Italy), but sea urchin and fish grazing strongly controlled seaweed biomass and community structure in a subtidal habitat off Methana (Greece), even though herbivore community composition changed dramatically as CO<sub>2</sub> levels increased.

Direct effects of carbon dioxide were mostly consistent between Vulcano and Methana. In both areas, bare substratum increased and CCA cover decreased with increasing CO<sub>2</sub>. Many crustose coralline algae are well known to be sensitive to ocean acidification (Kroeker *et al.*, 2010; Brodie *et al.*, 2014), and even tolerant species can be outcompeted by non-calcifying algae at elevated CO<sub>2</sub> levels (Kroeker *et al.*, 2013c). The “bare substratum” functional group used at Vulcano included biofilms, which are known to increase in high CO<sub>2</sub> environments (Johnson *et al.*, 2013; Kroeker *et al.*, 2013; Taylor *et al.*, 2014).

At Methana, fleshy brown and furoid algae also significantly increased near the seeps, whereas *Padina pavonica* abundance decreased with increased CO<sub>2</sub> at both sites. Furoid algae are commonly found in high abundances near volcanic



CO<sub>2</sub> seeps (Porzio *et al.*, 2011; Chapter 3), while fleshy brown algae may have increased in cover at high CO<sub>2</sub> after outcompeting *P. pavonica*. Recently, Johnson *et al.* (2012) reported increasing *P. pavonica* densities as CO<sub>2</sub> increased in shallow subtidal waters off Vulcano, possibly because of lower consumption by sea urchins. The decreased *P. pavonica* cover at elevated CO<sub>2</sub> found at Vulcano may be due to the different sampling depth (personal observation): *Paracentrotus lividus*, the species that mostly grazes on *P. pavonica* (Chiantore *et al.*, 2008), is easily dislodged by waves (Bulleri *et al.*, 1999). Intertidal shores off Vulcano are subject to high wave activity, so *P. pavonica* is not likely to be influenced by sea urchin grazing there. On the other hand, *P. pavonica* (the main calcifying brown alga) biomass decreased with increasing CO<sub>2</sub> at Methana, but only when herbivores were excluded, unlike in the study from Johnson *et al.* (2012). This probably happened because fleshy brown algae (*Dictyota* sp.) had a competitive advantage over the calcifier *P. pavonica* as CO<sub>2</sub> increased.

On the other hand, turf algae showed opposite responses to increased CO<sub>2</sub>, as their cover increased at Vulcano and decreased at Methana. Turf algae are often advantaged by increased CO<sub>2</sub>, as many turf species are extremely fast-growing and possibly carbon-limited (Connell *et al.*, 2013). However, a recent survey at seeps off Ischia reported decreased turf biomass at high CO<sub>2</sub> levels (Porzio *et al.*, 2011). Turf algae is a functional group that includes many species, some of which may be palatable to grazers (Falkenberg *et al.*, 2014), and therefore exhibit very different responses to ocean acidification.

Limpets exerted a weak top-down control on benthic communities off Vulcano in reference conditions, whereas carbon dioxide caused major changes in benthic community structure. Limpets have a decreasing influence on benthic

communities as latitude decreases (Coleman *et al.*, 2006), and in the Mediterranean Sea their effect is not consistent in space and time (Benedetti-Cecchi *et al.*, 2001). When consistent negative effects on filamentous algae were recorded in the Mediterranean Sea, limpet densities were much higher than in this study (Bulleri *et al.*, 2000). Moreover, when the physical environment is stressful limpet grazing has weaker effects on macroalgae (Bazterrica *et al.*, 2007); this could be the case at Vulcano, where communities are exposed to strong wave action.

At Vulcano, limpets influenced percent cover of the barnacle *Chthamalus stellatus* and of a red alga (*Laurencia* sp.). At the 600 ppm site, percent cover of both taxa decreased when limpets were present, while at elevated CO<sub>2</sub> limpets did not have any significant influence on the experimental plots. Limpets can reduce barnacle recruitment by dislodging young individuals (Menge *et al.*, 2010), but at elevated CO<sub>2</sub> levels barnacle recruitment was strongly reduced and no significant effect of limpets was detectable. This may be due to negative effects of elevated carbon dioxide on barnacles in a food-limited habitat (Pansch *et al.*, 2014), decreased limpet densities at elevated CO<sub>2</sub> (this study) or a combination of the above. The genus *Laurencia* is probably vulnerable to grazing, as limpets are thought to control the upper limit of the *Laurencia-Gigartina* belt found in some parts of Britain (Lewis, 1964). At the 1200 ppm site, *Laurencia* sp. percent cover was extremely low even in the control plots; consequently, lack of significant differences among experimental treatments at this site is probably due to this taxon response to elevated carbon dioxide rather than to decreased limpet grazing. So far, no experiments have been performed on this genus' response to ocean acidification, but at volcanic seeps off Ischia

*Laurencia obtusa* is only present at pH 8.1 and disappears even at moderate pCO<sub>2</sub> levels (Porzio *et al.*, 2011).

The slight increase in limpet length with high CO<sub>2</sub> confirms findings from seeps off Ischia (Hall-Spencer *et al.*, 2008) and could partly explain the trend towards increased grazing rates detected in this study. Increased feeding rates at elevated CO<sub>2</sub> levels have already been reported for some sea snails (Falkenberg *et al.*, 2013b), whereas other species decrease their food consumption at high CO<sub>2</sub> (Russell *et al.*, 2013). Changes in herbivore feeding rates may be due to altered food quality rather than to direct effects of CO<sub>2</sub> on their metabolism (Falkenberg *et al.*, 2013b; Poore *et al.*, 2013), and changes in macroalgal nutritional value at Vulcano could explain the higher feeding rates of limpets living at the 1200 ppm site. Increased calcification costs (Wood *et al.*, 2010), compensatory hyper-calcification at high CO<sub>2</sub> levels (Rodolfo-Metalpa *et al.*, 2011) and shifts in limpet shell mineralogy from calcite to the more energy-expensive aragonite (Langer *et al.*, 2014) could also explain increased limpet grazing rates.

Subtidal herbivore exclusion at Methana dramatically changed benthic communities grown on tiles after nine months. Previous studies show that herbivores have a greater influence on recruiting compared to established macroalgal communities (Korpinen *et al.*, 2008), and subtidal herbivores exert a stronger top-down control than limpets, whose effect is very variable (Benedetti-Cecchi *et al.*, 2001). Herbivore exclusion caused an increase in algal biomass regardless of site, but in the reference site only calcifying brown algae (*Padina pavonica*) colonised the caged tiles. On the other hand, every caged tile at the high CO<sub>2</sub> site was colonised by a different species (*Padina pavonica*, *Dictyota* sp. and erect brown algae). This confirms that non-calcifying algae become

more abundant as  $p\text{CO}_2$  increases, likely because of a decreased competitiveness of calcifying species (Porzio *et al.*, 2011; Kroeker *et al.*, 2013).

Herbivory is known to alter outcomes of macroalgal competition, favouring less palatable species (Hereu *et al.*, 2008). At Methana, herbivore-resistant encrusting algae became more abundant at both  $\text{CO}_2$  levels when herbivores were present. In addition, macroalgal communities at Methana showed smaller differences between  $\text{CO}_2$  levels when herbivores were present (Figure 5.10). Recent evidence shows that grazers can indeed dampen the effects of environmental changes on primary producers, both in terrestrial and in marine ecosystems (Post and Pedersen, 2008; Anthony *et al.*, 2011; Falkenberg *et al.*, 2014).

At Methana, both sea urchin species had reduced densities near the seeps regardless of sampling date, which is in accord with their predicted sensitivity to high  $\text{CO}_2$  resulting from laboratory experiments (Dupont *et al.*, 2010). There is a partial disagreement with results from Vulcano, where *P. lividus* densities decreased, but *A. lixula* densities increased with increasing  $\text{CO}_2$  (Calosi *et al.*, 2013a). Increased sea urchin densities near volcanic seeps have previously been correlated with low structural complexity of high- $\text{CO}_2$  habitats (Fabricius *et al.*, 2014); *A. lixula* may therefore tolerate moderate carbon dioxide enrichment, but high habitat complexity may prevent its colonisation at seeps off Methana. Sea urchins were replaced by herbivorous fish at high  $\text{CO}_2$  levels; functional redundancy of herbivores can maintain top-down control on macroalgal biomass and reduce the effects of multiple stressors on benthic communities (Blake and Duffy, 2010; Eriksson *et al.*, 2011). Fish, however, are highly mobile and could swim in and out of the high  $\text{CO}_2$  area (Riebesell, 2008), masking

potential negative effects of ocean acidification such as those on many species' neuroreceptors (Shaw *et al.*, 2013).

Coastal assemblages often have low functional redundancy, and the loss of a few species can negatively affect ecosystem functioning (Micheli and Halpern, 2005). Taxonomic diversity can help marine community resilience to increased temperatures (Allison, 2004), but there is no evidence this applies to community responses to ocean acidification. Here we show that taxonomic diversity helps improving resilience to ocean acidification: herbivorous fish kept grazing pressure high at elevated CO<sub>2</sub>, even though sea urchin densities decreased near the seeps. Overfishing of apex predators has led to higher abundances of Mediterranean sea urchins and herbivorous fish, as they are usually not targeted by commercial fisheries (Guidetti and Dulčić, 2007; Guidetti and Sala, 2007). High herbivore densities can often lead to impoverished macroalgal communities, very different from unexploited Mediterranean coastal ecosystems (Sala *et al.*, 2012). Thus, unvaried grazing pressure at different CO<sub>2</sub> levels may maintain suboptimal community structure. However, at a global level herbivorous fish abundance is strongly reduced by overfishing (Micheli and Halpern, 2005), and when this is combined with other herbivores disappearing (e.g. sea urchin mass mortality in Jamaica) benthic habitat can experience dramatic phase shifts (Hughes, 1994).

Bottom-up control (i.e. increase in CO<sub>2</sub>) seemed to be the main factor influencing benthic community structure regardless of herbivore consumption levels. Recent research has shown that indirect effects can be as important as the direct effects of CO<sub>2</sub> in shaping community responses to ocean acidification (Kroeker *et al.*, 2013). However, herbivores have the strongest effect and when they are present other indirect effects are reduced or disappear altogether

(Alsterberg *et al.*, 2013). Here we show that carbon dioxide still affects the specific composition of benthic communities in subtidal habitats even when herbivore pressure is strong, even though grazing reduced community structure variability. The most striking finding of this study is that herbivore functional redundancy can offset indirect effects of ocean acidification; this, however, is only possible in unpolluted ecosystems, as diversity is reduced in contaminated marine systems (Johnston and Roberts, 2009). Although neither of the sites studied in this Chapter is a nature reserve, these areas are not heavily impacted by human activities (author's personal observation); it is therefore possible that impacts of ocean acidification on benthic communities will be more severe in polluted areas. Managing local stressors (e.g. eutrophication, heavy metals) is thus essential to maintain high diversity and increase ecosystem resilience to environmental change (Ghedini *et al.*, 2013).

## **Chapter 6**

# **Seaweed acclimatisation to high pCO<sub>2</sub> at volcanic seeps**

## Abstract

Most experiments on organism responses to ocean acidification have been conducted for a relatively short time, so there is little evidence for most species' potential for long-term acclimatisation or adaptation, except for some species of phytoplankton. Volcanic seeps can expose benthic communities to increased CO<sub>2</sub> for centuries, and are starting to be used to study adaptive effects of elevated CO<sub>2</sub> on marine organisms. This chapter aims to determine whether dominant macroalgal species at volcanic seeps off Methana (Greece) show evidence of long-term acclimatisation. Ten thalli of the canopy-forming alga *Cystoseira corniculata* and ten thalli of the articulated coralline alga *Jania rubens* were transplanted within and between one high CO<sub>2</sub> and two reference sites, and their physiological performance was assessed after long-term transplants. Neither species showed signs of non-reversible acclimatisation to elevated CO<sub>2</sub> levels, since there were only very small differences between thalli depending on their site of origin. *C. corniculata* seemed to be favoured by increased CO<sub>2</sub>, as it had reduced epiphyte cover and higher rETR<sub>max</sub> (maximum relative electron transport rate) when transplanted near the seeps. At high CO<sub>2</sub>, this species also had increased chlorophyll *c* and antheraxanthin content, as well as increased C:N ratios. *Jania rubens* also showed an increase in some pigment concentrations (chlorophyll *a*, violaxanthin, zeaxanthin and phycocyanin) at high CO<sub>2</sub> levels, but in this species all other parameters were unaffected by the transplant. *Cystoseira corniculata* and *Jania rubens* appear not to permanently acclimatise to ocean acidification, but their different physiological responses may alter their competitive interactions. This would help explain the reduction in *J. rubens* cover recorded at increased CO<sub>2</sub> levels off Methana.



## 6.1 Introduction

Currently, most of the work on ocean acidification biological responses is performed using short-term experiments. As a consequence, there is limited information on the potential for marine algae to permanently acclimatise or adapt to ocean acidification, except for short-lived organisms. For example, the coccolithophore *Emiliania huxleyi* adapted after being exposed to high pCO<sub>2</sub> for about 500 generations. Actually, calcification rates at high CO<sub>2</sub> were always lower than those in reference conditions, but adapted organisms had much higher calcification rates than non-adapted ones (Lohbeck *et al.*, 2012). For longer-lived macroalgae a solution could be studying individuals from volcanic seeps, which can have high CO<sub>2</sub> levels for centuries (Dando *et al.*, 2000).

Genetic adaptation and non-reversible acclimatisation are thought to be rare in marine environments due to their connectivity, which increases the genetic exchange of adults, larvae or other propagules (Palumbi, 1994). However, there are only a few examples of gene flow preventing or slowing down local adaptation in marine species, whereas local adaptation, especially to temperature, is relatively common in marine environments (Sanford and Kelly, 2011). Connectivity of marine environments is therefore unlikely to be as high as previously thought, and speciation can occur over relatively short distances (e.g. Tellier *et al.*, 2011). Moreover, short dispersal distances decrease inter-population gene flow, increasing the likelihood of local adaptation (Endler, 1977). Dominant macroalgal species at seeps off Methana, *Cystoseira corniculata* and *Jania rubens*, have short dispersal distances (< 5 km; Jones and Moorjani, 1973; Susini *et al.*, 2007). It is thus possible that these two species have acclimatised to high and variable pCO<sub>2</sub>, as seeps off Methana influence approximately 10 km of shoreline (Baggini *et al.*, 2014).

Porzio (2010) showed that brown algae of the genus *Dictyota* had altered their morphology and had distinct genotypes when seawater pCO<sub>2</sub> was high. In fact, macroalgae exposed to other stressors on relatively short time scales can undergo permanent changes in their physiology or genome. For instance, exposure to low salinity in the Baltic Sea has led to the evolution of a new *Fucus* species after only a few thousand years (Bergström *et al.*, 2005). In another case, *Fucus serratus* individuals from copper-contaminated areas and their offspring are more tolerant to this heavy metal compared with individuals from more pristine areas, although it is not known whether this is transgenerational acclimation or genetic adaptation (Nielsen *et al.*, 2003).

Non-reversible acclimatisation to high pCO<sub>2</sub> might therefore occur in macroalgae, and studying species from volcanic seeps might give us an insight on the possible mechanisms. The aim of this chapter is to assess whether two dominant seaweed species growing near CO<sub>2</sub> seeps off Methana (Greece) were permanently acclimatised to high and variable pCO<sub>2</sub> conditions using reciprocal transplantations (Sanford and Kelly, 2011). Hypotheses tested were:

- 1) Growth rates and maximum quantum yield ( $F_v/F_m$ ) are higher in individuals transplanted at the same CO<sub>2</sub> level they are acclimatised to, since acclimatised populations perform best in their origin conditions (Leimu and Fischer, 2008);
- 2) Pigment content is higher in coralline algae acclimatised to reference conditions and decreases at elevated CO<sub>2</sub> (*Jania rubens*; Gao and Zheng, 2010), whereas chlorophyll in brown algae increases as seawater pCO<sub>2</sub> increases (*Cystoseira corniculata*; Johnson *et al.*, 2012);

- 3) Total phenolic compounds, such as phlorotannins, are higher in brown algae acclimatised to high pCO<sub>2</sub> (Swanson and Fox, 2007);
- 4) Carbon:nitrogen ratio increases in seaweed exposed to elevated carbon dioxide in the long term because there is more inorganic carbon available, not as a result of acclimatisation (Koch *et al.*, 2013);
- 5) Inorganic carbon content in *J. rubens* decreases in individuals from reference conditions transplanted to the high CO<sub>2</sub> area because of skeleton dissolution, whereas individuals from high CO<sub>2</sub> have higher inorganic carbon content when transplanted to the reference sites because of persistent hyper-calcification (Rodolfo-Metalpa *et al.*, 2011).

Evidence from macroalgae that have acclimatised to high CO<sub>2</sub> conditions is essential to assess the adaptation potential of macroalgae to ocean acidification. There is relatively little information on long-term responses of macroalgae to elevated CO<sub>2</sub>, and only a few studies have tackled the issue using field-based experiments. To date, evidence from laboratory experiments indicates that temperate macroalgal communities will change their specific composition as seawater pCO<sub>2</sub> increases; this has potential knock-on effects on marine food webs, nutrient cycling and carbon storage (Brodie *et al.*, 2014). However, there is very little information on macroalgal adaptation potential to increased CO<sub>2</sub>, meaning that coastal ecosystems might not change as much as anticipated if macroalgae can acclimatise to elevated pCO<sub>2</sub> levels (Sunday *et al.*, 2014).

## 6.2 Methods

### 6.2.1 Experimental design and field sampling

Two common and abundant macroalgal species in Methana were examined for signs of long-term acclimatisation to high pCO<sub>2</sub>; detailed sampling dates and sample sizes are reported in Table 1.1E. Ten thalli for each species were transplanted within and between one high CO<sub>2</sub> site (SEEP) and two reference sites (REF A and REF B) as shown in Figure 6.1; see Chapter 2 for site descriptions. All specimens were transplanted by detaching thalli with a small chip of rock still attached using hammer and chisel and attaching them to rocky substratum in the target site using epoxy putty (Z-Spar A-788 Splash Zone Epoxy Putty). This method has been previously used for *Cystoseira* in the Mediterranean Sea and has a good success rate (Sales *et al.*, 2011). Physiological parameters were then measured in the transplanted seaweeds and in seven unmanipulated thalli of each species per site; the number of unmanipulated thalli was selected to reflect the average number of transplanted thalli left at the end of the experiments.

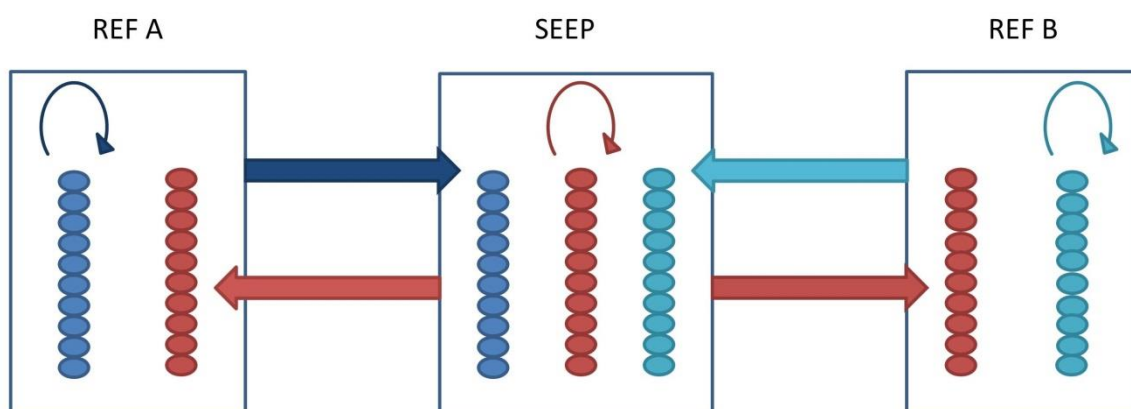


Figure 6.1. Scheme of the experimental design for reciprocal transplantations of *Cystoseira corniculata* transplanted from September 2012 to June 2013 and *Jania rubens* transplanted from June to September 2013. Ten individuals of each species from the high CO<sub>2</sub> site

(SEEP) and the two reference sites (REF A and REF B) were transplanted within their site of origin as procedural controls (round arrows). Ten individuals per species from both reference sites were also transplanted to the SEEP site (blue and light blue arrows). Furthermore, ten individuals per species originally from the high CO<sub>2</sub> site were transplanted to REF A and other ten individuals were transplanted to REF B (red arrows).

*Cystoseira corniculata* is a furoid alga and this genus is the main canopy-former in the Mediterranean Sea, where it indicates relatively pristine environmental conditions (Benedetti-Cecchi *et al.*, 2001). It was the dominant macroalgal species at seeps off Methana, and has a biomass maximum between May and June in the region (Haritonidis *et al.*, 1986). This species was transplanted in September 2012 and physiological parameters were measured in June 2013 (in correspondence to its biomass peak). The articulate coralline alga *Jania rubens* is an epiphytic thermophilic species and is extremely common in the study area in spring and summer, with a bloom around August (Beleggratis *et al.*, 1999). *Jania rubens* thalli were transplanted in June 2013 (when thalli are large enough to be visible) and their physiological parameters were measured in September 2013 (in correspondence to its biomass peak); thalli of this species transplanted to REF A were all lost due to stormy weather, so only samples from SEEP and REF B were analysed.

Difference in maximum thallus height between the beginning and the end of transplantation was measured placing thalli on graph paper (accuracy  $\pm 1$  mm) and used to calculate relative growth for *C. corniculata* and *J. rubens*. Tips were checked for grazer marks. While measuring growth, epiphyte cover of *C. corniculata* thalli was also evaluated using a scale from 1 (epiphyte cover < 25%) to 4 (epiphyte cover > 75%). Chlorophyll *a* fluorescence of the macroalgae was measured with a pulse amplitude modulated fluorometer

(Diving PAM, Walz, Effeltrich, Germany). The maximum photochemical quantum yield of photosystem II ( $F_v/F_m$ ) was measured after 15 minutes of dark adaptation (Schreiber *et al.*, 1995), then rapid light curves (eight points at 20 s intervals, E1=16, E2=24, E3=34, E4=52, E5=77, E6=118, E7=176 and E8=250  $\mu\text{mol photon m}^{-2} \text{s}^{-1}$ ) were conducted and non-photochemical quenching (NPQ) was measured. The physiological parameters  $I_k$ ,  $rETR_{\text{max}}$  and  $\alpha_{\text{ETR}}$  (saturation irradiance for ETR, maximum relative electron transport rate and photochemical efficiency, respectively) were calculated using a non-linear regression analysis (Eilers and Peeters, 1988).

Tissue samples for laboratory analyses were collected between 8:00 and 10:00 to avoid the confounding effect of mid-day photoinhibition on pigments (Häder *et al.*, 1996) and dried with silica gel for C:N ratio analysis (for all species), total phenolic compounds and tissue P analyses (*C. corniculata* only) and inorganic carbon content (*J. rubens*). More tissue was placed in liquid nitrogen and stored at -60 °C for pigment analysis (all species).

### **6.2.2 Laboratory analyses**

The total carbon and nitrogen content in dried samples of *C. corniculata* and the organic carbon and nitrogen content of *J. rubens* thalli was measured using a CHN Analyzer (CE Instruments EA1110 elemental analyser). Approximately 1-3 mg of tissue were ground to a powder and packed into aluminum capsules for analysis of total carbon and nitrogen. For organic carbon and nitrogen content of *J. rubens*, approximately 10 mg of sample was ground to powder, placed in silver capsules, acidified with 20  $\mu\text{l}$  of 2M HCl 12 times at 6-12 hours intervals and dried in an oven at 60°C. Separate tissue samples from *J. rubens* were dried for 72 h at 60°C, weighed to obtain dry mass and then put for 24h in a

muffle furnace at 400°C to burn all organic matter and obtain the mass of inorganic carbon. For total phenolic compounds analysis, ~100 mg of freeze-dried tissue was ground and extracted in methanol at 4°C for 24 h. Total phenolic compounds of *C. corniculata* were then analysed using a method modified from Kamal (2011). Seaweed extracts were diluted in distilled water (10% methanol extract, 90% distilled water) and absorbance at 765 nm was measured using a 96 well plate and a FLUOstar Omega microplate reader (BMG Labtech). Each well contained 20 µl 50% Folin-Ciocalteu reagent (Folin and Ciocalteu, 1927), 10 µl Na<sub>2</sub>CO<sub>3</sub> (1.5 M) and 10 µl sample solution. Phloroglucinol (1,3,5-trihydroxybenzene) was used as a standard. Plates were refrigerated overnight before measurement, and eight replicate measurements per sample were made.

Samples of *C. corniculata* and *J. rubens* for pigment analysis were freeze-dried in the dark for 24h, after which they were ground in pure acetone using a mortar and pestle. Extraction occurred at 4°C for 24 h in the dark. After extraction, samples were centrifuged at 4000 rpm for 15 min at 4°C. Pigment content was then analysed using the Gauss-Peak Spectra method (Küpper *et al.*, 2007). Samples were scanned in a dual-beam spectrophotometer from 350 nm to 750 nm at 1 nm steps. The absorbance spectra were introduced in the GPS fitting library, using SigmaPlot. The employment of this library allowed to identify and quantify Chlorophyll *a*, Chlorophyll *c*<sub>1</sub> and *c*<sub>2</sub>, Pheophytin *a*, Fucoxanthin, Antheraxanthin, β-carotene, Violaxanthin and Zeaxanthin for *C. corniculata* (Küpper *et al.*, 2007) and Chlorophyll *a*, Pheophytin *a*, β-cryptoxanthin, Antheraxanthin, β-carotene, Violaxanthin and Zeaxanthin for *J. rubens*. *J. rubens* carotenoids were selected based on Schübert *et al.* (2006). For phycobiliproteins in *J. rubens* samples approximately 0.5 g of tissue was

homogenised in 10 mL 0.1 M phosphate buffer (pH 6.8). After being left at 4°C in the dark overnight, extracts were centrifuged for 10 minutes at 1000g and then read in the spectrophotometer at the wavelengths determined by Beer and Eshel (1985).

### **6.2.3 Statistical analyses**

All data were checked for compliance with ANOVA assumptions (normality by visually inspecting data and homogeneity of variance using Levene's test) and transformed when necessary. Growth was analysed using a two way ANOVA with 'site of origin' and 'site of destination' as fixed factors. When a factor had a significant effect, a Tukey HSD pair-wise test was performed. Epiphyte cover of *C. corniculata* was analysed using a Kruskal-Wallis analysis with 'treatment' as a fixed factor. Separate MANOVAs were used to analyse treatment effects on C:N and N:P ratios (*C. corniculata*), C and N content (*J. rubens*), photochemical parameters (both species), pigments content (both species) and phycobilins (*J. rubens*). When the data did not meet Mauchly's test of sphericity, the degrees of freedom were corrected using Greenhouse-Geisser estimates of sphericity. CaCO<sub>3</sub> content (*J. rubens*) and total phenolic compounds (*C. corniculata*) were analysed using one-way ANOVAs with 'treatment' as a fixed factor.

## **6.3 Results**

### **6.3.1 Growth and epiphyte cover**

Relative growth of *C. corniculata* thalli was significantly different among transplantation sites (Table 6.1). Pairwise comparisons showed that thalli transplanted to the high CO<sub>2</sub> site (SEEP) and one reference site (REF A) grew more than those transplanted to the other reference site (REF B; Figure 6.2).



Different pCO<sub>2</sub> did not seem to have significant effects on linear growth of this macroalgal species.

Table 6.1. ANOVA results for transplanted *C. corniculata* thalli growth. The table shows main factors and their interactions and sum of squares (SS), degrees of freedom (df), Mean Squares (MS), F-ratios (F) and p values. Significant p values (< 0.05) are highlighted. The last row shows results from pairwise comparisons between sites of destination, different letters represent significantly different groups.

| Source               | Type III SS        | df                 | MS                | F- ratio | p                |
|----------------------|--------------------|--------------------|-------------------|----------|------------------|
| Origin               | 0.065              | 2                  | 0.032             | 1.028    | 0.368            |
| Destination          | 0.614              | 2                  | 0.307             | 9.766    | <b>&lt;0.001</b> |
| Origin * Destination | 0.114              | 2                  | 0.057             | 1.822    | 0.176            |
| Error                | 1.131              | 36                 | 0.031             |          |                  |
| Total                | 1.826              | 42                 |                   |          |                  |
| Post-hoc subsets     | REF B <sup>a</sup> | REF A <sup>b</sup> | SEEP <sup>b</sup> |          |                  |

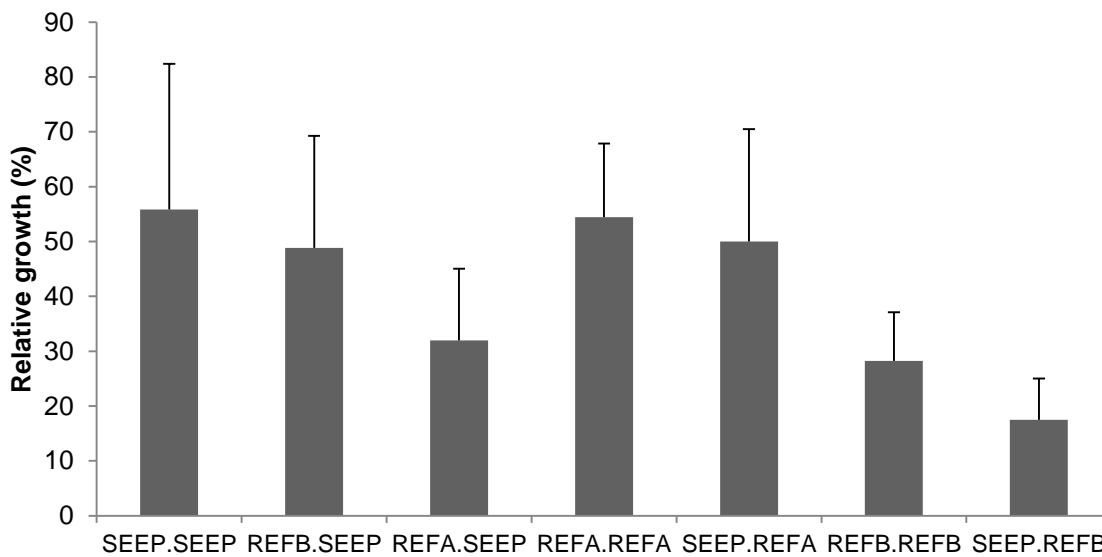


Figure 6.2. Mean relative growth ( $\pm$  SD, n=3-10) of *C. corniculata* thalli transplanted at Methana from September 2012 to June 2013 within and between two reference sites (REF A and REF B) and one high CO<sub>2</sub> site (SEEP). Treatments are shown as “Site of origin.Site of transplant”.

Transplanted *J. rubens* thalli did not exhibit any significant difference in relative growth (Table 6.2). Even though thalli transplanted from SEEP had higher

average growth than those transplanted from the reference site (REF A), the two groups did not show any significant difference as variability was very high (Figure 6.3).

Table 6.2. ANOVA results for transplanted *J. rubens* thalli growth. The table shows main factors and their interactions and sum of squares (SS), degrees of freedom (df), Mean Squares (MS), F-ratios (F) and p values.

| Source               | Type III SS | df | MS       | F-ratio | p     |
|----------------------|-------------|----|----------|---------|-------|
| Origin               | 1869.486    | 1  | 1869.486 | 2.312   | 0.149 |
| Destination          | 5.400       | 1  | 5.400    | 0.007   | 0.936 |
| Origin * Destination | 75.479      | 1  | 75.479   | 0.093   | 0.764 |
| Error                | 12130.052   | 15 | 808.670  |         |       |
| Total                | 14019.002   | 18 |          |         |       |

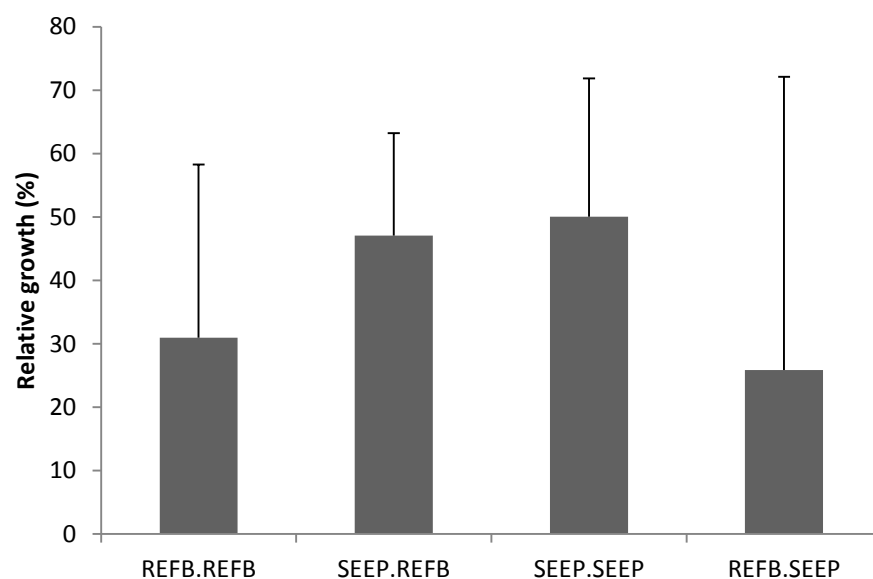


Figure 6.3. Mean relative growth ( $\pm$  SD, n=4-6) of *J. rubens* thalli transplanted at Methana from June 2013 to September 2013 within and between one reference site (REF B) and one high CO<sub>2</sub> site (SEEP). Treatments are shown as “Site of origin.Site of transplant”.

