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Global Change Effects on Seagrass Ecosystem

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A study on Global change effects on seagrass ecosystem

By

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Author's Declaration

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 Doctoral College Quality Sub-committee.

Work submitted for this research degree at the University of Plymouth has not formed part of any other degree either at the University of Plymouth or at another establishment.

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Global Change effects on Seagrass Ecosystem

Amrit Kumar Mishra

Abstract

Rising carbon dioxide (CO_2) concentrations in the atmosphere will increase the average pCO_2 level in the world oceans, which will have a knock-on effect on the marine ecosystem. Coastal seagrass communities one of the most productive marine ecosystems are predicted to benefit from the increase in CO_2 levels, but long-term effects of elevated CO_2 on seagrass communities are less understood. Population reconstruction techniques was used to investigate the population dynamics of Cymodocea *nodosa* meadows, exposed to long term elevated CO_2 at volcanic seeps off Greece and Italy. Effect of elevated CO_2 was noticed on the growth, morphometry, density, biomass and age structure at CO_2 seeps. Above to below ground biomass ratio of *C. nodosa* were higher at CO_2 seeps than at reference sites. The plastochrome interval were similar at all CO_2 seeps. The shoot age and shoot longevity of plants were lower at seeps than reference sites. The present recruitment (sampled year) of the seagrass were higher than long-term average recruitment of the communities near the seeps. Carbon to nitrogen ratios (%DW) of *C. nodosa* were higher in leaves at seeps. Annual leaf production was higher near the seeps. This study suggests increased production of *C. nodosa* under elevated CO_2 levels, but other co-factors such as nutrients, trace metal toxicity must also be taken into consideration while predicting effects of future CO_2 concentrations.

Volcanic CO_2 seeps are now being used as natural analogues for ocean acidification studies although these areas can be affected by trace element input and may alter ecosystem responses to gradient in carbonate chemistry. Here Fe and a range of trace elements (Cd, Co, Cu, Hg, Mn, Pb, Ni and Zn) were analysed from sediments and from the roots, rhizomes and leaves of seagrass at six CO_2 seeps and reference sites off Greece and Italy. There were higher metal levels in sediment and seagrasses at all CO_2 seeps than reference sites. Sediment Quality Guideline Quotient, a commonly used pollution index, indicated that some of the metals (Cd, Cu, Hg, Ni) were in high enough concentrations to have adverse biological effects, such as Cu at Ischia site and Hg at Vulcano. Higher accumulation of elements from sediments in roots and leaves at CO_2 seeps were found from Bio Sediment Accumulation Factor index. There were higher levels of Cu, Fe, Mn and Zn in leaves and rhizomes for *P. oceanica* and higher levels of Cd, Co, Cu, Fe and Zn in *C. nodosa* compartments at CO_2 seeps. Fe and Mn were found with positive correlation within sediment-roots and sediment-rhizomes, whereas Cd, Co and Pb were found with positive correlation in compartments of *C. nodosa*. In *P. oceanica* positive correlation were only observed for Cd within sediment-roots and plant compartments. Low pH and ocean acidification increased the concentration of elements at CO_2 seeps than reference sites. Thus, caution is needed, when using volcanic seep systems as analogue for the effects of rising CO_2, as metals can reach levels that are toxic to seagrass, masking any potential benefits of increased levels of carbon dioxide for seagrass productivity.

Net community production (NCP) and community respiration (CR) were measured under air exposed and CO_2 enriched conditions for intertidal *Z. noltei* meadows and unvegetated sediment communities during emersion in summer and winter seasons. Community production and respiration were measured in-situ using benthic chambers. CO_2 flux under air and CO_2 enriched conditions were measured over a series of short term incubations (30min) using an infra-red gas analyser. Incident photosynthetic active radiation (PAR) was recorded during the incubations covering the daily and seasonal variation. Linear regression model was used to test the effects of irradiance on net community production. NCP of *Z. noltei* community were higher under CO_2 enriched conditions than air exposed conditions in both summer and winter seasons. There was no effect of CO_2 on the CR rate of *Z. noltei* community in summer season. NCP of sediment community were higher in summer season and winter season under CO_2 enriched conditions. Sediment CR rates were higher in winter than summer season. The light compensation point of *Z. noltei* and sediment community were lower in both seasons under CO_2 enriched conditions. Seasonal budget of community production was higher in Z. noltei than sediment communities. A clear effect of PAR was noticed on the net community production of both communities. Higher PAR intensities resulted in higher NCP under CO_2 enriched conditions for both communities. CO_2 enrichment will have a positive effect on the intertidal communities during emersion.

Keywords: Seagrass, community production, reconstruction techniques, element toxicity, CO_2 seeps

Contents

Abbreviations list

ANOVA-Analysis of Variance

BEDS-Biological Effects Database for Sediments

BSAF-Bio- sediment accumulation factor

CH_4-Methane

Cu-Copper

Co-Cobalt

Cd-Cadmium

C-Carbon

CR-Community Respiration

Cs-Caesium

CO_2-Carbon dioxide

CO_3^{2-}- Carbonate

CO2Sys- Carbon Dioxide System calculation

DIC-Dissolved inorganic carbon

DW-Dry weight

ERL- Effects Range Low

ERM- Effects Range Median

Fe-Ferrous (Iron)

H_2S-Hydrogen Sulphide

HCO_3^- -Bicarbonate

HCl- Hydrogen chloride

Hg-Mercury

HNO_3- Nitric acid

GCP-Gross community production

IRGA- Infrared Gas Analyzer

IPCC- International Panel for Climate Change

ICP-MS- Induced couple plasms- Mass Spectrometry

ICP-AES- Induced Couple Plasma-Atomic Emission Spectroscopy

LCP-Light Compensation point

LGC-Laboratory for Government Chemistry

M- Mortality

Mn-Manganese

N-Nitrogen

NE-North East

NW-North West

Ni-Nickel

NCP-Net community production

OA-Ocean acidification

PAR-Photosynthetic active radiation

PEL-Probable Effects Level

Pb-Lead

$p\mathrm{CO}_2$-partial pressure of Carbon dioxide

PI-Plastochrome interval

R- Recruitment rate (long-term)

Ro- Recruitment rate (Present)

SCUBA-Self Contained Underwater Breathing Apparatus

SE-South East

SW-South West

SQG- Sediment Quality Guidelines

SQG-Q-Sediment Quality Guidelines Quotient

Sr-Strontium

TA-Total Alkalinity

TE- Trace elements

TEL-Threshold Effects Level

Zn-Zinc

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Chapter 1

General Introduction

General Introduction

1.1. Global changes

Many changes are underway on Earth including increased land and sea temperatures and increasing sea level, coupled with a host of secondary changes (IPCC, 2014). These changes are a result of increasing anthropogenic carbon dioxide levels (CO_2) in the Earth's atmosphere due to human activities such as deforestation, cement production and fossil fuel burning. Atmospheric CO_2 have increased 25% since the Industrial Revolution and include the concentration of other greenhouse gasses (e.g. methane, nitrous oxide, chlorofluorocarbons) which are also rising (Solomon et al., 2007). The ocean is the largest carbon reservoir with nearly fifty times more carbon storage capacity than either the atmosphere or terrestrial reservoirs (Solomon et al., 2007). Anthropogenic CO_2 enters the ocean across the air-sea interface and this facilitates the sequestration of anthropogenic carbon emissions through physical, chemical and biological processes (McLeod et al., 2011) into the ocean's carbon sink (Feely et al., 2001). Once assimilated into the ocean, carbon availability and water circulation partially regulate the biological productivity and biochemistry of the oceans globally (Libes, 2009). The role of the ocean as a sink for CO_2 is driven by two processes; the solubility pump and the biological pump (Sabina and Feely, 2007; Libes, 2009; McLeod et al., 2011). The solubility pump works because CO_2 is more soluble in cold seawater than warm. Cold deep waters are generally rich in dissolved inorganic carbon (DIC) and when these deep waters upwell into warmer equatorial regions, extensive outgassing of CO_2 occurs to the atmosphere due to reduced solubility (Le Quéré et al., 2009; McLeod et al., 2011). The biological carbon pump refers to the uptake of CO_2 by marine plankton form surface water through photosynthesis, thus a small fraction (1/3) of algal biomass produced is transferred to the deep ocean and buried in sediments (Boyce et al., 2010; McLeod et al., 2011). Both carbon pumps help in regulate the uptake of carbon from the atmosphere by the oceans.

Over 35 giga tonnes per year of anthropogenic CO_2 produced due to human activities ends up in the world oceans and this is rapidly changing seawater carbonate chemistry (Royal Society, 2005; IPCC, 2014,). When CO_2 dissolves in water, it forms carbonic acid (H_2CO_3), which further dissociates into hydrogen (H^+), bicarbonate (HCO_3^-) and carbonate (CO_3^{2-}) ions. The availability of more H^+ ions increases the acidity of the seawater and is known as "ocean acidification", (Caldeira & Wickett, 2003) which has reduced the pH levels of surface seawater by an average of 0.1 units, with a predicted further decrease of 0.3-0.4 units by the end of the

2100 century (IPCC, 2014). The oceans are well buffered through changes in the relative proportions of CO_3^{2-}, HCO_3^-, and CO_2. This DIC maintains the seawater pH within narrow limits although this buffering capacity diminishes as increased amounts of CO_2 are absorbed by the ocean. This is because when CO_2 dissolves, the chemical process that takes places reduces some carbonate ions (HCO_3^-) required for the ocean pH buffer (Royal Society, 2005).

Elevated atmospheric CO_2 is reducing carbonate and pH levels and increasing the concentration of aqueous CO_2 and bicarbonate (Doney et al.,2009). The decrease in surface pH is happening so rapidly that this may make it difficult for many organisms to adapt to a changing chemical environment (Milazzo et al., 2016). Reduced carbonate ion concentrations are known to impact calcified organisms with decreased aragonite and calcite saturation states causing skeletal or shell dissolution in extreme cases, so calcifying organisms are expected to be especially vulnerable to rising CO_2 levels (Kleypas et al., 1999; Andersson et al., 2007).

1.2. Seagrass ecosystem and Carbon sequestration

The coastal zones are characterized by several different ecosystems, each dominated by different suites of primary producers making costal habitats one of the most productive regions in the world. Primary productivity in coastal systems can be dominated by phytoplankton, benthic microalgae, seaweeds, kelp, seagrasses, tidal marsh plants or mangroves (Thom, 2001). Along with high production rates coastal zones are also characterized for nutrient cycling and biodiversity (Thom, 2001). These habitats are important for the ocean's biogeochemical cycles, and they play a crucial role in providing ecosystem services (Gattuso et al., 1998; Erwin et al., 2009). Their physical structure provides breeding grounds and nurseries for crustaceans, finfish and shellfish, as well as food for reptiles, fish, birds and mammals (Coles et al., 2007). Coastal marine algae and plants are the basis of a detrital food chain, they process nutrients and contaminants, stabilize sediments and act as storm buffers. Economically, they support food webs and contribute to the local economy (Conservation International, 2008)

One of the most important, productive and dynamic types of coastal vegetation are seagrasses. Seagrasses are a relatively small group of flowering plants with about 50 species in 13 Genera and 5 Families (Coles et al., 2007) that are found all over the world. Seagrass growth is characterized by repeated structural units that in combination form the body of the plant (Hemminga & Duarte, 2000). These units also make up the rhizomes (root- like stems) that extend below the sediments and create thick networks of detritus, leaves, old rhizomes and other organic matter, which can extend several meters below the surface. It is in these networks that carbon and nutrients are stored by seagrasses which makes seagrasses potential carbon sink (Hemminga& Duarte, 2000; Borum et al., 2004). These networks are called mattes which are mostly found in *P. oceanica.*

Seagrasses cover a very small area in the coastal systems and have low biomass with an average of 184 gCm^{-2} (Laffoley et al., 2009), but due to their high primary production they contribute 1% of the net primary production and 12% of the net ecosystem production in the ocean, around 600 gCm^{-2} (Fourqurean et al., 2012). This high productivity, combined with their global distribution, makes them stand out from other coastal systems and they bury 48 - 112 Tg C y^{-1} globally (McLeod et al., 2011), with some estimates that they account for nearly 10% of the yearly carbon burial in the oceans (Fourqurean et al., 2012). For example, *Posidonia oceanica*, is thought to be responsible for long term, large- scale carbon burial due to its long survival rate and large mattes formation (Borum et al., 2004), this species is endemic to and widespread throughout most of the coastal Mediterranean, and contains one of the largest coastal reservoirs of carbon in world (Lo-Iacono et al., 2008).

Seagrass meadows are thought to play an important role in the sequestration of carbon that would otherwise remain in the atmosphere and exacerbate climate change (Nellemann et al., 2010; Duarte et al., 2010; Kennedy et al., 2010). Seagrasses sequester carbon within their underlying sediments, within living biomass aboveground (roots), and within non-living biomass (leaf litter and dead shoots) and account for as much as 71% of the carbon storage in ocean sediments along with mangroves and saltmarsh ecosystems (Nellemann et al., 2009). “Blue carbon” is the carbon that is captured by the living organisms in the ocean and coastal ecosystems and stored in the form of biomass and in sediments of the oceans. This carbon is sequestered both short term (decennial) in biomass and long term (millennial) time scales in sediments (Duarte et al., 2005a; Lo lacono et al., 2008, McLeod et al., 2011). The carbon burial in the seagrass ecosystem is higher than other coastal ecosystems (Duarte et al., 2013) and due to their anaerobic sediment conditions and sediment dynamics, the buried carbon stays buried for millennia (Laffoley et al., 2009), for instance in *Posidonia oceanica* meadows, which are extremely efficient at both burying and storing carbon for millennia (Lo-Iacono et al., 2008; Fourqurean et al., 2012). The rate and success of carbon sequestration varies between and within seagrass species based on a whole suite of natural processes including herbivory, export of nutrients and decomposition (Gattuso et al., 1998).

Seagrass ecosystems will not become saturated with C deposits, as sediments will accrete vertically with response to rising sea levels, assuming ecosystem health is maintained (McKee et al., 2007). Therefore, the rate of sediment C sequestration and the size of the sediment C sink may continue to increase over time (Chmura et al., 2003), for example in *P. oceanica* meadows in Portlligat Bay, Spain (McKee et al., 2007). The global changes are likely to affect the C sequestration capacity of seagrass ecosystem, but exact prediction is difficult (McLeod et al., 2011). As such, global storage estimates differ between species, sources and measuring methods, despite these uncertainties, seagrass contribute a large portion of the ocean's ability to sequester carbon (Mc Leod et al., 2011; Mazarrasa et al., 2015).

1.3. Global Change Effects on Seagrasses

Global change is widely recognized as a real threat to our natural systems (Laffoley et al., 2009). Coastal zones and seagrasses are no exception. Seagrass meadows, while supporting a huge array of diversity, are often themselves not particularly diverse. Large areas of seagrass are often dominated by a single species (Green and Short, 2003; Ehlers et al., 2008). They are also exceptionally vulnerable to changes in water clarity, light availability and temperature (Bjork et al., 2008; Laffoley et al., 2009). The combination of genetic homogeneity and vulnerability to changes in climatic and environmental factors makes seagrasses particularly vulnerable to global warming (Ehlers et al., 2008; Jorda et al., 2012). In fact, some studies suggest that they are more vulnerable than many other ocean systems (Nellmann et al., 2009). Seagrass ecosystems can shift from being a net sink of C to a source of C because of changes in climate (e.g. changes in precipitation and temperature), atmospheric composition effects (e.g. CO_2 fertilization, nutrient deposition, damage by pollution), and land-use-change effects (e.g. deforestation, afforestation, agricultural practices; IPCC, 2007)

Climate change may exacerbate these factors and seagrass meadows are already under stress due to climate change (Short and Neckles, 1999; Duarte et al., 2008b; Waycott et al., 2009), particularly where maximum temperatures already approach their physiological limit (Short and Neckles, 1999). Increases in water temperature will directly affect seagrass metabolism and the maintenance of a positive carbon balance (Evans et al., 1986; Zimmerman et al., 1989; Collier and Waycott, 2014) thus affecting the growth rates (Koch et al., 2013). Temperature increase effects will be species -specific and result in increase/decrease of production at thermal tolerance levels (Short and Neckles, 1999; Collier and Waycott, 2014).

Heatwaves can lead to widespread seagrass shoot mortality (Reusch et al., 2005; Diaz-Almela et al., 2009; Rasheed and Unsworth, 2011; Repolho et al., 2017), for instance shoot mortality in *P. oceanica* in Mediterranean Sea (Marbà and Duarte, 2010). Increased temperature will also affect the seagrass distribution and abundance through direct effects on flowering events of seagrass (Duarko and Moffler, 1987; Short and Neckles, 1999; Marbà and Duarte, 2010; Diaz-Almela et al., 2007), whereas in other studies warming have shown to increase seagrass flowering but the larger recruitment rate is insufficient to compensate for the losses resulting from elevated temperature (Diaz-Almela et al., 2009). Before temperature tolerance levels are reached, increase in water temperature may increase productivity, but it may also increase the productivity of algae that shades seagrasses from sunlight (Watkiss &Bonjean, 2012; Collier and Waycott, 2014). Along with coastal developments, eutrophication events will increase turbidity (Burkholder et al. 2007), hence decreasing light intensities will have consequences on seagrass productivity (Short and Neckles, 1999; Bjork et al., 2008) and colonization depth (Duarte, 1991). Finally, an increase in the frequency or severity of storms may cause significant additional mechanical damage to the seagrass beds (Connolly, 2009; Watkiss& Bonjean, 2012).

Though other global change factors will affect seagrass ecosystem, increased CO_2 concentrations will play a major role on their growth and productivity and carbon cycling capacity in coastal areas (Duarte and Chiscano 1999; Hemminga and Duarte, 2000). Seagrass meadows are reported as one of few ecosystems that may benefit from rising CO_2 levels because their photosynthetic rates have been considered Ci-limited at current oceanic CO_2 levels (Beer and Koch 1996; Thom 1996; Zimmerman et al., 1997; Invers et al., 2001). Consequently, increase in seagrass production and growth may occur in a future high-CO_2 scenario. CO_2 enrichment may also affect nitrogen uptake and the assimilation process, as growth enhancement at high-CO_2 concentrations is expected to increase the nitrogen demand of plants (Stitt and Krapp, 1999; Ow et al., 2016). In addition, the relative uptake rates of ammonium and nitrate may be altered by the acidification of the seawater resulting from CO_2 enrichment (Short and Neckles, 1999; Ow et al., 2016), due to involvement of protons (H^+) in nitrogen transport across plasma membrane. From ionic balance perspective, lower pH levels in seawater may reduce the ammonium uptake rates of seagrasses, whereas nitrate uptake rates may be unaffected or even increased (Short and Neckles, 1999; Ow et al., 2016) or decreased Alexandre et al., 2012)

Both short and long term experiments of elevated CO_2 conditions on seagrass have shown that net photosynthetic rate and photosynthetic efficiency was positively affected by CO_2 enrichment of seawater for *Z. noltii* (Welsh et al., 1997; Invers et al., 2001; Alexandre et al., 2012) and *Z. marina* (Zimmerman et al. 1997; Palacios and Zimmerman, 2007; Pajusalu et al., 2016), whereas no direct effects of high CO_2 on plant biochemistry or population level traits were found for *Z. noltii* (Martinez-Crego et al., 2014). Plants exposed to CO_2-enriched conditions showed higher photosynthetic rates at saturating irradiances and were photosynthetically more efficient with higher reproductive output, below ground to above ground biomass and vegetative proliferation at limiting light intensities, when compared with plants exposed to the current CO_2 concentration (Welsh et al. 1997; Palacios and Zimmerman, 2007). This results are due to a higher carboxylation activity relatively to oxygenation activity from Rubisco in presence of a higher CO_2/O_2 ratio, as CO_2 and O_2 are competitive inhibitors for Rubisco's active site (Furbank 1998; Taiz and Zeiger, 2002, Pajusalu et al., 2016; Repolho et al., 2017). Significant effects of high CO_2, when combined with different factors (light, temperature and nutrients) have yielded different variation in results for *Z. noltii* (Alexandre et al., 2012; Martinez-Crego et al., 2014).

Experimental studies on *Cymodocea nodosa* and *Posidonia oceanica* have indicated, that increase in CO_2 concentrations will favour their utilization of inorganic carbon sources for higher photosynthetic activity (Invers et al., 2001, Apostolaki et al., 2014), whereas gene expression of *C. nodosa* photosynthetic genes expressed a down regulation and decrease in net primary production at elevated CO_2 levels (Olive et al., 2017). Short term mesocosm experiments with elevated CO_2 on *C. nodosa* have been observed with lower net community production (Khan et al., 2016). Lower density, biomass and above to below ground biomass was observed for *C. nodosa* at high CO_2 concentrations (Apostolaki et al., 2014), whereas increased density and biomass was observed for *C. rotundata* and *C. serrulata* (Takahashi et al., 2016). Carbon content and $\delta^{13}C$ of leaves declined with increasing pCO_2 for *C. nodosa* (Apostolaki et al., 2014) and *C. serrulata* and *C. rotundata* (Takahashi et al., 2016). High CO_2 and low pH conditions also reduces the concentration of phenolic protective substances of *C. nodosa* followed by higher grazing rates, which can alter the coastal carbon flux of seagrasses (Arnold et al., 2012). In case of *Posidonia oceanica* increase in CO_2 concentration was observed with increase in net carbon balance, which can also increase the depth limit of the seagrass (Invers et al., 2002; Vizzini et al., 2010) and shoot density (Hall Spencer et al., 2008), whereas limited effects on shoot productivity (Cox et al., 2015) and no effects of high CO_2 were observed on leaf biomass, biomass and photosynthesis (Cox et al., 2016).

Most seagrass species possess the ability to utilize HCO_3^- (Koch et al., 2013), as HCO_3^- represents higher percentage of the total DIC pool in seawater than CO_2 (Stum and Morgan, 1981). So, carbon limitation of seagrasses in the current scenario is mitigated by shifting towards use of HCO_3^- , although use of HCO_3^- is species specific for seagrasses (Invers et al., 2001). Irrespective of photosynthetic mechanisms, a key issue in predicting the response to rising CO_2 is whether photosynthesis and growth are saturated by seawater DIC under present-day conditions of inorganic C speciation, pH, O_2 and temperature. In many cases an increase in DIC will result in higher photosynthetic and growth rates (Invers et al., 2001)

Seagrass photosynthesis appears limited by current DIC, due to the slow diffusive supply of CO_2 to the leaves, and possibly a less effective use of HCO_3^- when compared to many macroalgae (Beer et al. 1989, Beer and Rehnberg, 1997; Zimmerman et al., 1997), for instance *Posidonia* and *Cymodocea* sp. with higher capacity for HCO_3^- use can have higher production than compared to *Zostera sp.* with lower efficiency for HCO_3^- utilization (Duarte, 1991; Invers et al. 2001; Beer et al., 2002). Several seagrass species have also exhibited higher photosynthesis, increased reproduction, below ground biomass, and greater production of non-structural carbohydrates, as well as lower leaf-N and chlorophyll under elevated CO_2 (Durako, 1993; Zimmerman et al., 1997; Beer & Koch, 1996; Palacios & Zimmerman, 2007; Jiang et al., 2010). Thus, CO_2 enrichment can shift C allocation to carbohydrates and away from N-containing compounds, such as Rubisco while increasing efficiency in seagrass production (Hemminga and Duarte, 2000; Vizzini et al., 2010), like findings in terrestrial C3 species (Leakey et al., 2009). Consequently, an increase in dissolved CO_2 could be positive for photosynthesis and growth in seagrasses that are presently under saturated with respect to DIC, regardless of their capacity to utilize HCO_3^-. Seagrasses through their metabolic activity can alter the chemical properties of the meadow (Hendriks et al., 2014a), thus making the organisms within the meadow less vulnerable to ocean acidification due to large diel pH changes. The increased productivity during the day may buffer the impacts of ocean acidification, particularly for calcifiers by providing a daily window of maximum calcium carbonate saturation where calcification can be more efficient and finally ocean acidification could stimulate seagrass productivity and thus increase buffering capacity (Shaw et al., 2013; Hendriks et al., 2013; Hendriks et al., 2014b).