Epiphytes cover of *C. corniculata* thalli was significantly different among treatments according to a Kruskal-Wallis test. In general, thalli collected from or transplanted to the high CO<sub>2</sub> site (SEEP) had lower epiphytes cover compared

to those collected from or transplanted to the two reference sites (REF A and REF B). Macroalgae transplanted from high CO<sub>2</sub> levels to the reference sites (i.e. SEEP.REFA and SEEP. REFB) have lower epiphytes cover than those transplanted within the same reference site (i.e. REFA.REFA and REFB.REFB; Figure 6.4).

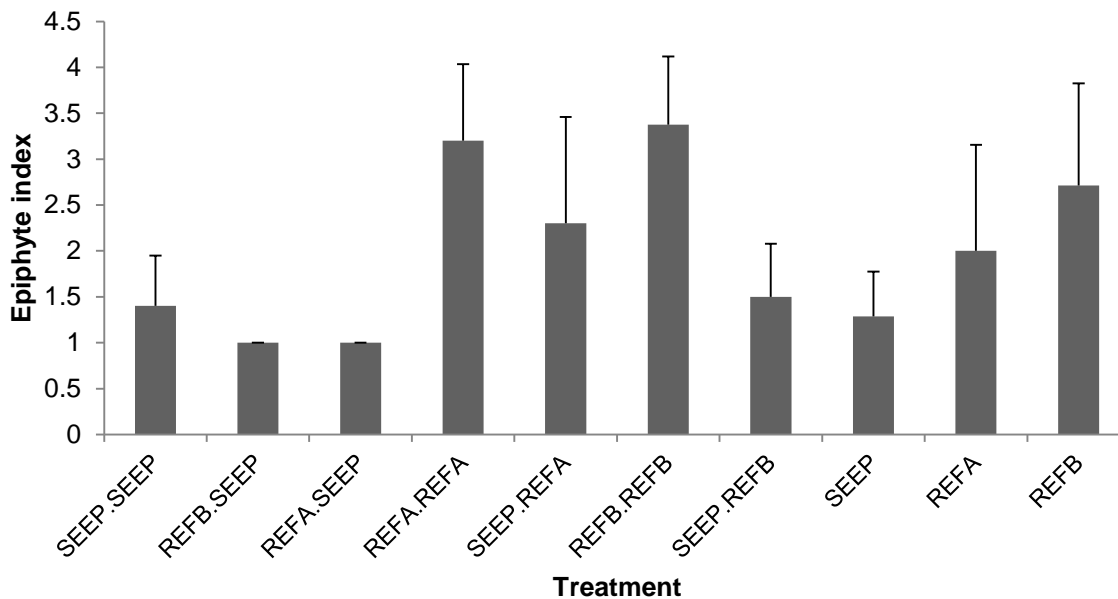


Figure 6.4. Mean epiphyte cover ( $\pm$  SD, n=3-10) of *C. corniculata* thalli transplanted at Methana from September 2012 to June 2013 within and between two reference sites (REF A and REF B) and one high CO<sub>2</sub> site (SEEP). Treatments are shown as “Site of origin.Site of transplant”; the last three treatments (SEEP, REF A and REF B) are unmanipulated thalli collected in June 2013.

### 6.3.2 Photosynthetic parameters

Maximum relative electronic transport rate ( $rETR_{max}$ ) and saturation irradiance for ETR ( $I_k$ ) were significantly different among treatments for *C. corniculata* transplanted at Methana from September 2012 to June 2013 (Table 6.3). The main differences in relative electron transport rates were connected with site of transplant, with thalli transplanted to SEEP generally having higher values of

rETR<sub>max</sub> than those transplanted to the reference sites (Table 6.4). On the other hand, I<sub>k</sub> did not show any consistent trend related to the experimental treatments.

Table 6.3. MANOVA on photosynthetic parameters in *C. corniculata* thalli transplanted at Methana from September 2012 to June 2013 within and between two reference sites (REF A and REF B) and one high CO<sub>2</sub> site (SEEP). F-ratios (F) and p values are reported when significant (p<0.05).

| Response variable              | Treatment             |
|--------------------------------|-----------------------|
| rETR <sub>max</sub>            | F(9,44)=4.01; p=0.001 |
| I <sub>k</sub>                 | F(9,44)=2.46; p=0.023 |
| α                              | -                     |
| NPQ                            | -                     |
| F <sub>v</sub> /F <sub>m</sub> | -                     |

Table 6.4. Mean photosynthetic parameters (± SD, n=3-9) of *C. corniculata* thalli transplanted at Methana from September 2012 to June 2013 within and between two reference sites (REF A and REF B) and one high CO<sub>2</sub> site (SEEP). Treatments are shown as “Site of origin.Site of transplant”; the last three treatments (SEEP, REF A and REF B) are unmanipulated thalli collected in June 2013.

| Origin.Destination | rETR <sub>max</sub><br>(μmol e m <sup>-2</sup> s <sup>-1</sup> ) | I <sub>k</sub><br>(μmol photons m <sup>-2</sup> s <sup>-1</sup> ) | α <sub>ETR</sub> | NPQ           | F <sub>v</sub> /F <sub>m</sub> |
|--------------------|--|---|------------------|---------------|--------------------------------|
| SEEP.SEEP          | 21.7 ± 12.2  | 87.4 ± 44.1   | 0.247 ± 0.052    | 0.065 ± 0.104 | 0.644 ± 0.053                  |
| REFB.SEEP          | 19.3 ± 11.4  | 66.0 ± 37.2   | 0.305 ± 0.059    | 0.104 ± 0.109 | 0.665 ± 0.053                  |
| REFA.SEEP          | 15.5 ± 3.4   | 62.2 ± 5.2  | 0.247 ± 0.036    | 0.231 ± 0.041 | 0.664 ± 0.022                  |
| REFA.REFA          | 9.6 ± 3.6  | 53.5 ± 22.1   | 0.185 ± 0.033    | 0.379 ± 0.240 | 0.594 ± 0.035                  |
| SEEP.REFA          | 17.0 ± 13.5  | 100.7 ± 94.3  | 0.194 ± 0.078    | 0.280 ± 0.292 | 0.587 ± 0.083                  |
| REFB.REFB          | 13.3 ± 25.7  | 108.0 ± 248.3   | 0.369 ± 0.417    | 0.313 ± 0.192 | 0.672 ± 0.069                  |
| SEEP.REFB          | 7.3 ± 3.7  | 67.6 ± 37.8   | 0.132 ± 0.072    | 0.219 ± 0.240 | 0.590 ± 0.123                  |
| SEEP               | 30.5 ± 16.7  | 133.3 ± 62.5  | 0.225 ± 0.055    | 0.293 ± 0.193 | 0.628 ± 0.064                  |
| REFA               | 13.3 ± 5.3   | 60.4 ± 33.0   | 0.364 ± 0.374    | 0.311 ± 0.206 | 0.610 ± 0.098                  |
| REFB               | 3.8 ± 0.7  | 17.2 ± 7.0  | 0.242 ± 0.075    | 0.176 ± 0.097 | 0.588 ± 0.041                  |

No significant differences among treatments were detected using a MANOVA for *J. rubens* transplanted from June to September 2013 (Table 6.5). The

measured (NPQ,  $F_v/F_m$ ) and calculated ( $rETR_{max}$ ,  $I_k$ ,  $\alpha_{ETR}$ ) parameters for *J. rubens* are reported in Table 6.6, and all parameters are generally lower in *J. rubens* than in *C. corniculata* (Table 6.4).

Table 6.5. MANOVA on photosynthetic parameters in *J. rubens* thalli transplanted at Methana from June 2013 to September 2013 within and between one reference site (REF B) and one high CO<sub>2</sub> site (SEEP). F-ratios (F) and p values are reported when significant ( $p < 0.05$ ).

| Response variable | Treatment |
|-------------------|-----------|
| $rETR_{max}$      | -         |
| $I_k$             | -         |
| $\alpha$          | -         |
| NPQ               | -         |
| $F_v/F_m$         | -         |

Table 6.6. Mean photosynthetic parameters ( $\pm$  SD,  $n=3-4$ ) of *J. rubens* thalli transplanted at Methana from June 2013 to September 2013 within and between one reference site (REF B) and one high CO<sub>2</sub> site (SEEP). Treatments are shown as "Site of origin.Site of transplant"; the last three treatments (SEEP, REF A and REF B) are unmanipulated thalli collected in September 2013.

| Origin.Destination | $rETR_{max}$<br>( $\mu\text{mol e m}^{-2} \text{s}^{-1}$ ) | $I_k$<br>( $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ ) | $\alpha_{ETR}$    | NPQ               | $F_v/F_m$         |
|--------------------|--|---|-------------------|-------------------|-------------------|
| SEEP.SEPE          | 5.2 $\pm$ 5.2  | 39.9 $\pm$ 44.7   | 0.136 $\pm$ 0.031 | 0.095 $\pm$ 0.083 | 0.485 $\pm$ 0.018 |
| REFB.SEPE          | 5.4 $\pm$ 4.1  | 57.7 $\pm$ 47.0   | 0.163 $\pm$ 0.126 | 0.147 $\pm$ 0.140 | 0.441 $\pm$ 0.078 |
| REFB.REFB          | 10.1 $\pm$ 13.5  | 76.4 $\pm$ 83.7   | 0.110 $\pm$ 0.038 | 0.094 $\pm$ 0.162 | 0.454 $\pm$ 0.054 |
| SEEP.REFB          | 6.9 $\pm$ 4.8  | 128.7 $\pm$ 155.2   | 0.489 $\pm$ 0.826 | 0                 | 0.394 $\pm$ 0.119 |
| SEEP               | 3.5 $\pm$ 3.2  | 48.4 $\pm$ 21.4   | 0.069 $\pm$ 0.041 | 0.110 $\pm$ 0.056 | 0.383 $\pm$ 0.080 |
| REFA               | 2.4 $\pm$ 0.5  | 35.4 $\pm$ 16.1   | 0.077 $\pm$ 0.032 | 0.158 $\pm$ 0.106 | 0.397 $\pm$ 0.042 |
| REFB               | 3.1 $\pm$ 0.4  | 30.7 $\pm$ 18.4   | 0.136 $\pm$ 0.089 | 0.253 $\pm$ 0.116 | 0.458 $\pm$ 0.046 |

### 6.3.3 Pigment contents

Chlorophyll *c* and antheraxanthin content of *C. corniculata* transplanted from September 2012 to June 2013 significantly differed among treatments, while all other pigments were not significantly affected (Table 6.7). Thalli transplanted to

reference sites had lower chlorophyll *c* and antheraxanthin content than those transplanted to the high CO<sub>2</sub> site (Figure 6.5). Unmanipulated thalli, however, did not show a similar pattern, with very small differences among sites.

Table 6.7. MANOVA on pigments content in *C. corniculata* thalli transplanted at Methana from September 2012 to June 2013 within and between two reference sites (REF A and REF B) and one high CO<sub>2</sub> site (SEEP). F-ratios (F) and p values are reported when significant (p<0.05).

| Response variable | Treatment             |
|-------------------|-----------------------|
| Chl a             | -                     |
| Chl c             | F(9,53)=3.552 p=0.002 |
| Pheophytin a      | -                     |
| β-carotene        | -                     |
| Fucoxanthin       | -                     |
| Violaxanthin      | -                     |
| Antheraxanthin    | F(9,53)=4.117 p<0.001 |
| Zeaxanthin        | -                     |

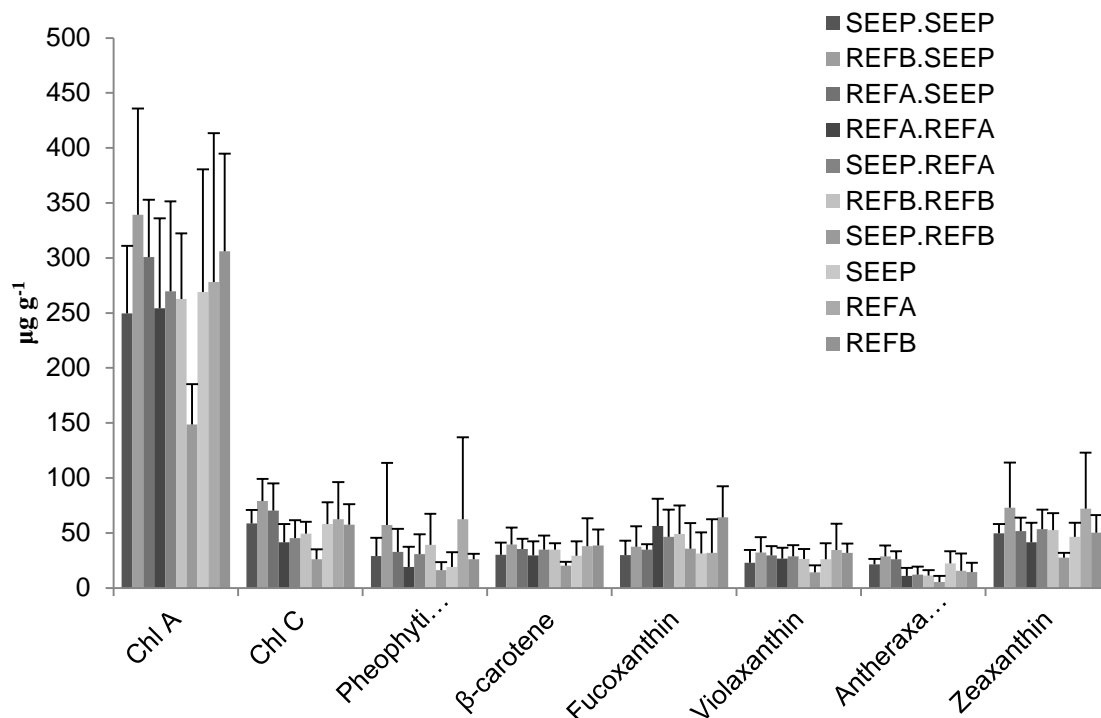


Figure 6.5. Mean pigments content ( $\pm$  SD, n=3-10) of *C. corniculata* thalli transplanted at Methana from September 2012 to June 2013 within and between two reference sites (REF

A and REF B) and one high CO<sub>2</sub> site (SEEP). Treatments are shown as “Site of origin.Site of transplant”; the last three treatments (SEEP, REF A and REF B) are unmanipulated thalli collected in June 2013.

MANOVA on log-transformed pigments content for *J. rubens* transplanted at Methana from June to September 2013 showed that treatment had a significant effect on all pigments analysed except for  $\beta$ -cryptoxanthin and antheraxanthin (Table 6.8). Chlorophyll *a*, violaxanthin and zeaxanthin content was higher in thalli transplanted to the high CO<sub>2</sub> site (SEEP), especially in those which also came from the high CO<sub>2</sub> site (SEEP.SEPE) and the unmanipulated thalli collected from that site (Figure 6.6). Pheophytin *a* and  $\beta$ -carotene were mostly present in higher quantities in unmanipulated thalli or in those transplanted to their site of origin (e.g. SEEP.SEPE) compared to those transplanted to a different site.

Table 6.8. MANOVA on pigments content in *J. rubens* thalli transplanted at Methana from June 2013 to September 2013 within and between one reference site (REF B) and one high CO<sub>2</sub> site (SEEP). F-ratios (F) and p values are reported when significant ( $p < 0.05$ ).

| Response variable      | Treatment                  |
|------------------------|----------------------------|
| Chl <i>a</i>           | F(6,33)=8.874 $p < 0.001$  |
| Pheophytin <i>a</i>    | F(6,33)=14.414 $p < 0.001$ |
| $\beta$ -cryptoxanthin | -                          |
| $\beta$ -carotene      | F(6,33)=3.925 $p = 0.005$  |
| Violaxanthin           | F(6,33)=7.151 $p < 0.001$  |
| Antheraxanthin         | -                          |
| Zeaxanthin             | F(6,33)=10.702 $p < 0.001$ |

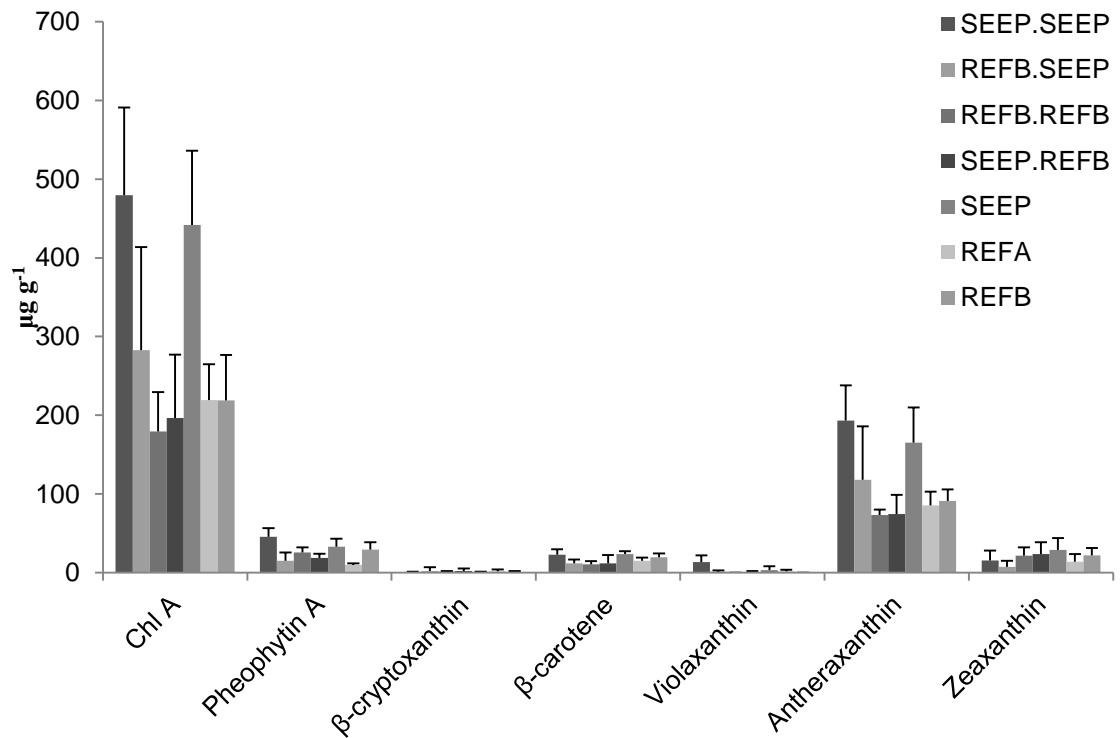


Figure 6.6. Mean pigments content ( $\pm$  SD,  $n=4-7$ ) of *J. rubens* thalli transplanted at Methana from June 2013 to September 2013 within and between one reference site (REF B) and one high CO<sub>2</sub> site (SEEP). Treatments are shown as “Site of origin.Site of transplant”; the last three treatments (SEEP, REF A and REF B) are unmanipulated thalli collected in September 2013.

MANOVA on log-transformed phycobilins content for *J. rubens* transplanted at Methana from June to September 2013 showed that treatment had a significant effect on phycocyanin (Table 6.9). Similarly to some pigments, phycocyanin content was higher in thalli transplanted to the high CO<sub>2</sub> site or unmanipulated thalli collected from SEEP (Figure 6.7).



Table 6.9. MANOVA on phycobilins content in *J. rubens* thalli transplanted at Methana from June 2013 to September 2013 within and between one reference site (REF B) and one high CO<sub>2</sub> site (SEEP). F-ratios (F) and p values are reported when significant (p<0.05).

| Response variable | Treatment             |
|-------------------|-----------------------|
| Phycoerythrin     | -                     |
| Phycocyanin       | F(6,32)=5.542 p<0.001 |

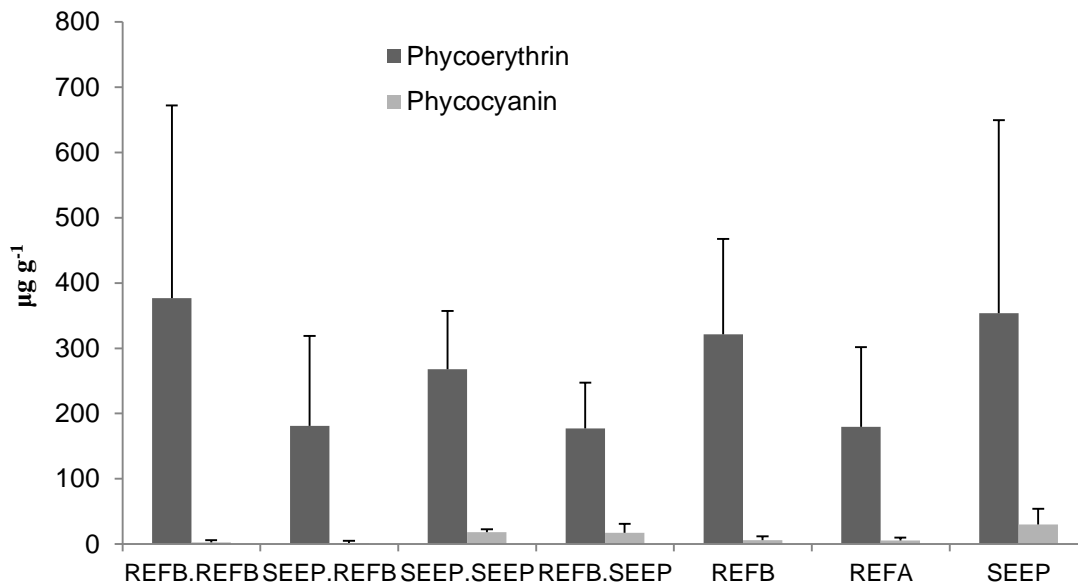


Figure 6.7. Mean phycobilins content ( $\pm$  SD, n=3-7) of *J. rubens* thalli transplanted at Methana from June 2013 to September 2013 within and between one reference site (REF B) and one high CO<sub>2</sub> site (SEEP). Treatments are shown as “Site of origin.Site of transplant”; the last three treatments (SEEP, REF A and REF B) are unmanipulated thalli collected in September 2013.

### 6.3.4 Carbon, nitrogen and phosphorus content

There was a significant difference among treatments for C:N ratio of *C. corniculata* thalli transplanted at Methana from September 2012 to June 2013, but not for N:P ratio (Table 6.10). C:N ratio was in fact higher in unmanipulated thalli collected at SEEP compared to those collected at reference sites (Figure

6.8). Similarly, thalli transplanted to the high CO<sub>2</sub> site had higher C:N ratio than those transplanted to the reference sites.

Table 6.10. MANOVA on C:N and N:P ratios in *C. corniculata* thalli transplanted at Methana from September 2012 to June 2013 within and between two reference sites (REF A and REF B) and one high CO<sub>2</sub> site (SEEP). F-ratios (F) and p values are reported when significant (p<0.05).

| Response variable | Treatment             |
|-------------------|-----------------------|
| C:N               | F(9,44)=5.404 p<0.001 |
| N:P               | -                     |

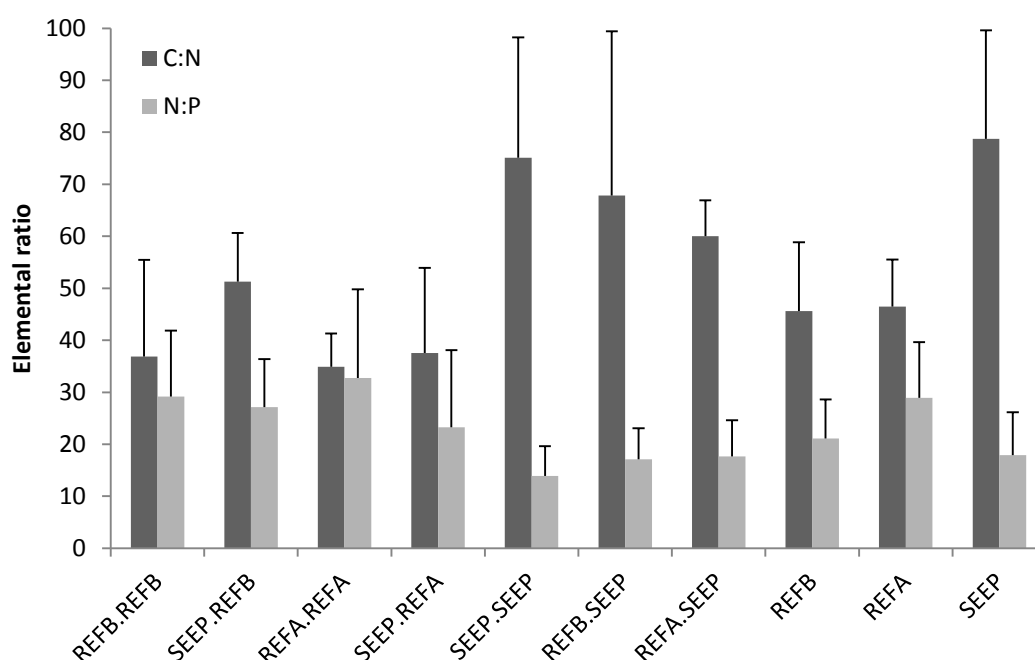


Figure 6.8. Mean C:N and N:P ratios ( $\pm$  SD, n=3-9) of *C. corniculata* thalli transplanted at Methana from September 2012 to June 2013 within and between two reference sites (REF A and REF B) and one high CO<sub>2</sub> site (SEEP). Treatments are shown as “Site of origin.Site of transplant”; the last three treatments (SEEP, REF A and REF B) are unmanipulated thalli collected in June 2013.

Calculating C:N ratios was not possible for many *J. rubens* samples because the nitrogen content was below detection limit; C and N were therefore analysed separately using a MANOVA, and results are reported in Table 6.11. Nitrogen content was significantly different among treatments, and it was higher in thalli

transplanted within the high CO<sub>2</sub> site and in unmanipulated thalli collected at SEEP (Figure 6.9).

Table 6.11. MANOVA on C and N content in *J. rubens* thalli transplanted at Methana from June 2013 to September 2013 within and between one reference site (REF B) and one high CO<sub>2</sub> site (SEEP). F-ratios (F) and p values are reported when significant (p<0.05).

| Response variable | Treatment              |
|-------------------|------------------------|
| C                 | -                      |
| N                 | F(6,22)=3.021; p=0.026 |

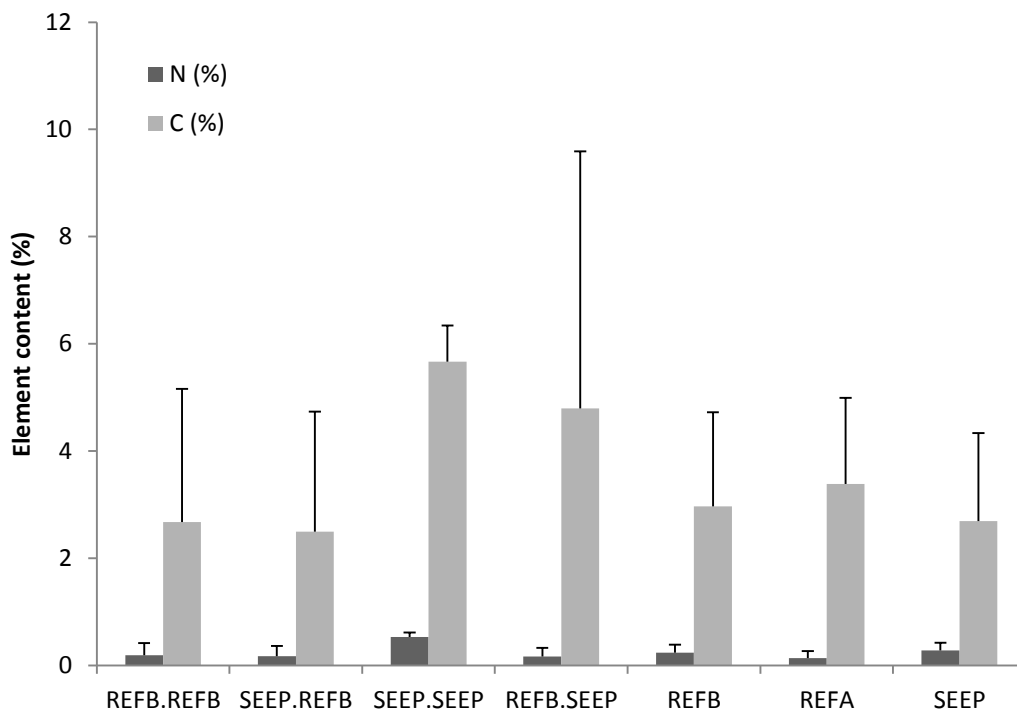


Figure 6.9. Mean C and N percent content ( $\pm$  SD, n=3-5) of *J. rubens* thalli transplanted at Methana from June 2013 to September 2013 within and between one reference site (REF B) and one high CO<sub>2</sub> site (SEEP). Treatments are shown as “Site of origin.Site of transplant”; the last three treatments (SEEP, REF A and REF B) are unmanipulated thalli collected in September 2013.

On the other hand, no significant differences among treatments were detected for inorganic carbon content of *J. rubens* transplants (Table 6.12). Percent

inorganic carbon content varies little among treatment, as it is around 80% for all of them (Figure 6.10).

Table 6.12. ANOVA results for transplanted *J. rubens* thalli inorganic carbon content. The table shows the source of variation and sum of squares (SS), degrees of freedom (df), Mean Squares (MS), F-ratios (F) and p values.

| Source    | Type III SS | df | MS     | F     | p     |
|-----------|-------------|----|--------|-------|-------|
| Treatment | 124.378     | 8  | 15.547 | 1.756 | 0.129 |
| Error     | 247.854     | 28 | 8.852  |       |       |
| Total     | 372.232     | 36 |        |       |       |

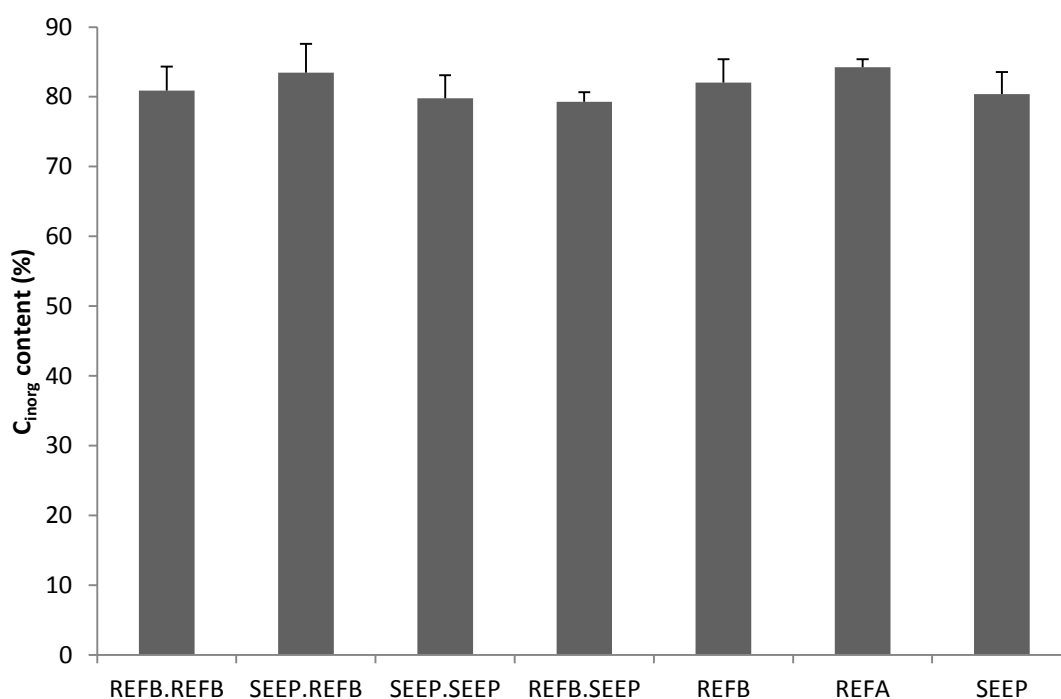


Figure 6.10. Mean inorganic carbon percent content ( $\pm$  SD,  $n=3-7$ ) of *J. rubens* thalli transplanted at Methana from June 2013 to September 2013 within and between one reference site (REF B) and one high CO<sub>2</sub> site (SEEP). Treatments are shown as “Site of origin.Site of transplant”; the last three treatments (SEEP, REF A and REF B) are unmanipulated thalli collected in September 2013.

### 6.3.5 Total phenolic compounds

Total phenolic compounds of *C. corniculata* thalli were significantly different among treatments (Table 6.13). In general, thalli transplanted to the high CO<sub>2</sub> site (SEEP) had higher phenols content compared to those transplanted to the two reference sites (REF A and REF B). However, macroalgae transplanted from high CO<sub>2</sub> levels to the reference sites (i.e. SEEP.REFA and SEEP. REFB) had higher phenols content than those transplanted within the same reference site (i.e. REFA.REFA and REFB.REFB), whereas unmanipulated thalli did not show clear patterns, possibly because of high within-site variability (Figure 6.11).