Saying that studies on short term effects of CO_2 enrichment on seagrasses have mostly done under experimental conditions and using ex-situ results for predicting the possible impacts of elevated CO_2 on seagrass is difficult as there are interactions between various factors (light, temperature, nutrients, epiphytic growth on leaf surface of seagrass) in-situ which determines the response of seagrass to CO_2 enrichments. This increases the need of short term CO_2 enrichment experiments in situ on seagrasses to have a better understanding on the effects of elevated CO_2. Similarly, to understand long term elevated CO_2 effects most studies have preferred volcanic CO_2 seeps where seagrass ecosystems are found. The response observed from various studies on seagrass at these CO_2 seeps are mostly species specific with some seagrasses showing positive responses, for example *C. serrulata* at CO_2 seeps of Papua New Guinea (Russel et al., 2013) whereas negative responses were observed for *C. nodosa* at Vulcano CO_2 seeps (Vizzini et al., 2013; Apostolaki et al., 2014). Both positive and negative responses at these CO_2 seeps can be related to other factor such as trace element levels which are higher at these CO_2 seeps due to continuous input from the seeps and can act as a confounding factor to seagrass ecosystem and associated biota due to their toxic effects. These element concentrations, their bioavailability and possible toxicity is going to increase in future ocean conditions due to low pH (Millero et al., 2009) and this can have negative impacts on the seagrass response to elevated CO_2 levels. So, while using natural CO_2 seeps for effects of elevated CO2 levels trace elements levels should be taken into consideration

.

Main Objectives

Considering the predicted changes to the marine environment, the importance of seagrass to the coastal ecosystem, the carbon metabolism of seagrass and the short and long term impacts of increased CO_2 on seagrass ecosystem, the main objectives of the thesis are

i) to understand the short-term impacts of elevated CO_2 on the diel and seasonal variations on the community metabolism intertidal seagrass *Zostera noltii* community with respect to light

ii) to understand the long -term impacts of elevated CO_2 on the growth, biomass, density, morphometry of seagrass *Cymodocea nodosa* ecosystem at CO_2 seeps using reconstruction techniques

iii) to understand the trace elements variation within sediments, their accumulation within the seagrass *Cymodocea nodosa* and *Posidonia oceanica*, possible impacts on seagrass with respect to future ocean conditions of elevated CO_2 and low pH at CO_2 seeps

1.4. References

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Chapter 2

Short term effects of CO_2 on the diel and seasonal responses of *Zostera noltii* community metabolism to light

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(To be submitted)

Short term effects of CO_2 on the diel and seasonal responses of *Zostera noltii* community metabolism to light

Abstract:

Net community production (NCP) and community respiration (CR) were measured under air exposed and CO_2 enriched conditions for intertidal *Z. noltei* meadows and unvegetated sediment communities during emersion seasonally. Community production and respiration were measured in-situ using benthic chambers under air and CO_2 enriched conditions over a series of short term incubations (30min) using an infra-red gas analyser. Incident photosynthetic active radiation (PAR) was recorded during the incubations covering the daily and seasonal variation. Linear regression model was used to test the effects of irradiance on net community production. NCP of *Z. noltei* community were higher under CO_2 enriched conditions than air exposed conditions in both summer and winter seasons. There was no effect of CO_2 on the CR rate of *Z. noltei* community in summer season. NCP of sediment community were higher in summer season and winter season under CO_2 enriched conditions. Sediment CR rates were higher in winter than summer season. The light compensation point of *Z. noltei* and sediment community were lower in both seasons under CO_2 enriched conditions. Seasonal budget of community production was higher in *Z. noltei* than sediment communities. A clear effect of PAR was noticed on the net community production of both communities. Higher PAR intensities resulted in higher NCP under CO_2 enriched conditions for both communities. CO_2 enrichment can have a positive effect on the intertidal communities during emersion.

Keywords: Intertidal, emersion, unvegetated sediments, regression model, CO_2 enrichment

2.1. Introduction

Current concentration of atmospheric carbon dioxide (CO_2) is predicted to be doubled by end of this century (IPCC, 2007). Rising atmospheric CO_2 will lead to an increase in oceanic CO_2 levels, as 30% of the emitted anthropogenic CO_2 is absorbed in the surface oceans (IPCC, 2014), causing an increased interest of the direct impacts of elevated CO_2 on the marine ecosystem (Gattuso et al., 1998; Feely et al., 2004; Guinotte and Fabry 2008; Hall-Spencer et al., 2008; Portner, 2008; Porzio et al., 2011). The increase of CO_2 in the world oceans is changing the carbonate chemistry of the seawater, lowering the pH resulting in ocean acidification. A further decrease of pH by 0.3–0.4 units is expected by the end of year 2100 (Royal Society, 2005; IPCC, 2014). The low pH of seawater will alter the carbonate chemistry in the relative proportions of the dissolved inorganic carbon species, i.e. carbon dioxide (CO_2), bicarbonate (HCO_3^-), and carbonate (CO_3^{2-}), shifting the total dissolved inorganic carbon away from CO_3^{2-}toward more HCO_3^- and $CO_{2\,(aq)}$ (Riebesell et al., 2007). This shift toward more HCO_3^- is expected to benefit species that use it as a carbon source for photosynthesis in addition to CO_2 (Beer et al., 2002; Mercado et al., 2003)

Seagrass ecosystems are distributed worldwide and play an important role in the carbon cycle of coastal areas (Duarte and Chiscano 1999; Hemminga and Duarte 2000). They act as marine carbon sinks and store about 12% of the total carbon storage in the ocean (Duarte et al., 1999, McLeod et al., 2011; Fourqurean et al., 2012). Saying that, the response of seagrass ecosystem to the rising CO_2 levels will be very important, as seagrasses are predicted to benefit from the increasing CO_2 levels (Beer and Koch 1996; Zimmerman et al., 1997; Invers et al., 2001) and overcome their photosynthetic limitation to under current carbon dioxide concentrations.

Most of the studies on elevated CO_2 levels on seagrass have focused on the effects of elevated CO_2 concentrations on seagrass productivity and light requirements (Beer and Koch, 1996; Thom, 1996; Zimmerman et al., 1997; Palacios and Zimmerman, 2007), their photosynthetic and leaf growth rate (Alexandre et al., 2012;) and the uptake rate of ammonium and nitrate (Short and Neckles, 1999; Alexandre et al., 2012; Ow et al., 2016).

However, these experiments of elevated CO_2 levels were carried out more on submerged seagrass ecosystems than air exposed and results have suggested that, seagrass meadows can utilize the increased CO_2 concentration from the water column and enhance their photosynthetic activity and community metabolism, through a regime shift towards utilizing more HCO_3^- (Frankignoulle and Disteche, 1984; Frankignoulle and Bouquegneau 1990; Invers et al., 1997). Higher photosynthetic activity of submerged meadows was observed in shallow sites, influenced by light penetration in water column and diurnal variation (Leuschner et al., 1998; Runice and Duarko, 2004). The productivity decreased with increase in depth from shallow sites implying light availability being the important factor for seagrass productivity (Biber et al., 2005) along with other factors such as temperature, diurnal variations and nutrient availability (Lee et al., 2007) in both shallow and intertidal habitats (Jimenez et al., 1987, Kenworthy and Fonseca, 1996; Bach et al., 1998)

However, seagrass meadows in the intertidal habitats, are subjected to pronounced shifts in light and temperature, produced by the combined rhythms of tides and daily irradiance (Short et al., 2001; Vermaat, 2009; Van der Heide et al., 2010). In these intertidal flats, during emersion, light intensity is always higher than during immersion, whereas the nutrient sources are reduced to sediment pore water (Davoult et al. 2011). Intertidal seagrasses are also exposed to varying concentration of inorganic carbon such as gaseous CO_2 during emersion and dissolved CO_2, HCO_3^- and CO_3^{2-} during immersion (Ouisse et al. 2011; Bahlmann et al., 2015). The seagrass *Zostera noltei* that inhabits the low intertidal zone, experience alternate daily periods of immersion and exposure to air for several hours especially during spring tides (Silva and Santos, 2003; Coyer et al., 2004; Ouisse et al. 2011). In this environment, the *Z. noltei* community have shown considerable photosynthetic activity, when exposed to the air during low tide periods (Leuschner et al., 1998; Silva et al., 2005).

Most of community-level photosynthetic activity estimates of intertidal macrophytes has been assessed from gas exchange methods based on infrared gas analysis (IRGA) of carbon dioxide (CO_2) fluxes, using flow- through systems or closed chambers for longer periods of time (Streever et al. 1998). Studies of community-level productivity involving CO_2 flux measurements are more common in salt marsh communities than in seagrass meadows. In the former systems, carbon fluxes have been measured either in individual leaves, incubated on special leaf chambers with temperature control and artificial light or in whole plants, using larger incubation chambers (Streever et al., 1998), but less in intact communities. Carbon flux measurements using IRGA in intertidal seagrasses are scarce, particularly on *Zostera noltei*. Most studies using carbon flux measurements on *Z. noltei* are performed in lab conditions (Leuschner and Rees, 1993, Perez-Llorens and Neil, 1994; Leuschner et al. 1998), whereas few measurements have been performed in-situ (Leuschner et al., 1998; Silva et al., 2005: Ouisse et al., 2010, 2011). So, in this context, we aim to measure the effects of high CO_2 concentration (future scenarios of 750 ppm) on the net community production (NCP) and community respiration (CR) of air exposed Z. *noltei* community at Ria Formosa Lagoon, Portugal, where the species dominates the intertidal zone. The effects of vegetation on the intertidal ecosystem metabolism will be assessed by comparison with unvegetated areas.

2.2. Materials and methods

Study sites:

Ria Formosa lagoon (37°00′N, 7°58′ W) is a mesotidal system located in the southern coast of Portugal, characterized by large intertidal flats (Andrade, 1990). *Zostera noltei* is the most abundant seagrass species in Ria Formosa coastal lagoon in the lower intertidal zone, distributed within a bathymetric range of about 2 m and plays a major role in the lagoon's metabolism (Santos et al. 2004). The *Z. noltei* meadows regularly experience alternate daily periods of immersion and exposure (Site A, Fig 1) withstanding long periods of air exposure up to 4.5 to 6 hours in each tidal cycle. The unvegetated sediment beds are at the same bathymetric zone experiencing the daily tidal fluctuations. These beds are located on the north-west direction of seagrass beds approximately 600 m in distance (Site B, Fig 1).

Gas Exchange Measurements:

Measurements were made in-situ, through carbon flux measurements for short durations, using Infrared gas analyser (IRGA) and Plexiglas chambers, with respect to light variations daily and seasonally and the community response of *Z. noltei* was compared with adjacent un-vegetated sediments. This experiment was carried out in summer (June, July, August) 2014 and winter (November, December, January) 2014-15 seasons. Incubation chambers made of UV-transparent Plexiglas, providing closed environment were used for incubations. These chambers comprised of two separated parts, a basal ring (30 cm in diameter) and a dome-shaped lid to close the chamber. The basal ring was sharpened for easier sediment insertion, usually down to 5–10 cm deep. Once the lid was set in place, both parts were secured by power clamps (Fig. 2). A special latex ring installed in a flange insures the chamber was sealed. The chambers were provided with three ports, one in the ring (2), one on the top of the dome (1) and the third (3) on the base 5-10 cm above the above the sediment insertion line for the CO_2 gas to be fluxed into the chamber. All ports were fitted with Tygon® ST gas-impermeable tubing. The global operating volume of the chambers was between 22 and 26 L, depending on how deep the ring was inserted into the sediment.

Short term field incubations using these chambers were conducted in *Z. noltei* meadows and bare sediment beds during several low tides at different times of day along the season, so that the diel variation of light was captured. Each incubation lasted for a maximum of 30 minutes. Each incubation was first conducted under natural atmospheric CO_2 concentration (397-399 ppm) followed by CO_2 enrichment to 750 ppm, of the same *Z. noltei* community. During the incubation air entered the chamber through the ring port (2) and was out through the dome port (1), allowing the homogenization of air within the chamber medium. A low-volume (approx. 25 ml) closed air circuit is established between the chamber and an external non-dispersive infrared gas analyser (IRGA) (EGM-4, PP Systems, UK) that measures CO_2 concentrations with a precision of 1 µatm. The gas analyser had a built-in pump that pulled the air from the chamber, passed through a desiccation column (Anhydrous calcium sulphate, Drierite) which removed humidity from the air to avoid interferences in CO_2 measurements and sends it back after passing through the infrared cell, keeping a continuous air flow between the incubation chamber and the analyser. The CO_2 partial pressure inside the chamber is then continuously monitored (1 min steps) over a period, typically between 5 and 15 min for each incubation, or enough for a 30–40 µatm drop in the CO_2 partial pressure. During this time the port (3) was clamped, so that no air passes through the tubes. After completion of the air incubation, the clamp was removed and CO_2 gas was fluxed slowly into the chamber through the port (3), from a CO_2 gas enriched tank till the CO_2 concentration inside the chamber reached 750 ppm, recorded by IRGA. The clamp was closed again. The CO_2 partial pressure was measured, same as described previously. Following each incubation, the seagrass was collected, washed and dried at 60° C for 24 hours and the dry weight was recorded. Photosynthetic active radiation (PAR) inside the chambers was measured with a Li-Cor system (Li-Cor, 250 A meter, USA) at both the start and end of each incubation. Instantaneous PAR measurements were continuously done at the Ramalhete field station (Fig.1) every 30-minute using the same sensor to record daily and seasonal light variation. Light attenuation inside the chambers was estimated to be approximately 10%. Temperature was recorded using a thermometer at start and end of each air and CO_2 incubation (Fig. 2). Similar incubations were also performed for unvegetated sediment beds.

NCP and CR measurements

Light and dark incubations were conducted for both net community production (NCP) and community respiration (CR) respectively. The net flux (F) of CO_2 (µmol C m^{-2} h^{-1}) for both air and CO_2 enriched incubations was computed as:

$$F= s \times mv \times V/A \times 60/1000$$

where s (ppm CO_2 min^{-1} = µmol CO_2 mol air^{-1} min^{-1}) is the slope of the linear change in CO_2 concentration over time during the incubation period, V (L) is the chamber volume, A (m^2) is the benthic exchange area, and mv (mol L^{-1}) is the molar volume or number of gas mole per volume unit, calculated from the ideal gas law ($mv= P/(R \times T)$), where P (atm) is the gas pressure, T (K) the temperature, and R (0.082 atm LK^{-1} mol^{-1}) is the universal gas constant. Thus, the net CO_2 flux will be a direct measure of the NCP for the given light and temperature conditions. Similarly, the net flux of CO_2 in dark represents the community respiration, CR.

A linear regression model was fitted to the NCP versus PAR data to assess the effects of PAR on NCP,

$$Y= -b\ x +a,$$

Where Y is NCP, -b represent the rate ofCO_2 uptake by the community, X is the PAR value and 'a' intercept, representing community respiration. The daily PAR variation reaching the *Z. noltei* community when exposed to the air, was used to estimate the daily NCP variation using the above model. Tidal variations (emersion time) for the study site (*Z. noltei* meadows and sediment beds) for each season (summer and winter) was estimated using a tidal model (Carrasco et al. unpublished data). The emersion time derived from the model for each day along the month and seasons for seagrass meadows and sediment beds was used to calculate the daily NCP, GCP and CR for each season.

Linear regression was used to model the effects of PAR on NCP. The summer and winter NCPs were calculated based on the average daily PAR of the period the plants were exposed to the air and the linear models of NCP response to irradiance. Seasonal NCP (g C m^{-2} $season^{-1}$) is the average of all summer or winter days.

The daily NCP and CR during emersion was estimated by adding every 30 minute NCP and CR values along the day when the seagrass beds were exposed to the air. Gross community production (GCP) was obtained by adding the NCP and CR during light hours. The seasonal NCP, CR and GCP were obtained by considering the variability of exposure times related to the alternation of spring and neap tidal cycles and the daily cycle of irradiance.

Results are expressed as mean ± Standard error (S.E). A t-test was used to detect the significant differences of NCP, GCP and CR between natural and CO_2 enriched conditions. Data was tested for normality and constant variance before testing for significant difference. Significant levels were tested at $p<0.05$.

2.3. Results

NCP of *Zostera noltei* and sediment communities were higher under CO_2 enriched conditions than natural conditions in both summer and winter seasons (Fig. 3). The differences were only apparent at light intensities above the light compensation for community production (higher than 200 µmol m^{-2} s^{-1}), i.e. when production is higher than respiration. NCP of sediments was lower than *Z. noltei* within PAR ranges of 800 µmol m^{-2} s^{-1} to 1600 µmol m^{-2} s^{-1}, which was most evident in the summer (Fig. 4). In the summer season under higher light (1680µmol m^{-2} s^{-1}) the NCP of sediments was higher than *Z. noltei* under natural conditions, whereas under CO_2 enriched conditions the NCP of *Z. noltei* was higher than unvegetated sediment community (Fig. 4). Mean plant biomass of *Z. noltei* was 1.80 ± 0.89 g DW m^{-2} in summer and 1.68 ± 0.80 g DW m^{-2} in winter season.

Significant differences were observed in the net community production of *Z. noltei* and sediment communities under natural and CO_2 enriched conditions (Fig. 5). The NCP of *Z. noltei* was higher in both seasons under CO_2 enriched conditions and community respiration was lower and consequently gross community production was even higher under CO_2 enriched conditions than natural conditions (Fig. 5a). Similarly, under CO_2 enriched conditions sediment NCP was higher in both season, whereas the CR was higher in summer and lower in winter (Fig. 5b). GCP of sediment community was higher in summer and lower in winter season under CO_2 enriched conditions (Fig. 5b).

Seasonal budget of *Z. noltei* and unvegetated sediment community were higher under CO_2 enriched conditions than natural conditions in both seasons (Table 1). However, seasonal budget of NCP for *Z. noltei* was 3.9 -fold higher and seasonal GCP 4.9-fold higher in summer season than winter season under CO_2 enriched conditions (Table 1). In case of sediment communities, the increase of seasonal budget of NCP was 3.5-fold in summer season, whereas the decrease of GCP was 7.5-fold in winter season under CO_2 enriched conditions (Table 1). Similar seasonal budget of NCP for *Zostera noltei* and sediment community was observed in winter season under CO_2 enriched conditions, whereas seasonal GCP of *Z. noltei* was higher than sediments communities (Table 1). Seasonal budget of CR was higher in sediment communities than *Z. noltei* under CO_2 enriched conditions in winter season (Table 1).

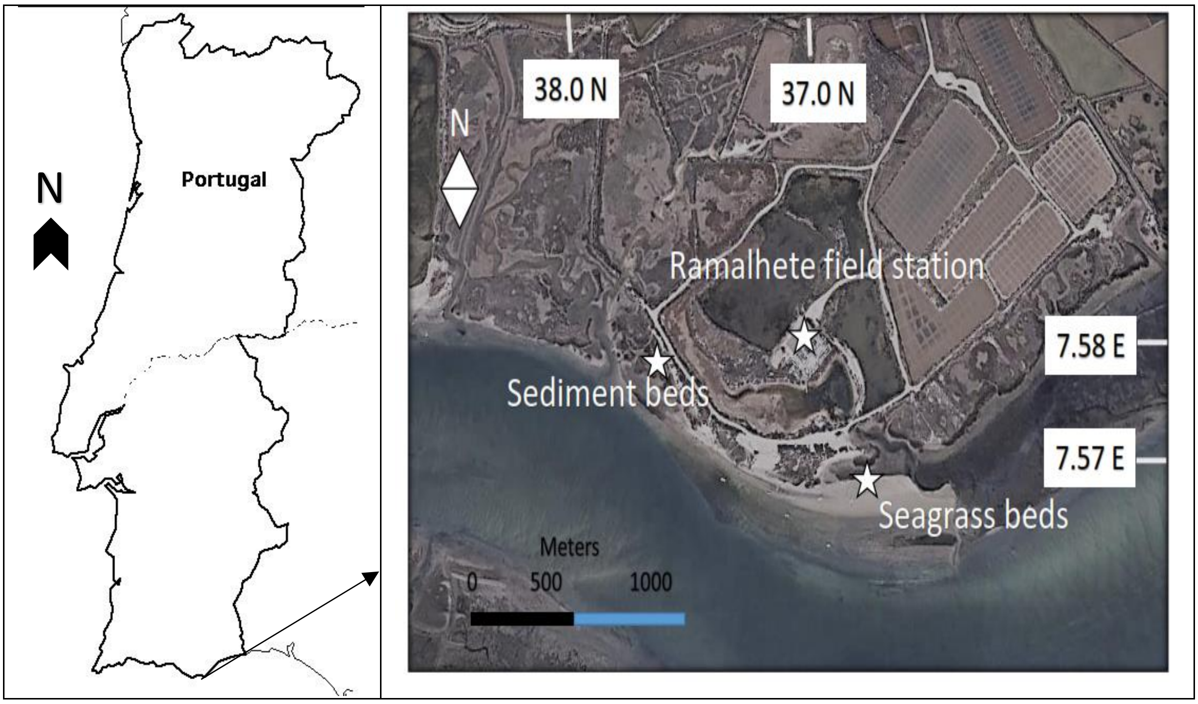


Fig. 1. Study area showing *Zostera noltei* beds and unvegetated sediment beds at Ria Formosa Lagoon.