Table 6.13. ANOVA on phenols content of *C. corniculata* thalli transplanted at Methana from September 2012 to June 2013 within and between two reference sites (REF A and REF B) and one high CO<sub>2</sub> site (SEEP). The table shows the source of variation and sum of squares (SS), degrees of freedom (df), Mean Squares (MS), F-ratios (F) and p values.

| Source    | Type III SS | df | MS    | F     | p            |
|-----------|-------------|----|-------|-------|--------------|
| Treatment | 10.922      | 9  | 1.214 | 3.202 | <b>0.004</b> |
| Error     | 18.189      | 48 | 0.379 |       |              |
| Total     | 29.111      | 57 |       |       |              |

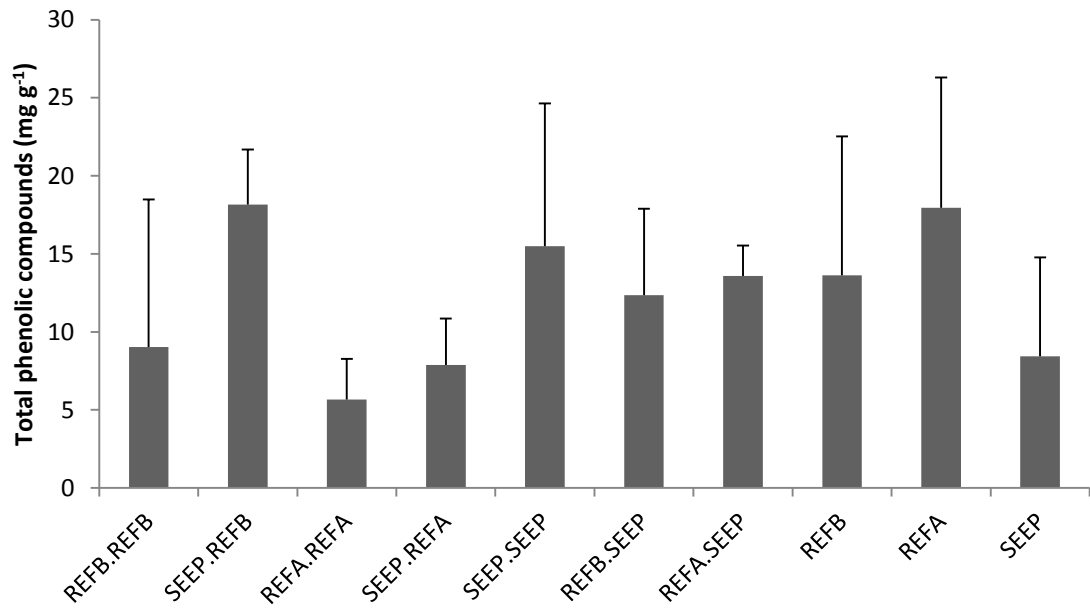


Figure 6.11. Mean phenols concentration ( $\pm$  SD,  $n=3-10$ ) of *C. corniculata* thalli transplanted at Methana from September 2012 to June 2013 within and between two reference sites (REF A and REF B) and one high CO<sub>2</sub> site (SEEP). Treatments are shown as “Site of origin.Site of transplant”; the last three treatments (SEEP, REF A and REF B) are unmanipulated thalli collected in June 2013.

## 6.4 Discussion

For the first time, long-term acclimatisation of macroalgae to ocean acidification has been assessed using reciprocal transplantations within and between areas with reference and elevated CO<sub>2</sub> levels. Results from these experiments suggest that phenotypic plasticity allowed *C. corniculata* and *J. rubens* to alter their physiological performance depending on the carbonate chemistry at the site of transplant. Although both species could survive  $p\text{CO}_2$  levels up to 1700  $\mu\text{atm}$ , *C. corniculata* seemed to be favoured at elevated CO<sub>2</sub>; this might lead to reduced abundances of *J. rubens* and increased abundances of *C. corniculata* at elevated  $p\text{CO}_2$  as a consequence of altered inter-specific competition.

In contrast with the initial hypothesis, linear growth and photosynthetic performance of both species were not higher in thalli transplanted within  $p\text{CO}_2$  levels. No effects of site were detected for *J. rubens* growth, while *C. corniculata* growth was significantly lower at one of the reference sites (REF B). These findings are in contrast with recent studies reporting decreased growth of articulated coralline algae (Gao and Zheng, 2010; Hofmann *et al.*, 2011; Hofmann *et al.*, 2012; Cornwall *et al.*, 2014; Johnson *et al.*, 2014) and fucoid algae (Gutow *et al.*, 2014) at elevated  $\text{CO}_2$  levels. However, responses to ocean acidification in macroalgae present high inter-specific variability (Kroeker *et al.*, 2013a), and fucoid algae can show no change or increase in growth rates as  $\text{CO}_2$  increases (Swanson and Fox, 2007; Chen and Zou, 2014). In addition, experiments have mostly been conducted in laboratories for relatively short periods of time, whereas this field experiment lasted three and nine months for *J. rubens* and *C. corniculata*, respectively. In the field, many macroalgae do not reach their maximum potential frond size as their linear growth is limited by external factors, such as wave motion or nutrient limitation (Fisher and Martone, 2014). As *C. corniculata* dry biomass showed an increasing trend with increasing seawater  $p\text{CO}_2$  at Methana (see Chapter 4), biomass of transplanted seaweeds might have shown a response to  $p\text{CO}_2$  levels. However, measuring biomass change was not possible in this study, as the seaweeds were permanently attached to the substratum using epoxy putty, which remained on the transplanted thalli after they were collected at the end of the experiment.

Photosynthetic performance of *J. rubens* was unaffected by the transplant, whereas *C. corniculata* had higher  $r\text{ETR}_{\text{max}}$  when transplanted to elevated  $\text{CO}_2$ . This is in accord with recent findings by Johnson *et al.* (2014), who did not find strong and consistent effects of ocean acidification on algal photophysiology.

Previous studies on articulated coralline algae have shown that although photosynthetic parameters are often not affected by increased CO<sub>2</sub> (Hofmann *et al.*, 2012), oxygen production can be negatively affected by ocean acidification (Hofmann *et al.*, 2011). However, oxygen production in articulated coralline algae is less affected by increased CO<sub>2</sub> compared to crustose forms (Noisette *et al.*, 2013b), and algae acclimated to elevated CO<sub>2</sub> in tidal pools do not show changes in their productivity as carbon dioxide increases (Egilsdottir *et al.*, 2013). As for brown algae, maximum quantum yield ( $F_v/F_m$ ) is normally not affected by elevated CO<sub>2</sub>, although it can decrease in combination with elevated temperature (Johnson *et al.*, 2012; Olabarria *et al.*, 2013). Similarly to *C. corniculata*, increased photosynthetic capacity (rETR<sub>max</sub>) at high CO<sub>2</sub> has been reported for *Padina pavonica* at seeps off Vulcano (Johnson *et al.*, 2012).

Both macroalgal species showed an increase in some pigment contents when transplanted to elevated CO<sub>2</sub> levels. This is in accord with the initial hypothesis for *C. corniculata*, but not for *J. rubens*, possibly because most information about coralline algal responses to ocean acidification comes from laboratory studies, while there are a few field studies on brown algae. *Cystoseira corniculata* thalli transplanted to the seeps had increased chlorophyll *c* and antheraxanthin, although unmanipulated thalli did not show any CO<sub>2</sub>-dependent pattern. Antheraxanthin is an intermediate and volatile compound in the xanthophyll cycle (Goss and Jakob, 2010); its increase is thus unlikely to have physiological significance, especially since de-epoxidation state did not change significantly among treatments (data not shown). *J. rubens* thalli transplanted to SEEP had higher chlorophyll *a*, violaxanthin, zeaxanthin and phycocyanin content. Some pigments of *J. rubens* were affected by the transplant: thalli transplanted to their site of origin had higher pheophytin *a* and β-carotene



content than those transplanted to another site. Few studies have examined changes in macroalgal pigment content with increased CO<sub>2</sub>; laboratory experiments performed so far have not found significant effects of ocean acidification on pigments in brown and red algae (Egilsdottir *et al.*, 2013; Noisette *et al.*, 2013a; Noisette *et al.*, 2013b; Yildiz *et al.*, 2013; Bender *et al.*, 2014). However, the only study examining macroalgal pigment content at volcanic seeps found increased chlorophyll *a* and *c* content in *Padina pavonica* grown at elevated CO<sub>2</sub> (Johnson *et al.*, 2012). Most laboratory experiments were conducted over a relatively short time (up to three months); it is then possible that macroalgae increase some species' pigment production at increased CO<sub>2</sub> levels, but only in the long term. However, the crustose coralline alga *Lithophyllum cabiochae* did not show significant changes in chlorophyll *a* concentration after being exposed to elevated CO<sub>2</sub> for one year (Martin *et al.*, 2013); more studies are therefore needed to test this phenomenon.

Epiphyte cover of *C. corniculata* was lower in thalli transplanted near the seeps, and was slightly lower in thalli transplanted from the SEEP site than in thalli from reference sites. This could be partly explained by the increase in total phenolic compounds in thalli transplanted to SEEP and the smaller phenols increase in those transplanted from SEEP; increased total phenolic compounds with elevated CO<sub>2</sub> have already been found in other brown algae, although not all species exhibit this pattern (Swanson and Fox, 2007; Poore *et al.*, 2013). Although phenolic compounds, mostly phlorotannins, have been proven to inhibit grazers and protect brown seaweeds from UV radiation (Halm *et al.*, 2010), their effect on seaweed epiphyte load is not clear (Jennings and Steinberg, 1997; Brock *et al.*, 2008). In fact, epiphyte settlement on macroalgae

is likely controlled by other factors, including macroalgal morphology and polar secondary metabolites such as terpenoids (Jennings and Steinberg, 1997).

Phenols are carbon-dense compounds, and their increase could explain the increased C:N ratio in *C. corniculata* thalli transplanted to SEEP. The increase in C:N ratio was caused by increased carbon and decreased nitrogen content in thalli transplanted to SEEP. This is in contrast with other studies reporting decreased C:N ratio in macroalgae exposed to high CO<sub>2</sub>; in one case this was caused by decreased nitrogen content with increased CO<sub>2</sub> (Falkenberg *et al.*, 2013b; Gutow *et al.*, 2014). Decreased nitrogen content with increased CO<sub>2</sub> is also reported by Kübler *et al.* (1999) for the red alga *Lomentaria articulata*, but coupled with decreased carbon content. However, increased CO<sub>2</sub> did not change carbon and nitrogen content in many brown and red macroalgae (Olabarria *et al.*, 2013; Poore *et al.*, 2013). The strong grazing pressure at Methana could have driven an enhanced carbon-dense chemical defences production at SEEP thanks to increased CO<sub>2</sub> availability (Connell *et al.*, 2013); chemical defences of brown algae and seagrasses may therefore be differently affected by ocean acidification, as the latter had lower defensive compound contents at elevated pCO<sub>2</sub> levels (Arnold *et al.*, 2012).

Unaltered inorganic carbon content of *J. rubens* among treatments suggests that this species is not locally adapted to elevated pCO<sub>2</sub> and can maintain calcification rates at high CO<sub>2</sub> and counter increased dissolution rates at volcanic seeps. Work to date has shown that articulated coralline algal calcification is less affected by increased CO<sub>2</sub> than that of crustose forms; although net calcification rates of articulated coralline algae can decrease at elevated CO<sub>2</sub>, crustose coralline algae often start dissolving at pCO<sub>2</sub> levels above 1000 µatm (Hofmann *et al.*, 2011; Noisette *et al.*, 2013b; Johnson *et al.*,

2014). However, maintaining calcification rates at decreased calcium carbonate saturation is energetically expensive (Bradassi *et al.*, 2013), and *J. rubens* thalli grown at reference sites might not be able to maintain them when exposed to elevated CO<sub>2</sub> for longer periods. Some coralline algae exposed to high CO<sub>2</sub> for two months or more have lower proportions of very soluble high-Mg calcite in their skeletons (Egilsdottir *et al.*, 2013; Diaz-Pulido *et al.*, 2014). *Jania rubens* transplanted near seeps at Methana could therefore have modified mineralogy or increased the production of calcification-inducing compounds to improve survival at elevated CO<sub>2</sub> levels (Koch *et al.*, 2013). However, articulated coralline algae appear to increase their Mg content with increasing seawater temperatures (Williamson *et al.*, 2014). The concurrent increase in seawater pCO<sub>2</sub> levels and temperatures predicted for 2100 may therefore impair the ability of articulated coralline algae to modify their mineralogy to better resist to ocean acidification.

To summarise, the two seaweeds examined changed their physiology depending on the environmental conditions at the site of transplant. The possibility of genotypic differentiation among populations of these macroalgal species depending on their acclimatisation to elevated CO<sub>2</sub> cannot be excluded until genetic studies are performed. Species with high phenotypic plasticity could in fact have genetically adapted to elevated CO<sub>2</sub> levels, but show similar physiological performances, a phenomenon named “phenotypic buffering” (Sunday *et al.*, 2014). Changes in the physiological performance of dominant macroalgal species are likely to alter the outcome of competition between them; this is reflected by the macroalgal community composition near seeps off Methana. Here, *J. rubens* cover decreased, whereas *C. corniculata* cover increased with increasing pCO<sub>2</sub> (Chapter 3; Figure 6.12). This study therefore

shows that even after centuries of exposure to high CO<sub>2</sub> levels, two dominant macroalgal species did not appear to have permanently acclimatised to elevated carbon dioxide levels. This is likely to heavily influence temperate coastal ecosystems, as the observed changes in benthic community structure are likely to indirectly influence upper trophic levels and ecosystem processes such as nutrient cycling or carbon sequestration.

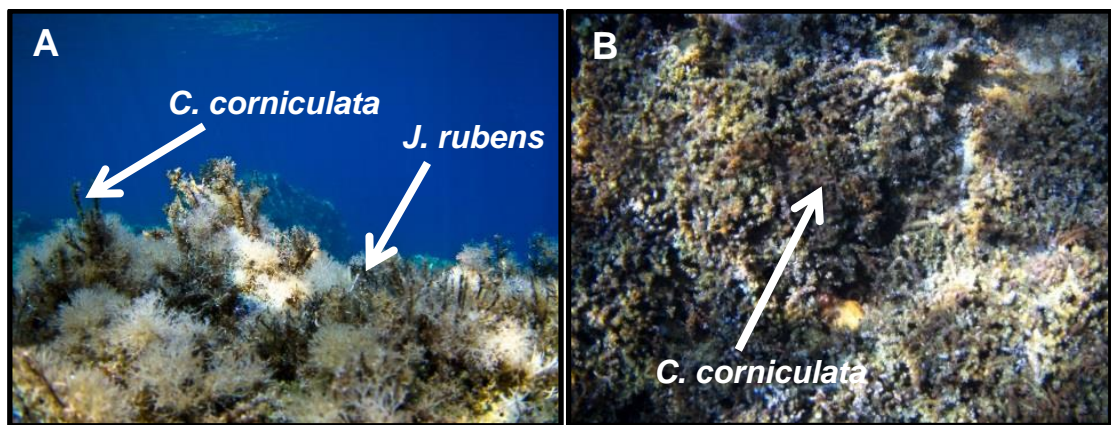


Figure 6.12. Typical macroalgal assemblage off Methana in September 2013 at reference sites (A) with abundant *J. rubens* overgrowing *C. corniculata*, and near the seeps (B), where *C. corniculata* could grow undisturbed; photos by Maria Salomidi.

## **Chapter 7**

**A short-term copper pulse affects macroalgal copper accumulation and indirectly alters epifaunal colonisation at elevated pCO<sub>2</sub>.**

## Abstract

Ocean acidification is expected to interact with other anthropogenic stressors to modify marine ecosystems. Copper is toxic to marine organisms, and copper pulses are a common source of pollution in coastal areas. In this study, calcifying and non-calcifying seaweeds acclimatised to high CO<sub>2</sub> at volcanic seeps or from reference sites were exposed to elevated CO<sub>2</sub> and a 36-hour copper pulse in two field experiments. Invertebrate re-colonisation of a fucoid alga exposed to copper at different pCO<sub>2</sub> levels was also assessed. *Cystoseira corniculata* and *Jania rubens* accumulated more copper in high CO<sub>2</sub> conditions. *Jania rubens* grown near the seeps accumulated more copper than those transplanted from reference sites. These changes had no effect on maximum quantum yield of both species. *Cystoseira corniculata* pigment contents changed little, but total carotenoids decreased in *J. rubens* thalli exposed to copper at both sites. Phycoerythrin content in *J. rubens* slightly increased in thalli exposed to copper at the reference site, whereas it decreased following copper exposure at elevated CO<sub>2</sub> levels. Previous acclimatisation to high CO<sub>2</sub> did not influence seaweed responses to copper. However, copper accumulation at high CO<sub>2</sub> altered epifaunal community structure near the seeps, but not in reference conditions. Thus, multiple stressors can interact and increase the magnitude of changes in benthic communities.

## 7.1 Introduction

Macroalgal responses to increased CO<sub>2</sub> differ in calcifying and non-calcifying species (Porzio *et al.*, 2011). Carbon concentrating mechanisms (CCMs) are used by most macroalgae to convert bicarbonate ions into carbon dioxide; these are energy expensive, so increased dissolved CO<sub>2</sub> decreases the energy needed to obtain the substratum for photosynthesis (Cornwall *et al.*, 2012). On the other hand, calcifying algae face increased energetic costs of calcification as calcium carbonate saturation levels fall due to ocean acidification (Bradassi *et al.*, 2013; Koch *et al.*, 2013). The combination of increased carbon availability and higher energetic cost of calcification may cause shifts from coralline-dominated to fleshy seaweed communities as atmospheric CO<sub>2</sub> increases (Connell *et al.*, 2013), not only because of reduced coralline algae growth (Küffner *et al.*, 2008; Martin *et al.*, 2008; Kroeker *et al.*, 2010), but also because of altered competitive interactions between calcified and non-calcified algae (Kroeker *et al.*, 2013c; Short *et al.*, 2014).

Acclimatisation or adaptation to high pCO<sub>2</sub> could improve the ecological performance of calcifying algae as oceans become acidified (Hofmann *et al.*, 2010). Algae living in high-CO<sub>2</sub> environments could therefore be helpful in determining the potential for acclimatisation in these organisms. So far, very few studies have tackled this issue. For instance, the green microalga *Chlamydomonas reinhardtii* changes its physiology when exposed to elevated carbon dioxide over multiple generations, showing reduced CO<sub>2</sub> uptake using carbon-concentrating mechanisms (Collins *et al.*, 2006) and a marine coccolithophore has the potential to adapt to future CO<sub>2</sub> concentrations (Lohbeck *et al.*, 2012). Unpublished work at volcanic seeps off Ischia (Italy)

indicates that the genome of the brown macroalga *Dictyota* sp. changes at high pCO<sub>2</sub>, resulting in the dominance of a stress-resistant form (Porzio, 2010).

Although there is scant information regarding macroalgal acclimatisation to elevated CO<sub>2</sub>, their response to other changing abiotic conditions is widely studied. For example, there are species-specific differences in the tolerance of macroalgae to changes in salinity (Ryan *et al.*, 2004), temperature (Collén and Davison, 2001) and light (Bischof *et al.*, 2006) depending on the natural variability of their habitat. In some cases, intraspecific differences in responses to environmental stressors have been detected (Pearson *et al.*, 2009). These differences can have a hereditary component (Nielsen *et al.*, 2003) and environmental isolation can lead to rapid speciation, such as for *Fucus* spp. in the Baltic Sea (Bergström *et al.*, 2005).

Epifaunal communities are affected by increased CO<sub>2</sub> as well, with decreased abundance of molluscs and an increase of some crustacean taxa (Kroeker *et al.*, 2011). The above responses mostly conform to predictions based on laboratory experiments (Kroeker *et al.*, 2013a), but communities studies have also found unexpected community changes due to altered inter-specific interactions (Hale *et al.*, 2011). Invertebrates can also exhibit enhanced sensitivity to ocean acidification if they are concurrently exposed to another stressor, such as increased temperature or low food availability (Rodolfo-Metalpa *et al.*, 2011; Kroeker *et al.*, 2013a; Thomsen *et al.*, 2013).

Since ocean acidification is only one of the changes humans are causing in the marine realm, we have to consider that several abiotic factors are acting interdependently, with interactive and sometimes unexpected results (Shears and Ross, 2010; Gaylord *et al.*, 2014). Even if calcifying macroalgae have the



potential to acclimatise or adapt to high CO<sub>2</sub>, they could be more sensitive to any additional stressors due to the increased energetic cost of calcification (Bradassi *et al.*, 2013). On the other hand, non-calcifying algae could be more resistant to additional stressors as they have more energy available after reducing their use of carbon concentrating mechanisms (CCMs; Cornwall *et al.*, 2012), leading to more drastic community changes compared to the effects of CO<sub>2</sub> alone.

In coastal waters, copper is a common pollutant as it is mined in many regions (Figure 7.1); however, copper can also derive from urban runoff (Pitt, 1995), industrial waste (Apte and Day, 1998) or antifouling paints (Paulson *et al.*, 1989). Copper is extremely toxic at high concentrations, especially before binding to organic material (Hall *et al.*, 1998). As a consequence, copper pulses are common in coastal waters near human settlements and industries. Copper accumulates in macroalgal tissues and can strongly inhibit photosynthesis by damaging photosystem II (Schröder *et al.*, 1994). Many invertebrate taxa are negatively affected by copper as well (Johnston *et al.*, 2002), and seaweed epifauna is more strongly affected because they are exposed to copper through the macroalgae they live in and feed upon (Roberts *et al.*, 2006). Other effects of elevated copper concentrations on marine flora and fauna include reduced growth and calcification, altered osmoregulatory processes and oxidative damage (Thurberg *et al.*, 1973; Kangwe *et al.*, 2001; Collén *et al.*, 2003), although there is large among-taxa variability in copper sensitivity (Mayer-Pinto *et al.*, 2010). In addition, some organisms can be more sensitive to copper exposure at elevated CO<sub>2</sub> levels because of the higher energetic cost of maintaining physiological processes (Roberts *et al.*, 2013).

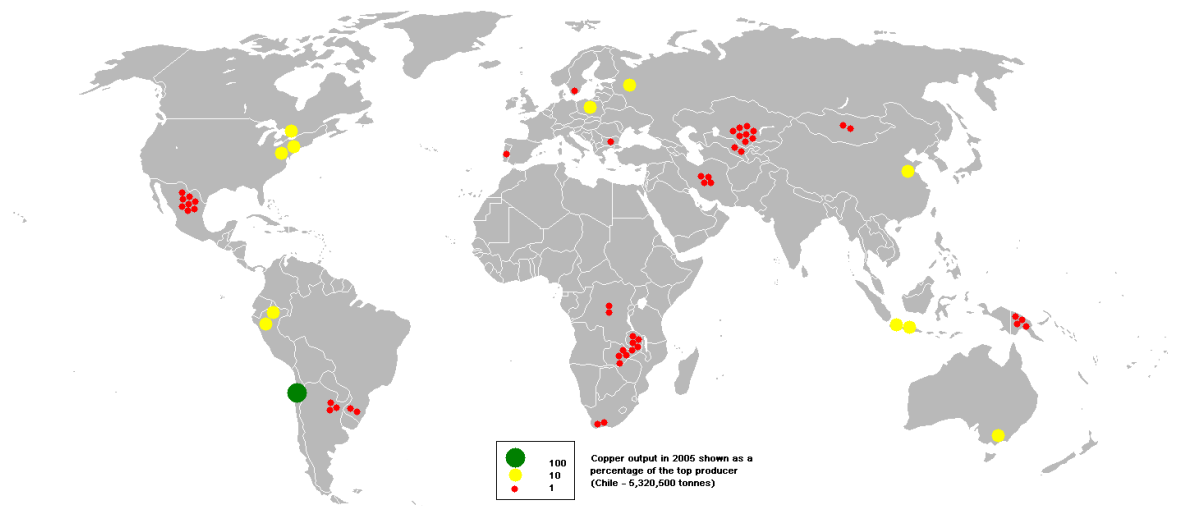


Figure 7.1. Copper production in 2005 shown as a percentage of the top producer (Chile, 5320500 tonnes). Data from British Geological Survey.

This study investigated how populations of a calcifying and a non-calcifying alga from high or reference pCO<sub>2</sub> areas responded to a short-term copper pulse *in situ*, and assesses how re-colonisation by seaweed epifauna was affected by copper exposure at different pCO<sub>2</sub> levels. The hypotheses tested were:

- 1) A non-calcifying alga (*Cystoseira corniculata*) is more resistant to short-term copper stress in high CO<sub>2</sub> conditions;
- 2) A calcifying alga (*Jania rubens*) is less resistant to short-term copper stress in high pCO<sub>2</sub> conditions, but this effect is reduced for algae acclimatised to high CO<sub>2</sub>;
- 3) *Cystoseira corniculata* epifaunal colonisation is negatively affected by copper exposure, and the effect of copper is stronger at high CO<sub>2</sub>.

## **7.2 Methods**

### **7.2.1 Study area**

Experiments were carried out in June and September 2013 at two sites (see Chapter 2, Figure 2.1), one characterised by high pCO<sub>2</sub> due to hydrothermal activity (SEEP) and a reference site (REF A); detailed sampling dates and sample sizes are reported in Table 1.1F. Macroalgae were also collected from another reference site (REF B). A geochemical survey of the area revealed that none of the study sites was contaminated with respect to copper, making the area suitable for testing the effects of copper pollution on non-adapted organisms (Chapter 2).

### **7.2.2 Experimental design**

Two common macroalgal species were chosen to test the combined effects of elevated carbon dioxide and copper, the brown alga *Cystoseira corniculata* in June 2013 and the articulated coralline *Jania rubens* in September 2013. Similarly sized thalli were collected from three sites, one characterised by high pCO<sub>2</sub> (SEEP) and two reference sites (REF A and REF B). The algae were kept in coolers and transported from their site of origin to the sites REF A and SEEP, and attached with cable ties on plastic attached to concrete blocks and deployed at the same depth the thalli were collected from (<0.5 m). Individuals from REF A and SEEP were transplanted both in their site of origin and in the other site, while individuals from REF B were transplanted to REF A and SEEP. Ten individuals per species per treatment were attached to nets, left 48 h to acclimatise to the new conditions and then half of them were exposed for three days to increased copper levels via plaster blocks containing copper attached to

their nets. A scheme of the experimental design is shown in Figure 7.2, and transplanted *J. rubens* is shown in Figure 7.3.

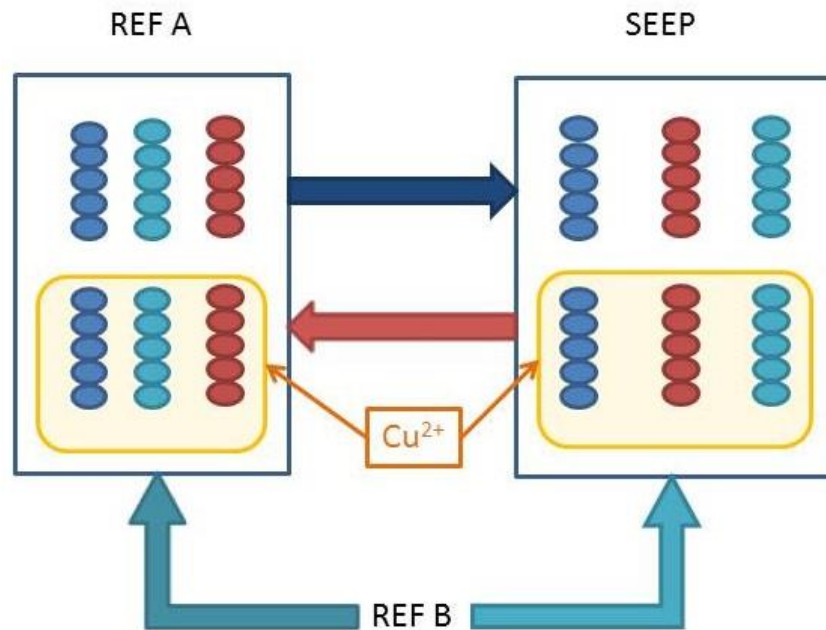


Figure 7.2. Off Methana (Greece), ten individuals from SEEP (red circles), REF A (blue circles) and REF B (light blue circles) were transplanted to SEEP and REF A (blue rectangles) in June (*C. corniculata*) and September 2013 (*J. rubens*). After 48 h, half were exposed to 36 h copper pulses (shaded).

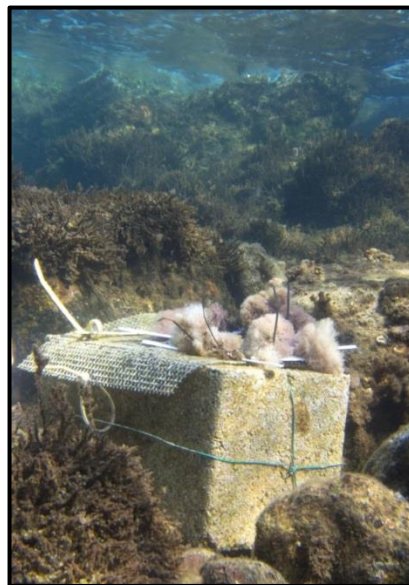


Figure 7.3. *Jania rubens* transplanted near seeps off Methana (Greece) in September 2013 before being exposed to copper; the individual thalli were attached to plastic nets using

cable ties, and the net was attached to a concrete block deployed on the rocky shore at depth < 0.5 m.

For these experiments, CuSO<sub>4</sub> (copper II sulfate anhydrous) was used as a reference toxicant following methods described by Johnston and Webb (2000). 3.2 g of CuSO<sub>4</sub> were dissolved in 13 g of deionized water and refrigerated at 4°C for 60 min. Fifteen grams of dental plaster were refrigerated for 60 min, then mixed with the cool copper solution. The plaster was poured into 4 cm diameter plastic cups and left to dry for seven days. The same process was used to make control blocks except for the CuSO<sub>4</sub> addition. Plaster was changed daily and the removed blocks were air dried and weighed to check that all macroalgae were exposed to a comparable amount of copper.

In June 2013, an additional experiment was performed to test how *C. corniculata* re-colonisation by invertebrates was influenced by copper at different pCO<sub>2</sub> levels. Ten similarly sized *C. corniculata* thalli per site were detached from the rocky substratum using hammer and chisel, briefly rinsed with fresh water to remove all mobile invertebrates and attached to the nets used for the macroalgal physiology experiment, resulting in five copper-exposed and five control thalli per site.

### **7.2.3 Environmental parameters monitoring**

Environmental parameters were measured daily for the duration of the experiments. Temperature, salinity and pH were monitored in both sites with a multiprobe (YSI Professional Series, Professional Plus) and total alkalinity was sampled twice per site on the first and the last day of the experiment using borosilicate bottles. Total alkalinity samples were treated and analysed according to the procedures detailed in Chapter 2. The other carbonate

chemistry parameters were calculated from pH and total alkalinity using CO2Sys software (Lewis and Wallace, 1998).

#### 7.2.4 Sampling and laboratory analyses

Physiological responses of transplanted thalli of both species were assessed by measuring *in vivo* chlorophyll *a* fluorescence associated with Photosystem II by using a portable pulse amplitude modulated fluorometer Diving-PAM (Diving PAM, Walz, Effeltrich, Germany). The maximum quantum yield ( $F_v/F_m$ ) of apical shoots was measured after 10 minutes of dark acclimation before transplanting the seaweeds, after the acclimation period and after copper exposure. Appropriate duration of dark acclimation was determined by measuring  $F_v/F_m$  of ten thalli per species after 5, 10, 15 and 20 minutes in the dark. Maximum quantum yield ( $F_v/F_m$ ) relates the capacity for photochemical quenching ( $F_v = F_m - F_0$ , where  $F_0$  is the basal fluorescence of dark-adapted thalli and  $F_m$  is the maximal fluorescence after a saturation light pulse of  $> 4000 \mu\text{mol m}^{-2} \text{s}^{-1}$ ) to the total fluorescence emission of closed PSII reaction centres ( $F_m$ ).  $F_v/F_m$  is then directly proportional to the quantum efficiency of PSII photochemistry (Butler 1978), and its reduction from maximal values is an indicator of stress responses, and specifically of metal stress (Mallick and Mohn, 2003).

After 36 h of copper exposure, *C. corniculata* thalli were collected and transported to the field laboratory in coolers. There their apical parts were gently scrubbed of epiphytes, rinsed with distilled water and immediately frozen in liquid nitrogen. The samples were subsequently stored at  $-80^\circ\text{C}$  until they were analysed for copper content and pigment composition. In June 2013, *C. corniculata* thalli for the invertebrate re-colonisation experiment were covered with plastic zip-lock bags, taken from the site and transported to the field

laboratory, where they were sieved (200 µm mesh) and stored in 70% Industrial Methylated Spirit (IMS). Samples were later sorted and mobile invertebrates identified to the lowest possible taxonomic level. Macroalgal thalli were dried in an oven at 50 °C for 72h and weighed ( $\pm 1$  mg accuracy) to determine dry mass (DW).

Samples for copper concentration were freeze-dried for 24h and ground with pestle and mortar; approximately 0.1 g of each sample was weighed in acid-washed Teflon tubes with a high precision digital scale (0.1 mg accuracy). Two ml of concentrated nitric acid were then added; the tube containing the digestant was then placed in a high-Throughput Microwave Reaction System Run (MARSXpress, CEM Corporation, Matthews, USA) and gently heated to boiling for at least 1 h to ensure full digestion. Samples were allowed to cool, quantitatively transferred into pre-cleaned 10 ml volumetric flasks and diluted to volume with Milli-Q water. Blanks were prepared following the same procedure, but omitting the sample; the digested samples were then analysed using inductively coupled plasma optical emission spectrometry (ICP-OES).

Samples for pigment analysis were freeze-dried in the dark for 24h, after which they were grinded in pure acetone using mortar and pestle. Extraction occurred at 4°C for 24 h in the dark. After extraction samples were centrifuged at 4000 rpm for 15 min at 4°C. Pigment content was then analysed using the Gauss-Peak Spectra method (Küpper *et al.*, 2007). Samples were scanned in a dual-beam spectrophotometer from 350 nm to 750 nm at 1 nm steps. The absorbance spectra were introduced in the GPS fitting library using SigmaPlot. The employment of this library allowed to identify and quantify Chlorophyll a, Chlorophyll c1 and c2, Pheophytin a, Fucoxanthin, Antheraxanthin,  $\beta$ -carotene, Violaxanthin and Zeaxanthin for *C. corniculata* and Chlorophyll a, Pheophytin a,

$\beta$ -cryptoxanthin, Antheraxanthin,  $\beta$ -carotene, Violaxanthin and Zeaxanthin for *J. rubens*. For phycobiliproteins approximately 0.5 g of tissue was homogenised in 10 mL 0.1 M phosphate buffer (pH 6.8). After being left at 4°C in the dark overnight, extracts were centrifuged for 10 minutes at 1000g and read in the spectrophotometer at the wavelengths determined by Beer and Eshel (1985).

### 7.2.5 Statistical analyses

All data were tested for normality and homogeneity of variances by visual evaluation of boxplots and residuals and using Levene's test, respectively. Analysis of pH data was performed using a non-parametric analysis (Kruskal-Wallis ANOVA); the two study periods were analysed separately. Mass loss of plaster blocks containing copper was performed using a one-way ANOVA with site as fixed factor. Copper content in seaweed tissues and changes in  $F_v/F_m$  following transplant and copper exposure were analysed using three-way ANOVAs with three fixed factors (site of origin, site of transplant, copper exposure). The analysis of hydrophilic pigments and phycobilins (only for September 2013) was performed through three-way MANOVAs with the same factors of previous analyses. When the data did not meet Mauchly's test of sphericity, the degrees of freedom were corrected using Greenhouse-Geisser estimates of sphericity. All of the analyses above were performed using SPSS v. 19 (IBM, USA).

Mobile invertebrates community composition and abundance was tested using a two-factor PERMANOVA with "site" and "copper" as fixed factors and "biomass" as a covariate. A square-root transformation was used to reduce the influence of abundant taxa in the community, and Type I sums of squares with 9,999 permutation of residuals under a reduced model was used. Variance



derived from significant interactions was then decomposed to determine which factor determined the significant interaction, and pairwise tests were performed when necessary. A nMDS plot was also used to visually inspect the similarities among samples. A SIMPER analysis was then used to determine the contribution of each taxon to the average Bray-Curtis dissimilarity between levels of a factor if the PERMANOVA analysis was significant. The SIMPER analysis was performed on broad taxonomic categories for ease of interpretation. All analyses above were performed using PRIMER 6 with PERMANOVA+ extension (Plymouth Routines In Multivariate Ecological Research, version 6).

## **7.3 Results**

### **7.3.1 Environmental parameters**

Data for environmental parameters monitored during the experiments are shown in Table 7.1. Statistical analyses of pH data revealed significant differences among sites in both seasons, and pH was lower at the SEEP site compared to the controls. On the other hand, average total alkalinity was similar among all sites and seasons. Temperature and salinity varied seasonally, but only showed small variability among sites.

Table 7.1. Mean ( $\pm$  SD) pH, total alkalinity (TA), temperature (T) and salinity (S) measured during the experiments, as well as parameters calculated with CO2SYS ( $p\text{CO}_2$ , bicarbonate ( $\text{HCO}_3^-$ ) and carbonate ( $\text{CO}_3^{2-}$ ) ions concentrations and saturation state of aragonite ( $\Omega_{\text{Ar}}$ ) and calcite ( $\Omega_{\text{Ca}}$ ); n=3-13.

| <b>June 2013</b>      |                          |                                   |                             |                     |                                       |   |   |                      |                      |
|-----------------------|--------------------------|-----------------------------------|-----------------------------|---------------------|---------------------------------------|---|---|----------------------|----------------------|
|                       | $\text{pH}_{\text{NBS}}$ | TA<br>(mmol<br>$\text{kg}^{-1}$ ) | T<br>( $^{\circ}\text{C}$ ) | S<br>(ppt)          | $p\text{CO}_2$<br>( $\mu\text{atm}$ ) | $\text{HCO}_3^-$<br>(mmol $\text{kg}^{-1}$ )    | $\text{CO}_3^{2-}$<br>(mmol<br>$\text{kg}^{-1}$ ) | $\Omega_{\text{Ar}}$ | $\Omega_{\text{Ca}}$ |
| SEEP                  | 7.59 $\pm$<br>0.06       | 2.785                             | 24.77 $\pm$<br>0.69         | 37.93 $\pm$<br>0.11 | 2205.9<br>$\pm$ 312.4                 | 2578.2 $\pm$<br>25.6                            | 86.6 $\pm$<br>10.7                                | 2.03 $\pm$<br>0.25   | 1.34 $\pm$<br>0.17   |
| REF A                 | 8.10 $\pm$<br>0.08       | 2.701                             | 24.12 $\pm$<br>0.43         | 38.20 $\pm$<br>0.42 | 575.1 $\pm$<br>134.0                  | 2148.9 $\pm$<br>78.5                            | 229.0<br>$\pm$ 32.1                               | 5.36 $\pm$<br>0.73   | 3.53 $\pm$<br>0.48   |
| REF B                 | 8.11 $\pm$<br>0.04       | 2.703                             | 22.96 $\pm$<br>0.45         | 38.16 $\pm$<br>0.45 | 543.4 $\pm$<br>54.9                   | 2156.9 $\pm$<br>24.8                            | 226.5<br>$\pm$ 10.3                               | 5.30 $\pm$<br>0.25   | 3.48 $\pm$<br>0.16   |
| <b>September 2013</b> |                          |                                   |                             |                     |                                       |   |   |                      |                      |
|                       | $\text{pH}_{\text{NBS}}$ | TA<br>(mmol $\text{kg}^{-1}$ )    | T<br>( $^{\circ}\text{C}$ ) | S<br>(ppt)          | $p\text{CO}_2$<br>( $\mu\text{atm}$ ) | $\text{HCO}_3^-$<br>(mmol<br>$\text{kg}^{-1}$ ) | $\text{CO}_3^{2-}$<br>(mmol<br>$\text{kg}^{-1}$ ) | $\Omega_{\text{Ar}}$ | $\Omega_{\text{Ca}}$ |
| SEEP                  | 7.65 $\pm$<br>0.03       | 2.785                             | 26.50<br>$\pm$ 0.17         | 39.60<br>$\pm$ 0.10 | 1913.9<br>$\pm$ 121.1                 | 2532.2<br>$\pm$ 14.2                            | 105.5<br>$\pm$ 3.2                                | 2.44 $\pm$<br>0.13   | 1.62 $\pm$<br>0.09   |
| REF A                 | 8.12 $\pm$<br>0.02       | 2.701                             | 25.87<br>$\pm$ 0.06         | 39.07<br>$\pm$ 0.35 | 534.2 $\pm$<br>33.4                   | 2097.6<br>$\pm$ 16.5                            | 249.8<br>$\pm$ 0.9                                | 5.81 $\pm$<br>0.23   | 3.86 $\pm$<br>0.15   |
| REF B                 | 8.10 $\pm$<br>0.04       | 2.703                             | 25.60<br>$\pm$ 0.36         | 38.90<br>$\pm$ 0.08 | 572.8 $\pm$<br>68.6                   | 2129.3<br>$\pm$ 39.1                            | 237.8<br>$\pm$ 1.8                                | 5.54 $\pm$<br>0.38   | 3.67 $\pm$<br>0.24   |

### 7.3.2 Copper exposure and accumulation

In June 2013, plaster blocks containing copper in the sites REF A and SEEP did not show significant differences in mass loss as they were deployed in the field ( $F_{1,7}=0.016$ ,  $p=0.903$ ); at both sites, plaster blocks lost approximately 68% of their initial mass, releasing comparable amounts of copper in seawater. Tissue copper content in *C. corniculata*, however, was significantly different between transplant sites, and copper had a significant effect as well (Table 7.2). Samples originating from REF B had to be removed from analysis due to high sample loss. Figure 7.4 shows that *C. corniculata* exposed to copper had much higher tissue copper concentration than control thalli, and that site of transplant had a major effect on copper accumulation, with individuals transplanted to SEEP accumulating 3-4 times more copper than those transplanted to REF A.

Table 7.2. ANOVA on log-transformed copper concentration in *C. corniculata* thalli measured at the end of the experiment of June 2013. The table shows main factors and their interactions and sum of squares (SS), degrees of freedom (df), Mean Squares (MS), F-ratios (F) and p values. Significant p values (< 0.05) are highlighted.

| Source                       | Type III SS | df | MS     | F ratio | p              |
|------------------------------|-------------|----|--------|---------|----------------|
| Copper                       | 89.456      | 1  | 89.456 | 209.475 | < <b>0.001</b> |
| Origin                       | 0.836       | 1  | 0.836  | 1.958   | 0.172          |
| Transplant                   | 22.459      | 1  | 22.459 | 52.591  | < <b>0.001</b> |
| Copper * Transplant          | 0.434       | 1  | 0.434  | 1.017   | 0.321          |
| Origin * Transplant          | 0.568       | 1  | 0.568  | 1.330   | 0.258          |
| Origin * Copper              | 1.087       | 1  | 1.087  | 2.545   | 0.121          |
| Origin * Transplant * Copper | 0.124       | 1  | 0.124  | 0.290   | 0.594          |
| Error                        | 13.239      | 31 | 0.427  |         |                |
| Total                        | 130.769     | 38 |        |         |                |

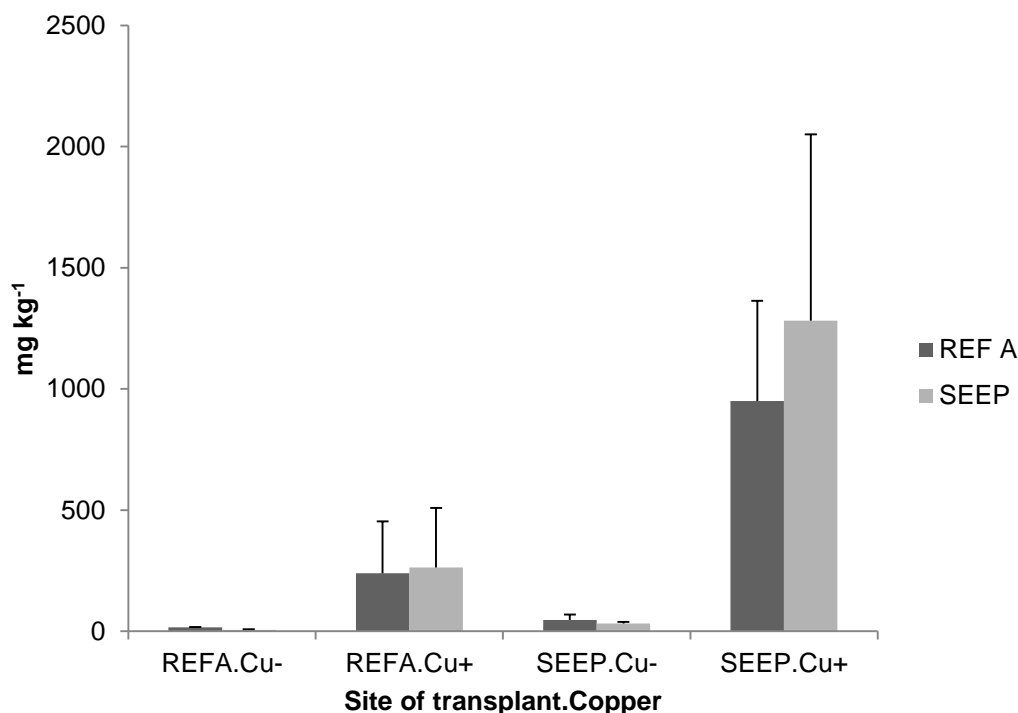


Figure 7.4. Mean ( $\pm$  SD, n=4-5) copper concentration ( $\text{mg kg}^{-1}$ ) of *C. corniculata* thalli transplanted from REF A or SEEP not exposed (Cu-) or exposed (Cu+) to copper at REF A and SEEP in June 2013.

In September 2013, percent mass loss of plaster blocks containing copper significantly differed between the sites REF A and SEEP ( $F_{1,7}=14.926$ ,  $p=0.008$ ); plaster blocks deployed at REF A lost approximately 78% of their initial mass, releasing more copper in seawater compared to the SEEP site, where plaster blocks only decreased in mass by 55%. Statistical analysis of tissue copper content in *J. rubens* showed that copper exposure caused different effects in the two sites (site of transplant \* copper interaction significant) and that copper concentration depended on the macroalgae origin as well (Table 7.3). Figure 7.5 shows that *J. rubens* exposed to copper had higher tissue copper concentration than control thalli, but this difference was much more evident at the SEEP site than at REF A. Furthermore, thalli originally from SEEP accumulated more copper than those originating from the reference sites.

Table 7.3. ANOVA on log-transformed copper concentration in *J. rubens* thalli measured at the end of the experiment of September 2013. The table shows main factors and their interactions and sum of squares (SS), degrees of freedom (df), Mean Squares (MS), F-ratios (F) and p values. Significant p values (< 0.05) are highlighted.

| Source                       | Type III SS | df | MS     | F ratio | p                 |
|------------------------------|-------------|----|--------|---------|-------------------|
| Origin                       | 1.896       | 2  | 0.948  | 6.652   | <b>0.003</b>      |
| Transplant                   | 1.243       | 1  | 1.243  | 8.719   | <b>0.005</b>      |
| Copper                       | 21.126      | 1  | 21.126 | 148.212 | <b>&lt; 0.001</b> |
| Origin * Transplant          | 0.209       | 2  | 0.105  | 0.734   | 0.485             |
| Origin * Copper              | 0.458       | 2  | 0.229  | 1.607   | 0.212             |
| Transplant * Copper          | 0.593       | 1  | 0.593  | 4.161   | <b>0.047</b>      |
| Origin * Transplant * Copper | 0.172       | 2  | 0.086  | 0.604   | 0.551             |
| Error                        | 6.557       | 46 | 0.143  |         |                   |
| Total                        | 32.106      | 57 |        |         |                   |

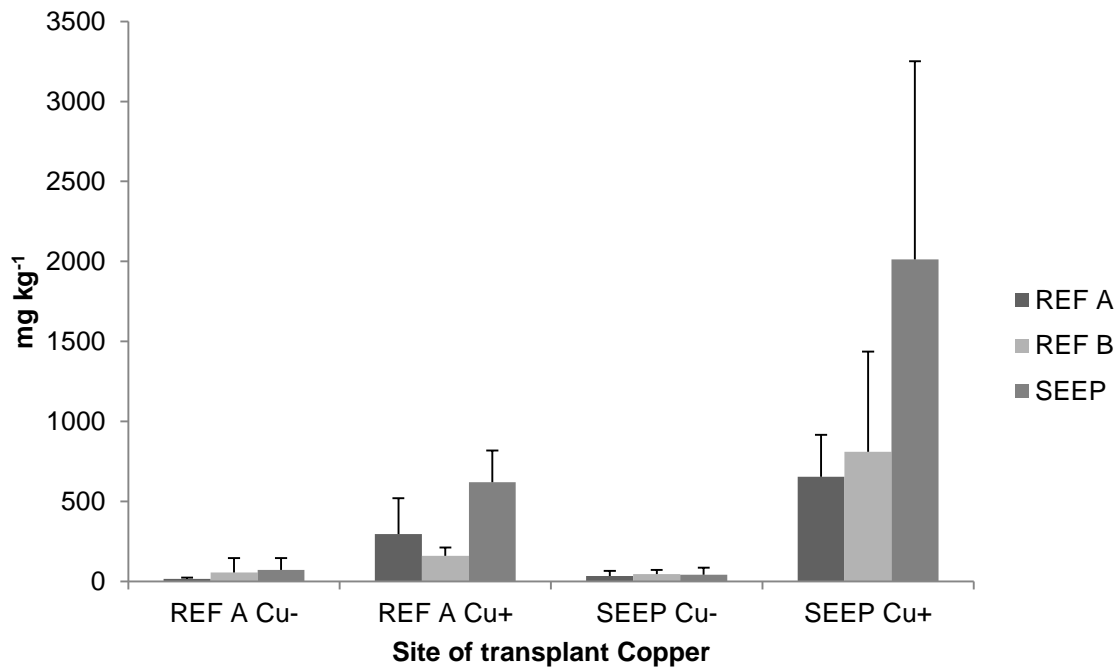


Figure 7.5. Mean ( $\pm$  SD,  $n=3-5$ ) copper concentration ( $\text{mg kg}^{-1}$ ) of *J.rubens* thalli transplanted from REF A, REF B or SEEP not exposed (Cu-) or exposed (Cu+) to copper at REF A and SEEP in September 2013.

### 7.3.3 Maximum quantum yield ( $F_v/F_m$ )

Change in maximum quantum yield in *C. corniculata* exposed to copper in June 2013 showed a significant interaction of origin and copper both after transplant and after copper exposure (Table 7.4). This was due to a sharp decrease in maximum quantum yield of the thalli from REF B that were to be exposed to copper; the same thalli recovered from the transplant later than the other groups, leading to a median increase in maximum quantum yield of about 0.2 after copper exposure, whereas all other groups showed no significant effects of copper (Figure 7.6).

Table 7.4. ANOVA on change in maximum quantum yield ( $F_v/F_m$ ) after transplant (upper part) and after copper exposure (lower part) for *C. corniculata* thalli in June 2013. The table shows main factors and their interactions and sum of squares (SS), degrees of freedom (df), Mean Squares (MS), F-ratios (F) and p values. Significant p values (< 0.05) are highlighted.

| <b>Effect of transplant:</b> |                    |           |           |                |              |
|------------------------------|--------------------|-----------|-----------|----------------|--------------|
| <b>Source</b>                | <b>Type III SS</b> | <b>df</b> | <b>MS</b> | <b>F ratio</b> | <b>p</b>     |
| Origin                       | 0.194              | 2         | 0.097     | 7.054          | <b>0.002</b> |
| Transplant                   | 0.065              | 1         | 0.065     | 4.731          | <b>0.035</b> |
| Copper                       | 0.048              | 1         | 0.048     | 3.488          | 0.069        |
| Origin * Transplant          | 0.051              | 2         | 0.025     | 1.842          | 0.171        |
| Origin * Copper              | 0.163              | 2         | 0.082     | 5.941          | <b>0.005</b> |
| Transplant * Copper          | <0.001             | 1         | <0.001    | 0.022          | 0.882        |
| Origin * Transplant * Copper | 0.002              | 2         | 0.001     | 0.072          | 0.931        |
| Error                        | 0.578              | 42        | 0.014     |                |              |
| Total                        | 1.046              | 53        |           |                |              |
| <b>Effect of copper:</b>     |                    |           |           |                |              |
| <b>Source</b>                | <b>Type III SS</b> | <b>df</b> | <b>MS</b> | <b>F ratio</b> | <b>p</b>     |
| Origin                       | 0.126              | 2         | 0.063     | 8.867          | <b>0.001</b> |
| Transplant                   | 0.005              | 1         | 0.005     | 0.658          | 0.422        |
| Copper                       | 0.020              | 1         | 0.020     | 2.782          | 0.103        |
| Origin * Transplant          | 0.016              | 2         | 0.008     | 1.091          | 0.345        |
| Origin * Copper              | 0.061              | 2         | 0.030     | 4.254          | <b>0.021</b> |
| Transplant * Copper          | 0.002              | 1         | 0.002     | 0.323          | 0.573        |
| Origin * Transplant * Copper | 0.002              | 2         | 0.001     | 0.158          | 0.854        |
| Error                        | 0.299              | 42        | 0.007     |                |              |
| Total                        | 0.519              | 53        |           |                |              |

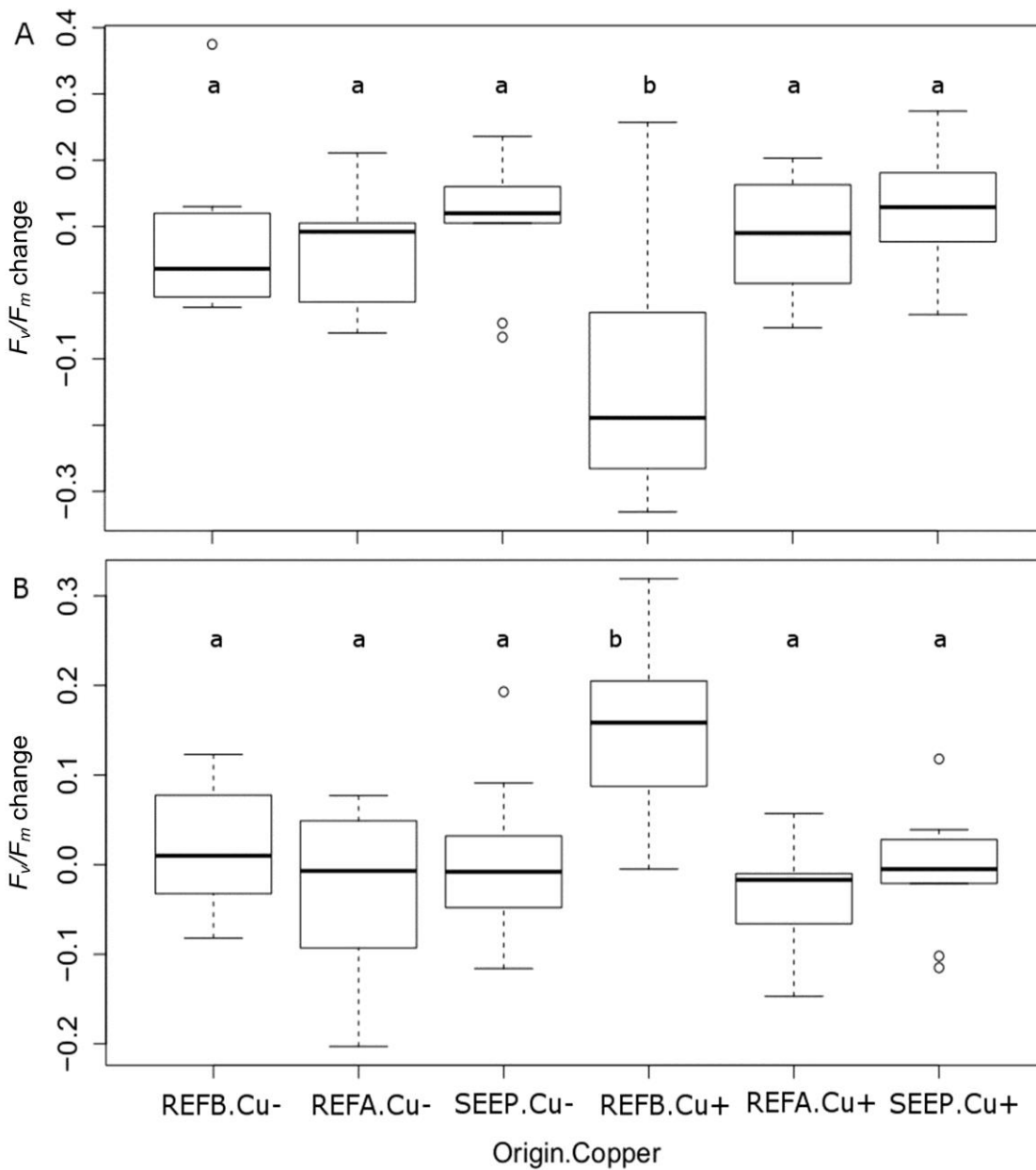


Figure 7.6. Changes in maximum quantum yield ( $F_v/F_m$ ) following *C. corniculata* thalli transplant (A) and copper exposure (B) in June 2013 depending on their site of origin (REF A, REF B or SEEP) and their exposure to copper (Cu- = no; Cu+ = yes);  $n = 7-10$ . Horizontal line = median, vertical boxes = 25th and 75th percentiles, whiskers = min/max values if smaller than 1.5 times the inter-quartile range and dots = outliers.

Change in maximum quantum yield in *J. rubens* exposed to copper in September 2013 showed a significant interaction of site of origin and site of transplant before copper exposure, but no significant effects of copper exposure (Table 7.5). This was due to a decrease in maximum quantum yield of the thalli

from REF A transplanted at SEEP and a concurrent increase in maximum quantum yield of thalli from the other reference site transplanted at SEEP (Figure 7.7).

Table 7.5. ANOVA on change in maximum quantum yield ( $F_v/F_m$ ) after transplant (upper part) and after copper exposure (lower part) for *J. rubens* thalli in September 2013. The table shows main factors and their interactions and sum of squares (SS), degrees of freedom (df), Mean Squares (MS), F-ratios (F) and p values. Significant p values (< 0.05) are highlighted.

| <b>Effect of transplant</b>  |                    |           |           |                |              |
|------------------------------|--------------------|-----------|-----------|----------------|--------------|
| <b>Source</b>                | <b>Type III SS</b> | <b>df</b> | <b>MS</b> | <b>F ratio</b> | <b>p</b>     |
| Origin                       | 0.036              | 2         | 0.018     | 1.162          | 0.323        |
| Transplant                   | 0.012              | 1         | 0.012     | 0.779          | 0.383        |
| Copper                       | 0.001              | 1         | 0.001     | 0.048          | 0.827        |
| Origin * Transplant          | 0.138              | 2         | 0.069     | 4.417          | <b>0.018</b> |
| Origin * Copper              | 0.010              | 2         | 0.005     | 0.326          | 0.724        |
| Transplant * Copper          | 0.015              | 1         | 0.015     | 0.962          | 0.333        |
| Origin * Transplant * Copper | 0.053              | 2         | 0.026     | 1.681          | 0.199        |
| Error                        | 0.627              | 40        | 0.016     |                |              |
| Total                        | 0.920              | 51        |           |                |              |
| <b>Effect of copper</b>      |                    |           |           |                |              |
| <b>Source</b>                | <b>Type III SS</b> | <b>df</b> | <b>MS</b> | <b>F ratio</b> | <b>p</b>     |
| Origin                       | 0.003              | 2         | 0.002     | 0.069          | 0.934        |
| Transplant                   | 0.004              | 1         | 0.004     | 0.171          | 0.681        |
| Copper                       | 0.032              | 1         | 0.032     | 1.275          | 0.266        |
| Origin * Transplant          | 0.093              | 2         | 0.046     | 1.871          | 0.167        |
| Origin * Copper              | 0.014              | 2         | 0.007     | 0.274          | 0.762        |
| Transplant * Copper          | 0.049              | 1         | 0.049     | 1.997          | 0.165        |
| Origin * Transplant * Copper | 0.014              | 2         | 0.007     | 0.275          | 0.761        |
| Error                        | 0.989              | 40        | 0.025     |                |              |
| Total                        | 1.208              | 51        |           |                |              |



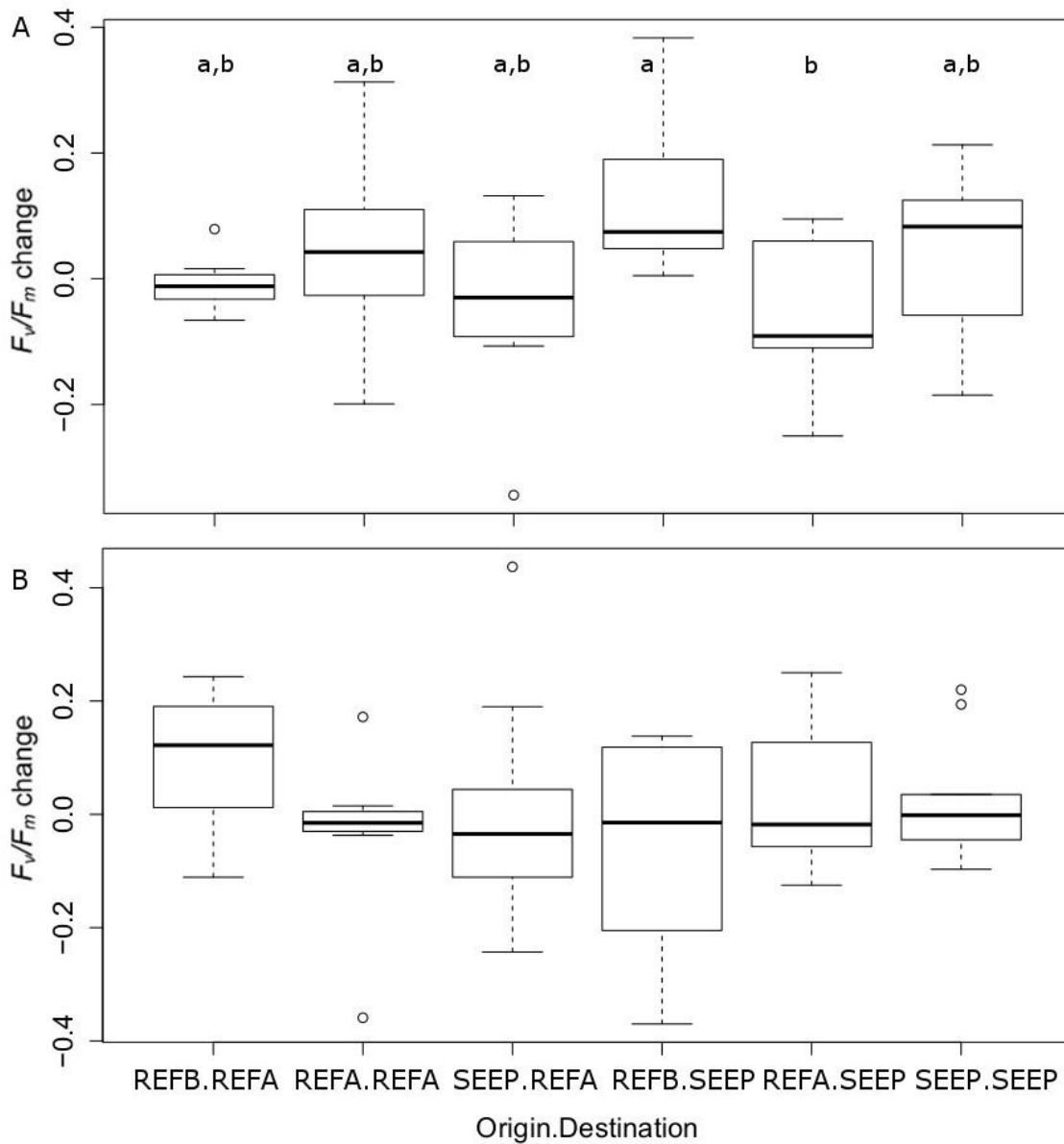


Figure 7.7. Changes in maximum quantum yield ( $F_v/F_m$ ) following *J. rubens* thalli transplant (A) and copper exposure (B) in September 2013 depending on their site of origin (REF A, REF B or SEEP) and their site of transplant (REF A or SEEP);  $n = 7-10$ . Horizontal line = median, vertical boxes = 25th and 75th percentiles, whiskers = min/max values if smaller than 1.5 times the inter-quartile range and dots = outliers.

### 7.3.4 Pigment contents

In the experiment performed in June 2013 on *C. corniculata*, copper had a significant effect on pheophytin *a* and antheraxanthin, while chlorophyll *c*, pheophytin *a* and fucoxanthin were significantly affected by the site of origin (Table 7.6). Figure 7.8 shows that thalli exposed to copper had higher antheraxanthin concentration, but lower pheophytin *a*. On the other hand, thalli originally from SEEP had higher chlorophyll *c* and lower pheophytin *a* and fucoxanthin compared with samples originally from REF A. Samples originating from REF B had to be removed from analysis due to high sample loss.

Table 7.6. MANOVA on pigment concentrations in *C. corniculata* thalli measured after the end of the experiment of June 2013. The table shows main factors and their interactions and dependent variables. F-ratios (F) and p values are reported when significant ( $p < 0.05$ ).

| Response variable   | Origin                      | Transplant | Copper                     | Origin * Transplant | Origin* Copper | Transplant * Copper | Origin * Transplant * Copper |
|---------------------|-----------------------------|------------|----------------------------|---------------------|----------------|---------------------|------------------------------|
| Chl <i>a</i>        | -                           | -          | -                          | -                   | -              | -                   | -                            |
| Chl <i>c</i>        | F(1,29)=<br>5.1<br>p=0.032  | -          | -                          | -                   | -              | -                   | -                            |
| Pheophytin <i>a</i> | F(1,29)=<br>25.8<br>p<0.001 | -          | F(1,29)=<br>6.0<br>p=0.021 | -                   | -              | -                   | -                            |
| β-carotene          | -                           | -          | -                          | -                   | -              | -                   | -                            |
| Fucoxanthin         | F(1,29)=<br>5.6<br>p=0.025  | -          | -                          | -                   | -              | -                   | -                            |
| Violaxanthin        | -                           | -          | -                          | -                   | -              | -                   | -                            |
| Antheraxanthin      | -                           | -          | F(1,29)=<br>7.8<br>p=0.009 | -                   | -              | -                   | -                            |
| Zeaxanthin          | -                           | -          | -                          | -                   | -              | -                   | -                            |

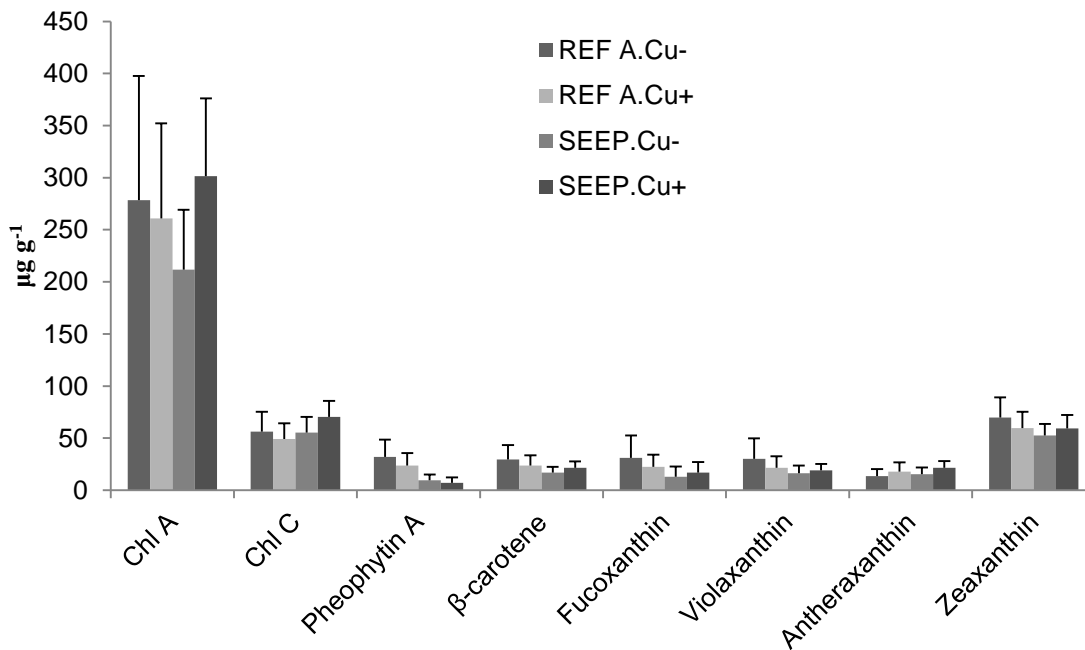


Figure 7.8. Mean ( $\pm$  SD,  $n=9-10$ ) pigment concentrations ( $\mu\text{g g}^{-1}$  dry mass) of *C. corniculata* thalli transplanted from REF A or SEEP not exposed (Cu-) or exposed (Cu+) to copper in June 2013.