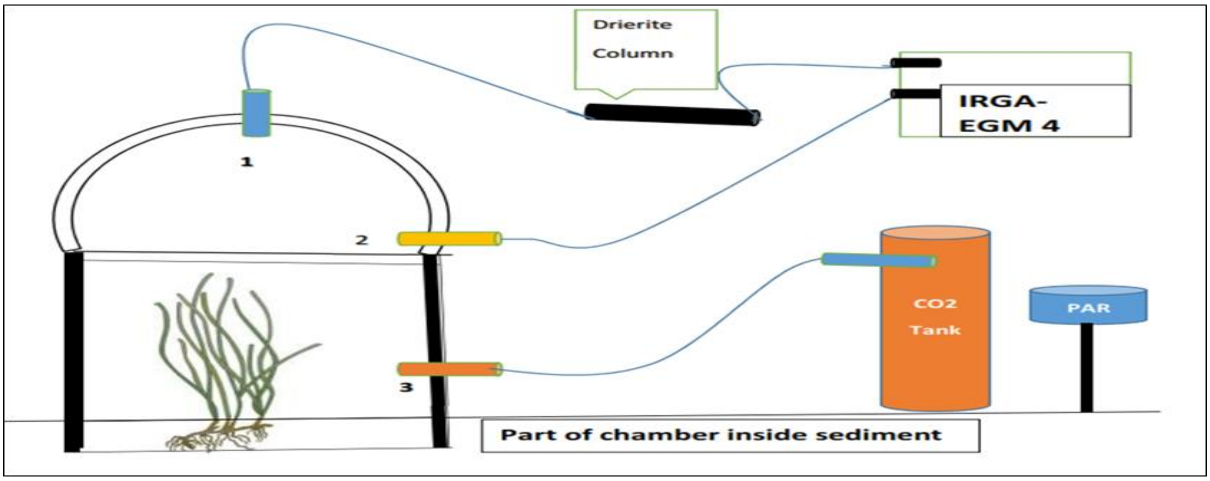
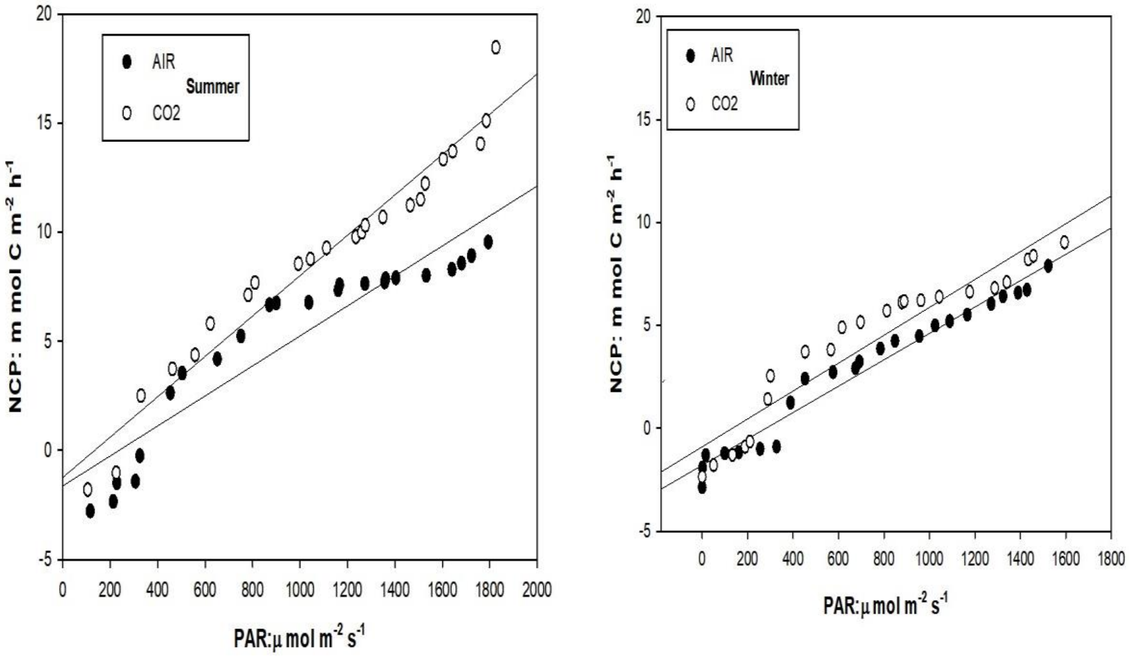
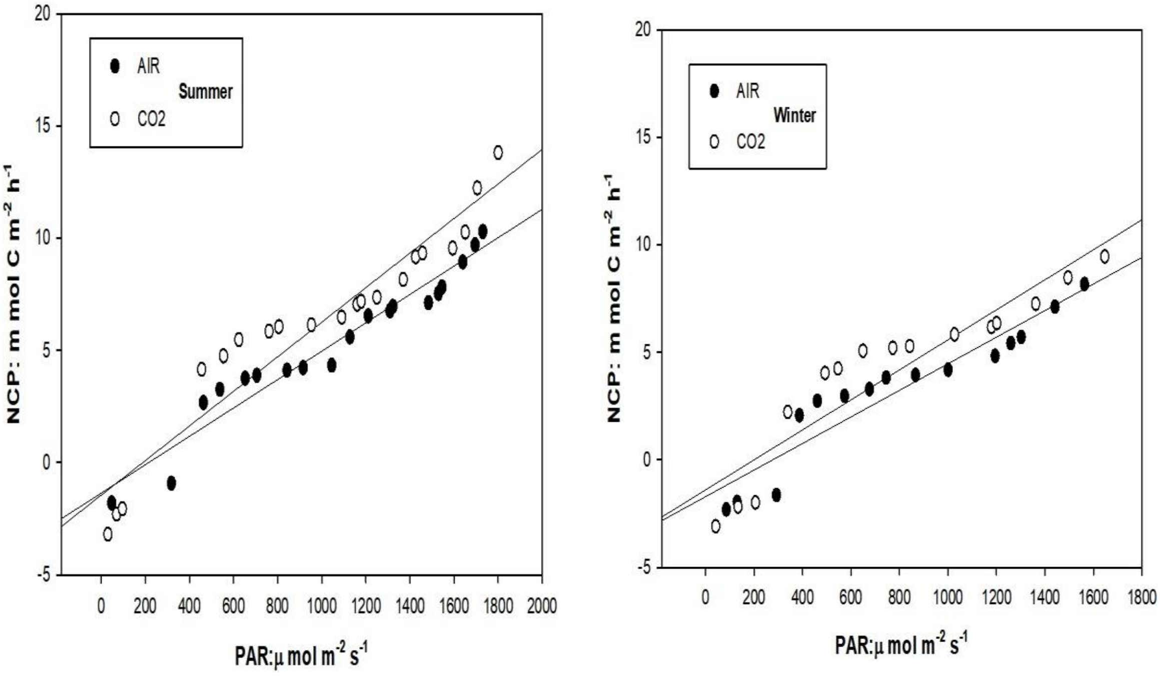


Fig. 2. A diagrammatic representation of the incubation work. 1. Port one on top of dome. 2. Port two on ring. 3. Port three for CO_2 flux



a. *Z. noltei* community

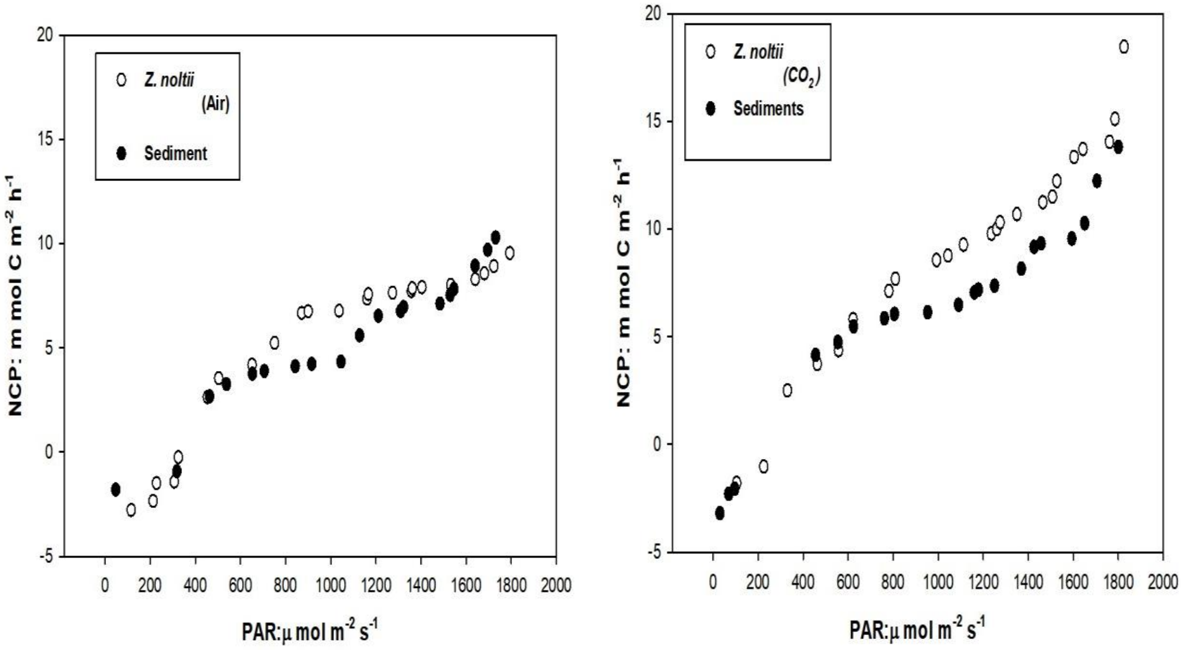
b. *Z. noltei* community



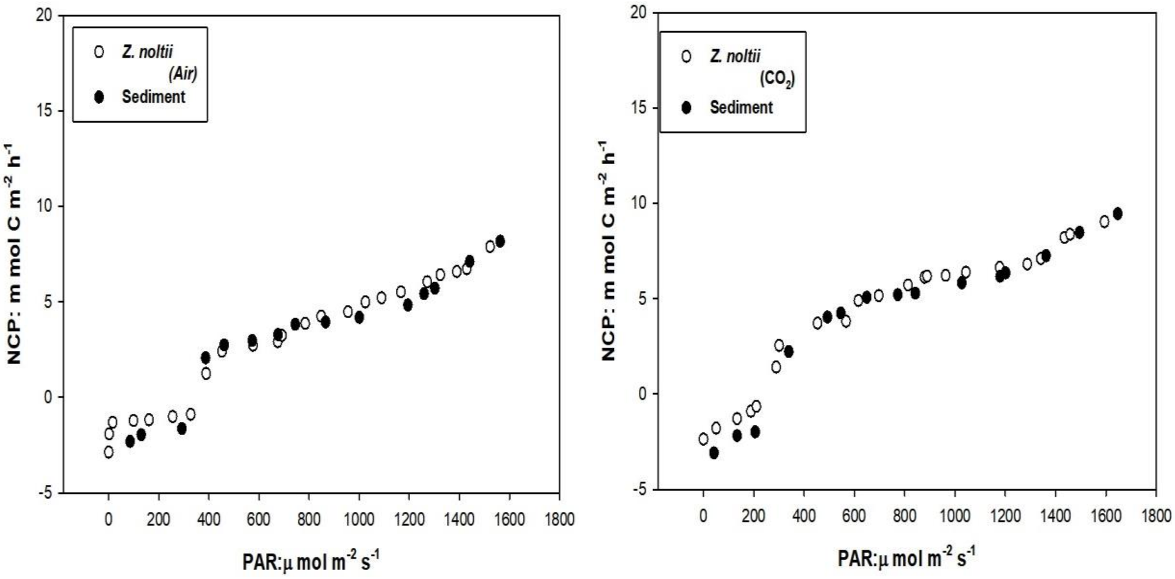
c. Sediment community

d. Sediment community

Fig. 3**.** Response of net community production (NCP) to irradiance (PAR) under natural and CO_2 enriched conditions in summer and winter for *Z. noltei*, (a, b) and sediments (c, d).

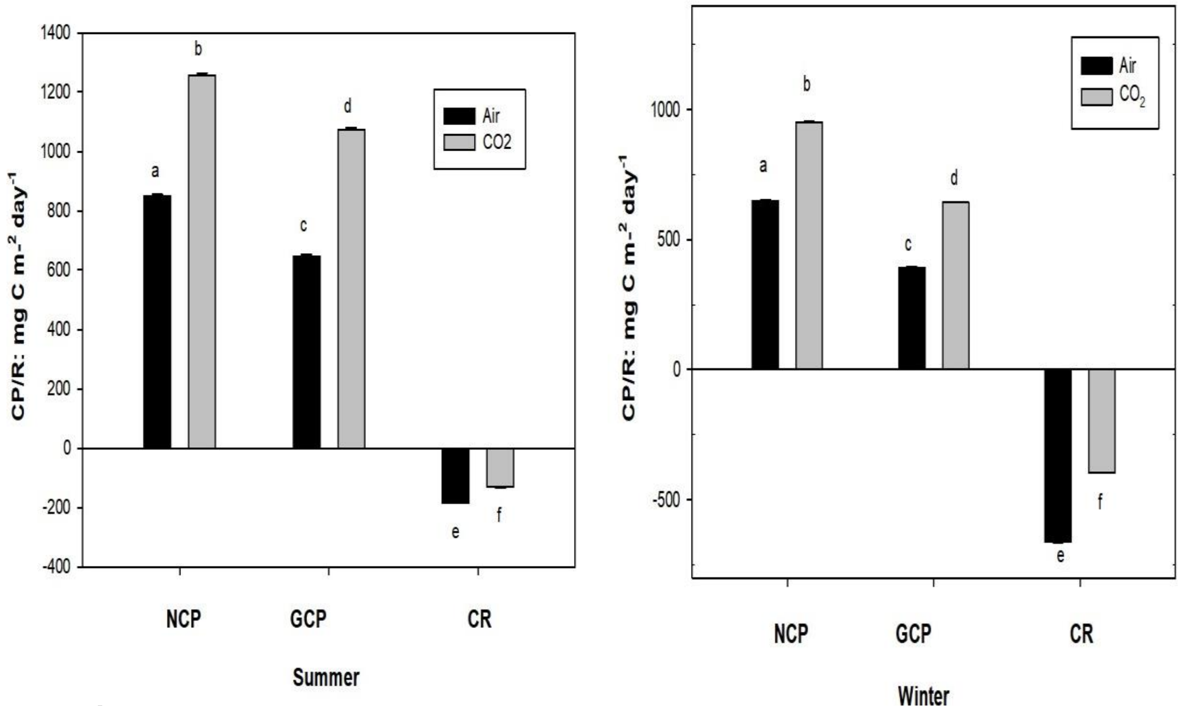


a. Summer b. Summer



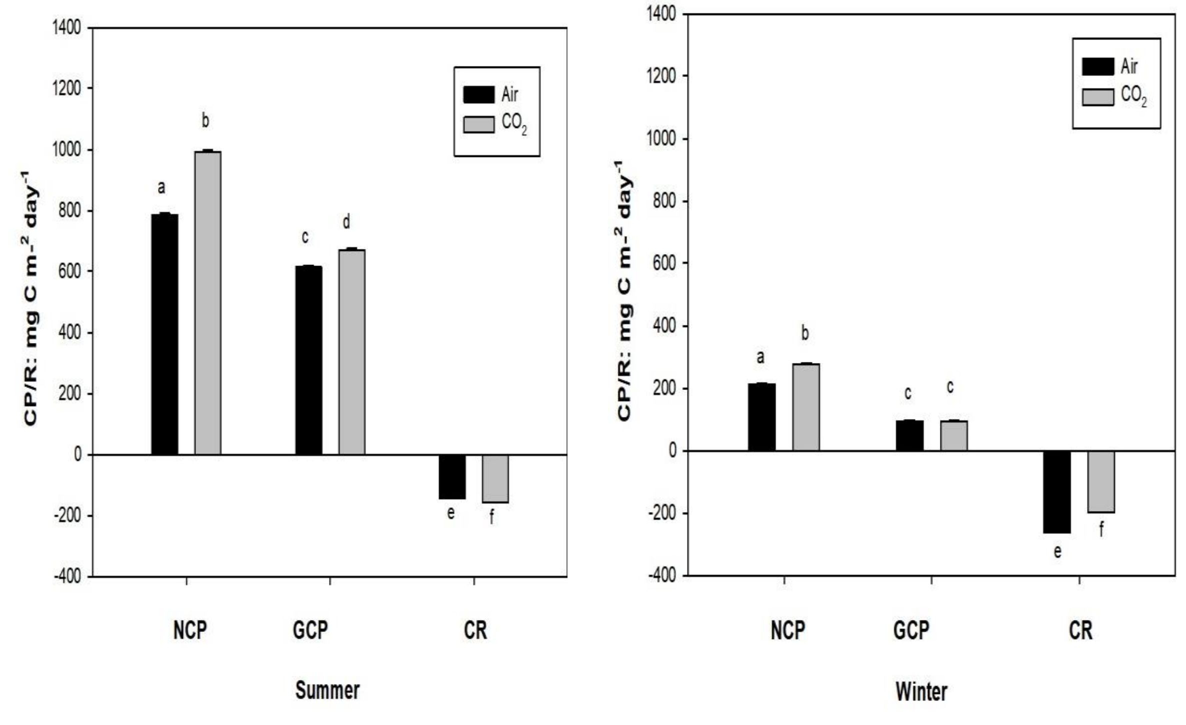
c. Winter d. Winter

Fig. 4. Comparison of community production and respiration of *Z. noltei* and sediment community for summer (a, b) and winter seasons (c, d) under natural (air) and CO_2 enriched conditions



a. *Z. noltei* community

b. *Z. noltei* community



c. Sediment community

d. Sediment community

Fig. 5. Mean (± se) community production and respiration (mg C m^{-2} day^{-1}) of *Z. noltei* (a, b) and unvegetated sediment community (c, d) in summer and winter seasons under natural and CO$_2$ enriched conditions. Different letters indicate significant difference between natural (air) and CO$_2$ enriched conditions. Error bars represent standard errors

Table 1.

Seasonal budget of NCP (g C m^{-2} $season^{-1}$), CR (g C m^{-2} $season^{-1}$) and GCP (g C m^{-2} $season^{-1}$) of Z. *noltei* and unvegetated sediment community under natural (air) and CO_2 enriched conditions in summer and winter seasons

		***Z. noltei* community**		
Season	**Condition**	**NCP**	**CR**	**GCP**
Summer	Air	78.21 ± 5.58	16.99 ±0.85	59. 56 ± 5.19
	CO_2	115.54 ± 7.58	12.12 ±0.67	98.65 ±7.44
Winter	Air	20.13 ±3.02	26.86 ±1.52	12.15 ±2.30
	CO_2	29.45 ±4.04	12.30 ±0.74	19.91 ±3.67
		Sediment community		
Summer	Air	72.26 ±5.08	13.24 ± 0.74	56.51 ±4.87
	CO_2	91.28 ±6.32	14.55 ±0.81	61.57 ±5.94
Winter	Air	19.78 ±2.98	24.36 ±1.50	8.75 ±1.99
	CO_2	25.83 ±4.01	18.45 ±1.27	8.60 ±3.54

Significant effect of PAR was observed on the NCP of *Z. noltei* and unvegetated sediments under natural and CO_2 enriched conditions in summer and winter seasons (Table 2). The slope, intercept and R^2 values of the linear regression model for both conditions and seasons are presented in Table 2. The light compensation point (LCP) were lower under CO_2 enriched conditions than natural conditions for Z. *noltei* and sediment community (Table 2). LCP of *Z. noltei* community was lower than unvegetated sediment community in both seasons under CO_2 enriched conditions (Table 2). Highest PAR recorded during the field experiments was 1840.45 to 1895.56 mol m^{-2} s^{-1} in summer season, and between 1522μ mol m^{-2} s^{-1} to 1594 μ mol m^{-2} s^{-1} in winter season for both communities (Fig. 3).

Table 2.

Slope (b), intercept (a), p, R^2 and light compensation point (LCP, μ mol m^{-2} s^{-1}) values derived from linear regression model between PAR and NCP under natural and CO_2 enriched conditions for *Z. noltei* community and unvegetated sediments community.

			***Z. noltei* community**			
Season	**Condition**	**b**	**a**	**PAR vs NCP (p value)**	**R^2**	**LCP**
Summer	Air	0.0069	-1.622	<0.001	0.87	247.50
	CO_2	0.0092	-1.233	<0.001	0.95	134.02
Winter	Air	0.0064	-1.796	<0.001	0.96	280.65
	CO_2	0.0068	-0.882	<0.001	0.90	129.70
			Sediment community			
Summer	Air	0.0062	-1.281	<0.001	0.95	206.61
	CO_2	0.0077	-1.436	<0.001	0.90	186.49
Winter	Air	0.0062	-1.707	<0.001	0.89	275.32
	CO_2	0.0070	-1.391	<0.001	0.86	198.71

2.4. Discussion

In our study, the linear regression model used to test the effect of irradiance on the net community production showed a significant correlation with both natural and CO_2 enriched conditions. The high coefficient of determination (R^2) in our datasets under natural and CO_2 enriched conditions (Table 1) indicates a closed fit of the model to the data, suggesting irradiance is key driver of NCP. The NCP followed a linear pattern of increase with increasing PAR variation and CO_2 enrichment, indicating higher CO_2 concentrations with higher irradiance levels will increase NCP of *Z. noltei* and sediment communities under future high CO_2 conditions. Lower light compensation point obtained from the model suggests that high CO_2 will compensate for low light and increase the carbon utilizing capacity of *Z. noltei* during low light conditions. The linear increasing trend of community production versus irradiance observed in our model differs from irradiance versus net community production models observed for *Z. noltei* elsewhere (Cavier et al., 2011, 2014) and unvegetated sediment community (Uthicke et al., 1998; Cavier et al., 2014).

The light compensation point derived from the model showed lower LCP for CO_2 enriched conditions and higher under natural conditions. The LCP for *Z. noltei* was in between 134μ mol quanta m^{-2} s^{-1} to 247 μ mol quanta m^{-2} s^{-1} for summer season and between 129μ mol quanta m^{-2} s^{-1} to 280μ mol quanta m^{-2} s^{-1} for winter season under CO_2 enriched and natural conditions respectively (Table 1). The LCP observed in our results were higher than previously reported for *Z. noltei* for both seasons under natural conditions (Gacia et al., 2005; Ouisse et al., 2010) and lower for CO_2 enriched conditions. The LCP for sediment community were between 186μ mol quanta m^{-2} s^{-1} to 275μ mol quanta m^{-2} s^{-1} for both seasons under CO_2 enriched and natural conditions, which were higher than previously observed for sediment communities (Uthicke& Klumpp, 1998; Clavier & Garrigue, 1999). The light compensation point of *Z. noltei* values for both summer and winter seasons suggest and effect of light on net community production for both *Z. noltei* and sediment community in the Ria Formosa. The LCP for CO_2 enriched experiments during emersion are presented for the first time for *Z. noltei* and sediment community.

Measurements of NCP vs PAR during emersion highlighted the continuous increase in net community production with PAR variation for both conditions in summer season, similar results were obtained for *Z. noltei* with no decrease in community production under natural conditions (Ouisse et al., 2010, 2011; Clavier et al., 2011). The increase in community production at higher light intensities can be related to tolerance of high light intensities by *Z. noltei* without damaging its pigment pool and dynamically down regulating the photosynthetic apparatus (Jimenez et al., 1987). This phenomenon was also observed in *Z. marina* (Zimmerman et al., 1997) and *Z. noltei* (Alexandre et al., 2012) where increase in CO_2 concentrations increased the light utilizing capacity of individual plants.

The lower range of LCP in CO_2 enriched conditions and higher net community production for *Z. noltei* in summer suggests that the community is more efficient in utilizing low light (Silva & Santos, 2003) and can start utilizing CO_2 for production in low light conditions without community production compensating for community respiration, similar pattern was observed for *Z. marina* in natural conditions (Ouisse et al., 2011). In case of sediment community, the LCP were higher than Z. *noltei* under CO_2 enriched conditions, but the community production was not, which suggests that even with higher CO_2 concentrations the microphytobenthos can't utilize carbon still a certain level of irradiance level is reached.

Net community production and community respiration of sediment community was lower than *Z. noltei* community in both summer and winter seasons under natural conditions (Fig. 5). Similar results were observed for *Z. noltei* community and sediment community at Bay of Morlaix (Ouisse et al., 2010) and Banco de Mauritania (Clavier et al., 2014).

The short-term CO_2 enrichment in our studies resulted in increase of net community production of *Z. noltei* community along the daily variation of PAR. During summer, the net community production of *Z. noltei* were higher and community respiration rates lower in CO_2 enriched conditions. This study showed that net community production of *Z. noltei* is affected by CO_2 enrichment and that PAR variation along the day and season plays an important role in the community production. Seagrass community exposed to CO_2 enriched conditions showed higher community production and were more efficient at low light intensities, when compared with seagrass community exposed to the current CO_2 concentration.

For summer season, NCP of *Z. noltei* community in air exposed community was higher than previously reported for *Z. noltei* and Z. *marina* air exposed community (Ouisse et al., 2011), whereas the CR observed in our results under natural conditions was lower than *Z. marina* community and higher than *Z. noltei* community response observed by Ouisse et al., (2011). Similarly, NCP under CO_2 enrichments experiments was higher than results reported for air exposed *Z. noltei* and *Z. marina* community (Ouisse et al., 2010, 2011; Clavier et al., 2014).

For summer season, GCP under natural conditions were lower to GCP obtained for *Z. noltei* community and higher to *Z. marina* community (Ouisse et al., 2011). Similarly, GCP under CO_2 enrichment was higher than values observed for air exposed *Z. noltei* and *Z. marina* community (Ouisse et al., 2011; Clavier et al., 2014). The GCP and CR of *Z. noltei* observed under air exposed conditions in winter season, were higher than previously reported for Z. *noltei* community by Clavier et at., (2011,2014) and Ouisse et al., (2011). Higher community production can be due to higher irradiance (1522to 1594 μ mol m^{-2} s^{-1}) levels observed in our studies than irradiance (829 μ mol m^{-2} s^{-1}) level observed by Ouisse et al., (2011) contributing to the community production and higher respiratory demand of the extended rhizome network of *Z. noltei* which requires higher production (Vermaat et al., 1987). Gross community production of *Z. noltei* communities were higher than previously reported by Silva et al., (2005) in Ria Formosa, under natural and CO_2 enriched conditions.

The community respiration of *Z. noltei* community under natural conditions was higher in winter than summer season (Fig.5), opposite trend of higher respiration rates in summer than winter was observed for *Z. noltei* community elsewhere (Plus et al., 2005; Ouisse et al., 2010). Higher community respiration rates in *Z. noltei* in winter can be due to low irradiance levels observed in our studies, followed by low community production, thus community respiration takes over the community production with CO_2 release and oxygen consumption, similar pattern of higher respiration was observed in *Z. marina* elsewhere (Rasmusson, 2015).