In the experiment performed in September 2013 on *J. rubens*, copper had a significant effect on  $\beta$ -cryptoxanthin,  $\beta$ -carotene and zeaxanthin, while pheophytin A was significantly affected by the site of transplant and zeaxanthin was significantly affected by the interaction of site of origin and site of transplant (Table 7.7). Figure 7.9 shows that thalli exposed to copper had lower  $\beta$ -carotene and zeaxanthin concentration, but higher  $\beta$ -cryptoxanthin. On the other hand, thalli transplanted to SEEP had higher pheophytin A than those transplanted to REF A. Zeaxanthin concentration was higher in thalli transplanted from their site of origin to a different site (i.e. REF A to SEEP and vice versa) compared to concentrations in thalli that remained in their site of origin.

Table 7.7. MANOVA on pigment concentrations in *J. rubens* thalli measured after the end of the experiment of September 2013. The table shows main factors and their interactions and dependent variables. F-ratios (F) and p values are reported when significant ( $p < 0.05$ ).

| Response variable     | Origin | Transplant                  | Copper                      | Origin * Transplant    | Origin * Copper | Transplant * Copper | Origin * Transplant * Copper |
|-----------------------|--------|-----------------------------|-----------------------------|------------------------|-----------------|---------------------|------------------------------|
| Chl a                 | -      | -                           | -                           | -                      | -               | -                   | -                            |
| Pheophytin a          | -      | F(1,26)=<br>15.3<br>p=0.001 | -                           | -                      | -               | -                   | -                            |
| $\beta$ cryptoxanthin | -      | -                           | F(1,26)=<br>13.5<br>p=0.001 | -                      | -               | -                   | -                            |
| Antheraxanthin        | -      | -                           | -                           | -                      | -               | -                   | -                            |
| $\beta$ -carotene     | -      | -                           | F(1,26)=<br>7.9<br>p=0.009  | -                      | -               | -                   | -                            |
| Violaxanthin          | -      | -                           | -                           | -                      | -               | -                   | -                            |
| Zeaxanthin            | -      | -                           | F(1,26)=<br>9.2<br>p=0.005  | F(1,26)=5.0<br>p=0.033 | -               | -                   | -                            |

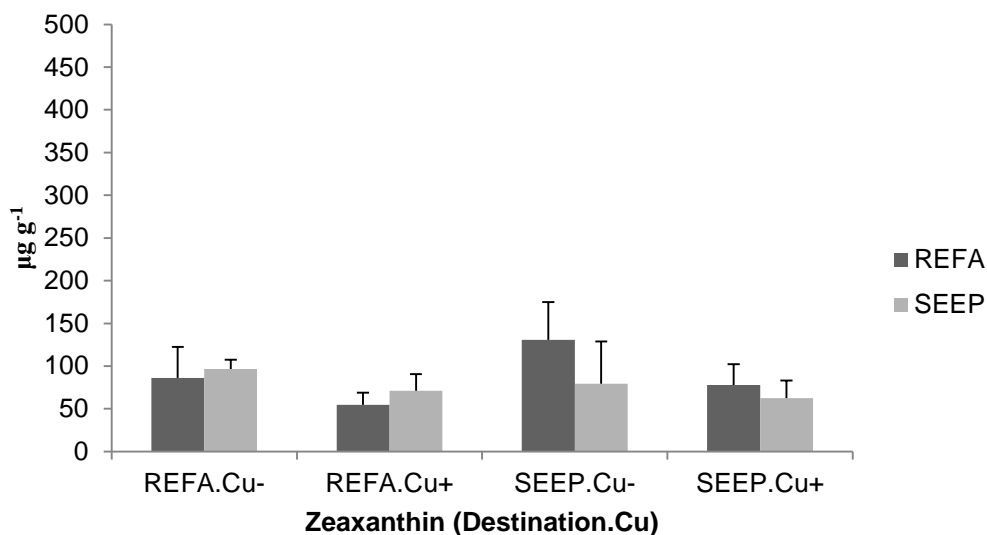
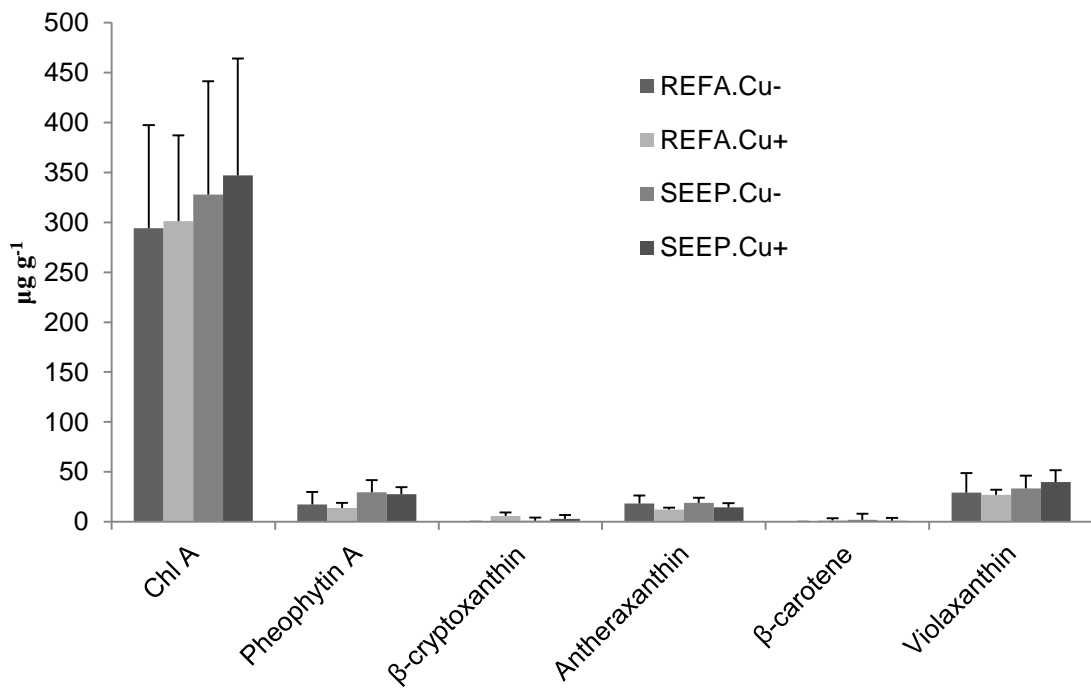


Figure 7.9. Upper part: mean ( $\pm$  SD,  $n=8-9$ ) pigment concentrations ( $\mu\text{g g}^{-1}$  dry mass) of *J. rubens* thalli transplanted to REF A or SEEP not exposed (Cu-) or exposed (Cu+) to copper in September 2013. Lower part: mean ( $\pm$  SD,  $n=3-5$ ) zeaxanthin concentration ( $\mu\text{g g}^{-1}$  dry mass) of *J. rubens* thalli transplanted to REF A or SEEP from REF A and SEEP not exposed (Cu-) or exposed (Cu+) to copper in September 2013.

With regards to phycobilins, only phycoerythrin (PE) showed any significant difference among treatments, with a significant site of transplant \* copper interaction, whereas phycocyanin (PC) seemed unaffected by the experiment (Table 7.8). At REF A, phycoerythrin concentration increased in thalli exposed to copper, while the opposite was true from *J. rubens* individuals at SEEP, where thalli exposed to copper had lower phycoerythrin concentration compared with controls (Figure 7.10).

Table 7.8. MANOVA on phycoerythrin (PE) and phycocyanin (PC) concentrations in *J. rubens* thalli measured after the end of the experiment of September 2013. The table shows main factors and their interactions and dependent variable, sum of squares (SS), degrees of freedom (df), Mean Squares (MS), F-ratios (F) and p values. Significant p values (< 0.05) are highlighted.

| Source                       | Dependent Variable | Type III SS | df | MS    | F-ratio | p            |
|------------------------------|--------------------|-------------|----|-------|---------|--------------|
| Origin                       | PE                 | 0.118       | 1  | 0.118 | 0.262   | 0.614        |
|                              | PC                 | 0.001       | 1  | 0.001 | 0.001   | 0.977        |
| Transplant                   | PE                 | 1.623       | 1  | 1.623 | 3.591   | 0.071        |
|                              | PC                 | 1.227       | 1  | 1.227 | 1.506   | 0.232        |
| Copper                       | PE                 | 0.005       | 1  | 0.005 | 0.011   | 0.919        |
|                              | PC                 | 1.627       | 1  | 1.627 | 1.997   | 0.171        |
| Origin * Transplant          | PE                 | 1.361       | 1  | 1.361 | 3.012   | 0.096        |
|                              | PC                 | 0.644       | 1  | 0.644 | 0.790   | 0.383        |
| Origin * Copper              | PE                 | 0.002       | 1  | 0.002 | 0.004   | 0.947        |
|                              | PC                 | 2.402       | 1  | 2.402 | 2.947   | 0.099        |
| Transplant * Copper          | PE                 | 2.572       | 1  | 2.572 | 5.692   | <b>0.026</b> |
|                              | PC                 | 1.409       | 1  | 1.409 | 1.729   | 0.202        |
| Origin * Transplant * Copper | PE                 | 0.016       | 1  | 0.016 | 0.035   | 0.853        |
|                              | PC                 | 0.989       | 1  | 0.989 | 1.213   | 0.282        |
| Error                        | PE                 | 10.394      | 23 | 0.452 |         |              |
|                              | PC                 | 18.742      | 23 | 0.815 |         |              |
| Total                        | PE                 | 15.965      | 30 |       |         |              |
|                              | PC                 | 26.690      | 30 |       |         |              |

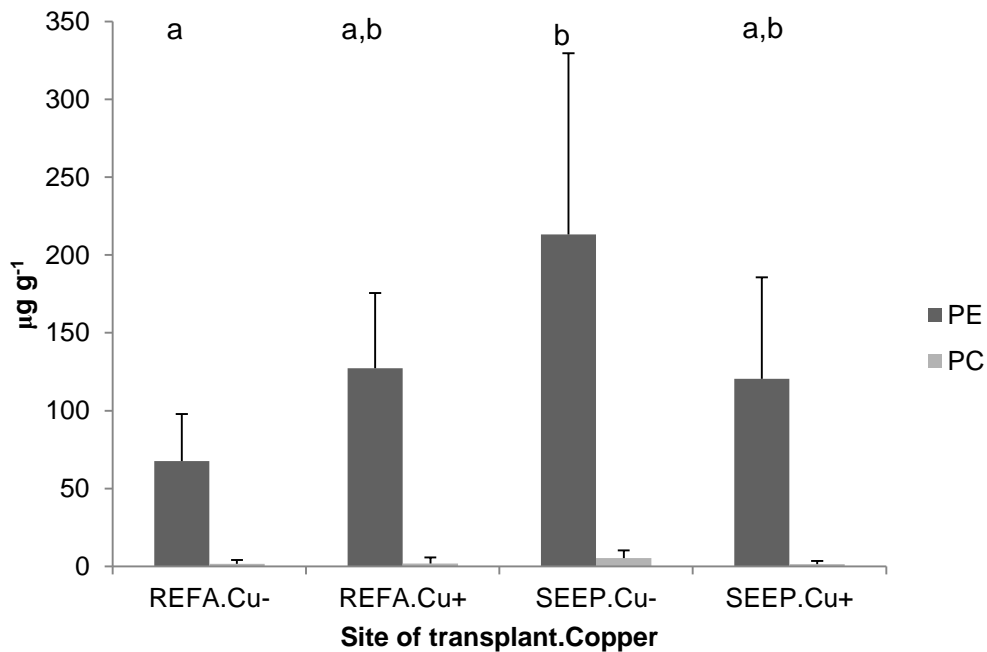


Figure 7.10. Mean ( $\pm$  SD, n=6-9) phycoerythrin (PE) and phycocyanin (PC) concentrations ( $\mu\text{g/g}$ ) of *J. rubens* thalli transplanted to REF A or SEEP not exposed (Cu-) or exposed (Cu+) to copper in REF A and SEEP in September 2013.

### 7.3.5 Invertebrate re-colonisation

Invertebrate communities inhabiting *C. corniculata* thalli were significantly affected by copper, but this effect was not consistent between sites (Site x Copper interaction significant), even after considering the effect of individual thalli biomass on invertebrate community structure (Table 7.9a). Pairwise comparisons showed that copper only had a significant effect near the seeps, but not in the reference site (Table 7.9b). This was also evident from the nMDS plot, where samples from REF A exposed and not exposed to copper are all grouped together, whereas samples from SEEP are separated from REF A samples, but also clearly grouped depending on their copper exposure (Figure 7.11).

Table 7.9. PERMANOVA analyses of square-root transformed invertebrate abundances in *C. corniculata* thalli from copper exposure experiment in June 2013. The first table shows main factors and their interactions and degrees of freedom (df), sum of squares (SS), pseudo-F, permutational p and unique permutations for each of them. The second table shows pair-wise comparisons between copper treatments at both sites. Significant p values (< 0.05) are highlighted.

| A) | Source                  | df | SS     | MS     | Pseudo-F | P(perm)       | Unique perms |
|----|-------------------------|----|--------|--------|----------|---------------|--------------|
|    | Biomass                 | 1  | 2193.4 | 2193.4 | 3.2403   | <b>0.0029</b> | 9937         |
|    | Site                    | 1  | 5429.8 | 5429.8 | 8.0213   | <b>0.0001</b> | 9916         |
|    | Copper                  | 1  | 1510.2 | 1510.2 | 2.2310   | <b>0.0116</b> | 9920         |
|    | Biomass x Site          | 1  | 1036.5 | 1036.5 | 1.5312   | 0.1236        | 9930         |
|    | Biomass x Copper        | 1  | 441.99 | 441.99 | 0.6529   | 0.7933        | 9926         |
|    | Site x Copper           | 1  | 1373.5 | 1373.5 | 2.0291   | <b>0.0244</b> | 9933         |
|    | Biomass x Site x Copper | 1  | 554.56 | 554.56 | 0.8192   | 0.6289        | 9938         |
|    | Residual                | 11 | 7446.1 | 676.92 |          |               |              |
|    | Total                   | 18 | 19986  |        |          |               |              |

| B) | Within level 'REF A' of factor 'Site' |        |               |              |
|----|---------------------------------------|--------|---------------|--------------|
|    | Groups                                | t      | P(perm)       | Unique perms |
|    | Cu+, Cu-                              | 1.2129 | 0.1598        | 9805         |
|    | Within level 'SEEP' of factor 'Site'  |        |               |              |
|    | Groups                                | t      | P(perm)       | Unique perms |
|    | Cu+, Cu-                              | 1.6212 | <b>0.0102</b> | 9917         |



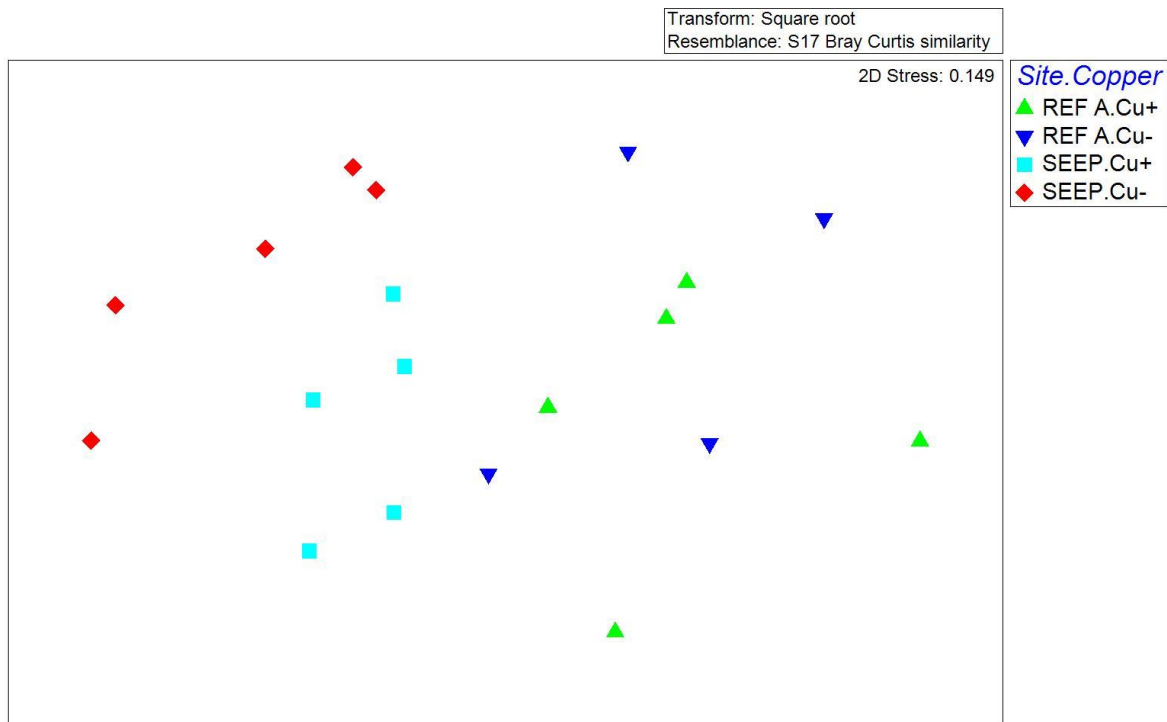


Figure 7.11: MDS plot of invertebrate assemblages on *C. corniculata* thalli placed at REF A or SEEP, and not exposed (Cu-) or exposed (Cu+) to copper in June 2013.

SIMPER analysis showed that crustaceans, molluscs and polychaetes were the main taxa driving differences between sites and copper treatments (Table 7.10). Mean abundance of these taxa is shown in Figure 7.12, showing that all crustacean groups and ophiuroids consistently increased near the seeps when seaweed thalli were not exposed to copper; copper exposure had a negligible effect on their abundance in the reference site and a dramatic negative effect at SEEP. On the other hand, polychaetes were largely unaffected by changes in pCO<sub>2</sub>, but their abundance greatly increased in the thalli exposed to copper near the seeps. Gastropods exhibited another type of response, with their abundance decreasing near the seeps and further decreasing when exposed to copper at elevated CO<sub>2</sub>. Bivalve and oligochaete abundances were very low and no clear pattern was detectable.

Table 7.10. SIMPER analysis showing the average dissimilarities between sites and copper treatments and which taxonomic groups contributes to the dissimilarity up to 90%. For each taxon, the average abundance in the two groups, their average dissimilarity, the dissimilarity to standard deviation ration and the taxon contribution and cumulative contribution are shown.

| <b>Groups REF A &amp; SEEP; Average dissimilarity = 32.05</b> |                 |                 |                |                |                 |              |
|---|-----------------|-----------------|----------------|----------------|-----------------|--------------|
| <b>Species</b>  | <b>REF A</b>    | <b>SEEP</b>     | <b>Av.Diss</b> | <b>Diss/SD</b> | <b>Contrib%</b> | <b>Cum.%</b> |
|   | <b>Av.Abund</b> | <b>Av.Abund</b> |                |                |                 |              |
| Copepods  | 5.46            | 8.52            | 6.53           | 1.28           | 20.36           | 20.36        |
| Gastropods  | 2.15            | 0.10            | 3.94           | 2.18           | 12.28           | 32.65        |
| Amphipods   | 3.49            | 5.34            | 3.86           | 1.23           | 12.04           | 44.68        |
| Polychaetes   | 3.72            | 4.06            | 3.57           | 1.12           | 11.13           | 55.81        |
| Tanaids   | 3.19            | 4.55            | 2.93           | 1.32           | 9.14            | 64.95        |
| Ostracods   | 1.72            | 2.37            | 2.29           | 1.25           | 7.16            | 72.10        |
| Isopods   | 1.98            | 2.56            | 2.11           | 0.90           | 6.59            | 78.69        |
| Bivalves  | 0.92            | 0.34            | 1.43           | 1.35           | 4.45            | 83.14        |
| Ophiuroids  | 0.16            | 0.72            | 1.34           | 0.79           | 4.19            | 87.33        |
| Oligochaetes  | 0.73            | 0.20            | 1.28           | 0.96           | 4.00            | 91.34        |

| <b>Groups Cu+ &amp; Cu-; Average dissimilarity = 27.61</b> |                 |                 |                |                |                 |              |
|--|-----------------|-----------------|----------------|----------------|-----------------|--------------|
| <b>Species</b>   | <b>Cu+</b>      | <b>Cu-</b>      | <b>Av.Diss</b> | <b>Diss/SD</b> | <b>Contrib%</b> | <b>Cum.%</b> |
|  | <b>Av.Abund</b> | <b>Av.Abund</b> |                |                |                 |              |
| Copepods   | 6.19            | 8.05            | 5.42           | 1.26           | 19.64           | 19.64        |
| Polychaetes  | 4.50            | 3.24            | 3.85           | 1.08           | 13.94           | 33.58        |
| Amphipods  | 4.10            | 4.87            | 3.14           | 1.38           | 11.38           | 44.97        |
| Isopods  | 1.70            | 2.93            | 3.10           | 1.78           | 11.21           | 56.18        |
| Ostracods  | 2.34            | 1.75            | 2.54           | 1.28           | 9.21            | 65.39        |
| Decapods   | 3.61            | 4.24            | 1.98           | 1.31           | 7.18            | 72.57        |
| Gastropods   | 1.11            | 1.03            | 1.39           | 0.70           | 5.02            | 77.59        |
| Ophiuroids   | 0.24            | 0.69            | 1.35           | 0.80           | 4.89            | 82.47        |
| Oligochaetes   | 0.31            | 0.60            | 1.16           | 0.86           | 4.21            | 86.68        |
| Bivalves   | 0.54            | 0.69            | 1.00           | 0.92           | 3.63            | 90.31        |

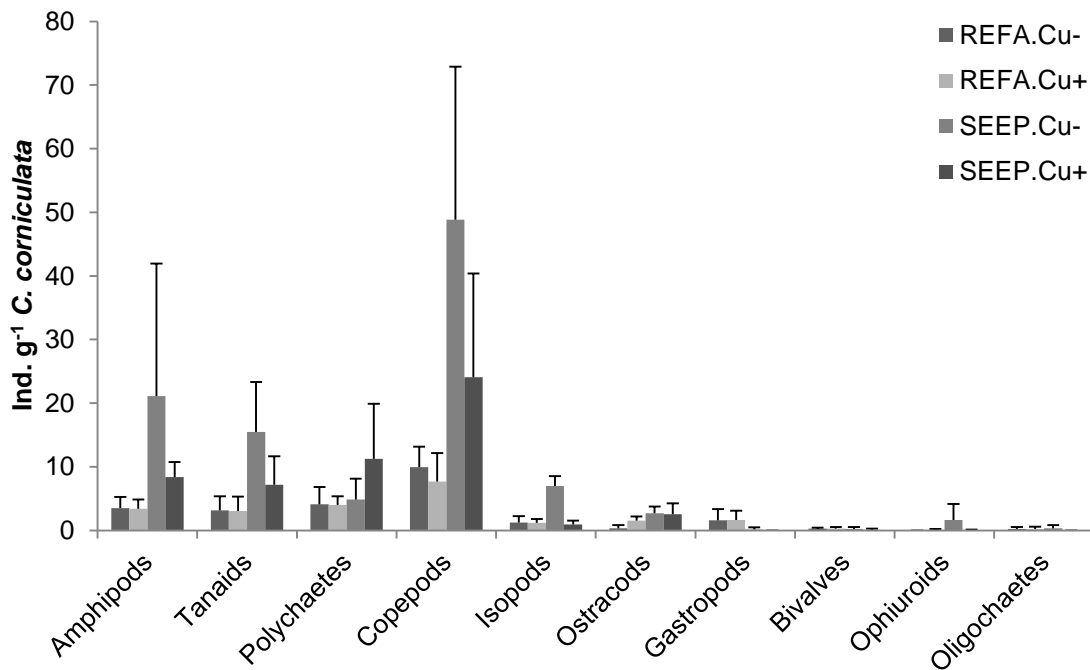


Figure 7.12. Mean ( $\pm$ SD, n=4-5) number of individuals for each of the main taxonomic groups. Abundances were normalised by *C. corniculata* dry mass; *C. corniculata* thalli were placed in REF A or SEEP and exposed (Cu+) or not exposed (Cu-) to copper for 36h in June 2013.

## 7.4 Discussion

This is the first investigation of the combined effect of elevated carbon dioxide and metal toxicity on macroalgal physiology and invertebrate communities. Copper accumulation in both *C. corniculata* and *J. rubens* increased at elevated pCO<sub>2</sub> levels, even though copper released from plaster blocks was the same (June 2013) or less (September 2013) near the seeps than at the reference site. Long-term acclimatisation effects on copper accumulation were evident for the coralline alga *J. rubens*, as thalli grown near the seeps accumulated significantly more copper than those transplanted from reference sites.

Copper bioavailability is expected to increase with decreased seawater pH (Richards *et al.*, 2011) and might explain increased copper accumulation at high

CO<sub>2</sub>. However, seaweeds accumulate copper for over 24 h after its addition to seawater (Connan and Stengel, 2011a). As Cu<sup>2+</sup> ions were not complexed immediately while plaster block continuously released them, copper was equally bioavailable at both sites. Copper uptake, however, is regulated by metabolic activity (Connan and Stengel, 2011a), so it is possible that macroalgae had more energy available after down-regulating carbon concentrating mechanisms (CCMs). Reduced use of CCMs following increased CO<sub>2</sub> availability has already been proven for some macroalgae (Olischläger and Wiencke, 2013), and this might explain the copper accumulation pattern observed in *C. corniculata*.

As elevated CO<sub>2</sub> increases the energetic cost of calcification (Bradassi *et al.*, 2013), *J. rubens* thalli just transplanted from reference sites probably used most of the surplus energy derived from increased CO<sub>2</sub> levels to maintain calcification rates. They thus showed a relatively small increase in copper accumulation. On the other hand, thalli grown at high CO<sub>2</sub> may have mechanisms in place to make calcification at high pCO<sub>2</sub> energetically sustainable in the long term. They are therefore likely to have higher metabolic activity and accumulate more copper than thalli not acclimatised to elevated CO<sub>2</sub> levels. Coralline algal calcification is controlled by alginic acid and sulfated polysaccharides (Koch *et al.*, 2013), which are also known to have a high affinity for metals (Raize *et al.*, 2004). Increased production of calcification-inducing compounds in *J. rubens* acclimatised to high CO<sub>2</sub> could enhance copper accumulation, potentially increasing its vulnerability to metal toxicity.

Physiological responses to copper examined in both macroalgal species were not influenced by acclimatisation to increased carbon dioxide (i.e. no significant site of origin \* copper interactions). In fact, maximum quantum yield ( $F_v/F_m$ ) was only affected by transplant, while some pigments showed effects of site of origin

and copper in *C. corniculata* and of site of transplant, copper and site of origin in *J. rubens*. Copper is known to decrease  $F_v/F_m$  in some species of red algae (Küpper *et al.*, 2002; Brown and Newman, 2003; Baumann *et al.*, 2009), whereas maximum quantum yield of brown algae is mostly unaffected at moderate copper levels (Nielsen *et al.*, 2003; Nielsen and Nielsen, 2005; Baumann *et al.*, 2009; Nielsen and Nielsen, 2010).

Copper exposure only caused a small decrease of pheophytin A and a small increase of antheraxanthin in *C. corniculata*. Since the latter was not associated with an increase in the xanthophyll cycle pool size, these pigments are unlikely to play a role in copper defence for this species. This is in accord with previous studies on *Fucus serratus*, where no change in xanthophyll pool size was found in light-adapted thalli after copper exposure (Nielsen *et al.*, 2003; Nielsen and Nielsen, 2010). On the other hand, *J. rubens* thalli exposed to copper showed a small increase in  $\beta$ -cryptoxanthin, but a bigger decrease in  $\beta$ -carotene and zeaxanthin, resulting in a decrease in total carotenoids. Decreased carotenoids concentrations were previously found in a red alga exposed to high copper concentrations (5-10 ppm; Gouveia *et al.*, 2013), whereas red algae exposed to  $\text{Cu}^{2+}$  concentrations of 0.2-0.5 ppm often increase their carotenoids content to counter copper toxicity (Brown and Newman, 2003; Collén *et al.*, 2003; Pinto *et al.*, 2011). *J. rubens*, however, did not show the reduction in chlorophyll *a* found in another red alga at high  $\text{Cu}^{2+}$  concentrations (Gouveia *et al.*, 2013). This suggests the experimental copper pulse exposed *J. rubens* to relatively high  $\text{Cu}^{2+}$  concentrations. There was a reduction in phycoerithrin content when *J. rubens* was exposed to copper at SEEP, but not at REF A. Decreased phycobilins concentration is considered a sensitive indicator of copper stress (Küpper *et al.*, 2002; Brown and Newman, 2003; Xia *et al.*, 2004). *J. rubens*

transplanted near the seeps might be more sensitive to copper exposure because of the increased energetic cost of maintaining calcification rates at elevated CO<sub>2</sub> (Bradassi *et al.*, 2013) or because of increased copper accumulation.

Macroalgae counter negative effects of copper exposure by synthesizing metal-binding compounds such as metallothioneins and phytochelatins (Lobban and Harrison, 1994) and by increasing the activity of antioxidant enzymes (Collén *et al.*, 2003). Synthesis of complex molecules is energy expensive, so growth is reduced in many macroalgal species even at low copper levels (Collén *et al.*, 2003; Brown and Newman, 2003; Nielsen *et al.*, 2003; Xia *et al.*, 2004). Fucoid and articulate coralline algae, however, are relatively slow-growing species whose growth rates are unlikely to be measurable after 36 hours. In brown algae, phlorotannins movement to the cell wall and exudation in the surrounding seawater provides additional defence against copper damage (Connan and Stengel, 2011b). Phlorotannins are polyphenols characteristic of phaeophytes that bind metal ions reducing their toxicity; their increased proportion in the cell walls of algae exposed to copper and increased release in the environment could have contributed to the lack of observed effects of copper in *C. corniculata*. However, it is possible that some effects of copper exposure have not been detected in this study. Lipid peroxidation, antioxidant enzymes activity and antioxidant compounds all increase following copper exposure of 48 to 96 hours (Collén *et al.*, 2003; Contreras *et al.*, 2005). In red algae, mycosporine-like amino-acids (MAAs) are important antioxidant compounds that are up-regulated after short exposure to oxidative stress (Karsten *et al.*, 1998). Further research is therefore needed before concluding that *C. corniculata* is not negatively affected by copper pulses at high CO<sub>2</sub> levels.

Although acclimatisation to elevated CO<sub>2</sub> had no effects on macroalgal responses to copper, *C. corniculata* thalli collected at SEEP had higher chlorophyll *c* content and slightly lower phaeophytin A and fucoxanthin contents compared to those collected at reference sites. On the other hand, pheophytin a content increased in *J. rubens* transplanted near the seeps, possibly a short-term response to increased CO<sub>2</sub>. *J. rubens* pigments also showed some transplant effect, with zeaxanthin content being higher in thalli transplanted to a different site (i.e. SEEP. REFA and REFA.SEED) compared to those transplanted to their site of origin (i.e. SEEP.SEED and REFA.REFA). Short-term and long-term effects of CO<sub>2</sub> on macroalgal physiology will be compared in the next Chapter.

Invertebrate colonisation was significantly affected by copper exposure, but only at the high CO<sub>2</sub> site. This is probably due to very high copper accumulation by *C. corniculata* at elevated CO<sub>2</sub>, as copper release rates in seawater were similar between sites in June 2013. Some crustaceans are more sensitive to copper at elevated CO<sub>2</sub> levels (Roberts *et al.*, 2013), which could contribute to their observed decrease when exposed to copper near the seeps. The specific responses of invertebrate taxa are consistent with previous studies: many crustaceans increase in abundance with increased CO<sub>2</sub> (Kroeker *et al.*, 2011), but are negatively affected by copper (Roberts *et al.*, 2006). Gastropods abundance is negatively affected by both carbon dioxide (Hale *et al.*, 2011; Kroeker *et al.*, 2011) and copper (Roberts *et al.*, 2006), while many polychaete species are largely unaffected by both factors (Roberts *et al.*, 2006; Cigliano *et al.*, 2010). The increase in polychaete abundances in *C. corniculata* thalli exposed to copper at the high CO<sub>2</sub> site was therefore unexpected, but it is possible they had more space and resources available following the marked

decrease in crustaceans. A similar pattern has already been detected by Hale *et al.* (2011), who found increased nematode abundance following a decrease in the abundance of sensitive taxa as CO<sub>2</sub> increased. Copper exposure combined with ocean acidification could however affect polychete larval stages (Lewis *et al.*, 2012; Campbell *et al.*, 2014).

Overall, *J. rubens* appeared more sensitive to copper than *C. corniculata*, especially at elevated CO<sub>2</sub>, as well as showing changes in copper accumulation patterns following long-term exposure to high CO<sub>2</sub>. These species are currently the two main space occupiers on shallow rocky shores off Methana, but *J. rubens* is at competitive disadvantage with *C. corniculata* when CO<sub>2</sub> levels are high (see Chapter 3). This study shows that *J. rubens* is likely to be negatively affected by the interaction of ocean acidification and copper pollution, and therefore at risk of local extinction. Since only two species (a calcifying red alga and a non-calcifying brown alga) were examined in the present study, the results are not applicable to all competitive interaction between calcifying and non-calcifying algae, as red and brown algae have very different physiologies, which could also influence *J. rubens* and *C. corniculata* interactions (Lobban and Harrison, 1994).

Ocean acidification increased seaweed copper bioaccumulation, and had significant effects on their epifauna. This adds to a growing body of research showing that indirect effects of ocean acidification are at least as important as its direct effects (Kroeker *et al.*, 2013c). Decreased growth rates of coralline algae with increased CO<sub>2</sub> can make them less competitive and cause communities to become dominated by fleshy algae (Kroeker *et al.*, 2013c), or cause changes in herbivore performance through decreased food quality (Rossoll *et al.*, 2012; Poore *et al.*, 2013). Interactive negative effects of ocean



acidification and copper pollution on competitive ability of *J. rubens* and abundance of many epifaunal taxa, especially heavily calcified ones, suggest that benthic communities will dramatically change in copper-polluted areas. These areas are relatively common worldwide and include the copper mining regions illustrated in Figure 7.2; here, it is essential that local managers reduce copper pollution to reduce the negative effects of ocean acidification on macroalgal communities and the services they provide.

## **Chapter 8**

### **General discussion**

## 8.1 Main findings and implications for marine systems

Macroalgal beds are an extremely important habitat in temperate coastal environments, as they provide vital ecosystem services such as oxygen production, nutrient cycling, water depuration, fisheries production and shore protection from waves (Rönnbäck *et al.*, 2007). Information on macroalgal beds responses to ocean acidification at the community level that take into account biological interactions and adaptation potential are therefore needed to reliably forecast future ecosystem state and take appropriate measures to adapt to or mitigate the possible reduction of ecosystem services provided by those habitats. This thesis contributes to achieving this objective, and its main findings are illustrated in Figure 8.1. All this thesis' objectives were achieved; specifically:

- Geochemical surveys at seeps off Methana showed that this site is suitable to study the effects of high CO<sub>2</sub> on benthic communities, as no confounding gradients in temperature, salinity, total alkalinity, nutrients, hydrogen sulphide or heavy metals were found (Chapter 2).
- At Mediterranean CO<sub>2</sub> seeps off Italy and Greece, macroalgal communities greatly changed, with fucoid algal abundance increasing and coralline algal abundance decreasing as pCO<sub>2</sub> increased (Chapter 3). Epifaunal communities changed as well: at high CO<sub>2</sub> sites, abundance of heavily calcified taxa (e.g. gastropods, bivalves) decreased, while more resistant taxa (mainly polychaetes) abundances increased at high CO<sub>2</sub> (Chapter 4).
- Strength of herbivore top-down control did not appear to change at different pCO<sub>2</sub> levels, even though densities of calcifying intertidal and

subtidal herbivores (i.e. limpets and sea urchins) decreased at elevated  $p\text{CO}_2$  (Chapter 5).

- Non-reversible acclimatisation did not seem to play a role in benthic community changes with increased  $p\text{CO}_2$ , as the responses of transplanted calcified and non-calcified macroalgae to elevated  $\text{CO}_2$  did not depend on their history of  $p\text{CO}_2$  exposure (Chapter 6).
- Exposure to an additional stressor (i.e. copper pollution) had no additional negative effects on the physiology of a calcifying alga, but there were strong interactive effects on seaweed epifauna, reducing abundances of some taxa that were weakly affected or advantaged by exposure to elevated  $p\text{CO}_2$  alone (e.g. amphipods; Chapter 7).

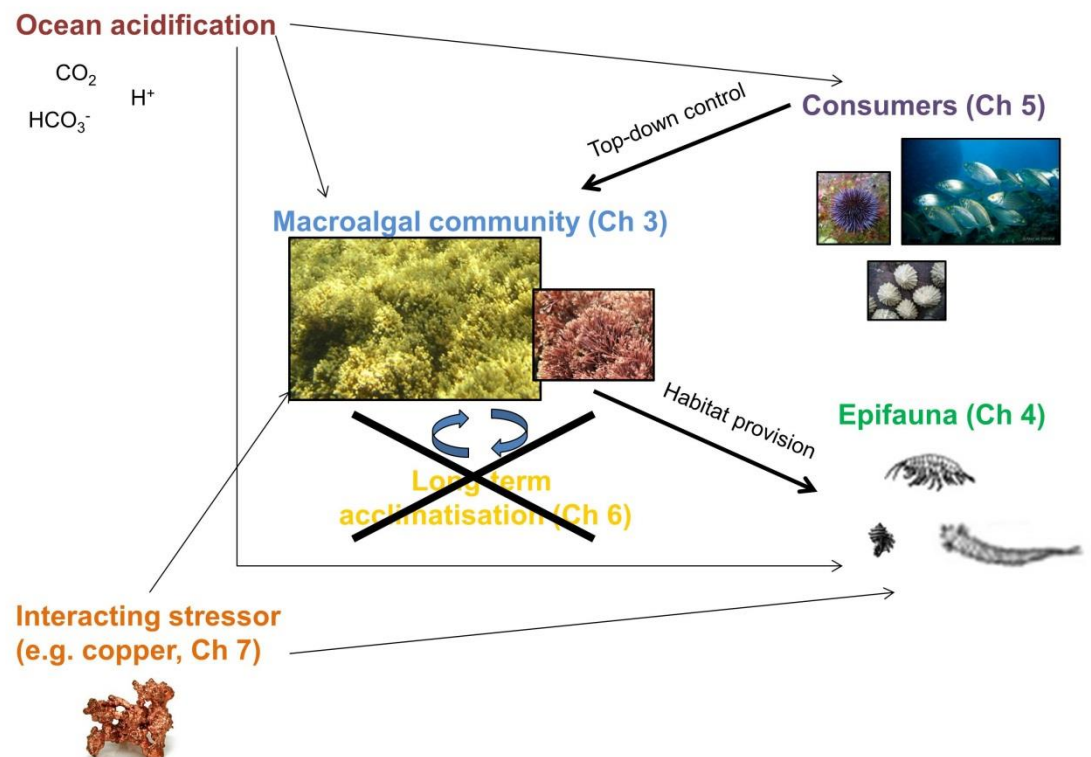


Figure 8.1. Visual abstract of thesis results; macroalgal communities (Chapter 3) and their epifauna (Chapter 4) change with increasing  $p\text{CO}_2$  levels, with heavily calcified macroalgae and invertebrates decreasing in abundance at elevated  $\text{CO}_2$ . Long-term acclimatisation to

elevated pCO<sub>2</sub> did not seem to have permanent effects on dominant macroalgal species, as no significant effects of origin site on macroalgal physiology were found (Chapter 6). I also found that decreased calcifying herbivores densities do not significantly affect the strength of top-down control on macroalgal communities (Chapter 5), and that ocean acidification and short-term copper pollution interact and produce larger negative effect on a dominant calcifying macroalga, but especially on seaweed epifauna (Chapter 7).

### **8.1.1 Benthic community responses to ocean acidification**

General patterns of benthic community changes with increasing pCO<sub>2</sub> levels at volcanic seeps off Italy and Greece were consistent with results from laboratory experiments and other ocean acidification analogues in that diversity and abundance of calcifying organisms decreased as CO<sub>2</sub> levels increased (Kroeker *et al.*, 2013a). Macroalgal communities responded in very similar ways at all Mediterranean seep sites studied so far, with a decrease in calcifying macroalgae and an increase in *Sargassum vulgare* abundance (Porzio *et al.*, 2011; Chapter 3). Epifaunal communities showed different patterns depending on the study area and the habitat studied (Chapter 4). While epifaunal communities of fucoid algae show a decrease of most invertebrate taxa at elevated pCO<sub>2</sub> at Methana, polychaete abundance increased at high CO<sub>2</sub> at Vulcano (Chapter 4). On the other hand, turf epifauna at seeps off Ischia showed an increase in crustaceans at elevated CO<sub>2</sub> (Kroeker *et al.*, 2011), seagrass-dwelling amphipods and polychaetes increase in abundance at elevated CO<sub>2</sub> levels off Ischia (Garrard *et al.*, 2014), and nematode abundance increased in Atlantic turf epifaunal communities exposed to ocean acidification conditions (Hale *et al.*, 2011). In addition, epiphyte communities of *Cystoseira corniculata* at Methana did not change significantly with CO<sub>2</sub> (Chapter 4), possibly because *C. corniculata* photosynthesis raised local pH and protected

epiphytes from the negative effects of increased CO<sub>2</sub> (Cornwall *et al.*, 2014). This clearly shows that site- and habitat-specific interactions among species result in different communities at high CO<sub>2</sub>.

Studies in nutrient-rich areas have shown that calcifiers can remain abundant in areas with naturally high pCO<sub>2</sub> if food is not limiting. For instance, barnacles and mussels are dominant in upwelling water off Kiel fjord, where pCO<sub>2</sub> reaches concentrations over 1000 µatm but nutrient levels are high (Thomsen *et al.*, 2010), while spirorbid worms from an upwelling area in the Baltic Sea are negatively affected by CO<sub>2</sub> only at levels over 3000 µatm (Saderne and Wahl, 2013). However, climate change is expected to reduce nutrient availability in surface waters due to increased water stratification (Sarmiento *et al.*, 2004), meaning that results from upwelling areas might underestimate the negative impacts of ocean acidification. Seeps off Methana and Vulcano are oligotrophic, and have similar nutrient concentrations (Chapter 2; Johnson, 2012). Although the Eastern Mediterranean is usually more oligotrophic than the Western basin (Siokou-Frangou *et al.*, 2010), the Saronikos Gulf has relatively high nutrient concentrations due to riverine inputs and urbanisation (Tsiamis *et al.*, 2013). In contrast, south-eastern Mediterranean coastal waters are ultra-oligotrophic ([Chl a] < 0.06 mg\*m<sup>-3</sup>; Shushkina *et al.*, 1997) during the warmest part of the year (Siokou-Frangou *et al.*, 2010); comparable chlorophyll concentrations are only found in the Northern Red Sea (Labiosa *et al.*, 2003) and in subtropical gyres (Kletou and Hall-Spencer, 2012). As low food availability impairs organisms' ability to cope with increased CO<sub>2</sub> (Thomsen *et al.*, 2013), these nutrient-poor ecosystems are probably highly vulnerable to ocean acidification. However, community responses to elevated CO<sub>2</sub> in ultra-oligotrophic coastal areas are virtually unstudied.

This thesis contributes to revealing general patterns of community responses to high CO<sub>2</sub>. However, general limitations of using volcanic seeps as ocean acidification laboratories, described in detail in Chapter 2, should be taken into account. In addition, more controlled studies (e.g. using mesocosms or field pCO<sub>2</sub> manipulations) would determine the exact CO<sub>2</sub> concentrations that trigger the observed community changes (Gattuso *et al.*, 2014). Insights could also be gained from the study of a wider range of habitats (e.g. soft substrata).

### **8.1.2 Changes in biological interactions at elevated pCO<sub>2</sub>**

Changes in biological interactions with increasing CO<sub>2</sub> are poorly known, although there is evidence that calcifying macroalgae become less competitive at elevated CO<sub>2</sub> (Kroeker *et al.*, 2013c; Short *et al.*, 2014) and reduced sea urchin grazing appears to favour increased macroalgal biomass (Johnson *et al.*, 2012). In this thesis, experiments on intertidal and subtidal rocky shores demonstrated that reduced abundances of calcifying herbivores at elevated CO<sub>2</sub> do not necessarily have a significant effect on sessile community composition (Chapter 5). At Vulcano, limpets had little effect on macroalgal communities in reference conditions, and their reduced densities with increasing CO<sub>2</sub> did not affect macroalgal communities, whereas carbon dioxide changed the specific composition and structure of intertidal communities. On the other hand, sea urchins strongly controlled macroalgal biomass on subtidal rocky reefs off Methana, but grazing control on macroalgal biomass was maintained at high CO<sub>2</sub> thanks to a marked increase in the abundance of herbivorous fish.

These results show that while ocean acidification can profoundly affect marine ecosystems, functional redundancy within trophic groups such as herbivores can reduce its effect. Since coastal environments have low functional redundancy, even when diversity is relatively high (Micheli *et al.*, 2014),

preserving diversity in marine ecosystems is essential for maintaining ecosystem function in the face of future environmental changes. In the Mediterranean Sea, overfishing of apex predators has led to higher abundances of sea urchins and herbivorous fish, as they are usually not targeted by commercial fisheries (Guidetti and Dulčić, 2007; Guidetti and Sala, 2007). High herbivore densities can often lead to impoverished macroalgal communities with much lower diversity biomass than unexploited Mediterranean coastal ecosystems (Sala *et al.*, 2012).

Thus, unvaried grazing pressure at different CO<sub>2</sub> levels may maintain suboptimal community structure. Figure 8.2A shows typical subtidal communities found at Methana at elevated pCO<sub>2</sub>, with high fish biomass and a *Cystoseira* belt reaching depths of up to 12 meters (author's personal observation). This suggests that in the long term, non-calcifying macroalgae benefit from increased CO<sub>2</sub> levels and overall primary productivity is likely higher near the seeps than at reference sites, where the biomass of macroalgae and fish is lower (Figure 8.2B).

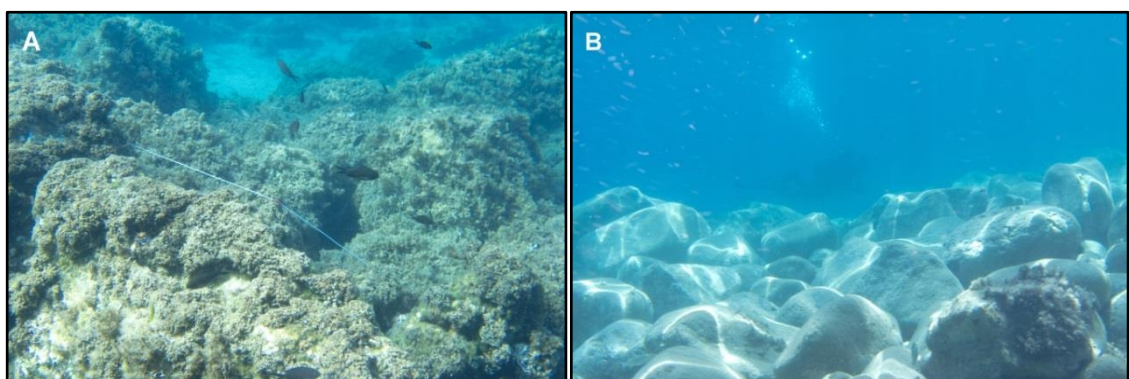


Figure 8.2. Typical seascape near seeps off Methana (A) and at reference sites (B) at ~ 3 m depth; areas near the seeps had higher macroalgal cover and higher fish biomass than reference sites, which were dominated by crustose coralline algae and sea urchins (photos by Maria Salomidi, September 2013).



Findings of this thesis have improved our knowledge on how herbivory will be affected by elevated CO<sub>2</sub>. There is still very little research on how other biological interactions, especially predator-prey interactions, will be influenced by ocean acidification in temperate systems, but Amaral *et al.* (2012) found that mussels grown at low pH are more vulnerable to crab predation. In tropical environments, recent studies show that changes in vulnerability to predators are size- and species-specific for fish, as some predators will be negatively affected by elevated pCO<sub>2</sub> as well (Ferrari *et al.*, 2011; Allan *et al.*, 2013). In addition, this thesis shows that changes in herbivory due to ocean acidification vary depending on the habitat studied, meaning that more research would be needed to predict responses of marine communities in a variety of habitats.

Both herbivore exclusion experiments reported in this thesis were performed at one reference and one high CO<sub>2</sub> site only. At Vulcano and Methana, pCO<sub>2</sub> is considered the main driver of change, with other environmental factors (e.g. temperature, salinity, heavy metals, hydrogen sulphide, wave exposure) not varying significantly between study sites (Boatta *et al.*, 2013; Chapter 2). Between-sites differences reported in Chapter 5 are therefore likely to be caused by changes in pCO<sub>2</sub> levels, but repeating these experiments in more than one reference site would improve their power and give more reliable results.

### **8.1.3 Adaptation potential to ocean acidification**

In this thesis, two dominant macroalgal species (the fucoid alga *Cystoseira corniculata* and the coralline alga *Jania rubens*) were transplanted between reference and high CO<sub>2</sub> sites for several months in order to assess whether they had permanently acclimatised to elevated pCO<sub>2</sub>, which might give an indication of their adaptation potential (Chapter 6). In addition, short-term effects

of elevated CO<sub>2</sub> were assessed on some physiological parameters in the same two species (Chapter 7). Comparison of short- and long-term effects of carbon dioxide on *C. corniculata* and *J. rubens* (Table 8.1) shows that there were very small effects on *C. corniculata* physiology in the short term, while in the long term it was evident that some pigments (chlorophyll *c* and antheraxanthin) concentration increase in thalli exposed to elevated CO<sub>2</sub> for several months. In addition, in the long term elevated carbon dioxide increased *C. corniculata* maximum electron transport rates (rETR<sub>max</sub>), C:N ratio and phlorotannin content, as well as decreasing epiphyte cover. *J. rubens* showed increases in some pigments concentration when transplanted to elevated CO<sub>2</sub>; in the short term, pheophytin *a* and phycoerithryin increased in thalli exposed to high CO<sub>2</sub>, whereas in the long term there was an increase in chlorophyll *a*, violaxanthin, zeaxanthin and phycocyanin. On the other hand, all other parameters measured in *J. rubens* did not change significantly after the thalli were transplanted near the seeps.

Table 8.1. Summary of the effect of elevated CO<sub>2</sub> on physiological parameters measured in thalli of *Cystoseira corniculata* and *Jania rubens* transplanted for 3 days (short term; Chapter 7) or 4-9 months (long term; Chapter 6). +: increase in parameter value; -: decrease in parameter value; n.s.: no significant effect; n.m.: parameter not measured.

| Response variable        | <i>Cystoseira corniculata</i> |           | <i>Jania rubens</i> |           |
|--------------------------|-------------------------------|-----------|---------------------|-----------|
|                          | Short-term                    | Long-term | Short-term          | Long-term |
| <b>Photochemistry</b>    |                               |           |                     |           |
| $F_v/F_m$                | n.s.                          | n.s.      | n.s.                | n.s.      |
| rETR <sub>max</sub>      | n.m.                          | +         | n.m.                | n.s.      |
| I <sub>k</sub>           | n.m.                          | n.s.      | n.m.                | n.s.      |
| α <sub>ETR</sub>         | n.m.                          | n.s.      | n.m.                | n.s.      |
| NPQ                      | n.m.                          | n.s.      | n.m.                | n.s.      |
| <b>Pigments</b>          |                               |           |                     |           |
| Chlorophyll a            | n.s.                          | n.s.      | n.s.                | +         |
| Chlorophyll c            | n.s.                          | +         | n.m.                | n.m.      |
| Pheophytin a             | n.s.                          | n.s.      | +                   | n.s.      |
| β-carotene               | n.s.                          | n.s.      | n.s.                | n.s.      |
| Fucoxanthin              | n.s.                          | n.s.      | n.m.                | n.m.      |
| Violaxanthin             | n.s.                          | n.s.      | n.s.                | +         |
| Antheraxanthin           | n.s.                          | +         | n.s.                | n.s.      |
| Zeaxanthin               | n.s.                          | n.s.      | n.s.                | +         |
| β-cryptoxanthin          | n.m.                          | n.m.      | n.s.                | n.s.      |
| Phycoerythrin            | n.m.                          | n.m.      | +                   | n.s.      |
| Phycocyanin              | n.m.                          | n.m.      | n.s.                | +         |
| <b>Growth</b>            | n.m                           | n.s.      | n.m                 | n.s.      |
| <b>Epiphyte cover</b>    | n.m                           | -         | n.m                 | n.m       |
| <b>C:N</b>               | n.m                           | +         | n.m                 | n.s.      |
| <b>C<sub>inorg</sub></b> | n.m                           | n.m       | n.m                 | n.s.      |
| <b>Phenol content</b>    | n.m.                          | +         | n.m.                | n.m.      |

Overall, both species seemed to change their physiology relatively quickly (more than three days, but less than four or nine months for *J. rubens* and *C. corniculata*, respectively), as the site of origin had very little effect on the physiology of long-term transplants. Macroalgae commonly show high phenotypic plasticity (Demes *et al.*, 2009), which could help calcifying species such as *J. rubens* to physiologically buffer negative effects of ocean acidification. However, phenotypic plasticity is known to slow down genetic adaptation by reducing selection gradients (Sunday *et al.*, 2014), meaning that if CO<sub>2</sub> will increase over *J. rubens*' current tolerance, this species may disappear.

Some physiological parameters were significantly different depending on the site of transplant in both species. While *C. corniculata* seemed to be favoured at high CO<sub>2</sub> levels (decreased epiphyte cover, higher phlorotannin content and maximum electron transport rates), *J. rubens* only showed increased concentration of some pigments. This probably leads to increased competitiveness of *C. corniculata*, as proven by the increase in its cover and the concurrent decrease of *J. rubens* cover as CO<sub>2</sub> increases (Chapter 3).

This highlights the importance of studying ocean acidification responses at the community level, as even calcifying algae that seem to cope relatively well with increased carbon dioxide, such as *J. rubens*, can be outcompeted by non-calcifying macroalgae that benefit from increased CO<sub>2</sub> levels, such as *C. corniculata*. This is in accord with a previous study showing that some calcifying algae can survive a moderate increase in pCO<sub>2</sub> levels, but their slower growth rates at high CO<sub>2</sub> reduce their competitive abilities (James *et al.*, 2014). Reduced growth rates at increased CO<sub>2</sub> have commonly been reported for coralline algae (Kroeker *et al.*, 2013a), although articulated coralline algae are less sensitive to ocean acidification than crustose forms (Johnson *et al.*, 2014).

*J. rubens* did not show significant differences in linear growth rates between sites, but it is possible that other life stages of this species are negatively affected by elevated CO<sub>2</sub>. Increased chemical defences or altered morphology of *C. corniculata* could have deterred *J. rubens* settlement at elevated CO<sub>2</sub> levels. Changes in macroalgal morphology or chemical defences influence the cover of their epiphytes, such as *J. rubens* (Jennings and Steinberg, 1997; Jones and Thornber, 2010). It is also possible that an episode of extremely high CO<sub>2</sub> drastically decreased *J. rubens* abundance. Recovery of *J. rubens*

population might be extremely difficult at elevated CO<sub>2</sub>, especially considering the limited distance its gametes travel (Jones and Moorjani, 1973). This hypothesis is supported by the fact that thalli of *J. rubens* transplanted to the seeps created areas of high *J. rubens* cover (Figure 8.3), suggesting that recruitment of this species is not impaired by moderate CO<sub>2</sub> enrichment, but recovery after extreme events is difficult because of the small distance its gametes travel.



Figure 8.3. *J. rubens* thalli transplanted near seeps off Methana are indicated by white plastic labels, and the high cover of *J. rubens* around them suggests this species' recruitment is not impaired by moderate pCO<sub>2</sub> enrichment.

Not all the physiological responses of these two macroalgal species may have been detected: many replicates were lost due to high wave action, leaving one treatment of *C. corniculata* with only three replicates and leaving only one usable reference site for *J. rubens* transplants. Low sample sizes reduce our

ability to detect small differences among treatments (Quinn and Keough, 2002), and reciprocal transplants to test for local adaptation should be performed using multiple reference sites (Sanford and Kelly, 2011). The species studied in this thesis should ideally be repeated in laboratory conditions (i.e. common garden experiment) to better assess the mechanisms of macroalgal responses to elevated CO<sub>2</sub>.

#### **8.1.4 Interaction with other stressors**

Studying the interaction of ocean acidification with other anthropogenic stressors is essential to reliably predict future conditions of marine ecosystems, as multiple stressors often interact synergistically; it is therefore very difficult to understand their combined effect from single-stressor experiments (Crain *et al.*, 2008). In this thesis, copper levels were manipulated *in situ* at different pCO<sub>2</sub> levels near volcanic seeps (Chapter 7). Results from these copper manipulation experiments showed that the combined effects of ocean acidification and copper pollution interact to increase copper accumulation in two macroalgal species (*Cystoseira corniculata* and *Jania rubens*), and this amplified the effects of ocean acidification on epifaunal communities. Interactive effects of ocean acidification and copper pollution have been largely overlooked by researchers so far, although it has been proven that these factors have synergistic negative effect on amphipods and polychaetes (Lewis *et al.*, 2012; Roberts *et al.*, 2013; Campbell *et al.*, 2014). The experiments in this thesis showed for the first time that tackling a local stressor such as copper pollution can help managing the impacts of a global stressor such as ocean acidification, similarly to what has already been demonstrated for other local stressors (e.g. eutrophication, sediment runoff; Ghedini *et al.*, 2013).

Many others anthropogenic stressors interact with ocean acidification to influence marine ecosystems. For instance, climate change is also causing an increase of temperature and UV radiation as well as decreased oxygen in marine systems (IPCC, 2014); further pressures on marine ecosystems include eutrophication, overfishing, invasive species and metal pollution (Halpern *et al.*, 2007). While ocean acidification and increased temperature are increasingly studied together (Kroeker *et al.*, 2013a), combinations of more than two stressors are rarely studied because of logistical constraints (Crain *et al.*, 2008). This thesis concentrated on one stressor only (ocean acidification) due to the difficulties in manipulating the environment *in situ*, but its only experiment concurrently investigating two factors highlighted that synergistic effects of anthropogenic stressors are likely and that future research needs to address the cumulative impacts of multiple variables.

## **8.2 Summary and direction for future research**

Ocean acidification has the potential to influence a wide range of physical, chemical and biological processes in the marine environment (Doney *et al.*, 2009). This thesis contributes to a growing body of research assessing the effects of ocean acidification on marine temperate rocky reefs. It is clear from these findings that temperate macroalgal communities and their epifauna change significantly with increasing CO<sub>2</sub>, and subtidal herbivore communities drastically shift from sea urchins to fish with increasing CO<sub>2</sub>. Experiments performed as part of this thesis have also shown that dominant macroalgal species at Methana have very high phenotypic plasticity, and change their physiology in a few months to acclimatise to pCO<sub>2</sub> levels up to 1700 µatm. Interactive effects of ocean acidification and copper pollution have also been proven to increase copper accumulation in macroalgae and indirectly affect their

epifauna. These findings have implications for the modelling of impacts of elevated CO<sub>2</sub> on marine ecosystems.

The findings from this thesis have highlighted that some marine taxa can tolerate, and sometimes thrive at, CO<sub>2</sub> levels up to 1700 µatm. Furoid algae were the main group to benefit from increased carbon dioxide, and at Methana *Cystoseira corniculata* could be found much deeper near the seeps than at the reference sites (~ 12 m vs ~ 2 m; author's personal observation). Since overall macroalgal biomass was higher at elevated CO<sub>2</sub>, it is very likely that primary productivity increases when CO<sub>2</sub> levels are high at Mediterranean rocky reefs. On the other hand, decreased diversity at elevated CO<sub>2</sub> has implications for ecosystem functioning of communities exposed to ocean acidification, especially if key species are lost. For instance, decreased coralline algal abundance and diversity near the studied seeps has the potential to influence carbon cycling in temperate systems. Coralline algae are one of the most important taxa for long-term carbon storage in the marine environment (Andersson *et al.*, 2008). Their decreasing abundance could therefore reduce the ability of temperate macroalgal beds to act as carbon sinks, even though an increase in furoid algal biomass might counter that effect (Chung *et al.*, 2011).

Our knowledge of ecosystem effects of ocean acidification is, however, still in its infancy. Using volcanic CO<sub>2</sub> seeps is a powerful approach to test hypotheses formulated following laboratory and mesocosm experiments, and can produce hypotheses to be verified in controlled conditions. Results from this thesis have revealed how temperate communities may change and how some organisms may change their physiology when exposed to ocean acidification. Patterns of rocky reef community changes are becoming clear after consistent results from mesocosm and field observations (Kroeker *et al.*, 2011; Porzio *et al.*, 2011;



Kroeker *et al.*, 2013a). Changes in ecosystem functions with increasing CO<sub>2</sub>, however, have rarely been tested in complex marine ecosystems. Ocean acidification is known to increase seagrass productivity at seeps off Vulcano (Apostolaki *et al.*, 2014), but macroalgal productivity responses are not as clear and have not been measured *in situ* so far (Hofmann *et al.*, 2011; Noisette *et al.*, 2013b; Olabarria *et al.*, 2013). Macroalgal communities not only contribute to coastal primary productivity, but offer other valuable ecosystem services, such as nutrient cycling, water depuration, fisheries production and shore protection from waves (Rönnbäck *et al.*, 2007). Responses of these processes to ocean acidification have scarcely been studied, and are a priority for future research.

Populations of *Cystoseira corniculata* and *Jania rubens* exposed for centuries to high and variable pCO<sub>2</sub> do not seem to have permanently acclimatised to ocean acidification, as their physiology was not influenced by their site of origin after a few months of acclimatisation to different pCO<sub>2</sub> levels. Very high phenotypic plasticity may slow down genetic adaptation, meaning that pCO<sub>2</sub> values above those physiological mechanisms can buffer could be detrimental to the two species studied in this thesis (Sunday *et al.*, 2014). However, it is possible that these species have in fact adapted to elevated pCO<sub>2</sub>, but their high phenotypic plasticity might have masked inter-population differences. Genetic studies are therefore needed to confirm that only phenotypic plasticity is at play in this instance. Furthermore, adaptation potential to ocean acidification is known for very few species and more studies are urgently needed to understand how marine organisms may adapt to ocean acidification (Reusch, 2014).

Predictions of community responses to ocean acidification are extremely important, but anthropogenic CO<sub>2</sub> emissions are causing other environmental changes, such as increased temperatures (IPCC, 2014). Moreover, human

pressures such as pollution and overfishing are contributing to degradation of marine ecosystems, and managing them is essential to improve the resilience of marine ecosystems to climate change (Ghedini *et al.*, 2013). Although laboratory experiments have been conducted to examine interactions between anthropogenic stressors, chiefly temperature (Kroeker *et al.*, 2013a), *in situ* studies are extremely rare because of the logistical difficulties in manipulating stressors in the field. Although complex, field experiments involving more than one stressor will be essential to understand how marine ecosystems will respond to future environmental changes, as interactive effects of stressors are often not predictable from single-stressor experiments (Gobler *et al.*, 2014).