Community production under natural conditions during emersion always varies with the community production during immersion. Higher community production during emersion than immersion was observed in the same study site for *Z. noltei* (Silva et al., 2005), whereas community production of *Z. noltei* lower during emersion and higher during immersion was observed elsewhere (Ouisse et al., 2011; Clavier et al., 2011).

The short-term gas exchange measurements with CO_2 enrichment conditions for *Z. noltei* community clearly indicated that community production (NCP and GCP) around similar irradiance level increased 1.5-fold and 1.6-fold respectively under CO_2 enriched conditions than natural conditions (Fig. 5). Higher community production under CO_2 enrichment suggest that community production of *Z. noltei* are currently carbon limited under the current atmospheric CO_2 concentration, confirming the same conclusions previously obtained for *Z. noltei* (Alexandre et al., 2012) and for *Z. marina* (Beer and Koch, 1996; Zimmerman et al., 1997; Invers et al., 2001). This results also suggest that *Z. noltei* community may benefit from future CO_2 enrichment by enhancing their net photosynthetic rates at higher CO_2 concentrations. The CO_2 stimulated increase in community production found here for *Z. noltei* is consistent with the findings reported for the temperate and tropical seagrass species, where positive community production responses to CO_2 enrichment were found *Z. marina* (Beer & Koch, 1996; Thom, 1996; Zimmerman et al., 1997) and *Thalassia hemprichii* (Jiang et al., 2010), whereas no response to CO_2 enrichment was observed in *Halophila uninervis* (Ow et al., 2016).

The key factor during emersion for higher production under CO_2 enriched conditions can be higher light availability during emersion periods and abundant CO_2 as substrate from the CO_2 enrichment. Increase in CO_2 as substrate resulting in higher production efficiency was observed in *Z. marina* where elevated CO_2 levels increased plant production (Zimmerman et al., 1997). Secondly sediment microtopography of *Z. noltei* community plays an important role in maintaining hydration for the whole emersion period, due to numerous small depressions in the sediment retaining water (Silva et al., 2005). Thirdly during emersion, a thin layer of water is trapped between the leaves of *Z. noltei* due to higher leaf density (Silva & Santos, 2003), helping the plants remain moist throughout the emersion and this thin layer of water also helps in transfer of CO_2 from the atmosphere to the leaf surface (Leuschner and Rees, 1993), whereas in our case this transfer of CO_2 takes place inside the chamber from CO_2 enrichment. Simultaneously, the desiccations in *Z. noltei* meadows are not severe (Silva & Santos, 2003) and the *Z. noltei* root system also supplies water to the leaves, which helps in maintaining a thin layer of water at the leaf surface (Leuschner et al., 1998). In these favourable conditions when CO_2 enrichment happens, a rapid diffusion of CO_2 across the thin film of water around the seagrass leaves occurs due to air-water CO_2 gradient, making the CO_2 readily available to the plants (Leuschner et al., 1998). Therefore, high CO_2 concentrations along with high irradiance levels during air exposure of the *Z. noltei* community, creates the ideal condition for high community production during emersion (Leuschner & Rees, 1993; Silva et al., 2005; Touchette& Burkholder, 2007).

Net community production of sediment community (785 mg Cm^{-2} day^{-1}) in our studies under natural conditions in summer was higher than previously reported NCP (681.5 mg Cm^{-2} day^{-1}) in natural at Tagus estuary, Portugal in summer season (Uthicke et al., 1998), whereas NCP (212.52 mg C m^{-2} day $^{-1}$) of sediment community in winter season in our results was lower than previously reported NCP (255 mg C m^{-2} day $^{-1}$). Average NCP and CR observed in our results for sediment community in winter season were higher than NCP and CR rates observed in the intertidal sediment community at Banco di Marituania (Clavier et al., 2014). Sediment community NCP were higher with CO_2 enrichment incubations in summer and winter than air incubations, which can be related to the presence of micro-phytobenthos in the sediments during intertidal exposure (Uthicke & Klumpp, 1998; Hubas et al., 2006; Clavier et al., 2014). The intertidal desiccation of the sediments was avoided due to the presence of a thin film of water trapped by the benthos community and water trapped by intertidal burrows (Michael & Mclntire, 1983).

Seasonal (summer and winter) variation of GCP (614.23 to 93.64 mg C m^{-2} day $^{-1}$) were higher than seasonal GCP (38 to 151 mg C m^{-2} day^{-1}) reported by Hubas et al., (2006). GCP of unvegetated sediment communities were lower in winter season that summer under CO_2 enriched conditions, due to higher community respiration rates (Fig.5). Higher community respiration can be related to the presence of other bacteria and infauna not contributing to the community production but respiration (Uthicke & Klumpp, 1998). Secondly lower irradiance intensities in winter means, less penetration of light into the sediment resulting in decrease of microphytobenthos biomass in the sediment (Pinckney & Zingmark, 1993b) and finally the presence of large content of detritus on the unvegetated sediment surface presents a shading effect on the fewer micro-phytobenthos community present in winter season (Ouisse et al., 2010). The incident PAR was also influenced by the length of the day in winter (11-hour daylight compared to 13-hour daylight in summer) and cloud cover which could have also affected the sediment community production (Parsons et al., 1984; Migné et al., 2004; Hubas et al., 2006).

Overall the short-term incubation method based on CO_2 enrichment using benthic chambers described here is reported for the first time for intertidal *Z. noltei* community. This method can be an important tool for intertidal lagoon community productivity. It provides fast and precise values of community carbon fixation during emersion periods for air exposed plants and sediment community. The use of small chambers and short-term incubation periods is an important feature in these experiments as the temperature and humidity levels remain constant. This method also allows repeatable in situ measurements of carbon uptake with minimum community disturbance. For these reasons, we recommend the use of this method to assess the contribution of different communities to the metabolism and carbon budget of complex tidal systems.

The results of a good correspondence observed from the linear regression model between carbon metabolism measurements of the *Z. noltei* community and sediment community with respect to light intensities across summer and winter seasons, provides evidence that community production rates derived from CO_2 enriched incubations are reflection of photosynthetic activity at ecologically relevant scales for future conditions of carbon dioxide. Positive response to elevated CO_2 levels and lower light requirements as observed in our results may at least partially offset negative responses to decreased light availability from sea level rise associated with global climate change.

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Population dynamics of *Cymodocea nodosa* in the vicinity of volcanic CO_2 seeps

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Population dynamics of *Cymodocea nodosa* in the vicinity of volcanic CO_2 seeps.

Abstract

Rising carbon dioxide (CO_2) concentrations in the atmosphere will increase the average pCO_2 level in the world oceans, which will have a knock-on effect on the marine ecosystem. Coastal seagrass communities are predicted to benefit from the increase in CO_2 levels, but long term effects of elevated CO_2 on seagrass communities are less understood. Population reconstruction techniques were used to investigate the population dynamics of *Cymodocea nodosa* meadows, exposed to long term elevated CO_2 at volcanic seeps off Greece and Italy. Effect of elevated CO_2 was noticed on the growth, morphometry, density, biomass and age structure at CO_2 seeps than reference sites. Above to below ground biomass ratio of *C. nodosa* were higher at CO_2 seeps. The plastochrome interval were similar at all CO_2 seeps. The shoot age and shoot longevity of plants were lower at seeps. The present recruitment (sampled year) of the seagrass were higher than long-term average recruitment of the communities near the seeps. Carbon to nitrogen ratios (%DW) of *C. nodosa* were higher in leaves at seeps. Annual leaf production was higher near the seeps. This study suggests increased production of *C. nodosa* under elevated CO_2 levels, but other co-factors such as nutrients, trace metal toxicity must also be taken into consideration while predicting effects of future CO_2 concentrations.

Keywords: Reconstruction techniques, plastochrome interval, elevated CO_2, carbon metabolism

3.1. Introduction

The ocean's absorption of anthropogenic CO_2 emissions (over 35 Giga tonnes of CO_2 per year, (IPCC, 2014) has already lowered the mean ocean surface pH by 0.1 units since preindustrial values, with a predicted further decrease of 0.3- 0.4 units by the end of this century (IPCC, 2014). These on-going changes are expected to intensify in the future with potentially significant, but variable effects on marine organisms depending on their sensitivity (Hendrick et al., 2010; Kroeker et al., 2010). Calcifying organisms are more susceptible to ocean acidification than non-calcifying organisms (Suggett et al., 2012), even though their responses to ocean acidification are also dependent on the taxonomic group and their developmental stages (Hendrick et al., 2010; Kroeker et al., 2010). Realisation of the key role of seagrass in coastal ecosystems has fostered ever growing efforts to quantify their annual productivity and growth dynamics. (Duarte et al., 1994). Seagrasses, as many macro algal species, are notably tolerant to CO_2 increases and may even benefit from it (Koch et al., 2013). In the current CO_2 levels, seagrasses are dissolved inorganic carbon (DIC) limited as they are inefficient in utilising bicarbonate (Invers et al., 2001).

Hence, under increased CO_2 conditions, it is expected seagrasses will increase utilization of CO_2 (Beer et al. 1996) resulting in increased photosynthesis and consequently growth and productivity (Koch et al., 2013, Russel et al., 2013). Despite these predictions, the real-time studies conducted overtime both ex-situ with high CO_2 and in-situ at natural CO_2 seeps on seagrasses not completely support this expectation. For example, the photosynthetic activity of *Cymodocea nodosa* was stimulated by the low pH at natural CO_2 seeps of Vulcano, with significant increase in chlorophyll-a content of leaves, maximum electron transport rate and compensation irradiance (Apostolaki et al., 2014). Low pH promoted productivity, but was not translated into biomass production, probably due to nutrient limitation, grazing or poor environmental conditions (Apostolaki et al., 2014). Similar results were obtained by Alexandra et al. (2012) for *C. nodosa* grown for five months under high CO_2 conditions. On the other hand, the biomass and net primary production of *Halophila ovalis* and *Cymodocea serrulata* increased near the CO_2 seeps, whereas the abundance of species increased only for *C. serrulata*, suggesting species specific response to elevated CO_2 (Russell et al., 2013). Long term elevated CO_2 experiments on *Zostera marina* for over one year showed greater reproductive outputs, increased below ground biomass and shoot density (Palacios and Zimmerman, 2007), whereas short term experiments resulted in increased photosynthetic rate and shoot productivity

(Zimmerman et al., 1997). In contrast, experiments on *Cymodocea serrulata* have shown no enhancement in productivity at higher CO_2 as they are carbon saturated in current CO_2 concentrations (Schwarz et al., 2000). Recent CO_2 enrichment studies on three tropical seagrass species showed a significant increase in net productivity with increase in CO_2 levels, but a different growth rate between species was noticed due to varying strategies of carbon allocation among species (Ow et al., 2015).

Studies on natural CO_2 seeps suggest that seagrass species can be adapted to survive and live under elevated CO_2 conditions (Hall Spencer et al., 2008, Fabricius et al., 2011 and Takahashi et al., 2016). Much of the natural CO_2 seeps of Europe are concentrated in the Mediterranean Sea (Dando et al.,1999). In the Mediterranean Sea, most known seeps are concentrated in the shallow waters of the active volcanic arcs in Aegean Seas and are usually of the gas hydrothermal type due to the large volume of gas released (Dando et al., 1999). These natural CO_2 seeps provide future oceanic conditions (Hall Spencer et al.,2008; Hall-Spencer and Rodolfo-Metalpa, 2009) and are expected to affect seagrass communities due to changes in the physical and chemical features of seawater and sediments with possibly large effects on functioning features (Vizzini et al., 2010). In these conditions the growth and age structure of the seagrass *Cymodocea nodosa* in these seeps have not been investigated. The seagrass *Cymodocea nodosa* is an endemic species that supports highly complex and biodiverse climax communities in the Mediterranean Sea (Mazzella et al., 1986). Nevertheless, the effects of hydrothermal CO_2 gas release associated to explosive volcanism activity on seagrass productivity has been studied (Vizzini et al., 2010). How these changes will affect higher levels of biological organization, such as seagrass population dynamics (e.g. shoot recruitment rate), is less studied.

The objective of this work is to assess for the first time the long -term responses of the population dynamics and production of seagrass, *C. nodosa* exposed to elevated CO_2 levels. Populations in the vicinity and away from the influence of volcanic seeps were compared. CO_2 seep sites have been used to assess the long-term effects of elevated CO_2 on benthic marine ecosystems and respective underlying mechanisms (Hall-Spencer et al., 2008, Fabricius et al., 2011, Vizzini et al., 2013, Enochs et al., 2015). However, other cofounding factors related to the volcanic seeps, such as the emissions of heavy metals (Dando et al., 1999, Vizzini et al., 2013, Kadar et al., 2013) and sulphide (Dando et al., 1999, Boatta et al.,2013) may influence the plants and population responses to elevated CO_2. These are very variable among seeps

(Dekov et al., 2004, Varnavas et al., 2005) whereas the major CO_2 composition of emissions is constant. To cope with possible confounding factors of the effects of CO_2 on the population dynamics of *C. nodosa,* we replicated the sampling effort in three seeps, two at the island of Milos in Greece and one at Vulcano island in Italy, to consider only the common responses as effects of elevated CO_2.

3.2. Methods

Study sites

Milos Islands, Greece

Paleochori Bay (36.67 N, 24.51 E) and Milos thermal stations (36.70 N, 24.46 E) are part of Milos island (Fig.1A). Extensive submarine venting occurs offshore, from the intertidal to depths of more than 100 m over a 34 km^2 area of seabed (Dando et al.,1999). The released gases are 95% CO_2 with some H_2S, CH_4 and H_2 (Dando et al., 1999).

Vulcano, Italy

We sampled Levante Bay (38.4 N, 15.0 E) off Vulcano island (Fig. 1B). Some parts of this bay are well-suited for studies of the effects of increased CO_2 levels (Boatta et al., 2013) despite areas with elevated H_2S and metals (Vizzini et al., 2013). The main underwater gas seeps are located along southern and western shores of the bay at <1 m depth (Boatta et al., 2013). Total CO_2 output is about 3.6 tonne d^{-1} (Inguaggiato et al., 2012), and the underwater gas emissions are 97-98% CO_2 with 2.2% H_2S close to the seeps, decreasing to less than 0.005% H_2S towards the north-eastern part of the bay, where most ocean acidification research has been located (Capaccioni et al., 2001; Milazzo et al., 2014). There is a step gradient in carbonate chemistry with pH 5.65 at the main gas seeps increasing to pH 8.1, which is typical for present day Mediterranean surface seawater, at >350 m from the seeps (Boatta et al., 2013). *Cymodocea nodosa* were absent at the main vents.

In each location, two sites were established where well developed populations of *C. nodosa* were present, a high CO_2 site near the seeps and a reference site away from the influence of the vent. Reference and CO_2 seep sites were established at the same depths, under similar hydrodynamics conditions. Overall, *C. nodosa* meadows sampled were at shallow (<5m) depth.

Water sampling:

Water samples were collected at CO_2 seeps and reference sites (n=5) in 100 ml Winkler bottles fixed with 20 µl mercury chloride in the field, stored in the dark and transported to the laboratory for total alkalinity (TA) and dissolved inorganic carbon (DIC) analysis. The pH (using pH meter, Titrino Methron) and temperature of the water samples were measured in the field immediately after collection. In the laboratory pH and temperature were measured again and 80 ml of water sample was used in Lab Titrino analyser (Dickson et al. 2007) for the analysis of TA. Temperature, pH and TA data were used to calculate the other carbonate parameters (pCO_2, HCO_3^-, aragonite saturation state, etc.) using CO_2Sys program (Pierrot and Wallace.2006).

Seagrass sampling and analysis:

The meadow density and biomass, the rhizome growth and production, the morphometric characteristics of the plants, the population age structure and derived population dynamics (long-term average recruitment, present recruitment and population growth rates) and the total C and N contents of plants were characterized in CO_2 seeps and reference sites. Reconstruction techniques, an indirect measure of plant growth history and population dynamics changes (Duarte et al., 1994; Fourqurean et al. 2003), were used to evaluate the *C. nodosa* responses to the different environmental conditions caused by the seeps. It was hypothesized that increased CO_2 promote higher plant growth, higher meadow production, and thus higher population dynamics.

Ten *C. nodosa* samples were collected from each site with a 20-cm diameter core (15 cm in Greece) into a depth of about 30 cm in May 2013 in Italy and May 2014 in Greece. The sediment was carefully rinsed off to prevent the modular sets disconnecting from each other and to keep the rhizome mat intact as required for the reconstruction of seagrass dynamics (Duarte et al., 1994). In each sample, the number of both shoots and apicals was counted to estimate the density of shoots and of physically independent individuals. The age of *C. nodosa* shoots was estimated by counting the number of leaf scars on the vertical rhizomes plus the number of leaves in each shoot multiplied by the leaf plastochrome interval (PI). To estimate the PI of each study site, i.e., the time needed to produce a new leaf, the sequence of average internodal length of *C. nodosa* shoots collected with the cores plus additional plants collected by hand was plotted. Then a 30% running average was applied to filter short-term seasonal variability and the difference in the number of vertical leaf scars between two consecutive length modes was counted. The modes represent annual growth periods and thus the average number of leaf scars produced between modes was averaged to estimate the leaf PI of the population (Short et al. 2001). The PI estimates of *Cymodocea nodosa* were 29.3 for all three locations off Greek and Italy islands.

To estimate the vertical and horizontal rhizome elongation rates the length of both the vertical and horizontal rhizomes between consecutive shoots was measured and the number of both vertical and horizontal internodes between consecutive shoots was counted (see Duarte et al., 1994 for details of method). The number of leaves per shoot were measured from intact shoots in each sample (n = 10). The horizontal and vertical rhizome production rates were estimated by multiplying the elongation rates (vertical or horizontal) by density (shoots or apicals), by the specific dry weight of rhizomes (vertical or horizontal) and by the dry weight contents of C (vertical or horizontal). Annual leaf production of each population was calculated as the product of the number of leaves annually produced per shoot, the shoot density, the mean specific dry weight of fully developed leaves and their C content.

The leaves, vertical rhizomes, horizontal rhizomes and roots were separated and dried for 48 h at 60º C for biomass and production estimates. Dried plant material (leaves, vertical rhizomes, horizontal rhizomes and roots) was grounded and analysed for CHN contents in a CHN analyser (EA 1110 Model, Elemental Microanalysis Ltd, Oakhampton, Devon, UK).

The long-term average recruitment (R) was estimated from the shoot age structure using the general model: $N_x = N_0\, e^{-Rx}$, where N_x is the number of shoots in age class x, N_0 is the number of shoots recruited into the population; assuming that mortality and recruitment have had no trend over the lifespan of the oldest shoots in the population, i.e. have remained constant over the lifespan of the oldest shoots, with year to year random variation around some mean value of mortality and recruitment (Fourqurean et al. 2003, Duarte et al. 2005). The recruitment for the current year of sampling (R_0) was estimated using the method described by Duarte et al., (1994). The population growth rate (r) was estimated as: $r = R_0 - M$, where M is the long-term mortality rate, which equals the long-term recruitment rate (R) under the assumptions of near steady state (Fourqurean et al., 2003). Population was considered growing if r is positive ($R_0 > R$), shrinking if r is negative ($R_0 < R$), or with the same trajectory pattern if R_0 is not significantly different from R (Fourqurean et al., 2003).

Statistical analysis:

Significant differences in biomass, density, production and plant morphometry among sites (CO_2 seeps and reference) and locations (Milos, Paleochori and Vulcano) were investigated using two-way ANOVA, after testing for homogeneity of variances and normality of distribution. The Tukey's multiple comparison test was applied to determine significant differences between factor levels. When ANOVA assumptions were not verified, comparison of data sets were performed using the non-parametric test of Kruskal-Wallis and the post hoc Dunn's test. Log transformations of variables were performed where needed.

The species vertical and horizontal rhizome elongation, and the population recruitment rates were obtained considering all replicates in each site. The t-test for the difference between two regression lines was used to compare the vertical rhizome elongation rates as these are equal to the slopes of the linear regression between age and size of rhizomes. Statistical analyses were not performed for the horizontal rhizome elongation rate, because just one value was obtained for each site. The confidence limits of the exponential decay regression model used to estimate the long-term average recruitment rate (R) allowed its statistical comparison to the present recruitment rate (R_0) as described in Fourqurean et al., (2003). Significant differences of the long-term recruitment rate among sites were tested using one-way ANOVA. Significance levels was considered at $p < 0.05$ (Sokal and Rohlf, 2012).

3.3. Results

Seawater carbonate chemistry values of the three locations Milos, Paleochori and Vulcano are presented in Table 1. At all locations, the *p*CO_2 concentration was higher (and pH lower) near the CO_2 seeps (Table 1). The reference sites lying further away from the CO_2 seeps (Reference) showed lower concentrations of CO_2. DIC and CO_2 concentrations were higher at Milos and lower at Vulcano.

Significant response of *C. nodosa* to increased CO_2 was noticed on density and biomass at all three CO_2 seeps. Both shoot and apex density, i.e. the number of physically independent plants was higher in all CO_2 seep sites. Both shoot and apex density was higher at Milos (Fig.2). Total biomass, leaf biomass, horizontal rhizome biomass and above ground-below ground biomass ratio of *C. nodosa* varied significantly at all three CO_2 seeps. Total biomass, leaf biomass and horizontal biomass was higher at seeps of Vulcano, whereas above ground-below ground biomass ratio was higher at seeps of Paleochori (Fig. 5).

Table 1:

Seawater carbonate chemistry measurements at Milos, Paleochori and Vulcano CO_2 seeps calculated with CO_2Sys programme, using constants from Dickson and Millero, 1987, and pH in NBS scale.

		Milos	
	pH (NBS scale)	**pCO_2 (µatm)**	**DIC (µ mol kg^{-1})**
CO_2 seeps	7.5 ± 0.04	2457.9 ± 1.87	3474.03 ± 4.55
Reference	8.2 ± 0.03	405.5 ± 1.65	1407.44 ± 51.25
		Paleochori	
CO_2 seeps	7.9 ±0.01	884.3 ± 3.07	2738.39 ± 16.49
Reference	8.2 ±0.01	402.9 ± 1.1	1396.14 ± 48.69
		Vulcano	
CO_2 seeps	7.98 ± 0.08	737 ± 158	2377 ± 37
Reference	8.17 ± 0.05	427 ± 68	2244 ± 40

Table 2.

Responses of *Cymodocea nodosa* biomass (means ± SE) at CO_2 seeps off Greek (Milos and Paleochori) and Italy (Vulcano) islands. Tukey's multiple comparison ($p<0.05$) test results are presented. Different letters indicate significant difference between CO_2 seeps and reference sites.

	CO_2 seeps	**Reference**
	Milos	
Vertical rhizome biomass (g DW m^{-2})	34.4± 5.3	28.6± 4.2
Root biomass (g DW m^{-2})	41.1± 1.1	31.5± 3.0
	Paleochori	
Vertical rhizome biomass (g DW m^{-2})	28.60 ± 5.9	14.4 ± 2.7
Root biomass (g DW m^{-2})	36.0 ± 2.6	30.3± 1.8
	Vulcano	
Vertical rhizome biomass (g DW m^{-2})	67.6 ± 10.0	124.4 ± 17.6
Root biomass (g DW m^{-2})	135.9 ± 27.4	154.6 ± 18.7

The vertical rhizome biomass was significantly lower near the seeps of Vulcano but not in the other seeps (Table 2). Significant effects of CO_2 on plant morphology was observed on number of leaves and vertical rhizome. The number of leaves was higher at Milos and the vertical rhizome which was shorter near the seeps at all sites (Fig.3). Density, biomass and morphometry of *C. nodosa* varied significantly between Greek and Italian locations (Milos vs Vulcano, Paleochori vs Vulcano) but not between Greek locations (Milos vs Paleochori), except for number of leaves which was significant between Greek locations and shoot density that did not vary among locations (see Appendix). Significant differences between apex density was found between Milos vs Vulcano and Vulcano vs Paleochori (See Appendix). Apex density was lower at Vulcano (Fig.2) whereas the total and plant part biomass were higher (Table 2). Leaf number (Fig.3) and horizontal rhizome length were lower at Vulcano (see Appendix), whereas vertical rhizome length was higher (Fig.3). Significant differences were found for vertical rhizome length and number of leaves between Milos vs Vulcano and Vulcano vs Paleochori, whereas the only significant difference in morphometry were found for number of leaves at Milos vs Paleochori (see Appendix). Vertical rhizome length in Vulcano was higher than Milos and Paleochori (Fig.3). Significant differences were found for vertical elongation rate and the vertical elongation rate was higher at CO_2 seeps at all locations (see Appendix).

Horizontal elongation rate was higher at CO_2 seep sites at all locations except for Vulcano CO_2 seep site (See Appendix).

Table 3

Age structure and population dynamics values of *Cymodocea nodosa* shoots at CO_2 seeps and reference sites off Greek (Milos and Paleochori) and Italy (Vulcano) islands. Mean ± Standard errors are presented for the shoot age. The exponential coefficient ± standard errors of the exponential decay regression are presented for the long-term average recruitment rate (R). Different letters indicate significant difference between CO_2 seeps and reference sites, ns= not significant. P values obtained from one way ANOVA for Milos (p=0.116), Paleochori (p=0.013) and Vulcano (p= 0.105)

	Milos	
	CO_2 seeps	**Reference**
Shoot longevity (years)	3.21	3.53
Long term avg. recruitment (R, $year^{-1}$)	1.03 ± 0.05^{ns}	0.90 ± 0.05^{ns}
Present recruitment rate (R_o, $year^{-1}$)	1.08	0.94
Population growth rate (r, $year^{-1}$)	0.05	0.04
	Paleochori	
	CO_2 seeps	**Reference**
Shoot longevity (years)	2.41	2.81
Long term avg. recruitment (R, $year^{-1}$)	1.41 ± 0.08^{a}	0.42 ± 0.23^{b}
Present recruitment rate (R_o, $year^{-1}$)	1.54	0.52
Population growth rate (r, $year^{-1}$)	0.07	0.09
	Vulcano	
	CO_2 seeps	**Reference**
Shoot longevity (years)	7.14	8.27
Long-term average recruitment rate (R, $year^{-1}$)	0.64 ± 0.11^{ns}	0.37 ± 0.05^{ns}
Present recruitment rate (R_0, $year^{-1}$)	0.65	0.36
Population growth rate (r, $year^{-1}$)	0.01	-0.01

Significant effects of elevated CO_2 were also observed on *C. nodosa* production. The vertical and horizontal rhizome productions were higher at all three CO_2 seep sites (Fig.6) The vertical and horizontal rhizome production was higher at Milos (Fig.6). Significant differences were found for vertical rhizome production between Vulcano vs Milos and Milos vs Paleochori sites, whereas for horizontal rhizome production significant differences were found at all three

CO_2 seep sites (see Appendix). Significant effects of elevated CO_2 were observed on the annual leaf production with higher leaf production at CO_2 seeps off all locations (Fig.7). Within the three locations significant differences were found for Milos vs Vulcano and Vulcano vs Paleochori (see Appendix).

The elemental content of carbon and C: N ratios of *C. nodosa* plant compartments (leaves, rhizomes and roots) showed that whenever there are significant differences, these reflect a carbon enrichment of tissues near CO_2 seeps (Fig.4). Significantly higher carbon content was observed near the seeps in the leaves and roots at Milos, leaves at Paleochori and leaves and rhizomes at Vulcano. The only significant difference found in the tissues N content was in the rhizomes of Paleochori, where it was lower near the seeps (Fig.4). Significant differences in C: N ratios were observed in roots off Milos, rhizomes off Paleochori and roots and rhizomes off Vulcano (Fig.4). Significant differences of C content of leaves among locations was found at Milos vs Vulcano and Vulcano vs Paleochori, whereas for C content of roots significant differences were found at Milos vs Paleochori and Milos vs Vulcano (see Appendix). Significant differences of N content among locations was found in leaves at Vulcano vs Paleochori and Milos vs Vulcano, whereas for N content of roots significant differences among locations was found at Milos vs Paleochori and Vulcano vs Paleochori (see Appendix). For C: N content significant differences was found in the leaves and rhizomes at Milos vs Paleochori and Vulcano vs Paleochori (see Appendix).

The age structure of *C. nodosa* was significantly affected at CO_2 seeps, as both the average shoot age (Fig.8) and shoot longevity, were lower near the seeps at all locations (Table 3). The lifespan of the oldest shoots was between seven to eight years at Vulcano, whereas at Paleochori and Milos it varied between three to four years (Fig. 9). The present and long term recruitments were always higher near the seeps, even though the differences were not statistically significant, except for Paleochori (Table 3). These results suggest higher dynamics of populations near the CO_2 seeps as the turnover of plants are higher near the seeps plants die earlier and recruitment is higher. Supporting this, the age frequency distribution of *C. nodosa* showed a significant higher number of younger plants (1 and 2 years older) at the CO_2 seeps, whereas the number of older plants (more than 3 years) were higher near the reference sites at all three locations (Fig.9).

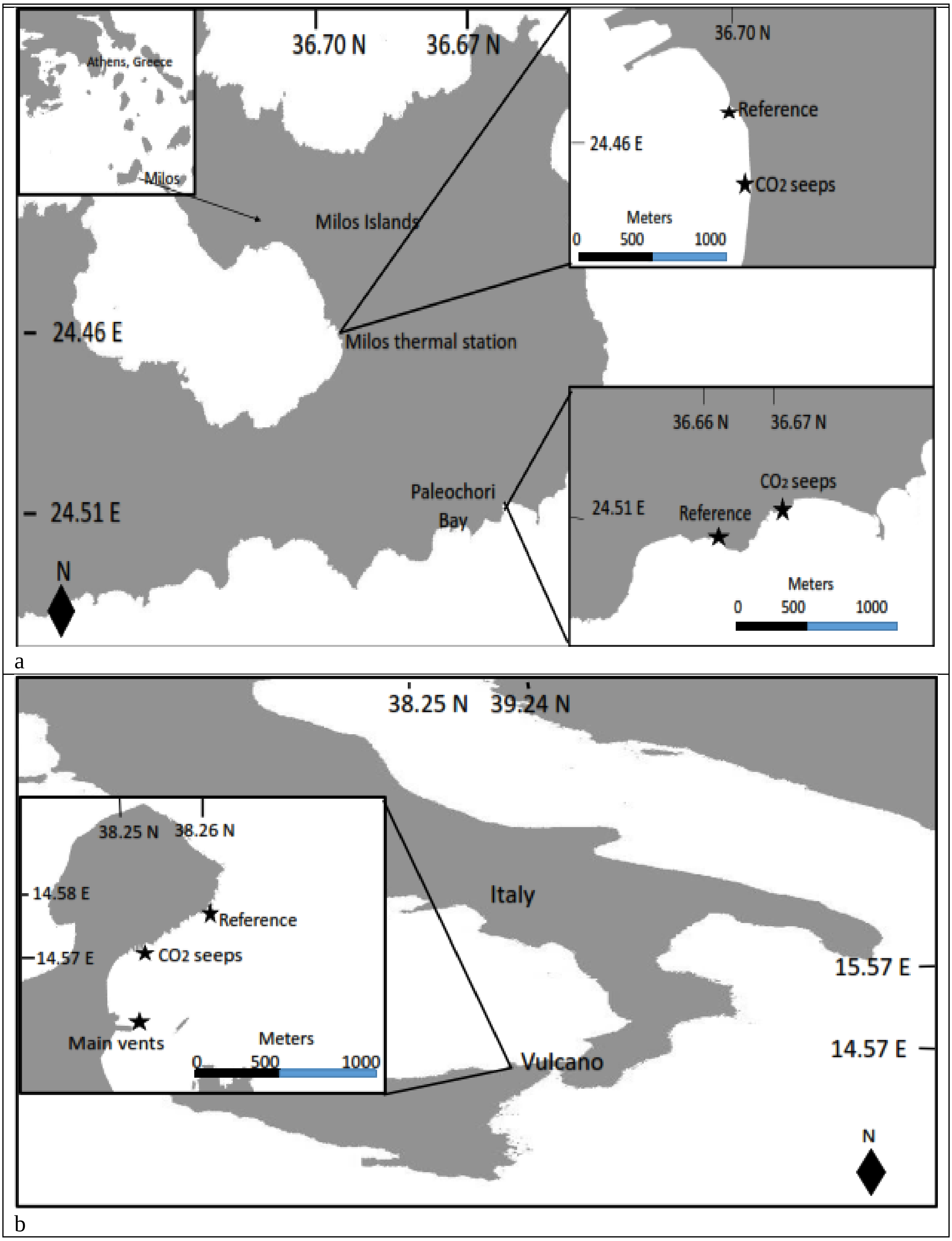

Fig. 1. Study sites with location of CO_2 seeps and Reference sites, A) Milos and Paleochori off Greece and B) Vulcano island off Italy

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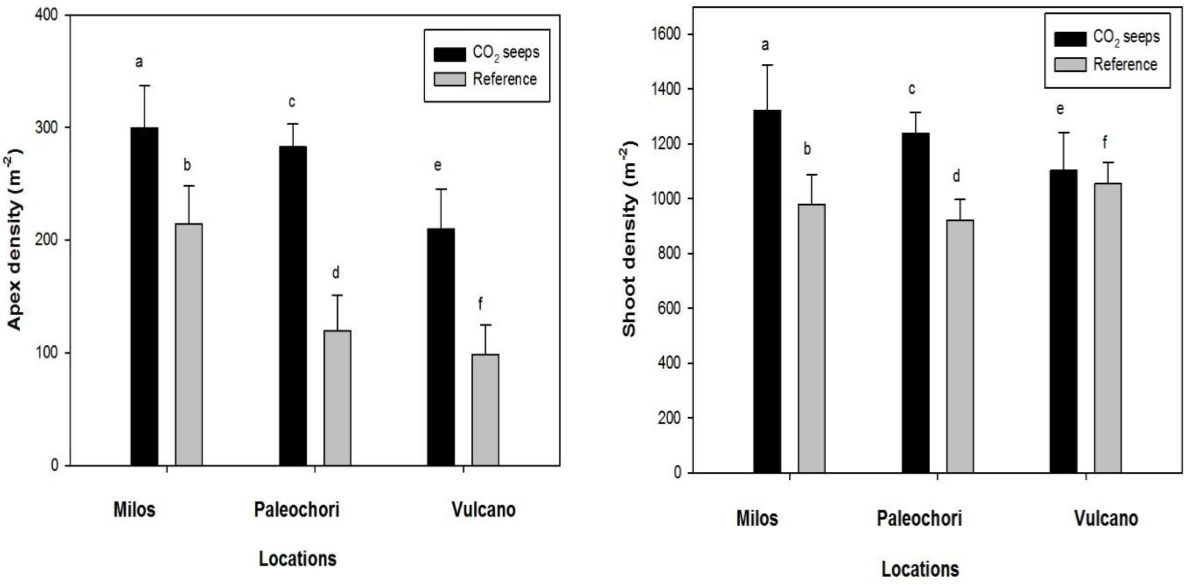
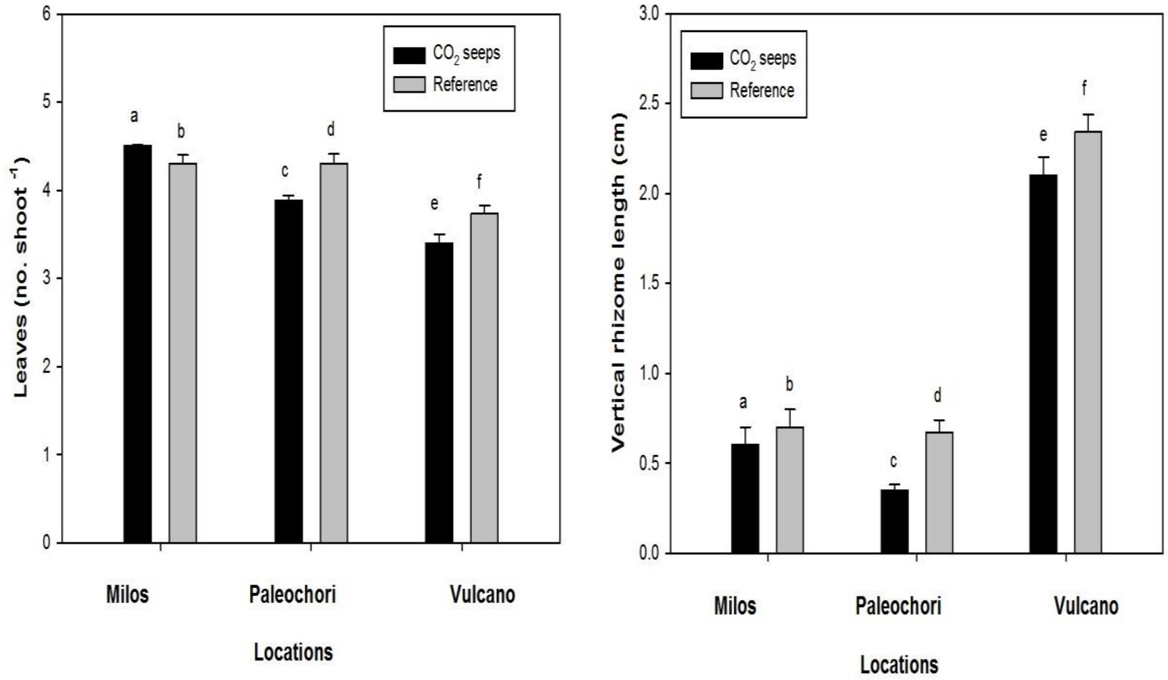


a

b

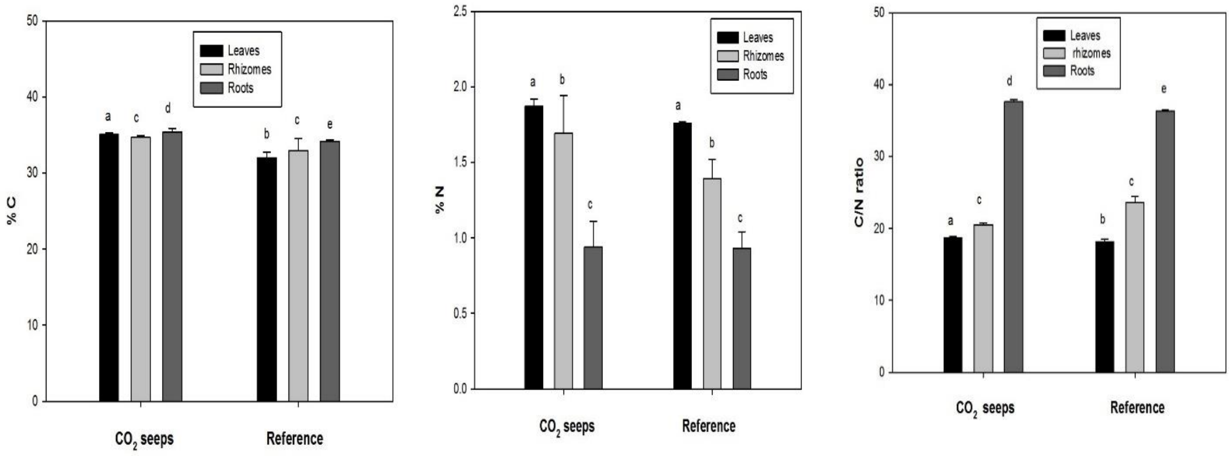
Fig.2. Density (Apex and Shoot) of *Cymodocea nodosa* at the CO_2 seeps off the Greek (Milos and Paleochori) and Italy (Vulcano) islands. Error bars represent standard errors. Significant differences between CO_2 seeps and reference sites are indicated by different letters.



a

b

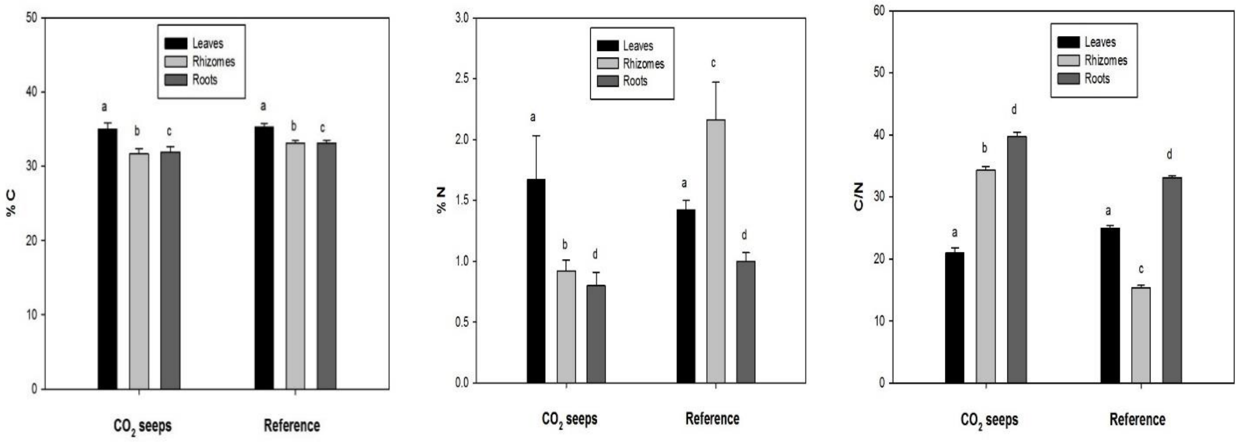
Fig.3. Morphometry (Number of Leaves and Vertical rhizome length) of *Cymodocea nodosa* at CO_2 seeps and reference sites off Greek (Milos and Paleochori) and Italy (Vulcano) islands. Error bars represent standard errors. Significant differences between CO_2seeps and reference sites are indicated by different letters.



a. (%C)

b. (%N)

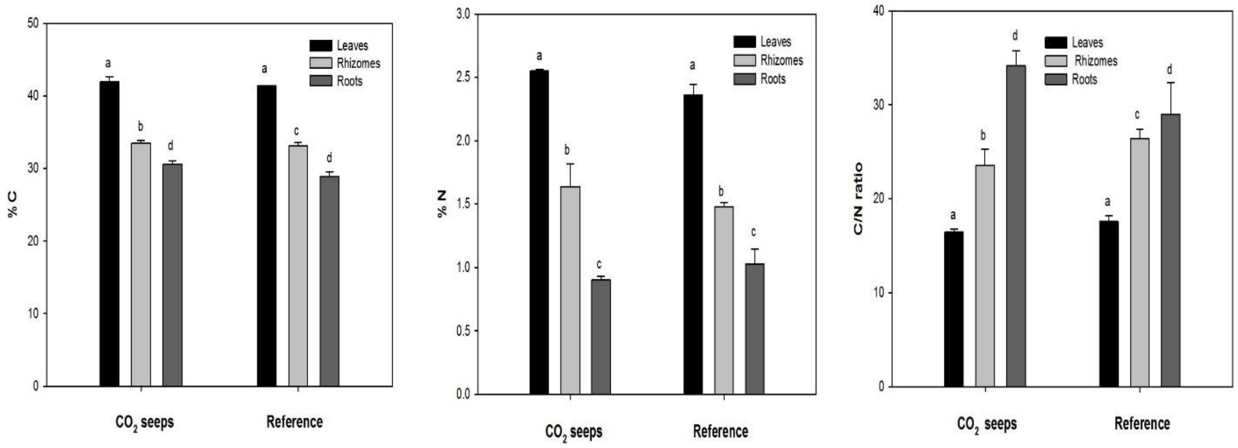
c. (C: N)



d. (%C)

e. (%N)

f. (C: N)



g. (%C)

h. (%N)

i. (C: N)

Fig. 4. Carbon, nitrogen elemental contents and its ratio for Milos (a, b, c), Paleochori (d, e, f) and Vulcano (g, h, i) in leaves, rhizomes and roots of *Cymodocea nodosa* plants at CO_2 seeps and reference sites off Greece and Italy respectively, (mean ± SE, n = 5). Error bars represent standard errors. Significant differences between CO_2seeps and reference sites are indicated by different letters.

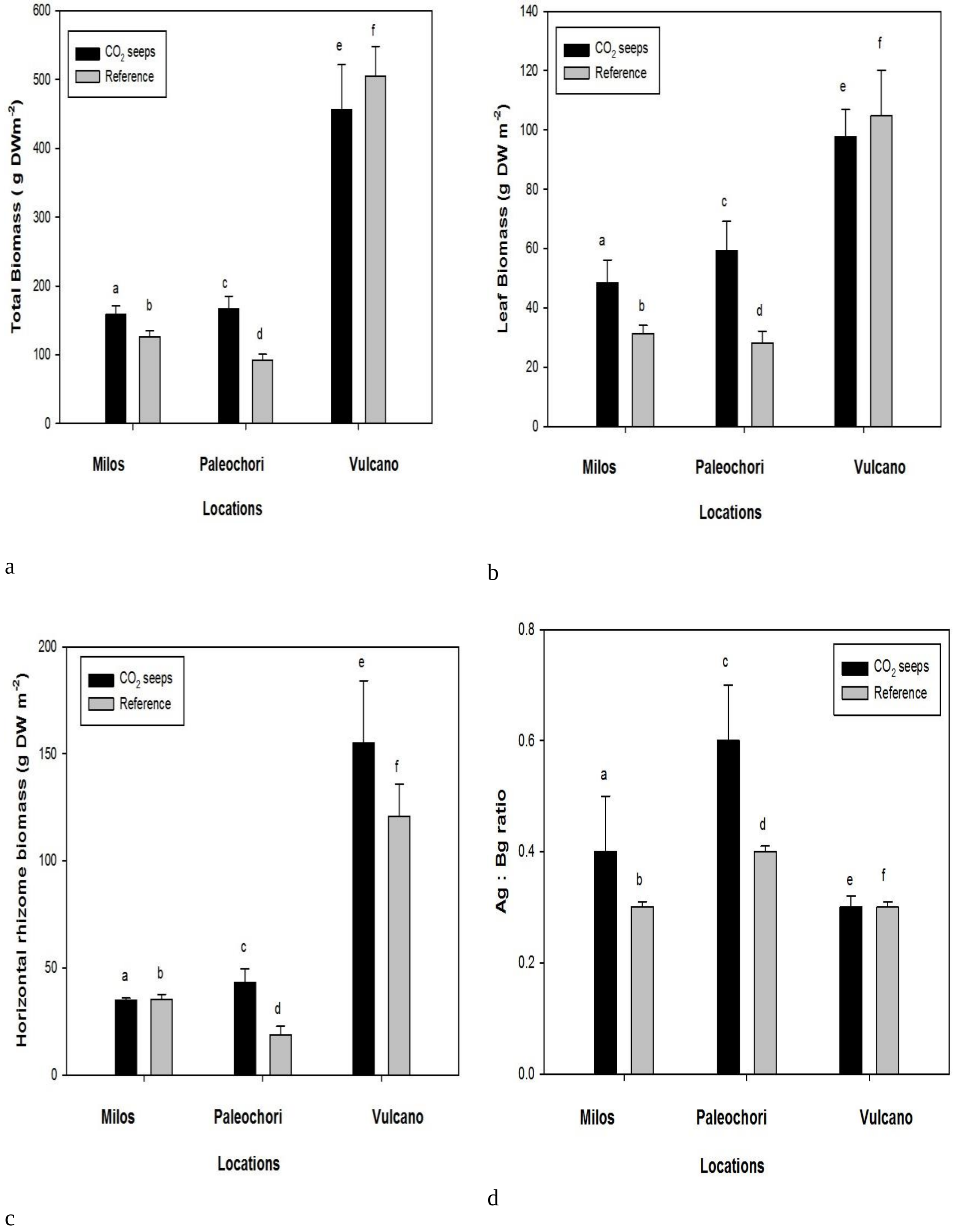
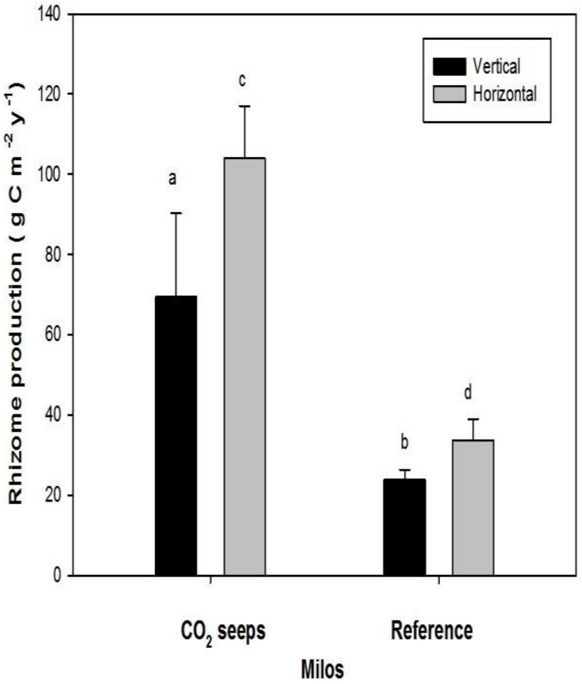
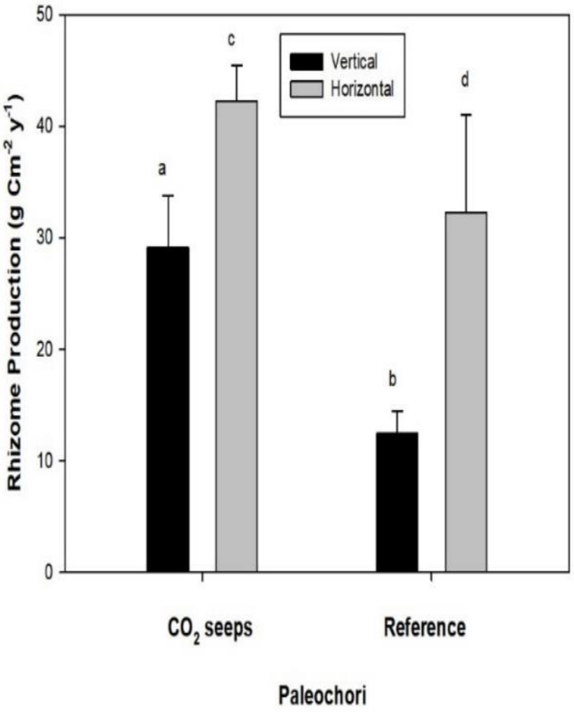