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## Appendix A:

### Benthic percent cover at Methana and biomass at Vulcano (Chapter 3)

Appendix A1: mean ( $\pm$  SE, n=3) percent cover of benthic organisms at Methana in May and September 2012.

| May 2012<br>Taxon                      | Site                 |                      |                      |                      |                      |
|--|----------------------|----------------------|----------------------|----------------------|----------------------|
|  | REF A                | REF B                | 200 E                | 200 W                | SEEP                 |
| <i>Cystoseira corniculata</i>          | 51.43 $\pm$<br>14.00 | 47.71 $\pm$<br>15.47 | 62.57 $\pm$<br>13.36 | 61.43 $\pm$<br>12.57 | 31.57 $\pm$<br>15.29 |
| <i>Cystoseira amentacea</i>            | 7.86 $\pm$<br>4.98   | 0                    | 0                    | 0                    | 0                    |
| <i>Dictyota</i> sp.                    | 21.14 $\pm$<br>8.78  | 14.29 $\pm$<br>5.71  | 10.71 $\pm$<br>4.94  | 11.43 $\pm$<br>4.59  | 14.43 $\pm$<br>9.44  |
| <i>Cladostephus spongiosus</i>         | 0                    | 0                    | 0                    | 7.71 $\pm$<br>3.96   | 3.14 $\pm$<br>3.14   |
| <i>Padina pavonica</i> (non-calcified) | 0                    | 0                    | 1.71 $\pm$<br>1.71   | 1.57 $\pm$<br>1.57   | 15.29 $\pm$<br>10.18 |
| <i>Padina pavonica</i> (calcified)     | 5.29 $\pm$<br>3.21   | 5.00 $\pm$<br>1.89   | 0                    | 0                    | 0                    |
| <i>Sargassum vulgare</i>               | 2.86 $\pm$<br>2.86   | 8.57 $\pm$<br>8.57   | 4.00 $\pm$<br>1.65   | 2.86 $\pm$<br>1.49   | 36.29 $\pm$<br>16.25 |
| <i>Cladophora</i> sp.                  | 0                    | 0                    | 0                    | 0                    | 0.57 $\pm$<br>0.57   |
| <i>Halimeda tuna</i>                   | 0                    | 0.14 $\pm$<br>0.14   | 0                    | 0.14 $\pm$<br>0.14   | 0.14 $\pm$<br>0.14   |
| <i>Halopteris scoparia</i>             | 1.43 $\pm$<br>1.43   | 11.43 $\pm$<br>7.69  | 0.57 $\pm$<br>0.57   | 0                    | 0                    |
| CCA                                    | 0.86 $\pm$<br>0.70   | 3.43 $\pm$<br>1.41   | 19.29 $\pm$<br>12.56 | 9.86 $\pm$<br>9.21   | 0                    |
| Porifera (black)                       | 0                    | 0                    | 1.14 $\pm$<br>1.14   | 0                    | 0                    |
| Bare substratum                        | 7.71 $\pm$<br>3.27   | 2.14 $\pm$<br>1.49   | 0                    | 0                    | 0                    |
| <i>Jania rubens</i>                    | 2.14 $\pm$<br>1.49   | 0.86 $\pm$<br>0.70   | 0                    | 0                    | 0                    |
| <i>Sargassum</i> sp.                   | 0                    | 5.71 $\pm$<br>4.14   | 0                    | 0                    | 0                    |
| <i>Caulerpa racemosa</i>               | 0                    | 0.71 $\pm$<br>0.71   | 0                    | 0                    | 0                    |
| <i>Falkenbergia</i> sp.                | 0                    | 0                    | 0                    | 0                    | 0                    |
| Porifera (orange)                      | 0                    | 0                    | 0                    | 0                    | 0                    |
| <i>Amphiroa</i> sp.                    | 0                    | 0                    | 0                    | 0                    | 0                    |
| <i>Corallina caespitosa</i>            | 0                    | 0                    | 0                    | 0                    | 0                    |
| Turf algae                             | 0                    | 0                    | 0                    | 0                    | 0                    |
| Hydrozoa                               | 0                    | 0                    | 0                    | 0                    | 0                    |

| September 2012                         | Site             |                  |                  |                  |                  |
|--|------------------|------------------|------------------|------------------|------------------|
|  | REF A            | REF B            | 200 E            | 200 W            | SEEP             |
| <i>Cystoseira corniculata</i>          | 18.33 ±<br>7.15  | 24.17 ±<br>12.41 | 47.50 ±<br>11.74 | 39.67 ±<br>14.02 | 57.67 ±<br>17.98 |
| <i>Cystoseira amentacea</i>            | 0                | 0                | 0                | 0                | 0                |
| <i>Dictyota</i> sp.                    | 0                | 0                | 0                | 0                | 0                |
| <i>Cladostephus spongiosus</i>         | 0                | 0                | 0                | 10.83 ±<br>7.24  | 0                |
| <i>Padina pavonica</i> (non-calcified) | 0                | 0                | 0                | 0                | 0                |
| <i>Padina pavonica</i> (calcified)     | 0                | 1.17 ±<br>0.83   | 0                | 0                | 0                |
| <i>Sargassum vulgare</i>               | 0                | 0                | 0                | 0                | 0.17 ±<br>0.17   |
| <i>Cladophora</i> sp.                  | 0                | 0                | 0                | 0                | 3.33 ±<br>3.33   |
| <i>Halimeda tuna</i>                   | 0                | 0                | 0.17 ±<br>0.17   | 0                | 0.33 ±<br>0.33   |
| <i>Halopteris scoparia</i>             | 5.00 ±<br>5.00   | 0                | 0                | 5.00 ±<br>5.00   | 0                |
| CCA                                    | 15.00 ±<br>4.83  | 17.83 ±<br>8.11  | 13.67 ±<br>4.01  | 7.50 ±<br>5.59   | 0.33 ±<br>0.33   |
| Porifera (black)                       | 0                | 0                | 0                | 0                | 0                |
| Bare substratum                        | 4.17 ±<br>3.27   | 11.67 ±<br>6.67  | 0                | 5.83 ±<br>3.75   | 8.67 ±<br>3.38   |
| <i>Jania rubens</i>                    | 55.83 ±<br>10.83 | 21.67 ±<br>8.43  | 35.67 ±<br>10.02 | 23.33 ±<br>8.91  | 1.67 ±<br>1.05   |
| <i>Sargassum</i> sp.                   | 0                | 16.17 ±<br>12.99 | 0                | 3.00 ±<br>1.63   | 25.83 ±<br>15.78 |
| <i>Caulerpa racemosa</i>               | 0                | 0                | 0                | 0                | 0                |
| <i>Falkenbergia</i> sp.                | 0                | 0                | 0                | 0                | 2.00 ±<br>0.93   |
| Porifera (orange)                      | 0.83 ±<br>0.83   | 0                | 1.67 ±<br>1.67   | 0                | 1.67 ±<br>1.67   |
| <i>Amphiroa</i> sp.                    | 0                | 0.67 ±<br>0.49   | 0.50 ±<br>0.50   | 0                | 0                |
| <i>Corallina caespitosa</i>            | 0                | 0                | 0.83 ±<br>0.83   | 0                | 0                |
| Turf algae                             | 0                | 3.00 ±<br>3.00   | 0                | 5.00 ±<br>5.00   | 0                |
| Hydrozoa                               | 0.83 ±<br>0.83   | 0.83 ±<br>0.83   | 0                | 0                | 0                |

Appendix A2: mean ( $\pm$  SE, n=4) macroalgal biomass (grams of dry weight) at Vulcano in May 2010.

| Taxon                           | Site                |                      |                       |
|---------------------------------|---------------------|----------------------|-----------------------|
|                                 | REF A               | Mid pCO <sub>2</sub> | High pCO <sub>2</sub> |
| <i>Cystoseira</i> sp.           | 26.724 $\pm$ 10.946 | 4.373 $\pm$ 1.460    | 27.083 $\pm$ 29.746   |
| <i>Flabellia petiolata</i>      | 0.192 $\pm$ 0.083   | 0.770 $\pm$ 0.557    | 7.192 $\pm$ 5.994     |
| <i>Caulerpa prolifera</i>       | 0                   | 0.358 $\pm$ 0.265    | 2.061 $\pm$ 0.880     |
| Turf algae                      | 0.912 $\pm$ 0.415   | 2.648 $\pm$ 0.391    | 4.651 $\pm$ 3.093     |
| <i>Sargassum</i> sp.            | 0.629 $\pm$ 0.627   | 0                    | 3.353 $\pm$ 3.915     |
| <i>Caulerpa racemosa</i>        | 0                   | 0.454 $\pm$ 0.343    | 0.022 $\pm$ 0.045     |
| <i>Nitophyllum punctatum</i>    | 0                   | 0                    | 0.005 $\pm$ 0.010     |
| <i>Chylocladia pelagosae</i>    | 0                   | 0                    | 0                     |
| CCA                             | 0.919 $\pm$ 0.634   | 1.531 $\pm$ 0.633    | 0.372 $\pm$ 0.665     |
| <i>Rytiphloea tinctoria</i>     | 0.016 $\pm$ 0.016   | 0.028 $\pm$ 0.028    | 0.008 $\pm$ 0.015     |
| <i>Peyssonnelia</i> sp.         | 0                   | 1.491 $\pm$ 1.385    | 0.015 $\pm$ 0.030     |
| <i>Halopteris scoparia</i>      | 1.121 $\pm$ 0.753   | 0.374 $\pm$ 0.374    | 0                     |
| Gigartinales                    | 0                   | 0.010 $\pm$ 0.010    | 0                     |
| Cladophorales                   | 0                   | 0.006 $\pm$ 0.006    | 0                     |
| <i>Dictyopteris membranacea</i> | 2.760 $\pm$ 1.676   | 0.403 $\pm$ 0.140    | 0                     |
| <i>Ulothrix</i> sp.             | 0                   | 0.002 $\pm$ 0.002    | 0                     |
| <i>Ceramium</i> sp.             | 0                   | 0.002 $\pm$ 0.002    | 0                     |
| <i>Acetabularia acetabulum</i>  | 0.110 $\pm$ 0.110   | 0                    | 0                     |
| <i>Dictyota</i> sp.             | 0.821 $\pm$ 0.300   | 1.032 $\pm$ 0.541    | 0.669 $\pm$ 0.309     |
| Articulated coralline           | 0.363 $\pm$ 0.166   | 0                    | 0                     |
| <i>Cladophora</i> sp.           | 0                   | 0.044 $\pm$ 0.041    | 0.043 $\pm$ 0.055     |
| Ceramiales                      | 0.002 $\pm$ 0.002   | 0                    | 0.044 $\pm$ 0.088     |
| <i>Rhodymenia ligulata</i>      | 0                   | 0                    | 0.042 $\pm$ 0.024     |
| <i>Stilophora tenella</i>       | 0                   | 0.031 $\pm$ 0.031    | 0.005 $\pm$ 0.009     |
| <i>Halopteris</i> sp.           | 0.006 $\pm$ 0.005   | 0.056 $\pm$ 0.032    | 0                     |
| <i>Anadyomene stellata</i>      | 0                   | 0.005 $\pm$ 0.005    | 0.026 $\pm$ 0.052     |
| <i>Pterocladia</i> sp.          | 0                   | 0.033 $\pm$ 0.021    | 0                     |
| <i>Taonia atomaria</i>          | 0.133 $\pm$ 0.082   | 0.054 $\pm$ 0.043    | 0.463 $\pm$ 0.926     |
| <i>Osmundea truncata</i>        | 0                   | 0.002 $\pm$ 0.001    | 0                     |
| <i>Padina pavonica</i>          | 0.001 $\pm$ 0.001   | 0.087 $\pm$ 0.025    | 0                     |
| <i>Dictyota fasciola</i>        | 0.332 $\pm$ 0.295   | 0                    | 0                     |



## Appendix B:

### Epifaunal abundance at Methana and Vulcano (Chapter 4)

Appendix B1: mean ( $\pm$  SE, n=3) abundance of epifaunal invertebrates at Methana in May 2012.

| OTU                          | REF A                 | REF B                 | 200 E               | 200 W              | SEEP                |
|------------------------------|-----------------------|-----------------------|---------------------|--------------------|---------------------|
| <b>Foraminifera</b>          |                       |                       |                     |                    |                     |
| <i>Amphistegina lobifera</i> | 220.00 $\pm$<br>95.55 | 173.33<br>$\pm$ 25.18 | 5.67 $\pm$<br>3.28  | 7.33 $\pm$<br>5.04 | 0.67 $\pm$<br>0.21  |
| Agglutinated                 | 119.00 $\pm$<br>83.92 | 28.33 $\pm$<br>10.73  | 0                   | 0                  | 0                   |
| Calcified sp. 1              | 2.67 $\pm$<br>0.33    | 3.33 $\pm$<br>1.76    | 0                   | 0                  | 0                   |
| Calcified sp. 2              | 0                     | 0                     | 2.00 $\pm$<br>1.00  | 0                  | 0                   |
| <b>Sipuncula</b>             |                       |                       |                     |                    |                     |
|                              | 7.00 $\pm$<br>1.53    | 15.33 $\pm$<br>6.17   | 1.67 $\pm$<br>0.67  | 8.67 $\pm$<br>3.84 | 15.00 $\pm$<br>0.32 |
| <b>Platyhelminthes</b>       |                       |                       |                     |                    |                     |
|                              | 0                     | 0                     | 2.00 $\pm$<br>1.53  | 1.33 $\pm$<br>0.88 | 2.00 $\pm$<br>0.37  |
| <b>Bryozoa</b>               |                       |                       |                     |                    |                     |
|                              | 0.01 $\pm$<br>0.01    | 0.02 $\pm$<br>0.01    | 0.16 $\pm$<br>0.08  | 0.01 $\pm$<br>0.01 | 0                   |
| <b>Mollusca</b>              |                       |                       |                     |                    |                     |
| Bivalvia                     |                       |                       |                     |                    |                     |
| <i>Arca noeae</i>            | 0.67 $\pm$<br>0.67    | 0                     | 0                   | 0.67 $\pm$<br>0.67 | 0                   |
| Arcidae sp. 1                | 1.33 $\pm$<br>0.33    | 0.33 $\pm$<br>0.33    | 0                   | 0.33 $\pm$<br>0.33 | 0                   |
| Arcidae sp. 2                | 0.33 $\pm$<br>0.33    | 0                     | 0                   | 0                  | 0                   |
| Arcidae sp. 3                | 0.33 $\pm$<br>0.33    | 0                     | 0                   | 0                  | 0                   |
| Bivalvia sp. 1               | 35.33 $\pm$<br>15.34  | 41.67 $\pm$<br>12.55  | 11.67 $\pm$<br>4.33 | 5.00 $\pm$<br>1.53 | 0                   |
| Bivalvia sp. 2               | 0                     | 0.67 $\pm$<br>0.67    | 0.33 $\pm$<br>0.33  | 0.33 $\pm$<br>0.33 | 0                   |
| <i>Cardita</i> sp.           | 0                     | 0.67 $\pm$<br>0.33    | 0                   | 0                  | 0                   |
| Ostreoidea sp.               | 0                     | 0                     | 0.33 $\pm$<br>0.33  | 0                  | 0                   |
| Gastropoda                   |                       |                       |                     |                    |                     |
| <i>Alvania cimex</i>         | 0                     | 0.33 $\pm$<br>0.33    | 0                   | 0                  | 0                   |
| <i>Alvania geryonia</i>      | 0.33 $\pm$<br>0.33    | 0                     | 0                   | 0                  | 0                   |
| <i>Alvania lactea</i>        | 0                     | 0.33 $\pm$<br>0.33    | 0                   | 0                  | 0                   |

|                                  |                |                |                |                |   |
|----------------------------------|----------------|----------------|----------------|----------------|---|
| <i>Cerithiopsis</i> sp. 1        | 0.33 ±<br>0.33 | 0.67 ±<br>0.33 | 0              | 0              | 0 |
| <i>Cerithiopsis</i> sp. 2        | 0              | 1.00 ±<br>1.00 | 0              | 0              | 0 |
| <i>Cerithiopsis tubercularis</i> | 0              | 0.33 ±<br>0.33 | 0              | 0              | 0 |
| <i>Cerithium vulgatum</i>        | 0              | 0.33 ±<br>0.33 | 0              | 0              | 0 |
| <i>Columbella rustica</i> var. 1 | 0.33 ±<br>0.33 | 1.00 ±<br>0.58 | 1.67 ±<br>0.88 | 0.33 ±<br>0.33 | 0 |
| <i>Columbella rustica</i> var. 2 | 0              | 0.33 ±<br>0.33 | 0              | 0              | 0 |
| <i>Columbella rustica</i> var. 3 | 0              | 0              | 0              | 0.33 ±<br>0.33 | 0 |
| <i>Columbella rustica</i> var. 4 | 0              | 0              | 1.00 ±<br>1.00 | 0              | 0 |
| <i>Columbella</i> sp.            | 0              | 0.33 ±<br>0.33 | 0              | 0.33 ±<br>0.33 | 0 |
| <i>Diodora graeca</i>            | 0.33 ±<br>0.33 | 0              | 0              | 0              | 0 |
| Gasteropoda sp. 1                | 0.33 ±<br>0.33 | 1.33 ±<br>0.88 | 0              | 0              | 0 |
| Gasteropoda sp. 2                | 0              | 0.33 ±<br>0.33 | 0              | 0              | 0 |
| Gasteropoda sp. 3                | 0              | 0.33 ±<br>0.33 | 0              | 0              | 0 |
| Gasteropoda sp. 4                | 0.67 ±<br>0.33 | 0              | 0              | 0              | 0 |
| Gasteropoda sp. 5                | 0.67 ±<br>0.67 | 0              | 0              | 0              | 0 |
| Gasteropoda sp. 6                | 0.33 ±<br>0.33 | 0              | 0              | 0              | 0 |
| Gasteropoda sp. 7                | 0              | 0.33 ±<br>0.33 | 0              | 0              | 0 |
| <i>Hinia costulata</i>           | 1.00 ±<br>0.58 | 0              | 0              | 0.67 ±<br>0.67 | 0 |
| <i>Jujubinus</i> sp.             | 0              | 0.33 ±<br>0.33 | 0              | 0              | 0 |
| <i>Jujubinus striatus</i>        | 0              | 0.33 ±<br>0.33 | 0              | 0              | 0 |
| Muricidae sp.                    | 0              | 0              | 0.33 ±<br>0.33 | 0              | 0 |
| Omalogyridae sp.                 | 0.33 ±<br>0.33 | 4.33 ±<br>2.19 | 0              | 0              | 0 |
| <i>Patella</i> sp.               | 0.33 ±<br>0.33 | 0.33 ±<br>0.33 | 0              | 0              | 0 |
| <i>Pusillina</i> sp.             | 1.00 ±<br>1.00 | 1.00 ±<br>0.58 | 0              | 0              | 0 |
| <i>Rissoina</i> sp.              | 0              | 0.33 ±<br>0.33 | 0              | 0              | 0 |

|                              |                   |                   |                       |                    |                   |
|------------------------------|-------------------|-------------------|-----------------------|--------------------|-------------------|
| <i>Setia maculata</i>        | 0                 | 2.00 ±<br>1.53    | 0                     | 0                  | 0                 |
| <i>Triphora perversa</i>     | 0                 | 0.33 ±<br>0.33    | 0                     | 0                  | 0                 |
| <b>Polychaeta</b>            |                   |                   |                       |                    |                   |
| Polychaeta                   | 107.67 ±<br>1.67  | 85.00 ±<br>19.52  | 217.00<br>± 37.27     | 490.00 ±<br>149.29 | 176.33<br>± 4.49  |
| Serpulidae                   | 1.33 ±<br>0.33    | 1.67 ±<br>0.33    | 3.00 ±<br>1.00        | 0.67 ±<br>0.67     | 0                 |
| <b>Crustacea</b>             |                   |                   |                       |                    |                   |
| <b>Amphipoda</b>             |                   |                   |                       |                    |                   |
| <i>Amphilocus</i> sp.        | 1.67 ±<br>0.33    | 2.00 ±<br>0.58    | 2.00 ±<br>1.53        | 1.67 ±<br>1.20     | 2.00 ±<br>0.37    |
| <i>Ampithoe</i> sp.          | 22.33 ±<br>2.19   | 9.33 ±<br>4.37    | 351.00<br>± 28.02     | 159.00 ±<br>59.18  | 150.67<br>± 14.63 |
| Aoridae sp.                  | 16.67 ±<br>11.20  | 41.33 ±<br>17.25  | 32.00 ±<br>20.13      | 14.00 ±<br>2.52    | 22.33 ±<br>5.15   |
| <i>Apherusa</i> sp.          | 10.67 ±<br>4.10   | 10.33 ±<br>6.84   | 0                     | 0.67 ±<br>0.67     | 0.67 ±<br>0.21    |
| <i>Peltocoxa</i> sp.         | 0                 | 1.00 ±<br>0.58    | 0                     | 0                  | 0                 |
| <i>Dexamine spiniventris</i> | 0                 | 1.00 ±<br>1.00    | 0                     | 0.33 ±<br>0.33     | 0                 |
| <i>Hyale</i> sp.             | 204.00 ±<br>55.77 | 68.33 ±<br>26.57  | 681.33<br>±<br>357.19 | 837.33 ±<br>211.58 | 367.67<br>± 13.45 |
| <i>Gammaropsis</i> sp.       | 80.00 ±<br>20.53  | 34.33 ±<br>8.29   | 574.00<br>±<br>158.35 | 70.00 ±<br>35.34   | 245.33<br>± 12.72 |
| <i>Microprotopus</i> sp.     | 0                 | 0.33 ±<br>0.33    | 0                     | 0                  | 0                 |
| <i>Ericthonius</i> sp.       | 8.33 ±<br>2.67    | 1.67 ±<br>1.20    | 325.67<br>± 77.62     | 323.33 ±<br>170.95 | 180.00<br>± 22.10 |
| <i>Ischyrocerus</i> sp.      | 0.67 ±<br>0.67    | 1.67 ±<br>1.20    | 13.00 ±<br>8.14       | 1.33 ±<br>0.88     | 10.00 ±<br>2.21   |
| <i>Jassa</i> sp.             | 74.00 ±<br>18.03  | 9.00 ±<br>5.51    | 145.00<br>± 93.74     | 29.00 ±<br>14.01   | 39.00 ±<br>5.48   |
| <i>Leucothoe</i> sp.         | 1.00 ±<br>1.00    | 0                 | 9.67 ±<br>5.93        | 0                  | 1.00 ±<br>0.18    |
| <i>Elasmopus</i> sp.         | 39.00 ±<br>11.68  | 38.67 ±<br>0.67   | 54.33 ±<br>9.84       | 37.00 ±<br>3.61    | 73.33 ±<br>10.88  |
| <i>Maera</i> sp.             | 21.33 ±<br>16.34  | 36.00 ±<br>5.86   | 17.00 ±<br>9.29       | 5.67 ±<br>4.18     | 31.33 ±<br>5.56   |
| <i>Pereionotus</i> sp.       | 0                 | 20.33 ±<br>5.24   | 4.33 ±<br>1.20        | 62.3 3 ±<br>48.84  | 33.33 ±<br>4.42   |
| <i>Podocerus</i> sp.         | 57.00 ±<br>8.50   | 124.33<br>± 27.94 | 180.33<br>± 12.84     | 7.33 ±<br>3.18     | 84.33 ±<br>4.96   |
| <i>Stenothoe</i> spp.        | 142.67 ±<br>83.79 | 340.33<br>± 61.50 | 80.00 ±<br>26.95      | 4.33 ±<br>1.86     | 6.00 ±<br>0.63    |

|                             |                  |                  |                   |                   |                  |
|-----------------------------|------------------|------------------|-------------------|-------------------|------------------|
| <i>Caprella</i> sp.         | 11.33 ±<br>5.24  | 35.33 ±<br>2.96  | 12.33 ±<br>4.91   | 2.33 ±<br>1.33    | 0                |
| Ostracoda                   | 1.00 ±<br>0.58   | 19.00 ±<br>13.05 | 2.67 ±<br>1.45    | 4.33 ±<br>2.33    | 0.33 ±<br>0.11   |
| Copepoda                    |                  |                  |                   |                   |                  |
| Harpacticoida               | 4.00 ±<br>2.52   | 93.67 ±<br>51.27 | 14.00 ±<br>6.11   | 17.00 ±<br>7.77   | 6.33 ±<br>1.19   |
| Cirripedia                  | 0.67 ±<br>0.67   | 0                | 4.00 ±<br>2.08    | 0                 | 0                |
| Tanaidacea                  |                  |                  |                   |                   |                  |
| <i>Leptochelia savignyi</i> | 30.67 ±<br>3.84  | 14.33 ±<br>4.98  | 175.33<br>± 38.89 | 215.00 ±<br>52.74 | 126.00<br>± 7.77 |
| <i>Tanais dulongii</i>      | 2.33 ±<br>0.33   | 72.00 ±<br>8.02  | 5.00 ±<br>1.15    | 5.33 ±<br>4.33    | 0                |
| <i>Araphura brevimanus</i>  | 0                | 1.33 ±<br>0.33   | 0                 | 0                 | 0                |
| Isopoda                     |                  |                  |                   |                   |                  |
| Asellota                    | 61.00 ±<br>32.73 | 81.00 ±<br>60.18 | 160.67<br>± 35.93 | 25.67 ±<br>2.31   | 84.67 ±<br>7.98  |
| Sphaeromatidea              | 5.33 ±<br>3.38   | 12.00 ±<br>5.29  | 1.67 ±<br>0.88    | 0.67 ±<br>0.67    | 4.00 ±<br>0.63   |
| Decapoda                    | 2.66 ±<br>1.33   | 8.00 ±<br>2.60   | 0.66 ±            | 0                 | 0                |
| <b>Pycnogonida</b>          | 1.67 ±<br>0.88   | 0.67 ±<br>0.33   | 10.00 ±<br>5.20   | 1.33 ±<br>0.33    | 1.33 ±<br>0.28   |
| <b>Echinodermata</b>        |                  |                  |                   |                   |                  |
| <i>Asterina gibbosa</i>     | 0                | 0                | 0                 | 0                 | 0.33 ±<br>0.11   |
| Ophiuroidea                 | 2.67 ±<br>2.19   | 2.67 ±<br>1.76   | 37.00 ±<br>17.58  | 44.33 ±<br>16.29  | 3.33 ±<br>0.64   |

Appendix B2: mean ( $\pm$  SE) abundance of epifaunal invertebrates at Vulcano in June 2013.

| Macroalgal host                    | <i>Sargassum vulgare</i> |                    | <i>Cystoseira</i> spp. |                    |
|------------------------------------|--------------------------|--------------------|------------------------|--------------------|
|                                    | 600 ppm<br>(n=9)         | 1200 ppm<br>(n=10) | 600 ppm<br>(n=15)      | 1200 ppm<br>(n=14) |
| <b>Polychaeta</b>                  |                          |                    |                        |                    |
| Filter feeder                      | 4.56 ±<br>2.43           | 159.00 ±<br>36.96  | 1.07 ±<br>0.27         | 4.64 ±<br>1.28     |
| Non filter feeder                  | 13.33 ±<br>3.15          | 145.30 ±<br>25.53  | 7.13 ±<br>1.55         | 43.71 ±<br>8.07    |
| <b>Mollusca</b>                    |                          |                    |                        |                    |
| Polyplacophora                     |                          |                    |                        |                    |
| <i>Acanthochitona fascicularis</i> | 0                        | 0                  | 0.07 ± 0.07            | 0                  |
| Bivalvia                           |                          |                    |                        |                    |
| <i>Cardita calyculata</i>          | 0                        | 0                  | 0.07 ± 0.07            | 0                  |

|  |             |              |              |               |
|--|-------------|--------------|--------------|---------------|
| <i>Musculus discors</i>                | 0           | 0.10 ± 0.10  | 0.40 ± 0.19  | 0             |
| <i>Musculus</i> sp. juv.               | 0           | 0            | 0            | 0.07 ± 0.07   |
| <i>Mytilaster minimus</i>              | 0           | 0            | 0.53 ± 0.47  | 0             |
| Gasteropoda                            |             |              |              |               |
| <i>Alvania cfr hirta</i>               | 0           | 0            | 0            | 0.07 ± 0.07   |
| <i>Ammonicera fischeriana</i>          | 0           | 0            | 0.40 ± 0.29  | 0.43 ± 0.20   |
| <i>Barleeia rubra</i>                  | 0           | 0            | 0.13 ± 0.13  | 0             |
| <i>Barleeia rubra</i> juv.             | 0           | 0            | 0.07 ± 0.07  | 0             |
| <i>Cerithium lividulum</i>             | 0.22 ± 0.15 | 0            | 0.07 ± 0.07  | 0             |
| <i>Cerithium</i> cfr <i>scabridum</i>  | 0           | 0            | 0.07 ± 0.07  | 0             |
| <i>Columbella rustica</i> juv          | 0           | 0.10 ± 0.10  | 1.07 ± 1.07  | 0.14 ± 0.14   |
| <i>Columbella rustica</i>              | 0.11 ± 0.11 | 0            | 0.20 ± 0.14  | 0             |
| <i>Eatonina cossurae</i>               | 2.00 ± 0.88 | 0            | 3.07 ± 0.88  | 0.07 ± 0.07   |
| <i>Eatonina cossurae</i> juv           | 1.33 ± 0.90 | 0            | 0.13 ± 0.13  | 0             |
| Gastropoda indet. juv.                 | 0.22 ± 0.22 | 0            | 0.07 ± 0.07  | 0.43 ± 0.29   |
| <i>Gibbula ricketti</i>                | 0           | 0            | 0.07 ± 0.07  | 0             |
| <i>Gibbula varia</i>                   | 0           | 0            | 0.07 ± 0.07  | 0             |
| <i>Gibbula</i> sp. juv.                | 0           | 0            | 0.60 ± 0.40  | 0.07 ± 0.07   |
| <i>Jujubinus</i> sp. juv               | 0.11 ± 0.11 | 0            | 0.07 ± 0.07  | 0             |
| <i>Omalogyra simplex</i>               | 0.11 ± 0.11 | 0            | 0.20 ± 0.11  | 0             |
| <i>Pollia</i> cfr <i>dorbignyi</i> juv | 0           | 0.10 ± 0.10  | 0            | 0             |
| <i>Rissoa auriscalpium</i>             | 0           | 0            | 0            | 0.07 ± 0.07   |
| <i>Rissoa guerinii</i>                 | 0.11 ± 0.11 | 0            | 0            | 0             |
| <i>Rissoa variabilis</i> juv.          | 0           | 0            | 1.13 ± 0.61  | 0.14 ± 0.10   |
| <i>Rissoa</i> sp juv                   | 0.11 ± 0.11 | 0.10 ± 0.10  | 0.13 ± 0.09  | 0.29 ± 0.22   |
| Rissoidea indet.                       | 0           | 0            | 0            | 0.07 ± 0.07   |
| <i>Setia</i> cfr <i>maculata</i>       | 0.22 ± 0.22 | 0            | 0            | 0             |
| <i>Setia</i> cfr <i>amabilis</i>       | 1.11 ± 0.75 | 0            | 0.07 ± 0.07  | 0             |
| <i>Setia</i> sp. juv.                  | 1.00 ± 0.78 | 0            | 0.07 ± 0.07  | 0.07 ± 0.07   |
| <b>Crustacea</b>                       |             |              |              |               |
| Copepoda (Harpacticoida)               | 0           | 0            | 0            | 0.21 ± 0.15   |
| Amphipoda                              |             |              |              |               |
| <i>Amphilocheus neapolitanus</i>       | 0.33 ± 0.17 | 0            | 0.07 ± 0.07  | 0             |
| <i>Ampithoe</i> sp.                    | 0           | 0            | 0            | 0.07 ± 0.07   |
| <i>Ampithoe ferox</i>                  | 0.11 ± 0.11 | 0.20 ± 0.20  | 0.13 ± 0.09  | 0             |
| <i>Ampithoe helleri</i>                | 0           | 0            | 0.07 ± 0.07  | 0.21 ± 0.11   |
| <i>Ampithoe ramondi</i>                | 1.89 ± 0.59 | 33.20 ± 8.53 | 2.40 ± 0.58  | 5.57 ± 3.03   |
| <i>Ampithoe riedli</i>                 | 0           | 0            | 0            | 0.07 ± 0.07   |
| <i>Ampithoe spuria</i>                 | 8.89 ± 2.47 | 49.80 ± 5.44 | 49.73 ± 8.30 | 73.36 ± 19.57 |
| <i>Cymadusa crassicornis</i>           | 3.89 ± 0.90 | 0            | 5.07 ± 1.02  | 1.14 ± 0.54   |
| Aoridae sp.                            | 0.33 ± 0.24 | 6.40 ± 2.48  | 0            | 0.14 ± 0.14   |
| <i>Apherusa</i> sp.                    | 7.78 ± 1.79 | 0.50 ± 0.27  | 0.67 ± 0.35  | 0.29 ± 0.22   |

|                                       |                   |               |              |              |
|---------------------------------------|-------------------|---------------|--------------|--------------|
| <i>Dexamine spiniventris</i>          | 0.33 ± 0.24       | 0             | 0.40 ± 0.24  | 0.07 ± 0.07  |
| <i>Dexamine spinosa</i>               | 0.11 ± 0.11       | 0             | 0            | 0            |
| <i>Hyale</i> sp.                      | 5.89 ± 1.88       | 35.00 ± 18.63 | 8.40 ± 3.85  | 1.86 ± 0.61  |
| <i>Hyale camptonyx</i>                | 0.11 ± 0.11       | 1.90 ± 1.28   | 1.00 ± 0.59  | 1.43 ± 0.54  |
| <i>Hyale crassipes</i>                | 0                 | 0             | 0            | 0.36 ± 0.25  |
| <i>Hyale perieri</i>                  | 0                 | 0.10 ± 0.10   | 0.20 ± 0.14  | 0            |
| <i>Hyale schmidti</i>                 | 4.33 ± 1.35       | 12.30 ± 3.33  | 4.33 ± 1.94  | 4.86 ± 1.73  |
| <i>Erichthonius</i> sp.               | 102.00 ±<br>31.51 | 98.10 ± 17.22 | 16.07 ± 5.89 | 20.21 ± 3.17 |
| <i>Ischyrocerus</i> sp.               | 0                 | 0             | 0            | 0.07 ± 0.07  |
| <i>Jassa marmorata</i>                | 0                 | 0.10 ± 0.10   | 0.13 ± 0.13  | 0            |
| <i>Lysianassa costae</i>              | 0.11 ± 0.11       | 0.20 ± 0.13   | 0            | 0.07 ± 0.07  |
| <i>Elasmopus</i> sp.                  | 0                 | 0             | 0.07 ± 0.07  | 0            |
| <i>Maera inaequipes</i>               | 0                 | 0             | 0.07 ± 0.07  | 0            |
| <i>Pereionotus testudo</i>            | 1.89 ± 0.98       | 0.20 ± 0.13   | 0.60 ± 0.35  | 0.21 ± 0.11  |
| <i>Podocerus variegatus</i>           | 0.89 ± 0.39       | 0             | 0.47 ± 0.27  | 0            |
| <i>Stenothoe</i> sp.                  | 15.44 ± 5.86      | 0.50 ± 0.22   | 7.20 ± 4.62  | 0.29 ± 0.16  |
| <i>Urothoe elegans</i>                | 0.11 ± 0.11       | 0             | 0            | 0            |
| <i>Caprella</i> sp.                   | 6.56 ± 2.75       | 15.00 ± 5.70  | 1.93 ± 0.96  | 0.36 ± 0.17  |
| Amphipod asp.                         | 10.00 ± 2.19      | 35.20 ± 6.11  | 14.87 ± 2.82 | 15.29 ± 3.37 |
| Tanaidacea                            |                   |               |              |              |
| <i>Araphura brevimanus</i>            | 2.22 ± 0.85       | 0.10 ± 0.10   | 0            | 0.07 ± 0.07  |
| <i>Leptochelia savignyi</i>           | 41.22 ± 12.59     | 14.20 ± 6.36  | 2.47 ± 0.56  | 2.29 ± 1.33  |
| <i>Tanais dulongii</i>                | 0.11 ± 0.11       | 0.30 ± 0.15   | 0.80 ± 0.38  | 0.14 ± 0.14  |
| Isopoda                               |                   |               |              |              |
| Sphaeromatidea                        | 0.11 ± 0.11       | 1.70 ± 0.67   | 0.27 ± 0.15  | 0.36 ± 0.36  |
| Asellota                              | 0                 | 0             | 0.13 ± 0.13  | 0            |
| Decapoda                              | 0                 | 0             | 0.07 ± 0.07  | 0            |
| <i>Pagurus</i> sp.                    | 0.11 ± 0.11       | 0             | 0            | 0            |
| <b>Pycnogonida</b>                    | 0.11 ± 0.11       | 0             | 0            | 0            |
| <b>Acarina</b>                        | 0.22 ± 0.15       | 0             | 0            | 0            |
| <b>Echinodermata</b><br>(Ophiuroidea) | 0                 | 0             | 0.13 ± 0.13  | 0            |