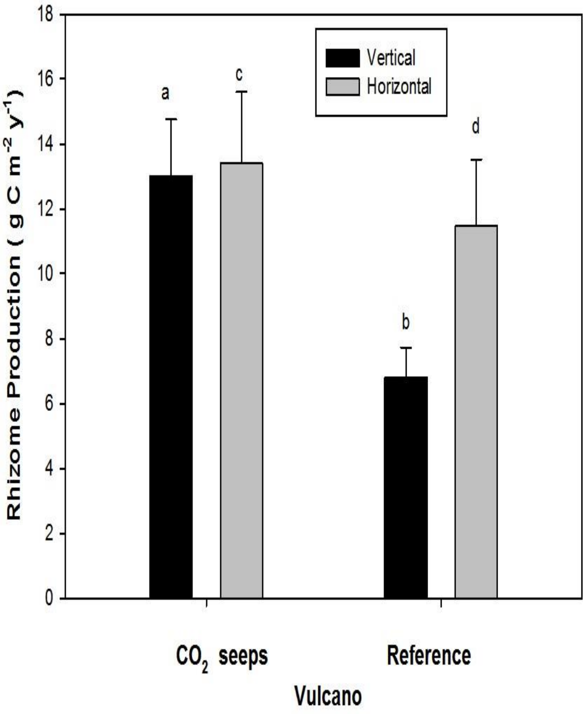
Fig. 5. Biomass of *Cymodocea nodosa* at the CO_2 seeps and reference sites off Greek (Milos and Paleochori) and Italy (Vulcano) islands. Error bars represent standard errors. Significant differences between CO_2 seeps and reference sites are indicated by different letters. (Ag: Bg; Above ground biomass: Below ground biomass)



a



b



c

Fig.6. Vertical and horizontal rhizome productions of *Cymodocea nodosa* at the CO_2 seeps and reference sites off Greek (Milos and Paleochori) and Italy (Vulcano) islands. Error bars represent standard errors. Significant differences between CO_2 seeps and reference sites are indicated by different letters

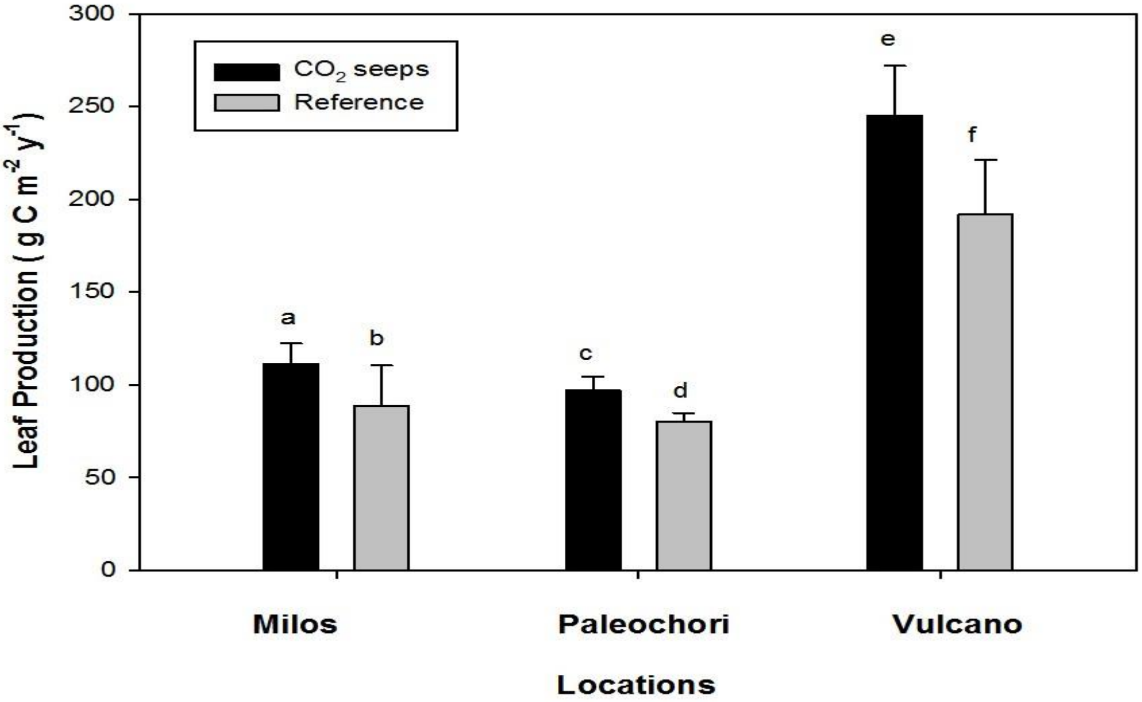


Fig.7. Annual leaf Production of *Cymodocea nodosa* at the CO_2 seeps and reference sites off Greek (Milos and Paleochori) and Italy (Vulcano) islands. Error bars represent standard errors. Significant differences between CO_2 seeps and reference sites are indicated by different letters.

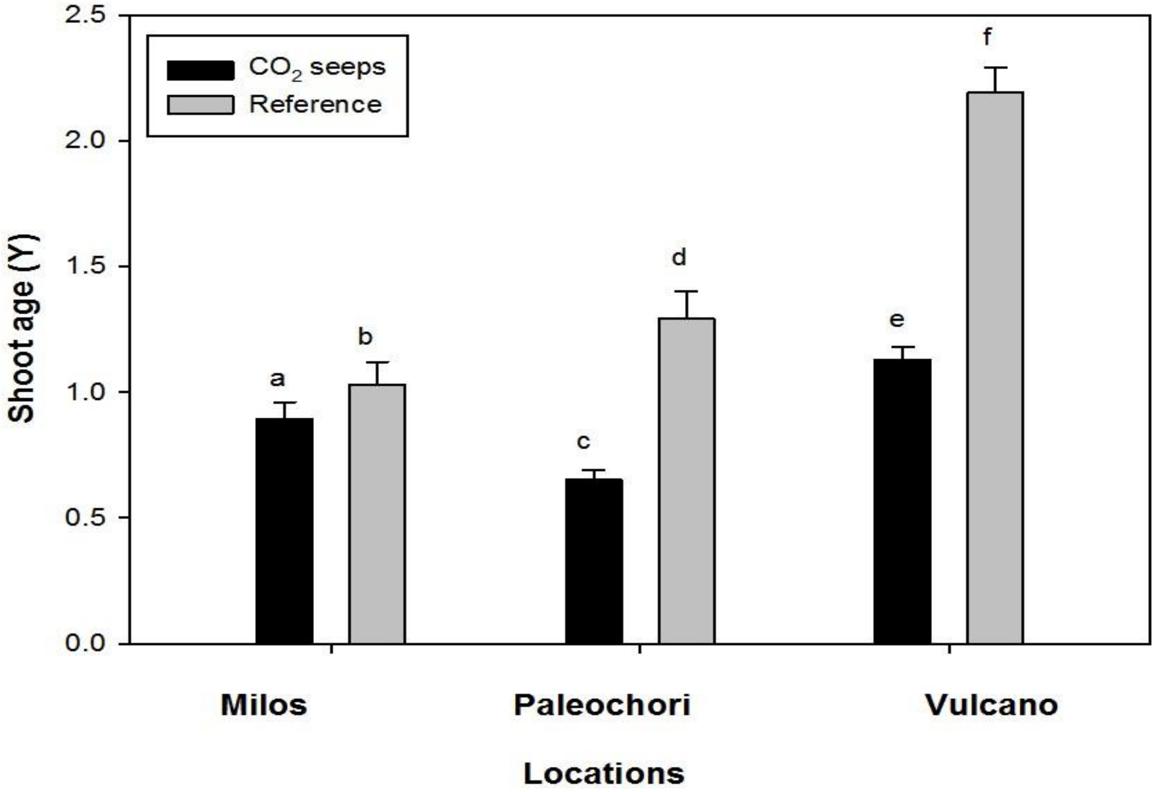
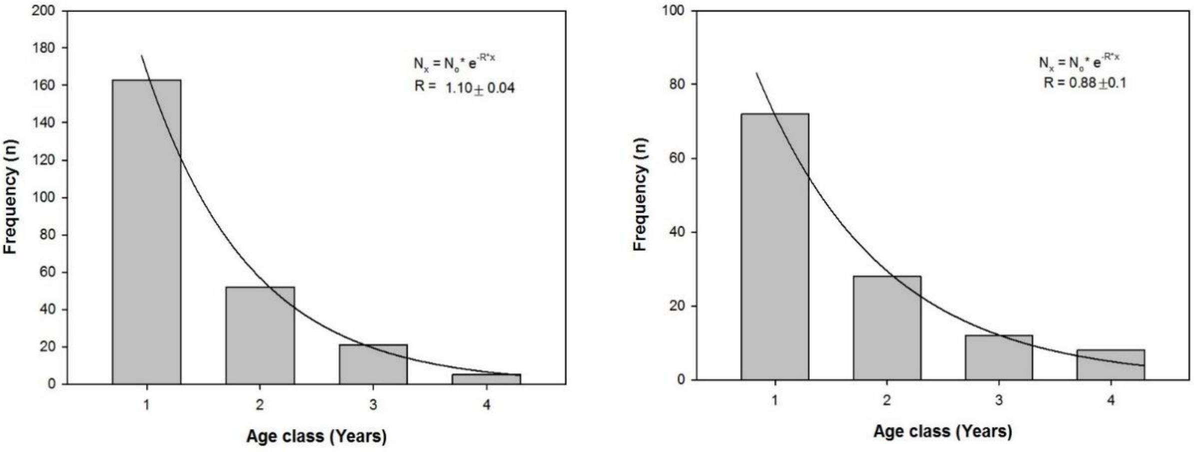
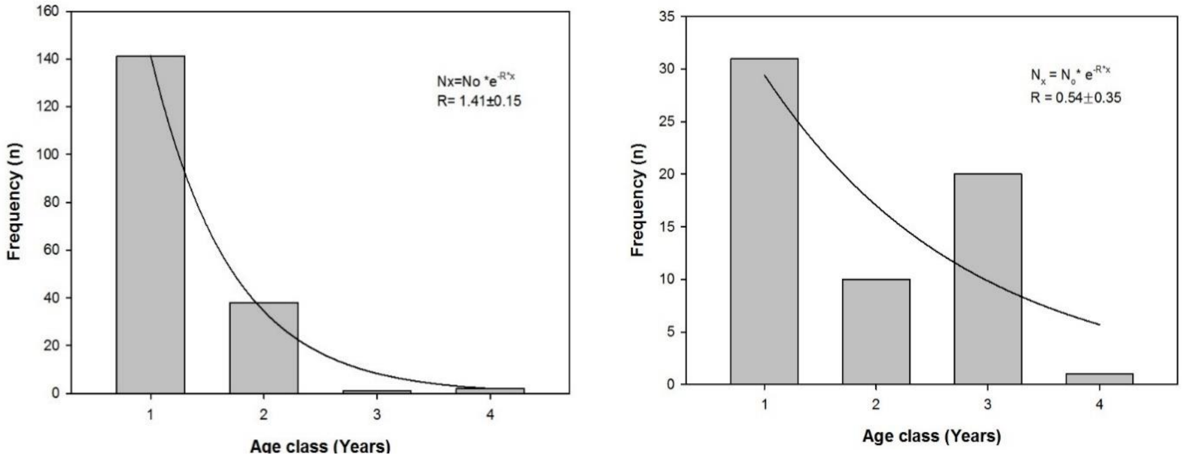


Fig.8. Shoot age of *Cymodocea nodosa* at the CO_2 seeps and reference sites off Greek (Milos and Paleochori) and Italy (Vulcano) islands. Error bars represent standard errors. Significant differences between CO_2 seeps and reference sites are indicated by different letters.



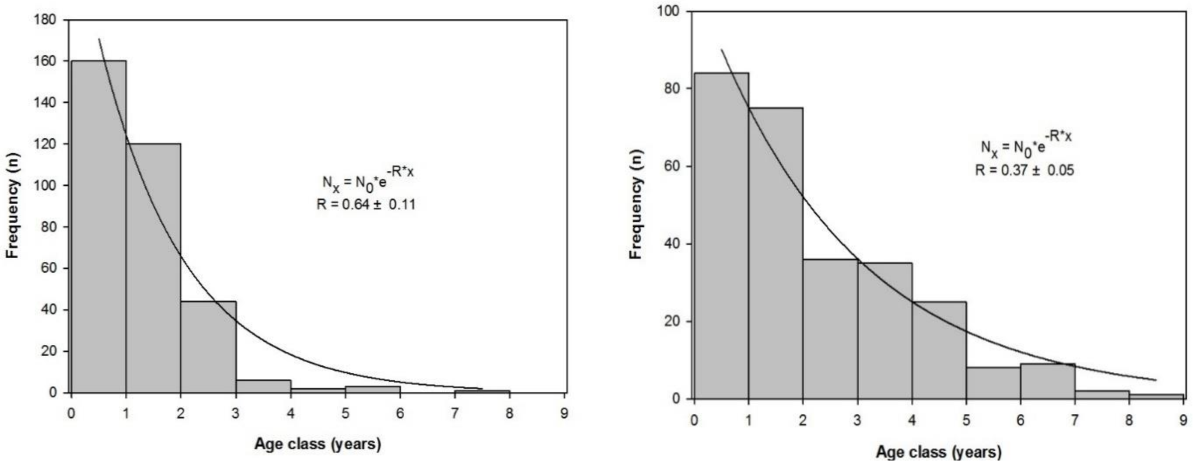
a. Milos (CO_2 seeps)

b. Milos (Reference)



c. Paleochori (CO_2 seeps)

d. Paleochori (Reference)



e. Vulcano (CO_2 seeps)

f. Vulcano (Reference)

Fig. 9. Age frequency distribution of *Cymodocea nodosa* population along the CO_2 seeps and reference sites off Milos (a, b), Paleochori (c, d) and Vulcano (e, f) respectively. The long-term average recruitment rate (R) was estimated from the exponential decay regression line fitted to age frequency distribution.

3.4. Discussion:

The shallow CO_2 volcanic seeps of the Greek (Milos and Paleochori) and Italy (Vulcano) islands have had elevated pCO_2 level for many years, providing insights about the effects of elevated CO_2 on seagrass communities. Despite the variation in pCO_2 at the seeps, the sites represented current (reference) and future 2100 (elevated CO_2) scenarios, enabling us to assess the long-term effects of elevated CO_2 on seagrass communities. Increase in CO_2 concentrations from the seeps, not only increase the dissolved CO_2 concentrations in the surrounding waters, but also increased the relative portion of dissolved CO_2 to HCO_3- (Short and Neckles. 1999) with a positive effect on seagrasses, as observed for the vertical and horizontal rhizome productivity, apex and shoot density and biomass of *C. nodosa* found at Milos, Paleochori and Vulcano CO_2 seeps.

C. nodosa communities showed an increased plant growth and production, and higher population dynamics characterized by higher shoot recruitment and younger shoots under increased CO_2 at Milos, Paleochori and Vulcano sites. Plants developing near the CO_2-seeps off Milos, Paleochori and Vulcano express an increase in fitness confirming that seagrasses are enable to thrive in predicted scenarios of global CO_2/OA changes (Hall-Spencer et al. 2008; Russell et al. 2013). Increased growth and production with proximity to CO_2-seeps suggests increase in CO_2 concentrations will favour the utilization of inorganic carbon sources for seagrass as plants were C limited (Invers et al., 2001; Apostolaki et al., 2014)

The significant spatial variability in *C. nodosa* may be related to high level of patchiness of the seagrass meadow which was evident during sampling. Apex density of *C. nodosa* were higher at all three CO_2 seeps compared to reference sites, but the higher density was not transferred to significant patch expansion of seagrass meadows close to the seeps, even though *C. nodosa* can translocate itself to make meadows (Kraemer and Mazzella, 1999). Lack of *C. nodosa* meadows near the CO_2 seeps can also be related to the associated geochemical features (e.g. toxic levels of trace elements) of individual CO_2 seeps affecting the migration of meadow spatially. Increase in density at all three CO_2 seeps and biomass (only at Greek CO_2 seep sites) for *C. nodosa* from our studies coincides with a range of seagrass species, for instance density and biomass increased with increase in CO_2 levels, for *P. oceanica* (Hall-Spencer et al., 2008), *C. rotundata* and *C. serrulata* (Fabricius et al., 2011b; Russell et al., 2013) and *Zostera marina* (Palacios and Zimmerman, 2007), whereas decrease in biomass for *C. nodosa* in our studies in

Vulcano CO_2 seeps coincides with the results of Russell et al., (2013) for *Halophila ovalis* implying species-specific response towards increased CO_2 levels in future oceans. Higher density at all three CO_2 seeps and higher horizontal rhizome biomass of *C. nodosa* at Vulcano among three CO_2 seeps, suggest, *C. nodosa* at these seeps are adjusted to their carbon utilization capacity to the dissolved inorganic carbon (both $CO_{2\,(aq)}$ and HCO_3^-) concentrations of the surrounding water, similar results were obtained for *T. testudinum* in field and laboratory conditions (Durako.,1993). The low pH conditions were followed by increase in density (shoot and apex) of *C. nodosa* at all three CO_2 seeps, compared to the reference sites, coinciding with results observed for *Cymodocea rotundata* and *Cymodocea serrulata* at Papua New Guinea CO_2 seeps (Takahashi et al., 2016) and differing from results of *C. nodosa* at CO_2 seeps of Vulcano (Eugenia et al., 2014), whereas decrease of total biomass in Vulcano agrees to the results of *C. nodosa* at CO_2 seeps of Vulcano (Eugenia et al., 2014). The above ground biomass was above 20% for Greek and Italy CO_2 seep sites, which agrees with findings for *C. nodosa* at Ischia seeps (Gianluigi et al., 2002), suggesting lower below ground biomass (lower than 80%) of seagrass meadows in elevated CO_2 levels. Our results of lower below ground biomass didn’t match with results from other seagrass (*Zostera marina*) in CO_2 enriched conditions (Welsh et al. 1997; Palacios and Zimmerman, 2007).

Carbon content in *C.nodosa* leaves increased at the CO_2 seep sites for Paleochori and Milos sites, and remained invaribale for Vulcano sites, which agrees with experimental results from *T. Testudinum* and *T. Hemprichii* that showed that carbon content remained invaribale in CO_2 enriched conditions (Campbell and Fourqurean et al., 2013: Jiang et al., 2010). The N content of the *C. nodosa* leaves (1.67 % DW ± 0. 36) were low at Paleochori CO_2 seep and were below the threshold (1.8% DW) for N limitation in seagrass (Duarte. 1990) suggesting that nitrogen deficiency may have limited overall plant growth, whereas N content of *C.nodosa* leaves at Milos (1.85% DW ± 0.05) were not along the thresehold level and may have added to higher growth and density of plants at the Milos CO_2 seeps. In case of *C. nodosa* leaves at Vulcano even with higher N content, growth and density remained lower, similar results were obtained for *C. nodosa* in experimental CO_2 and nutrient enrichment conditions (Khan et al., 2016). Simultaneously lower growth can attributed to the epiphytic microalgae on seagrass leaves, which gets dominant during high N content, compete with the seagrass in production and eventually shade the seagrass leaves (Valiela et al., 1997). Lower C: N ratios in leaves of the plants at Milos, Paleochori and Vulcano (Fig.4) suggests that plants near the seeps utilize the available nitrogen rapidly relative to their carbon content (Duarte. 1990). As nutrient availability increases seagrass tissues, must have been become progressively enriched in nitrogen relative to the carbon content implying decreasing C: N ratios, as found in rhizomes of all three locations and roots of Vulcano CO_2 seeps, whereas the opposite trends were observed in the roots of *C. nodosa* at Greek CO_2 seeps (Fig.4).

Additionally, the Milos, Paleochori and Vulcano seeps, apart from CO_2 supplies, may also be enriched volcanic inputs such as trace elements, which above certain levels can be toxic (Vizzini et al. 2013) along with low pH and depleted oxygen levels can deteriorate the chemical properties of nearby water and sediment and in turn affect the seagrass productivity negatively. It is also likely that responses of seagrasses to elevated levels of CO_2 depends on other factors, such as interactions with temperature, nutrients and light of that ecosystem (Palacios and Zimmerman, 2007).

The low rhizome elongation (ca. 4.27 – 10.6 cm year^{-1}) for *C. nodosa* at Milos, Paleochori and Vulcano (ca.7.91 cm year^{-1}) CO_2 seeps suggested a reduced recruitment (Duarte and Sand-Jensen, 1990) compared to other populations elsewhere (40 cm year^{-1}; Marbà and Duarte, 1998). Increased growth (vertical and horizontal elongation) and production (vertical rhizome, horizontal rhizome and leaf) with proximity to CO_2 seeps than reference sites at all three locations suggests plants were C limited in current conditions in the world oceans (Zimmerman et al., 1997; Invers et al., 2001). Our results comply with what has been observed for other seagrass species at natural CO_2-seeps elsewhere, for instance *Cymodocea serrulata* and *Halophila ovalis* in Papua New Guinea (Russell et al. 2013) and for *Posidonia oceanica* in Ischia, Italy (Hall-Spencer et al., 2008). Similar results were also obtained under experimental CO_2 enrichment for *Zostera marina* (Palacios and Zimmerman, 2007) and *Zostera noltii* (Alexandre et al. 2012).

Our results represent an early assessment of seagrass *C. nodosa* response under long term CO_2 enrichment using population reconstruction techniques as a tool. This is first of its kind to represent seagrass growth, production at the CO_2 seeps of Milos, Paleochori and Vulcano off Greece and Italy. Comparison to similar studies of *C. nodosa* at other seeps suggests that seagrass response to naturally acidified conditions is not so straight forward as the response is species-specific and depends on the biogeochemical characteristics of the site. Clearly, further research of seagrass growth and production using reconstruction techniques in CO_2 seeps in world oceans is necessary, keeping in mind the ecosystem services seagrass provides, their capacity to act as global carbon sink and their major role in mitigation of the elevated levels of CO_2 in future oceans.

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3.6. Appendix

Annex-A

Response of *Cymodocea nodosa* morphometry (mean ± S.E) at the volcanic CO_2 seeps and Reference sites off Greece and Italy. Kruskal- Wallis P values are presented. Different letters indicate significant difference between CO_2 seeps and reference sites.

	CO_2 seeps	**Reference**
	Milos	
Horizontal rhizome length (cm)	4.2 ±0.52	3.4 ±0.51
	Paleochori	
Horizontal rhizome length (cm)	4.8 ± 0.39	5.2 ± 0.52
	Vulcano	
Horizontal rhizome length (cm)	1.6 ± 0.1	1.8 ± 0.2

Annex-B

Two-way ANOVA results of *C. nodosa* morphometry, Sample: 2 variables (CO_2 seeps, Reference) and Site: 3 levels (Milos (M), Paleochori (P) and Vulcano (V)) off Greece and Italy. Post hoc Tukey's test ($p<0.05$) indicate significant differences among sites. Tukey's test with no values are represented by (-).

Variable	**Source of Variation**	**p value**	**Tukey's test (p values)**		
			M vs P	**M vs V**	**V vs P**
No. of leaves	Sample	0.019			
	Site	<0.001	0.002	<0.001	<0.001
	Sample x site	<0.001			
Horizontal rhizome length	Sample	0.118			
	Site	<0.001	-	<0.001	<0.001
	Sample x site	-			
Vertical rhizome length	Sample	<0.001			
	Site	<0.001	1.000	<0.001	<0.001
	Sample x site	0.063			

Annex-C

Two-way ANOVA results of *C. nodosa* density and biomass, (Sample: 2 variables (CO_2 seeps, Reference) and Site: 3 levels (Milos (M), Paleochori (P) and Vulcano (V)) off Greece and Italy. Post hoc Tukey's test (p<0.05) indicate significant differences among sites. Tukey's test with no values were represented by (-).

Variable	**Source of Variation**	**p value**	**Tukey's test (p values)**		
			M vs P	**M vs V**	**V vs P**
Shoot density	Sample	0.020			
	Site	0.929	-	-	-
	Sample x site	0.262			
Apex density	Sample	<0.001			
	Site	0.008	0.183	0.005	0.309
	Sample x site	0.452			
Total biomass	Sample	0.038			
	Site	<0.001	0.185	<0.001	<0.001
	Sample x site	0.019			
Leaf biomass	Sample	0.045			
	Site	<0.001	0.969	<0.001	<0.001
	Sample x site	0.062			
Vertical rhizome biomass	Sample	0.272			
	Site	<0.001	0.071	<0.001	<0.001
	Sample x site	0.136			
Horizontal rhizome biomass	Sample	0.040			
	Site	<0.001	0.461	<0.001	<0.001
	Sample x site	<0.001			
Root biomass	Sample	0.657			
	Site	<0.001	0.974	<0.001	<0.001
	Sample x site	0.950			
Above: below ground biomass	Sample	<0.001			
	Site	<0.001	1.000	<0.001	<0.001
	Sample x site	<0.001			

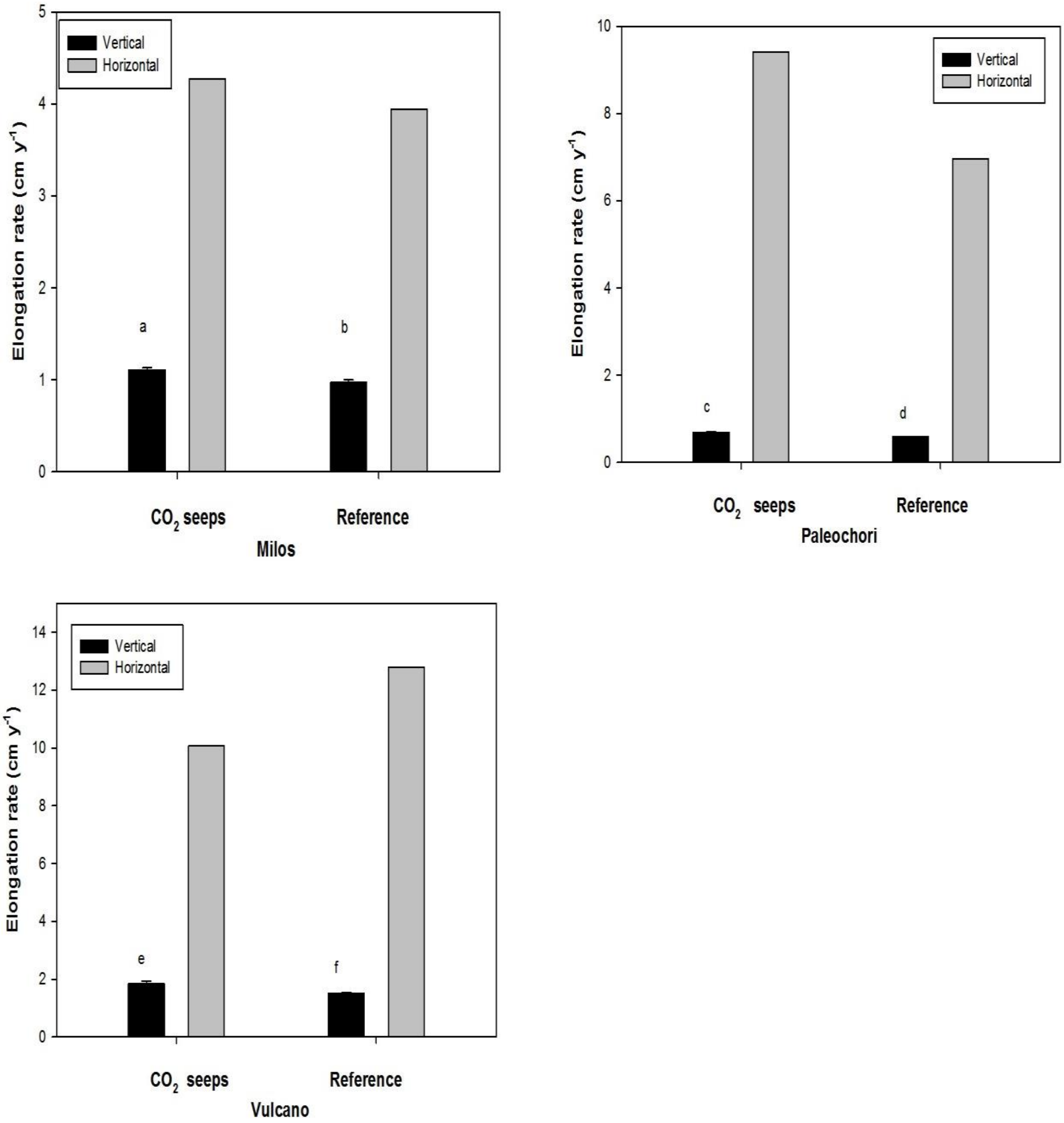
Annex-D

Two-way ANOVA results of *C. nodosa* carbon (C), nitrogen (N) and C: N ratios, (Sample: 2 variables (CO_2 seeps, Reference) and Site: 3 levels (Milos (M), Paleochori (P) and Vulcano (V)) off Greece and Italy. Post hoc Tukey's test ($p<0.05$) indicate significant differences among sites. Tukey's test with no values were represented by (-).

Variable	**Source of Variation**	**p value**	**Tukey's test (p values)**		
			M vs P	**M vs V**	**V vs P**
Leaves (%C)	Sample	0.023			
	Site (CO_2seeps)	<0.001	0.996	<0.001	<0.001
	Site (Reference)	<0.001	<0.001	<0.001	<0.001
	Sample x site	0.013			
Rhizomes (%C)	Sample	0.545			
	Site	0.196	-	-	-
	Sample x site	0.147			
Roots (%C)	Sample	0.176			
	Site (CO_2 seeps)	<0.001	<0.001	<0.001	0.235
	Site (Reference)	<0.001	0.300	<0.001	<0.001
	Sample x site	0.022			
Leaves (%N)	Sample	0.234			
	Site	<0.001	0.244	0.009	<0.001
	Sample x site	0.903			
Rhizomes (%N)	Sample	0.186			
	Site (CO_2 seeps)	0.042	0.043	0.289	0.003
	Site (Reference)	0.042	0.045	0.158	0.947
	Sample x site	0.003			
Roots (%N)	Sample	0.317			
	Site	0.897	-	-	-
	Sample x site	0.685			
Leaves (C: N)	Sample	0.608			
	Site	<0.001	0.003	0.711	0.002
	Sample x site	0.735			
Rhizomes (C: N)	Sample	0.059			
	Site (CO_2 seeps)	0.023	0.031	0.241	0.002
	Site (Reference)	0.023	0.073	0.143	0.999
	Sample x site	0.004			
Roots (C: N)	Sample	0.195			
	Site	0.343	-	-	-
	Sample x site	0.933			

Annex-E

Elongation rate of *C. nodosa* at CO_2 seeps and Reference sites off Greece and Italy. Error bars represent standard errors of vertical rhizome elongation rate. Different letters indicate significant differences between CO_2 seeps and reference sites.



Annex-F

Two-way ANOVA results of *C. nodosa* rhizome and leaf production, (Sample: 2 variables (CO_2 seeps, Reference) and Site: 3 levels (Milos (M), Paleochori (P) and Vulcano (V)) off Greece and Italy. Post hoc Tukey's test ($p<0.05$) indicate significant differences among sites. Tukey's test with no values were represented by (-).

Variable	**Source of Variation**	**p value**	**Tukey's test (p values)**		
			M vs P	**M vs V**	**V vs P**
Vertical rhizome production	Sample	0.002			
	Site	<0.001	0.032	<0.001	0.305
	Sample x site	0.096			
Horizontal rhizome production	Sample	<0.001			
	Site	<0.001	<0.001	<0.001	0.004
	Sample x site	-			
Annual leaf production	Sample	0.012			
	Site	<0.001	0.997	<0.001	<0.001
	Sample x site	0.583			
Vertical rhizome production rate	Sample	0.002			
	Site	<0.001	0.020	<0.001	0.328
	Sample x site	0.112			
Horizontal rhizome production rate	Sample	<0.001			
	Site	<0.001	<0.001	<0.001	0.006
	Sample x site	-			

Elevated trace elements in sediments and seagrass at six Mediterranean volcanic seeps

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(To be submitted)

Elevated trace elements in sediments and seagrass at six Mediterranean volcanic seeps

Abstract:

Volcanic CO_2 seeps are now being used as natural analogues for ocean acidification studies although these areas can be affected by trace element input and may alter ecosystem responses to gradient in carbonate chemistry. Here Fe and a range of trace elements (Cd, Co, Cu, Hg, Mn, Pb, Ni and Zn) were analysed from sediments and from the roots, rhizomes and leaves of seagrass at six CO_2 seeps and reference sites off Greece and Italy. There were higher metal levels in sediment and seagrasses at all CO_2 seeps than reference sites. Sediment Quality Guideline Quotient, a commonly used pollution index, indicated that some of the metals (Cd, Cu, Hg, Ni) were in high enough concentrations to have adverse biological effects, such as Cu at Ischia site and Hg at Vulcano. Higher accumulation of elements from sediments in roots and leaves at CO_2 seeps were found from Bio Sediment Accumulation Factor index. There were higher levels of Cu, Fe and Zn in leaves and rhizomes for *P. oceanica* and higher levels of Cd, Cu, Fe and Zn in *C. nodosa* compartments at CO_2 seeps. Fe was found with positive correlation within sediment-roots and sediment-rhizomes, whereas Cd and Pb were found with positive correlation in compartments of *C. nodosa*. In *P. oceanica* positive correlation were only observed for Cd within sediment-roots and plant compartments. Low pH and ocean acidification increased the concentration of elements at CO_2 seeps than reference sites. Thus, caution is needed, when using volcanic seep systems as analogue for the effects of rising CO_2, as metals can reach levels that are toxic to seagrass, masking any potential benefits of increased levels of carbon dioxide for seagrass productivity.

Keywords: ocean acidification, metal toxicity, coastal habitats, diving science, CO_2 seeps

4.1. Introduction

Earth's climate is undergoing significant changes due to anthropogenic CO_2 emissions (IPCC, 2014). Around 30% of these CO_2 emissions dissolve into the surface ocean causing the pH to fall and altering seawater carbonate chemistry in a process called ocean acidification (Caldeira and Wickett, 2003). These chemical changes have raised concerns about the knock-on effects on marine organisms (Doney et al., 2009; Fabricius et al., 2014). Ocean acidification is expected to reduce marine biodiversity and alter trophic interactions (Kroeker et al., 2013; Sunday et al., 2016) which will impact a range of ecosystem services (Lemasson et al., 2017).

There are concerns that changes in ocean carbonate chemistry will also exacerbate the harmful effects of metal pollution which is a widespread problem in coastal ecosystems (Ivanina et al, 2015; Lewis et al., 2016), particularly in the Mediterranean (Soliman et al., 2015). Ocean acidification is expected to increase the bioavailability and toxicity of elements like Cd, Cu, Ni, Zn by altering their speciation (Zeng et al.,2015). However, the ecological risk posed by the effects of ocean acidification on metal contaminated sediments is difficult to assess, due to the complex behaviour of trace elements in marine environment (Millero et al., 2009).

Trace elements occur naturally in very low concentrations in the environment and can be either essential (e.g. Co, Cu, Mn) or non-essential (e.g. Cd, Cs, Sr) to living organisms (Alloway, 1995). Trace elements such as arsenic, copper, lead and mercury can be harmful to biota in coastal waters (Stumm Morgan, 1995). The toxicity of these elements depends on their form, so arsenic, for example is toxic when found in its metalloid form, whereas mercury and lead are toxic as free ions and copper is toxic when reduced to Cu(I) and is widely used in antifouling paints for this reason (Tchounwou et al., 2014).

Submarine hydrothermal activity is of ecotoxicological interest as this can cause natural gradients in both ocean acidity and trace elements, offering opportunities to assess their combined effects (Monia Renzi et al., 2011; Kadar et al., 2012; Vizzini et al., 2013). Some trace elements become more toxic if seawater pH falls from 8.1 to 7.8, which is a concern since this is the shift expected in mean surface seawater pH this century. Lead and zinc both become more toxic over this range (Millero et al., 2009; Dong et al., 2016). Trace metal toxicity depends on concentration and speciation of the element as well as interactions at receptor sites that determine uptake by organisms (Batley et al., 2004). Increased seawater acidity as well as lower oxygen levels helps release of metals that are bound to sediments (Atkinson et al., 2007).

Metal toxicity increases at submarine hydrothermal vents with copper, for example, vent fluids lower the pH which releases non-toxic Cu (II) but this is then reduced to the toxic form Cu (I) as hydrothermal fluids are low in oxygen.

Decreases in seawater pH due to increases in pCO_2 levels can therefore increase the toxic concentration of certain metals in sediments (Roberts et al., 2013) and the water column (Millero et al., 2009). Ocean acidification is expected to increase the free ion concentration of Cu by 115% (Pascal et al., 2010; Richards et al., 2011), whereas the free ion concentration of other metals such as Cd may decrease or be unaffected (Lacoue-Labarthe et al., 2012; Pascal et al., 2010). Overall greater metal toxicity is predicted under ocean acidification (Lacoue-Labarthe et al., 2009; 2012; Pascal et al., 2010). Most studies on the bioavailability of trace metals at elevated CO_2 have been carried out in simplified laboratory conditions (Besar et al., 2008; Richir et al., 2013; Bravo et al., 2016) so there is limited understanding of the interplay between trace elements and ocean acidification in the field.

The behaviour, speciation and bioavailability of many trace elements in seawater is strongly dependent on seawater chemistry, with Cd, Co, Cu, Hg, Ni, Pb and Zn all known to be sensitive to speciation changes within the surface seawater pH range projected due to anthropogenic CO_2 emissions (Byrne et al., 1998; Richards et al., 2011). Most studies of the combined effects of ocean acidification and metal toxicities have been focused on marine fauna in laboratory settings (e.g. mussels and invertebrates) (Lewis et al., 2014; Shi et al., 2016). Lewis et al., (2014) observed toxic effects of Cu on sperm DNA damage of *Arenicola marina,* whereas Shi et al., (2016) observed damage of epithelia in *Mytilus edulis* due to Cd toxicity. There are opportunities to investigate the combined effects of metal toxicity on habitat forming primary producers since macroalgae and seagrasses can be abundant at CO_2 seeps (Apostolaki et al.,2014; Vizzini et al., 2010, 2014).

Shallow water volcanic seeps are widely used as natural analogues for future coastal ecosystems as they can have areas of seabed where entire communities of marine organisms are exposed to the shifts in carbonate chemistry that are expected due to continued anthropogenic CO_2 emissions (Hall-Spencer et al., 2008; Enochs et al., 2015; Connell et al., 2017). At such seeps, there are often elevated levels of hydrogen sulphide (H_2S) and trace elements (Kadar et al., 2012; Boatta et al., 2013; Vizzini et al., 2013). Thus, care is needed when using CO_2 seeps as analogues for ocean acidification due to confounding factors that may be harmful to marine biota (Barry et al., 2010). While relationships among organisms, environmental factors and trace elements have received much attention at deep sea hydrothermal vents (Kadar et al., 2007; Cravo et al., 2007), those of shallow marine CO_2 seeps are still little understood. Observations at multiple shallow CO_2 seeps show contrasting effects on seagrasses. These plants form lush stands at some seeps, e.g. off Ischia in the Mediterranean and at several sites around Papua New Guinea (Hall-Spencer et al., 2008; Russell et al., 2013). Yet at other CO_2 seeps seagrasses do poorly e.g. off Panarea and Vulcano in Italy, where H_2S and elevated trace elements are thought to be toxic to the plants (Vizzini et al., 2010, 2013).

Seagrass are important coastal habitats due to their high productivity and biodiversity (Thom, 2001). They provide various ecosystem services, such as food and nurseries for fish, turtles and mammals (Coles et al., 2007), they can be major carbon sinks and can sequester contaminants such as excess nutrients and metals (Orth et al., 2006; Fourqurean et al., 2012). Seagrass productivity is predicted to increase as CO_2 levels continue to rise if temperature increases do not become too stressful (Koch et al., 2013; Brodie et al., 2014). Seagrasses accumulate trace elements and so are used as bioindicator in coastal ecosystems (Catsiki and Panayotidis, 1993). The plants take in trace elements via the roots, rhizomes or the leaves and can translocate them between these tissue compartments (Ralph et al., 2006). The rate of uptake varies between essential and non-essential trace elements and between tissues and this introduces these substances into the food web via grazing and decomposition (Lewis and Devereux, 2009). For instance, *Cymodocea serrulata* transfers trace elements to detrital feeders (Klumpp et al., 1989).

Studies in laboratory mesocosms using high CO_2 have shown toxic effects of elevated concentrations of Cu, Pb and Zn on the physiology of the seagrasses *Zostera capricorni* (Ambo-Rappe et al., 2007) and *Halophila ovalis* (Ambo-Rappe et al., 2011). Although seagrass meadows are found at many CO_2 seeps around Greece and Italy (Hall-Spencer et al., 2008; Vizzini et al., 2010; Apostolaki et al., 2014) little is known about the influence of CO_2 seeps on seagrass trace metal contents. Vizzini et al., (2013) reported on Fe and trace element (As, Cd, Co, Cr, Cu, Hg, Mn, Mo, Ni, Pb, V and Zn) concentrations at Vulcano CO_2 seeps, where they found a wide variability in trace element concentrations in sediment and seagrass and reported on the possible bioavailability of harmful trace elements in areas with lower pH.

Here we expand on the work undertaken by Vizzini et al., (2013) to quantify the concentrations of Fe and trace elements, in sediments and seagrass at multiple seep sites around the Mediterranean. Our aim was to find out whether increased levels of trace elements near seeps correlate with increases in trace elements in seagrass roots, rhizomes and leaves. We also test the hypothesis that low pH will increase bioavailability of certain elements and we consider whether natural CO_2 seeps have potential for the study of ocean acidification on metal toxicity.

4.2. Methods

Study sites

We surveyed six sites in the Mediterranean Sea, all of which had seagrasses (*Posidonia oceanica or Cymodocea nodosa*) growing on sandy bottom in high salinity, high alkalinity low nutrient conditions (Table 1). At each location, we sampled a high CO_2 site and a reference site between May-July, 2014. The annual temperature range was around 18-22° C for all six sites and the CO_2 seeps were at 0-10 m depth with a tidal range of 0.30-0.50 m.

Vulcano, Italy

We sampled Levante Bay (38.4 N, 15.0 E) off Vulcano island (Fig. 1A). Some parts of this bay are well-suited for studies of the effects of increased CO_2 levels (Boatta et al., 2013) despite areas with elevated H_2S and metals (Vizzini et al., 2013). The main underwater gas seeps are located along southern and western shores of the bay at <1 m depth (Boatta et al., 2013). Total CO_2 output is about 3.6 tonne d^{-1} (Inguaggiato et al., 2012), and the underwater gas emissions are 97-98% CO_2 with 2.2% H_2S close to the seeps, decreasing to less than 0.005% H_2S towards the north-eastern part of the bay, where most ocean acidification research has been located (Capaccioni et al., 2001; Milazzo et al., 2014). There was a step gradient in carbonate chemistry with pH 5.65 at the main gas seeps increasing to pH 8.1, which is typical for present day Mediterranean surface seawater, at >350 m from the seeps (Boatta et al., 2013). *Cymodocea nodosa* was absent at the main vents so we collected it on the periphery of the CO_2 seeps at 1 m depth.

Ischia, Italy

At the Castello Aragonese, off Ischia (40°43'50.4"N; 13°57'48.2"E) CO_2 bubbles up from the seabed in shallow water (Fig.1A). The gas is 90–95% CO_2, 3–6% N_2, 0.6–0.8% O_2, 0.2–0.8% CH_4 and 0.08–0.1% air and at ambient temperature without sulphur (Tedesco, 1996). There is a pH gradient from 6.57 to 8.17 along a 300m stretch of rocky shore with a very shallow (0.5 m depth) *Posidonia oceanica* meadow (Hall-Spencer et al., 2008) where we took our samples (Fig.2a).

Panarea, Italy

Panarea island (38°38'12.2"N; 15°06'42.5"E) is to the east of the Aeolian Archipelago in the Southern Tyrrhenian Sea (Fig.1A). It is twelve miles south of the active Stromboli volcano, and emerges from a caldera of about 460km^2 that rises one thousand metres above the surrounding seabed (Gabianelli et al., 1990). On the main island and on the surrounding seafloor, tectonic faults have many gas seeps (Gabianelli et al., 1990; Voltattorni et al., 2009). These seeps have been used in several studies of the effects of CO_2 on the marine environment as well as field laboratories for the validation of monitoring and sampling techniques (Caramanna et al., 2011; Gofreddo et al., 2017). Here *Posidonia oceanica* was sampled at 5m depth.

Milos Islands, Greece

Paleochori Bay (36.67 N, 24.51 E) and Milos thermal stations (36.70 N, 24.46 E) are part of Milos island (Fig.1B). Extensive submarine venting occurs offshore, from the intertidal to depths of more than 100 m over a 34 km^2 area of seabed (Dando et al.,1999). The released gases are 95% CO_2 with some H_2S, CH_4 and H_2 (Dando et al., 1999). *Cymodocea nodosa* meadows were sampled at 4m depth at both stations (Fig.2b)

Methana, Greece

The Methana peninsula (37.638428 N; 23.359730 E) is the westernmost volcanic system of the northern Aegean Volcanic Arc (Fig.1B), derived from the subduction of the African tectonic plate beneath the Eurasian plate. We sampled the area described by Baggini et al., (2014) near Agios Nikolaos village on the NE part of the peninsula. The gases were 90% CO_2, with small amounts of nitrogen, carbon monoxide and methane (D'Alessandro *et al.*, 2008). Here we sampled *Posidonia oceanica* meadows at 8-10 m depth.

Water sampling

Water samples (n=5) were collected at each CO_2 seep and Reference site in 100 ml Winkler bottles fixed with 20 µl mercuric chloride in the field, stored in dark cool- boxes and transported to the laboratory for total alkalinity (TA) analysis. The pH_{NBS} (using pH meter, Titrino Methron) and temperature of the water samples were measured in the field immediately after collection. In the laboratory 80 ml water samples were analysed for TA using a Lab Titrino analyser following methods given by Dickson et al., (2007). Temperature, pH_{NBS} and TA data were used to calculate pCO_2 using CO_2SYS program following methods given by Pierrot and Wallace, (2006).