## Appendix C:

### Benthic functional groups cover at Vulcano and Methana (Chapter 5)

Appendix C1: mean ( $\pm$  SE, n=3-6) percent cover of benthic functional groups at Vulcano in 2012; C=control, P=procedural control, E=exclusion.

| May 2012                       |                      |                      |                     |                     |                      |                      |
|--------------------------------|----------------------|----------------------|---------------------|---------------------|----------------------|----------------------|
| Taxon                          | 600 C                | 600 P                | 600 E               | 1200 C              | 1200 P               | 1200 E               |
| <i>Padina pavonica</i>         | 4.17 $\pm$<br>3.00   | 25.00 $\pm$<br>12.58 | 8.33 $\pm$<br>2.71  | 1.67 $\pm$<br>1.67  | 4.17 $\pm$<br>3.00   | 1.67 $\pm$<br>0.83   |
| Brown turf algae               | 25.00 $\pm$<br>21.26 | 23.33 $\pm$<br>23.33 | 20.42 $\pm$<br>7.08 | 28.33 $\pm$<br>8.33 | 36.67 $\pm$<br>6.67  | 42.08 $\pm$<br>10.42 |
| Bare substratum                | 57.50 $\pm$<br>28.98 | 39.17 $\pm$<br>22.38 | 53.33 $\pm$<br>6.31 | 73.33 $\pm$<br>4.41 | 43.33 $\pm$<br>21.86 | 40.83 $\pm$<br>11.65 |
| Filamentous brown algae        | 0                    | 0                    | 0                   | 0                   | 0                    | 3.33 $\pm$<br>3.33   |
| Dictyotales                    | 8.33 $\pm$<br>6.01   | 5.00 $\pm$<br>2.89   | 4.58 $\pm$<br>1.87  | 1.67 $\pm$<br>1.67  | 0                    | 0                    |
| <i>Chtamalus stellatus</i>     | 0                    | 0                    | 1.25 $\pm$<br>0.85  | 1.67 $\pm$<br>1.67  | 0                    | 0                    |
| Green turf algae               | 0                    | 1.67 $\pm$<br>1.67   | 0.42 $\pm$<br>0.42  | 0                   | 15.83 $\pm$<br>15.83 | 9.58 $\pm$<br>7.76   |
| Filamentous green algae        | 0                    | 0                    | 0                   | 0                   | 0                    | 1.67 $\pm$<br>1.67   |
| <i>Acetabularia acetabulum</i> | 0                    | 1.67 $\pm$<br>0.83   | 0.42 $\pm$<br>0.42  | 0                   | 0                    | 0.42 $\pm$<br>0.42   |
| CCA                            | 0.83 $\pm$<br>0.83   | 0.83 $\pm$<br>0.83   | 3.33 $\pm$<br>1.67  | 0                   | 0                    | 0                    |
| <i>Laurencia</i> sp.           | 0                    | 0                    | 0.42 $\pm$<br>0.42  | 0                   | 0                    | 0                    |
| <i>Anadyomene stellata</i>     | 0                    | 0                    | 0.42 $\pm$<br>0.42  | 0                   | 0                    | 0                    |
| Encrusting brown algae         | 0.83 $\pm$<br>0.83   | 0.83 $\pm$<br>0.83   | 0                   | 0                   | 0                    | 0                    |
| <i>Dasycladus</i> sp.          | 0                    | 0.83 $\pm$<br>0.83   | 0.42 $\pm$<br>0.42  | 0                   | 0                    | 0                    |
| <i>Cystoseira</i> sp.          | 3.33 $\pm$<br>3.33   | 1.67 $\pm$<br>1.67   | 1.67 $\pm$<br>1.67  | 0                   | 0                    | 0                    |
| Serpulidae                     | 0                    | 0                    | 0.83 $\pm$<br>0.83  | 0                   | 0                    | 0                    |
| <i>Cladophora</i> sp.          | 0                    | 0                    | 0                   | 0                   | 0                    | 0                    |
| <i>Valonia utricularis</i>     | 0                    | 0                    | 0                   | 0                   | 0                    | 0                    |
| <i>Actinia equina</i>          | 0                    | 0                    | 0                   | 0                   | 0                    | 0                    |
| <i>Anemonia viridis</i>        | 0                    | 0                    | 0                   | 0                   | 0                    | 0                    |
| Articulated coralline algae    | 0                    | 0                    | 0                   | 0                   | 0                    | 0                    |

|                          |   |   |   |   |   |   |
|--------------------------|---|---|---|---|---|---|
| <i>Verrucaria</i> sp.    | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>Peyssonnelia</i> sp.  | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>Ralfsia verrucosa</i> | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>Caulerpa racemosa</i> | 0 | 0 | 0 | 0 | 0 | 0 |

July 2012

| Taxon                          | 600 C            | 600 P            | 600 E           | 1200 C           | 1200 P          | 1200 E           |
|--------------------------------|------------------|------------------|-----------------|------------------|-----------------|------------------|
| <i>Padina pavonica</i>         | 15.67 ±<br>6.98  | 34.67 ±<br>25.33 | 16.00 ±<br>4.68 | 1.00 ±<br>1.00   | 3.00 ±<br>2.08  | 4.50 ±<br>2.43   |
| Brown turf algae               | 18.67 ±<br>15.30 | 11.00 ±<br>9.54  | 17.17 ±<br>8.83 | 45.00 ±<br>17.79 | 22.00 ±<br>4.36 | 39.33 ±<br>13.79 |
| Bare substratum                | 36.67 ±<br>18.66 | 35.67 ±<br>19.06 | 36.50 ±<br>6.15 | 42.33 ±<br>15.59 | 65.67 ±<br>4.33 | 51.33 ±<br>12.66 |
| Filamentous brown algae        | 1.00 ±<br>1.00   | 0                | 0               | 1.00 ±<br>1.00   | 2.33 ±<br>2.33  | 0                |
| Dictyotales                    | 0.67 ±<br>0.67   | 1.00 ±<br>1.00   | 1.67 ±<br>1.67  | 3.33 ±<br>3.33   | 0               | 0.33 ±<br>0.33   |
| <i>Chtamalus stellatus</i>     | 0.67 ±<br>0.67   | 0.67 ±<br>0.67   | 6.17 ±<br>3.29  | 0.67 ±<br>0.67   | 1.33 ±<br>0.67  | 0.67 ±<br>0.42   |
| Green turf algae               | 0                | 0                | 0               | 0                | 0               | 0                |
| Filamentous green algae        | 0                | 0                | 0               | 0                | 0               | 0                |
| <i>Acetabularia acetabulum</i> | 0.67 ±<br>0.67   | 0.67 ±<br>0.67   | 1.67 ±<br>0.33  | 0                | 0               | 0.50 ±<br>0.50   |
| CCA                            | 18.33 ±<br>6.01  | 6.00 ±<br>4.58   | 11.00 ±<br>2.21 | 0.67 ±<br>0.67   | 1.67 ±<br>0.88  | 0.33 ±<br>0.33   |
| <i>Laurencia</i> sp.           | 0                | 0                | 2.17 ±<br>0.79  | 0                | 0               | 0.50 ±<br>0.50   |
| <i>Anadyomene stellata</i>     | 1.67 ±<br>0.88   | 1.00 ±<br>1.00   | 0.83 ±<br>0.54  | 4.00 ±<br>2.08   | 0.67 ±<br>0.67  | 1.17 ±<br>0.83   |
| Encrusting brown algae         | 0                | 0                | 0               | 0                | 0               | 0                |
| <i>Dasycladus</i> sp.          | 2.33 ±<br>2.33   | 3.33 ±<br>3.33   | 3.17 ±<br>1.30  | 0                | 0               | 0                |
| <i>Cystoseira</i> sp.          | 0.67 ±<br>0.67   | 1.67 ±<br>0.88   | 1.50 ±<br>0.50  | 0                | 0               | 0                |
| Serpulidae                     | 1.67 ±<br>0.88   | 0                | 1.50 ±<br>1.15  | 0.67 ±<br>0.67   | 0               | 0.67 ±<br>0.42   |
| <i>Cladophora</i> sp.          | 0                | 0                | 0.33 ±<br>0.33  | 0                | 1.67 ±<br>1.67  | 0                |
| <i>Valonia utricularis</i>     | 0                | 0                | 0               | 0                | 0               | 0.67 ±<br>0.67   |
| <i>Actinia equina</i>          | 0                | 0                | 0               | 0                | 1.00 ±<br>1.00  | 0                |
| <i>Anemonia viridis</i>        | 0                | 1.00 ±<br>1.00   | 0               | 0                | 0               | 0                |
| Articulated coralline algae    | 0                | 0.67 ±<br>0.67   | 0               | 0                | 0               | 0                |
| <i>Verrucaria</i> sp.          | 0                | 0.67 ±<br>0.67   | 0               | 0                | 0               | 0                |



|                          |   |                |                |   |   |   |
|--------------------------|---|----------------|----------------|---|---|---|
| <i>Peyssonnelia</i> sp.  | 0 | 1.00 ±<br>1.00 | 0.33 ±<br>0.33 | 0 | 0 | 0 |
| <i>Ralfsia verrucosa</i> | 0 | 0              | 0              | 0 | 0 | 0 |
| <i>Caulerpa racemosa</i> | 0 | 0              | 0              | 0 | 0 | 0 |

September 2012

| Taxon                          | 600 C            | 600 P            | 600 E           | 1200 C           | 1200 P          | 1200 E           |
|--------------------------------|------------------|------------------|-----------------|------------------|-----------------|------------------|
| <i>Padina pavonica</i>         | 0.67 ±<br>0.67   | 18.33 ±<br>9.53  | 12.00 ±<br>4.31 | 0                | 1.67 ±<br>0.88  | 2.00 ±<br>0.77   |
| Brown turf algae               | 48.33 ±<br>13.64 | 49.00 ±<br>11.79 | 52.00 ±<br>3.60 | 40.67 ±<br>21.30 | 80.33 ±<br>5.24 | 71.83 ±<br>14.50 |
| Bare substratum                | 20.67 ±<br>11.10 | 8.33 ±<br>4.41   | 8.67 ±<br>5.24  | 25.00 ±<br>9.07  | 8.33 ±<br>4.41  | 10.50 ±<br>9.91  |
| Filamentous brown algae        | 0                | 0                | 0               | 0                | 0               | 0                |
| Dictyotales                    | 5.67 ±<br>2.96   | 4.00 ±<br>1.00   | 2.17 ±<br>0.79  | 0                | 0               | 0.33 ±<br>0.33   |
| <i>Chtamalus stellatus</i>     | 2.33 ±<br>1.45   | 1.00 ±<br>1.00   | 6.83 ±<br>2.93  | 1.33 ±<br>0.67   | 0.67 ±<br>0.67  | 4.17 ±<br>3.39   |
| Green turf algae               | 0                | 0                | 0               | 0                | 1.00 ±<br>1.00  | 0.33 ±<br>0.33   |
| Filamentous green algae        | 0                | 0                | 0               | 0                | 0               | 0                |
| <i>Acetabularia acetabulum</i> | 0                | 0.67 ±<br>0.67   | 0.33 ±<br>0.33  | 0.67 ±<br>0.67   | 0               | 0.33 ±<br>0.33   |
| CCA                            | 15.00 ±<br>5.00  | 8.33 ±<br>7.36   | 11.33 ±<br>4.14 | 3.33 ±<br>0.88   | 3.33 ±<br>0.88  | 1.83 ±<br>0.87   |
| <i>Laurencia</i> sp.           | 0                | 0                | 0.83 ±<br>0.83  | 0                | 0               | 0.83 ±<br>0.83   |
| <i>Anadyomene stellata</i>     | 1.67 ±<br>0.88   | 3.00 ±<br>1.73   | 2.83 ±<br>0.65  | 3.33 ±<br>2.03   | 1.00 ±<br>1.00  | 1.67 ±<br>1.17   |
| Encrusting brown algae         | 0                | 0                | 0               | 0                | 0.67 ±<br>0.67  | 0                |
| <i>Dasycladus</i> sp.          | 3.33 ±<br>3.33   | 3.33 ±<br>3.33   | 1.33 ±<br>0.88  | 0                | 0.67 ±<br>0.67  | 0.50 ±<br>0.50   |
| <i>Cystoseira</i> sp.          | 0                | 1.00 ±<br>1.00   | 1.00 ±<br>0.63  | 8.00 ±<br>8.00   | 1.33 ±<br>0.67  | 1.17 ±<br>0.54   |
| Serpulidae                     | 0                | 1.00 ±<br>1.00   | 0.67 ±<br>0.42  | 0                | 0               | 0.33 ±<br>0.33   |
| <i>Cladophora</i> sp.          | 0                | 0                | 0               | 10.67 ±<br>10.67 | 0               | 0.50 ±<br>0.50   |
| <i>Valonia utricularis</i>     | 0                | 0                | 0               | 1.00 ±<br>1.00   | 0               | 0.33 ±<br>0.33   |
| <i>Actinia equina</i>          | 0                | 0                | 0               | 0                | 1.00 ±<br>1.00  | 0                |
| <i>Anemonia viridis</i>        | 0                | 0.67 ±<br>0.67   | 0               | 0                | 0               | 0                |
| Articulated coralline algae    | 0                | 0.67 ±<br>0.67   | 0               | 0                | 0               | 0                |
| <i>Verrucaria</i> sp.          | 0                | 0                | 0               | 0                | 0               | 0                |

| <i>Peyssonnelia</i> sp.        | 0                | 0                | 0               | 0                | 0      | 0                |
|--------------------------------|------------------|------------------|-----------------|------------------|--------|------------------|
| <i>Ralfsia verrucosa</i>       | 2.33 ±<br>2.33   | 0                | 0               | 5.33 ±<br>1.67   | 0      | 3.33 ±<br>3.33   |
| <i>Caulerpa racemosa</i>       | 0                | 0                | 0               | 0                | 0      | 0                |
| October 2012                   |                  |                  |                 |                  |        |                  |
| Taxon                          | 600 C            | 600 P            | 600 E           | 1200 C           | 1200 P | 1200 E           |
| <i>Padina pavonica</i>         | 1.67 ±<br>0.88   | 12.33 ±<br>11.35 | 7.33 ±<br>3.24  | 0                | lost   | 1.80 ±<br>1.36   |
| Brown turf algae               | 41.33 ±<br>7.69  | 49.67 ±<br>14.10 | 46.67 ±<br>6.64 | 45.67 ±<br>13.86 | lost   | 46.80 ±<br>15.56 |
| Bare substratum                | 20.00 ±<br>1.00  | 10.33 ±<br>6.06  | 4.17 ±<br>3.60  | 23.00 ±<br>5.57  | lost   | 28.80 ±<br>12.76 |
| Filamentous brown algae        | 0                | 0                | 0               | 0                | lost   | 0                |
| Dictyotales                    | 17.33 ±<br>11.85 | 0                | 4.17 ±<br>2.71  | 0.67 ±<br>0.67   | lost   | 2.00 ±<br>1.55   |
| <i>Chtamalus stellatus</i>     | 0                | 1.00 ±<br>1.00   | 4.33 ±<br>3.37  | 1.33 ±<br>0.67   | lost   | 1.20 ±<br>0.49   |
| Green turf algae               | 0                | 0                | 0               | 0                | lost   | 0                |
| Filamentous green algae        | 0                | 0                | 0               | 0                | lost   | 0                |
| <i>Acetabularia acetabulum</i> | 0                | 0.67 ±<br>0.67   | 0               | 0                | lost   | 0.40 ±<br>0.40   |
| CCA                            | 17.67 ±<br>3.71  | 13.33 ±<br>8.82  | 14.50 ±<br>4.75 | 5.00 ±<br>1.15   | lost   | 3.80 ±<br>2.46   |
| <i>Laurencia</i> sp.           | 0                | 0                | 0.83 ±<br>0.83  | 0                | lost   | 0                |
| <i>Anadyomene stellata</i>     | 0.67 ±<br>0.67   | 3.00 ±<br>0.58   | 1.83 ±<br>0.75  | 2.67 ±<br>1.45   | lost   | 2.00 ±<br>2.00   |
| Encrusting brown algae         | 0                | 0                | 0               | 0                | lost   | 0                |
| <i>Dasycladus</i> sp.          | 0                | 8.33 ±<br>8.33   | 4.33 ±<br>2.76  | 0                | lost   | 1.00 ±<br>1.00   |
| <i>Cystoseira</i> sp.          | 0                | 1.67 ±<br>1.67   | 10.33 ±<br>5.58 | 15.33 ±<br>15.33 | lost   | 7.80 ±<br>4.07   |
| Serpulidae                     | 0.67 ±<br>0.67   | 0.67 ±<br>0.67   | 0.50 ±<br>0.50  | 0                | lost   | 0.60 ±<br>0.60   |
| <i>Cladophora</i> sp.          | 0                | 0                | 0               | 2.33 ±<br>2.33   | lost   | 0.40 ±<br>0.40   |
| <i>Valonia utricularis</i>     | 0                | 0.67 ±<br>0.67   | 0.33 ±<br>0.33  | 0                | lost   | 0                |
| <i>Actinia equina</i>          | 0                | 0                | 0               | 0                | lost   | 0                |
| <i>Anemonia viridis</i>        | 0                | 1.00 ±<br>1.00   | 0               | 0                | lost   | 0                |
| Articulated coralline algae    | 0                | 0                | 0               | 0                | lost   | 0                |
| <i>Verrucaria</i> sp.          | 0                | 0                | 0               | 3.33 ±<br>3.33   | lost   | 3.40 ±<br>3.40   |
| <i>Peyssonnelia</i> sp.        | 0                | 0                | 0               | 0                | lost   | 0                |

|                          |   |   |                |   |      |   |
|--------------------------|---|---|----------------|---|------|---|
| <i>Ralfsia verrucosa</i> | 0 | 0 | 0              | 0 | lost | 0 |
| <i>Caulerpa racemosa</i> | 0 | 0 | 0.67 ±<br>0.42 | 0 | lost | 0 |

Appendix C2: mean ( $\pm$  SE, n=3-4) percent cover of benthic functional groups at Methana in June 2013; C=control, P=procedural control, E=exclusion.

| Functional group        | REF A_C          | REF A_P         | REF A_E          | SEEP_C          | SEEP_P           | SEEP_E           |
|-------------------------|------------------|-----------------|------------------|-----------------|------------------|------------------|
| Turf algae              | 49.50 ±<br>16.59 | 33.00 ±<br>6.51 | 29.25 ±<br>7.98  | 4.00 ±<br>1.83  | 25.33 ±<br>9.40  | 1.67 ±<br>1.67   |
| Fucoid algae            | 0                | 0.33 ±<br>0.33  | 0.50 ±<br>0.50   | 9.25 ±<br>4.11  | 9.67 ±<br>1.45   | 1.33 ±<br>1.33   |
| Fleshy brown algae      | 0                | 1.67 ±<br>1.67  | 0                | 0               | 0                | 33.33 ±<br>33.33 |
| Calcifying brown algae  | 0                | 0               | 50.75 ±<br>17.72 | 0               | 0                | 30.00 ±<br>28.02 |
| Encrusting black sponge | 0.25 ±<br>0.25   | 0               | 0                | 2.00 ±<br>2.00  | 0.33 ±<br>0.33   | 0                |
| Encrusting green algae  | 14.50 ±<br>10.27 | 8.33 ±<br>4.91  | 0.25 ±<br>0.25   | 27.25 ±<br>4.61 | 14.33 ±<br>9.77  | 0                |
| Erect brown algae       | 0                | 0.33 ±<br>0.33  | 2.00 ±<br>2.00   | 2.25 ±<br>2.25  | 0                | 24.33 ±<br>19.55 |
| Biofilm                 | 0                | 0               | 0.25 ±<br>0.25   | 5.00 ±<br>2.68  | 1.00 ±<br>0.58   | 4.00 ±<br>4.00   |
| Serpulids               | 0                | 0.33 ±<br>0.33  | 0                | 0               | 0                | 0                |
| CCA                     | 12.50 ±<br>2.90  | 8.00 ±<br>3.61  | 3.75 ±<br>2.25   | 1.25 ±<br>0.48  | 0.67 ±<br>0.67   | 0                |
| Bare substratum         | 23.25 ±<br>4.80  | 48.00 ±<br>9.17 | 13.25 ±<br>6.17  | 49.00 ±<br>5.35 | 48.67 ±<br>11.67 | 5.33 ±<br>3.53   |

## Appendix D:

### Epifaunal abundance at Methana (Chapter 7)

Appendix D1: mean ( $\pm$  SE) abundance of epifaunal invertebrates exposed or not exposed to copper at Methana in June 2013.

| Site                          | REF                  |                     | SEEP                  |                      |
|-------------------------------|----------------------|---------------------|-----------------------|----------------------|
| Copper                        | no<br>(n=4)          | yes<br>(n=5)        | no<br>(n=5)           | yes<br>(n=5)         |
| <b>Polychaeta</b>             |                      |                     |                       |                      |
| non calcifying                | 14.75 $\pm$<br>3.97  | 14.40 $\pm$<br>3.01 | 10.60 $\pm$<br>5.56   | 31.40 $\pm$<br>11.45 |
| Serpulidae                    | 0.25 $\pm$ 0.25      | 0.40 $\pm$ 0.40     | 0                     | 0                    |
| <b>Oligochaeta</b>            | 1.50 $\pm$ 0.96      | 1.00 $\pm$ 0.63     | 0.40 $\pm$ 0.24       | 0                    |
| <b>Sipuncula</b>              | 0.75 $\pm$ 0.48      | 0.20 $\pm$ 0.20     | 0                     | 0                    |
| <b>Mollusca</b>               |                      |                     |                       |                      |
| Gasteropoda                   |                      |                     |                       |                      |
| <i>Bittium reticulatum</i>    | 0.50 $\pm$ 0.29      | 0.40 $\pm$ 0.24     | 0                     | 0                    |
| <i>Columbella rustica</i>     | 0.50 $\pm$ 0.29      | 0                   | 0                     | 0                    |
| Gasteropoda sp. 1             | 0                    | 0.20 $\pm$ 0.20     | 0                     | 0                    |
| Gasteropoda sp. 2             | 0.25 $\pm$ 0.25      | 0.20 $\pm$ 0.20     | 0                     | 0                    |
| Gasteropoda sp. 3             | 0.25 $\pm$ 0.25      | 0                   | 0                     | 0                    |
| Gasteropoda sp. 4             | 0.25 $\pm$ 0.25      | 0                   | 0                     | 0                    |
| Muricidae sp 1                | 0.25 $\pm$ 0.25      | 0                   | 0                     | 0                    |
| <i>Odostomia acuta</i>        | 3.00 $\pm$ 1.78      | 4.60 $\pm$ 2.04     | 0                     | 0                    |
| <i>Rissoa</i> sp.             | 0                    | 0                   | 0.20 $\pm$ 0.20       | 0                    |
| Rissoidae sp. 1               | 0.75 $\pm$ 0.75      | 0                   | 0                     | 0                    |
| Bivalvia                      |                      |                     |                       |                      |
| <i>Arca noeae</i>             | 0.50 $\pm$ 0.50      | 0                   | 0.20 $\pm$ 0.20       | 0                    |
| Bivalvia sp. 1                | 0.25 $\pm$ 0.25      | 0.80 $\pm$ 0.37     | 0.20 $\pm$ 0.20       | 0.20 $\pm$ 0.20      |
| Bivalvia sp. 2                | 0.25 $\pm$ 0.25      | 0                   | 0.20 $\pm$ 0.20       | 0                    |
| <i>Ostrea</i> sp.             | 0.25 $\pm$ 0.25      | 0                   | 0                     | 0                    |
| <i>Striarca lactea</i>        | 0                    | 0.20 $\pm$ 0.20     | 0                     | 0                    |
| <b>Crustacea</b>              |                      |                     |                       |                      |
| Ostracoda                     | 2.25 $\pm$ 1.65      | 5.80 $\pm$ 1.56     | 6.80 $\pm$ 3.40       | 6.20 $\pm$ 1.56      |
| Copepoda                      | 41.00 $\pm$<br>12.21 | 25.40 $\pm$<br>6.36 | 110.40 $\pm$<br>56.21 | 64.20 $\pm$<br>20.12 |
| Amphipoda                     |                      |                     |                       |                      |
| <i>Elasmopus</i> sp.          | 2.25 $\pm$ 1.03      | 1.60 $\pm$ 0.68     | 3.60 $\pm$ 1.83       | 4.00 $\pm$ 1.14      |
| <i>Hyale perieri</i>          | 0                    | 0                   | 0                     | 0.20 $\pm$ 0.20      |
| <i>Hyale schmidtii</i>        | 0                    | 0                   | 8.00 $\pm$ 3.79       | 1.20 $\pm$ 0.49      |
| <i>Hyale camptonyx</i>        | 0                    | 0                   | 1.40 $\pm$ 0.75       | 0                    |
| <i>Hyale crassipes</i>        | 0.25 $\pm$ 0.25      | 0.20 $\pm$ 0.20     | 0.40 $\pm$ 0.24       | 0                    |
| <i>Apherusa chierieghinii</i> | 0.50 $\pm$ 0.29      | 0.40 $\pm$ 0.24     | 0                     | 0                    |
| <i>Dexamine spiniventris</i>  | 0                    | 0.20 $\pm$ 0.20     | 0                     | 0                    |

|                               |                 |                |                 |                 |
|-------------------------------|-----------------|----------------|-----------------|-----------------|
| <i>Erichtonius</i> sp.        | 0               | 0.20 ± 0.20    | 1.40 ± 0.98     | 0.80 ± 0.37     |
| <i>Pereionotus testudo</i>    | 0               | 0.20 ± 0.20    | 1.40 ± 0.98     | 1.00 ± 0.45     |
| <i>Peltocoxa gibbosa</i>      | 0               | 0              | 0.20 ± 0.20     | 0               |
| <i>Podocerus variegatus</i>   | 1.50 ± 1.19     | 0.60 ± 0.40    | 0.60 ± 0.24     | 0.80 ± 0.37     |
| <i>Stenothoe</i> sp.          | 5.75 ± 1.89     | 2.80 ± 0.80    | 0.80 ± 0.58     | 1.40 ± 0.93     |
| <i>Gammaropsis</i> sp.        | 0               | 0              | 0.20 ± 0.20     | 2.60 ± 1.66     |
| <i>Maera grossimana</i>       | 0               | 0              | 0.40 ± 0.24     | 0               |
| <i>Maera inaequipes</i>       | 0               | 0.80 ± 0.80    | 0.20 ± 0.20     | 1.60 ± 1.12     |
| <i>Maera</i> sp.              | 2.25 ±<br>0.95  | 3.60 ± 1.60    | 3.00 ± 1.58     | 4.60 ± 1.60     |
| <i>Ampithoe riedlii</i>       | 0               | 0              | 7.40 ± 1.89     | 1.60 ± 0.51     |
| <i>Ampithoe ramondi</i>       | 0               | 0              | 4.20 ± 2.18     | 1.20 ± 0.73     |
| Aoridae sp.                   | 1.00 ± 0.01     | 0              | 5.00 ± 2.59     | 2.40 ± 0.51     |
| Tanaidacea                    |                 |                |                 |                 |
| <i>Caprella acanthifera</i>   | 0.50 ± 0.50     | 0.40 ± 0.24    | 0               | 0.40 ± 0.24     |
| <i>Leptocheira savignyi</i>   | 11.00 ±<br>2.38 | 9.80 ±<br>3.40 | 25.00 ±<br>3.91 | 17.60 ±<br>2.64 |
| <i>Tanais cavolinii</i>       | 0.25 ± 0.25     | 0.80 ± 0.37    | 0               | 0               |
| <i>Leptognathia brevimanu</i> | 0.50 ± 0.50     | 0              | 0               | 0               |
| Isopoda                       |                 |                |                 |                 |
| Asellopoda                    | 3.75 ± 2.39     | 1.00 ± 0.45    | 13.40 ± 4.87    | 2.40 ± 0.40     |
| Flabellifera                  | 2.50 ± 0.96     | 2.80 ± 0.92    | 0.80 ± 0.37     | 0               |
| Cumacea                       | 0.50 ± 0.29     | 0              | 0               | 0               |
| Decapoda                      | 0.50 ± 0.29     | 0              | 0               | 0               |
| <b>Pycnogonida</b>            | 0.25 ± 0.25     | 0.20 ± 0.20    | 0               | 0.40 ± 0.24     |
| <b>Echinodermata</b>          |                 |                |                 |                 |
| <i>Amphiura</i> sp.           | 0               | 0.40 ± 0.40    | 2.80 ± 1.50     | 0.20 ± 0.20     |

## Appendix E:

### Publications

Appendix E1:

**C. Baggini**, M. Salomidi, E. Voutsinas, L. Bray, E. Krasakopoulou, J.M. Hall-Spencer (2014). Seasonality affects macroalgal community response to increases in pCO<sub>2</sub>. *PLoS ONE*, 9: e106520.

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C. Baggini, M. Salomidi, E. Voutsinas, L. Bray, E. Krasakopoulou, J.M. Hall-Spencer (2014). Seasonality affects macroalgal community response to increases in pCO<sub>2</sub>. *PLoS ONE*, 9: e106520.

Full text available at:

<http://journals.plos.org/plosone/article?id=10.1371/journal.pone.0106520>

Appendix E2:

L.C. Hofmann, K. Bischof, **C. Baggini**, A. Johnson, K. Koop-Jakobsen, M. Teichberg (2015). CO<sub>2</sub> and inorganic nutrient enrichment affect the performance of a calcifying green alga and its non-calcifying epiphyte. *Oecologia*, 177: 1157-1169.



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L.C. Hofmann, K. Bischof, C. Baggini, A. Johnson, K. Koop-Jakobsen, M. Teichberg (2015). CO<sub>2</sub> and inorganic nutrient enrichment affect the performance of a calcifying green alga and its non-calcifying epiphyte. *Oecologia*, 177: 1157-1169.

Full text available at: <http://link.springer.com/article/10.1007/s00442-015-3242-5>

Appendix E3:

G. Langer., G. Nehrke, **C. Baggini**, R. Rodolfo-Metalpa, J.M. Hall-Spencer, J. Bijma (2014). Limpets counteract ocean acidification induced shell corrosion by thickening of aragonitic shell layers. *Biogeosciences Discussions*, 11: 12571-12590.

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G. Langer., G. Nehrke, C. Baggini, R. Rodolfo-Metalpa, J.M. Hall-Spencer, J. Bijma (2014). Limpets counteract ocean acidification induced shell corrosion by thickening of aragonitic shell layers. *Biogeosciences Discussions*, 11: 12571-12590.

Full text available at:

<http://www.biogeosciences-discuss.net/11/12571/2014/bgd-11-12571-2014.html>

Appendix E4:

P. Calosi, S.P.S. Rastrick, M. Graziano, S.C. Thomas, **C. Baggini**, H.A. Carter, J. Hall-Spencer, M. Milazzo, J.I. Spicer (2013). Ecophysiology of sea urchins living near shallow water CO<sub>2</sub> vents: Investigations of acid-base balance and ionic regulation using *in-situ* transplantation. *Marine Pollution Bulletin*, 73: 470-484.

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P. Calosi, S.P.S. Rastrick, M. Graziano, S.C. Thomas, C. Baggini, H.A. Carter, J. Hall-Spencer, M. Milazzo, J.I. Spicer (2013). Ecophysiology of sea urchins living near shallow water CO<sub>2</sub> vents: Investigations of acid-base balance and ionic regulation using *in-situ* transplantation. *Marine Pollution Bulletin*, 73: 470-484.

Full text available at:

<http://www.sciencedirect.com/science/article/pii/S0025326X12005784>

Appendix E5:

S. Hahn, R. Rodolfo-Metalpa, E. Griesshaber, W.W. Schmahl, D. Buhl, J.M. Hall-Spencer, **C. Baggini**, K.T. Fehr, A. Immenhauser (2012). Marine bivalve shell geochemistry and ultrastructure from modern low pH environments: environmental effect versus experimental bias. *Biogeosciences*, 9: 1897-1914.

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S. Hahn, R. Rodolfo-Metalpa, E. Griesshaber, W.W. Schmahl, D. Buhl, J.M. Hall-Spencer, C. Baggini, K.T. Fehr, A. Immenhauser (2012). Marine bivalve shell geochemistry and ultrastructure from modern low pH environments: environmental effect versus experimental bias. *Biogeosciences*, 9: 1897-1914.

Full text available at:

<http://www.biogeosciences.net/9/1897/2012/bg-9-1897-2012.html>

Appendix E6:

**C. Baggini**, Y. Issaris, M. Salomidi, J.M. Hall-Spencer (2014). Herbivore diversity improves benthic community resilience to ocean acidification. *Journal of Experimental Marine Biology and Ecology* (accepted pending revisions).



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C. Baggini, Y. Issaris, M. Salomidi, J.M. Hall-Spencer (2014). Herbivore diversity improves benthic community resilience to ocean acidification. *Journal of Experimental Marine Biology and Ecology* (accepted pending revisions).