Sediment & seagrass sampling

Sediment samples were collected from CO_2 seep and Reference sites by SCUBA diving using a 10-cm long syringe with the tip cut off to collect the upper 5 cm of sand. The sediment samples were in Teflon tubes in dark boxes then analysed for the grain size following dry sieving at Half Phi intervals (Blott and Pye, 2001).

Samples of *Cymodocea nodosa* (from Vulcano, Milos and Paleochori islands) and *Posidonia oceanica* (from Ischia, Panarea and Methana) were collected by SCUBA diving at up to 10m depth at the CO_2 seep and Reference sites. The seagrasses were rinsed to remove sediments, air-dried and stored in polybags until analyses. *Cymodocea nodosa* and *Posidonia oceanica* leaves were scraped to remove epiphytes. Sediment samples and seagrass leaves, roots and rhizomes were oven dried at 40°C and powdered in a mortar.

Analytical Methods

Total trace element concentrations were determined by Aqua Regia Soluble Total method. Dried sediment (0.25 g) was put into digestion tubes (Tecator type). Cold and concentrated acids in the order: 4.5 mL Hydrochloric acid (HCl): 1.5 mL Nitric acid (HNO_3) was added to the tubes. The digestion tubes were left to pre-digest, for one hour then heated for 2 hours at 95 - 100°C. After cooling, the digest was filtered quantitatively into a volumetric flask and diluted using 2% HNO_3 (25 ml volume).

For dried seagrass (leaves, rhizomes and roots) samples, 0.25g of sample was dissolved in 6mL of HNO_3 and the volume made up to 25ml. Similarly, blanks and standards (Laboratory of the Government Chemist (LGC Reference Materials, UK) used for sediments and plants were prepared using the same method. Analysis of Cd, Co, Cu, Fe, Hg, Mn, Pb and Zn was performed using an ICP-MS (Thermo Scientific, iCAP 7000 Series) and an ICP-AES (Thermo Scientific, X Series-2) in duplicate.

Data Analysis

To assess the sediment quality of all six locations we used Sediment Quality Guidelines Quotient (SQG-Q, Long and MacDonald, 1998). Among the environmental quality indices in the literature, this was chosen for its simplicity, comparability and robustness as reported by Caeiro et al., (2005). The SQG-Q consists of two values: a threshold effects level (TEL) and a probable effect level (PEL) (MacDonald et al., 1996). TEL represents concentrations below which adverse biological effects are unlikely.

The SQG-Q was calculated as follows:

$$\text{SQG-Q} = (\Sigma^{n}{}_{i=1} \text{ PEL-Q}_i)/ \text{ n}$$

Where PEL-Q_i = contaminant/PEL. The PEL-Qi represents the probable effect level quotient (PEL-Q) of the i contaminant. Using the SQG-Q index, we put sediments into three categories as established by MacDonald et al. (2000). SGQ-Q $\leq$ 0.1- low potential for adverse biological effects; 0.1< SQG-Q<1- moderate potential for adverse biological effects; SQG-Q$\geq$1- high potential for adverse biological effects. Our data were compared with a biological effect database for sediments (BEDS) developed by Mac Donald et al. (1996), which comprises of 350 reports on the analysis of toxicity and/or benthic community composition of marine and estuarine sediments.

To understand bio-accumulation of elements, we calculated the Bio Sediment Accumulation Factor (BSAF), which is defined as the ratio between metal concentration in the organism and that in the sediment (Lau et al., 1998; Szefer et al., 1999), given by:

$$\text{BSAF} = \text{Mp} / \text{Ms}$$

Where Mp is the concentration of the element in the seagrass and Ms is the concentration of the element in the sediment (Fergusson. 1990). BSAF is an important factor in understanding how the increase or decrease of trace element concentrations affects the fate of trace elements in the plant compartments. Roots (the usual point of uptake) and leaves (the usual point of release) of trace elements (Weis & Weis, 2004) were used for BSAF analysis.

Statistics

A three-way ANOVA was used to analyse the variability in trace element concentration among plant compartments (leaves, rhizomes, roots), sediments and sites (reference, CO_2 seeps) between three locations (Ischia, Panarea and Methana) for *Posidonia oceanica* and Milos, Paleochori and Vulcano for *Cymodocea nodosa*. All data was pre-checked for normality and homogeneity of variances, when variances were not homogenous, data was ln(x+1) transformed. After transformation, some data was still not showing homogeneity of variances. In these cases, we analysed the data untransformed, since ANOVA is considered robust to lack of homogeneity of variances with balanced designs and considerable large amount of treatments (Underwood, 1997). In some cases, ANOVA main effect was difficult to interpret due to the presence of statistically significant interactions, but in mixed effect ANOVA's the test of the fixed main effect is potentially interpretable even in the presence of interaction (Quinn and Keough.2002). Holm-Sidak test was performed for a *posteriori* comparisons among levels to check significant main effects in ANOVA. Pearson's correlation co-efficient was applied to identify correlations between trace element concentration in sediment and seagrass compartments, after testing for normality of distribution on raw or log transformed data. When normality was not achieved, non-parametric Spearman's rank correlation coefficient was applied. All statistical tests were conducted with a significance level of $\alpha = 0.05$ and data were reported as mean ± standard error (SE).

4.3. Results

As expected pCO_2 concentration were highest (and pH lowest) at each of the seeps whereas reference sites had normal CO_2 and pH levels (Table 1). Salinity, temperature and total alkalinity were not affected by the seeps.

Grain size analysis showed that 99% of the particles sampled at all locations were sand in the very coarse, coarse, medium and fine fractions. Sediment samples from the seeps usually had higher levels of iron and several trace elements than at reference sites, but most were in such low concentrations that they were unlikely to be toxic (Table 2). Exceptions were Hg at Vulcano, Cu at Ischia as well as Ni and Cd at Paleochori (Table 2). These metals were at high enough concentrations in sediments at the seeps to likely have adverse biological effects on biota such as seagrasses. Fe, Co and Mn are excluded from these results as they have no SQG-Q values (Mac Donald et al., 1996) to compare with our results.

Table 1: Seawater salinity, temperature, total alkalinity, pH and $p\mathrm{CO_2}$ values (mean ± SE, n=5) at six Mediterranean $\mathrm{CO_2}$ seeps.

Site	**Salinity (psu)**	**Temp.(°C)**	**$\mathrm{pH_{NBS}}$**	**TA (µmol Kg $\mathrm{SW^{-1}}$)**	**$p\mathrm{CO_2}$**
Vulcano					
Reference	35.8	21.6	8.17 ± 0.05	2439	427 ± 6.8
$\mathrm{CO_2}$ seep	35.8	22.4	7.98 ± 0.08	2432	2928 ± 15.8
Ischia					
Reference	35.6	17.7	8.19± 0.06	2596	428± 2.3
$\mathrm{CO_2}$ seep	35.7	17.8	7.78±0.05	2589	1653± 10.2
Panarea					
Reference	36.0	20.5	8.18±0.05	2507	420± 4.6
$\mathrm{CO_2}$ seep	36.0	22.3	7.47±0.04	2500	3370± 2.3
Milos					
Reference	36.7	22.6	8.2 ± 0.03	2715	405.5 ± 1.6
$\mathrm{CO_2}$ seep	36.7	23.5	7.5 ± 0.04	2704	2457.9 ± 1.8
Paleochori					
Reference	36.0	22.6	8.2 ±0.01	2711	402.9 ± 1.1
$\mathrm{CO_2}$ seep	36.0	22.8	7.9 ±0.01	2706	1884.3 ± 3.0
Methana					
Reference	36.8	22.8	8.2±0.01	2715	460 ± 6.9
$\mathrm{CO_2}$ seep	36.8	23.0	7.8±0.02	2704	1980± 4.4

Table 2. Sediment Quality Guidelines-quotient (SQG-Q) of sediment calculated with Probable Effects Level for Reference and CO_2 seep sites in Greece and Italy. SQG-Q <0.1 (low effect), <0.1 SQG-Q>1 (moderate effect), SQG-Q>1 adverse biological effects. Numbers and letters in bold indicate possible adverse effects of trace elements.

		SQG-Q		**Effects**	
Location	**Element**	**Reference**	**CO_2 seeps**	**Reference**	**CO_2 seeps**
Vulcano	Cu	0.08	0.33	Low	Moderate
	Hg	0.32	**1.18**	Moderate	**Adverse**
	Ni	0.13	0.21	Moderate	Moderate
	Zn	0.09	0.13	Low	Moderate
Ischia	Cu	0.93	**1.06**	Moderate	**Adverse**
	Hg	0.64	0.86	Moderate	Moderate
	Pb	0.11	0.13	Moderate	Moderate
	Zn	0.12	0.10	Moderate	Moderate
Panarea	Cd	0.10	0.16	Low	Moderate
	Cu	0.06	0.11	Low	Moderate
	Hg	0.79	0.84	Moderate	Moderate
	Ni	0.03	0.18	Low	Moderate
	Pb	0.09	0.57	Low	Moderate
	Zn	0.05	0.12	Low	Moderate
Milos	Cd	0.21	0.21	Moderate	Moderate
	Ni	0.31	0.41	Moderate	Moderate
Paleochori	Cd	0.04	**1.84**	Low	**Adverse**
	Ni	0.71	**1.01**	Moderate	**Adverse**
Methana	Ni	0.11	0.16	Moderate	Moderate
	Pb	0.05	0.42	Low	Moderate

Table 3. Three-way ANOVA differences in Fe and trace element levels between Sample:2 variables (CO_2 seeps, Reference)) Site: 3 levels (Methana (M), Panarea(P) and Ischia (V)) and compartments :4 levels (Sediments (Sd), Rhizomes (Rh), Roots (R), Leaves (L)). Holm-Sidak significant test ($p<0.05$) is presented for locations, sediment versus plant compartments and *P. oceanica* compartments. Numbers (in bold) indicate differences that were not significant.

			Holm-Sidak p values								
			Location			**Sediment vs Compartment**			**Compartments**		
Element	**Variation**	**p value**	**M vs P**	**M vs V**	**V vs P**	**Sd vs R**	**Sd vs Rh**	**Sd vs L**	**R vs Rh**	**Rh vs L**	**R vs L**
Fe	Sample	<0.001	<0.001	<0.001	<0.001						
	Site	<0.001									
	Compt.	<0.001				<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Cd	Sample	<0.001	<0.001	<0.001	<0.001						
	Site	<0.001									
	Compt.	<0.001				<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Cu	Sample	<0.001	<0.001	<0.001	<0.001						
	Site	<0.001									
	Compt.	<0.001				<0.001	<0.001	**0.314**	<0.001	<0.001	<0.001
Ni	Sample	<0.001	<0.001	<0.001	<0.001						
	Site	<0.001									
	Compt.	<0.001				<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Pb	Sample	<0.001	<0.001	<0.001	<0.001						
	Site	<0.001									
	Compt.	<0.001				<0.001	<0.001	<0.001	<0.001	<0.001	**0.652**
Zn	Sample	<0.001	<0.001	<0.001	<0.001						
	Site	<0.001									
	Compt.	<0.001				**0.222**	<0.001	<0.001	<0.001	<0.001	<0.001

Table 4. Three-way ANOVA differences in Fe and trace element levels between Sample:2 variables (CO_2 seeps, Reference) Site: 3 levels (Milos (M), Paleochori (P) and Vulcano (V)) and compartments: 4 levels (Sediments (Sd), Rhizomes (Rh), Roots (R), Leaves (L). Holm-Sidak significant test ($p<0.05$) was significant is presented for locations, sediment versus plant compartments and *C. nodosa* compartments. Numbers (in bold) indicate differences that were not significant.

			Holm-Sidak p values								
			Location			**Sediment vs Compartment**			**Compartments**		
Element	**Variation**	**p value**	**M vs P**	**M vs V**	**V vs P**	**Sd vs R**	**Sd vs Rh**	**Sd vs L**	**R vs Rh**	**Rh vs L**	**R vs L**
Fe	Sample	<0.001	<0.001	<0.001	<0.001						
	Site	<0.001									
	Compt.	<0.001				<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Cd	Sample	<0.001	<0.001	<0.001	<0.001						
	Site	<0.001									
	Compt.	<0.001				<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Cu	Sample	<0.001	**0.626**	<0.001	<0.001						
	Site	<0.001									
	Compt.	<0.001				<0.001	**0.621**	<0.001	<0.001	<0.001	<0.001
Ni	Sample	<0.001	<0.001	**0.853**	<0.001						
	Site	<0.001									
	Compt.	<0.001				<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Pb	Sample	<0.001	<0.001	<0.001	**0.253**						
	Site	<0.001									
	Compt.	<0.001				<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Zn	Sample	<0.001	<0.001	<0.001	<0.001						
	Site	<0.001									
	Compt.	<0.001				<0.001	<0.001	<0.001	<0.001	**0.910**	<0.001

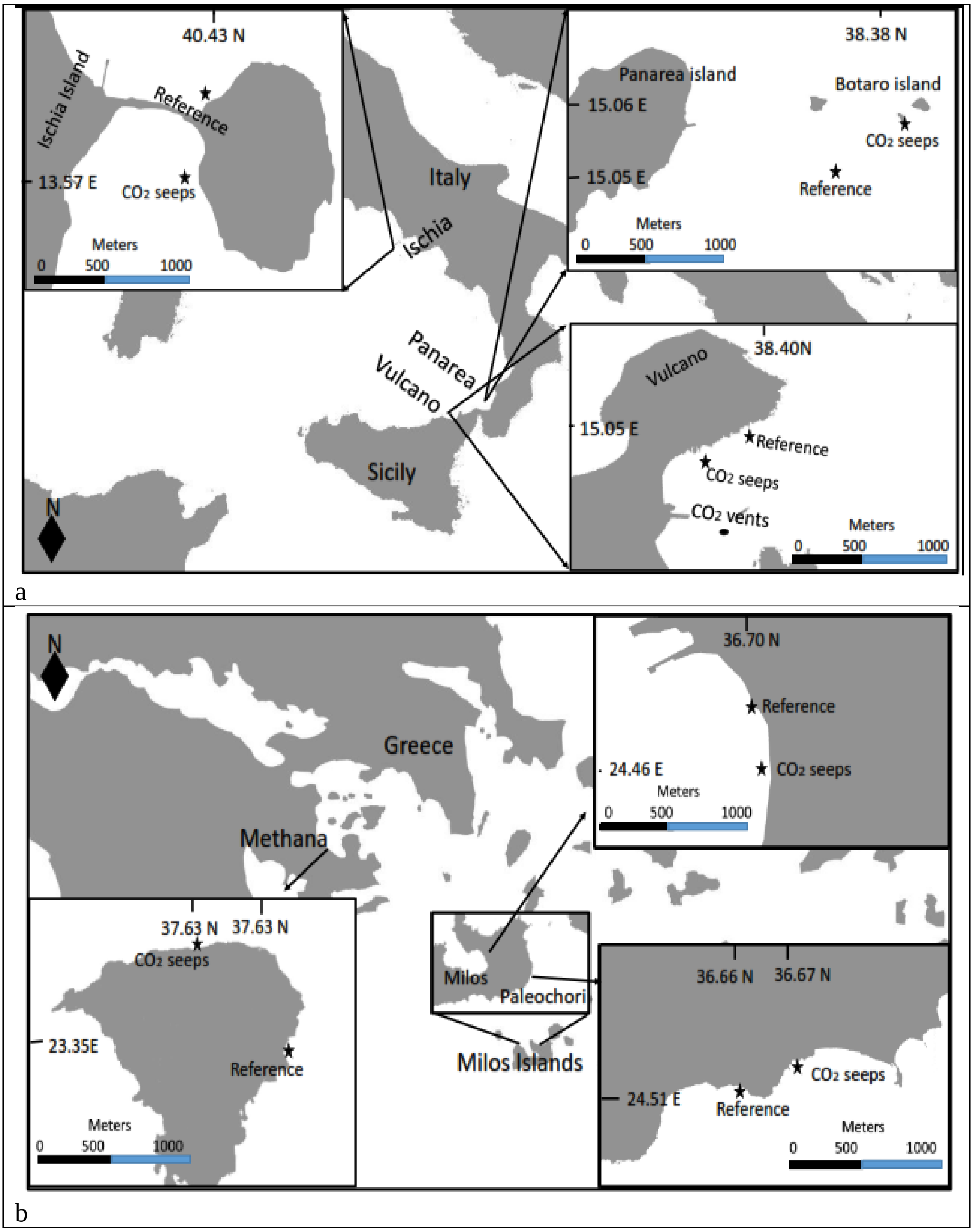


Fig.1. Study sites in Italy a) and b) Greece, showing reference and CO_2 seep sites which were all sampled between May to July 2014.

Fig. 2. a) *Posidonia oceanica* and b) *Cymodocea nodosa* meadows at CO_2 seeps off Ischia (Italy) and Paleochori (Greece)

Fe and trace element levels within sediments at all six CO_2 seeps were higher at seeps than reference sites, except at Methana (Figs. 3 and 4). Wide variation in concentration levels of Fe and trace elements were observed at all CO_2 seeps. Paleochori seep sediments were more significantly enriched with Cd than rest of the seeps. Sediments off Ischia had the highest Cu concentration among all seep locations, but this higher concentration was not reflected in seagrass compartments, so this copper may not be bioavailable. Mercury was only found in seep sediments off Italy, with highest level observed at Vulcano. Sediments at Paleochori seeps had a significantly higher concentration of Ni than at reference site, whereas all other CO_2

seeps had lower levels. Zinc concentration in sediments were similar at Vulcano, Milos, Ischia and Panarea seeps, but were low at Panarea. Levels of Pb were high and significant in the sediments of Ischia CO_2 seeps than all other seeps, but this was not reflected in the seagrass compartments (Figs.3 and 4).

Fe was significantly different within with the roots, rhizomes and leaves in *P. oceanica* at all three CO_2 seeps except in leaves at Ischia and Panarea (Fig.3). Fe was observed with highest concentration followed by Cu and Zn in *P. oceanica* compartments (Fig.3). Trace element concentration were high and significant within roots (Cd, Cu, Hg and Zn), rhizomes (Cd, Cu, Hg, Ni and Zn) and leaves (Cu, Hg and Zn) of *P. oceanica* at Ischia than Panarea and Methana respectively (Fig.3). Variation in concentration of Fe and trace elements within location (Ischia vs Panarea, Ischia vs Methana and Methana vs Panarea) were significant at all three locations (Table 3). The concentration of elements between sediments and those found in plant compartments were significantly different at all three locations except for Cu (sediment-leaves) and Zn (sediment-roots). Within *P. oceanica* compartments all elements analysed were significantly different except for Pb (roots-leaves) at all three seeps (Table 3). The variation in concentration of elements were higher in roots followed by leaves and rhizomes in *P. oceanica* at the CO_2 seeps off Ischia, Panarea and Methana (Fig.3).

Iron levels were significantly different within the roots, rhizomes and leaves of *C. nodosa* at Vulcano, in rhizomes at Paleochori and in roots at Milos CO_2 seeps (Fig. 4). Trace elements concentration were high and significant within the roots (Cd, Hg, Ni and Zn), rhizomes (Hg and Zn) and leaves (Cd, Cu, Hg, Ni and Zn) of *C. nodosa* at Vulcano than at Milos and Paleochori (Fig.4). Variation in Fe and trace elements concentration within locations (Milos vs Paleochori, Paleochori vs Vulcano, Vulcano vs Milos) was significant except for Cu at Milos vs Paleochori, Ni at Vulcano vs Milos (Table 4). Element concentrations found in sediment and that in *C. nodosa* compartments were different significantly except for Cu (sediment vs rhizomes) at all three locations (Table 4). Within the plant compartments (roots vs rhizomes, rhizomes vs leaves, roots vs leaves) Fe and trace elements concentration were significantly different except Zn (rhizomes vs leaves) and Cd (rhizomes vs leaves) at all three locations (Table 4). For *C. nodosa,* variation in concentration of elements were high and significant in roots followed by rhizomes and leaves off Milos, Paleochori and Vulcano CO_2 seeps (Fig.4). Iron had the highest concentration followed by Cu and Ni in *C. nodosa* compartments (Fig. 4)**.**

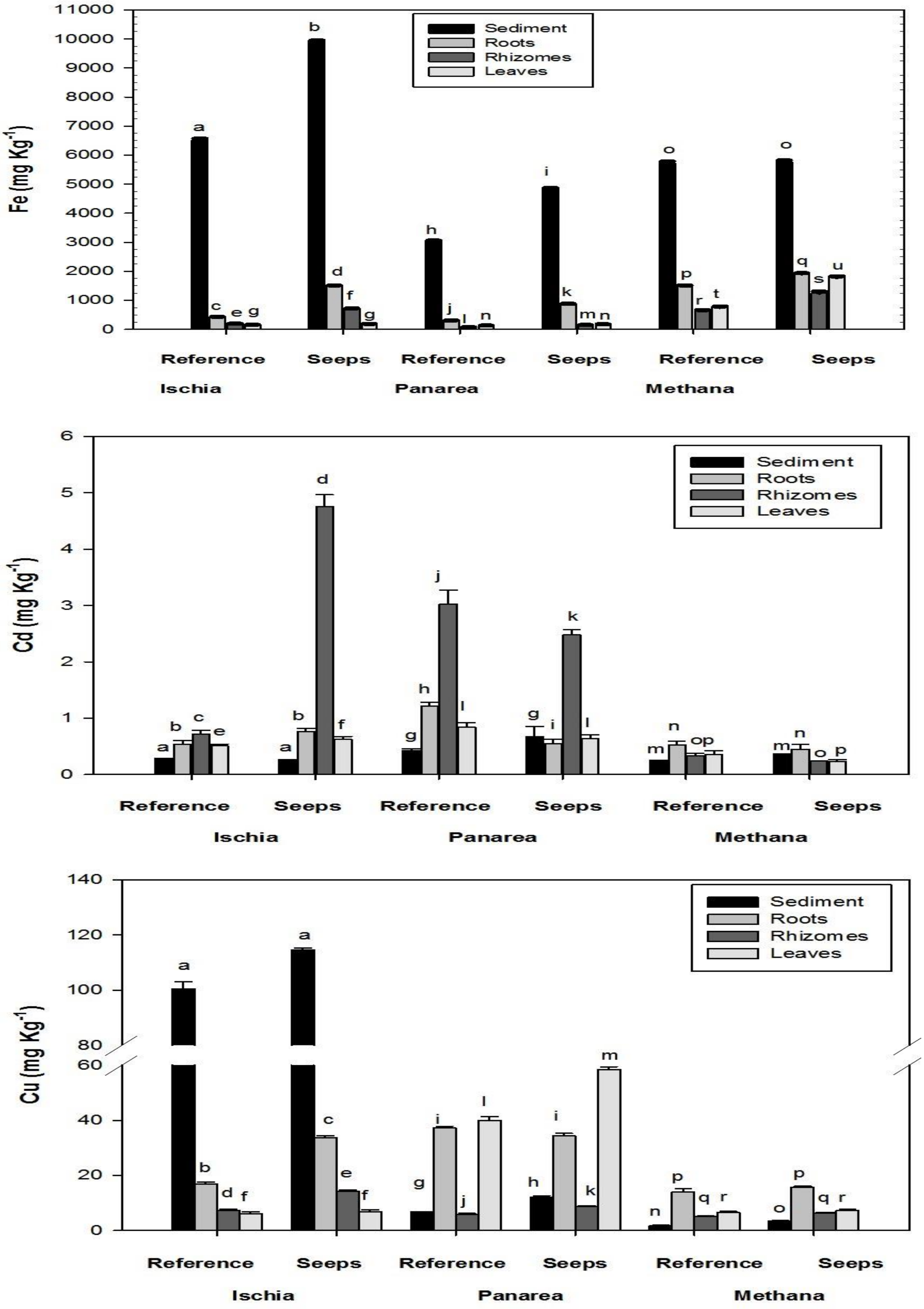
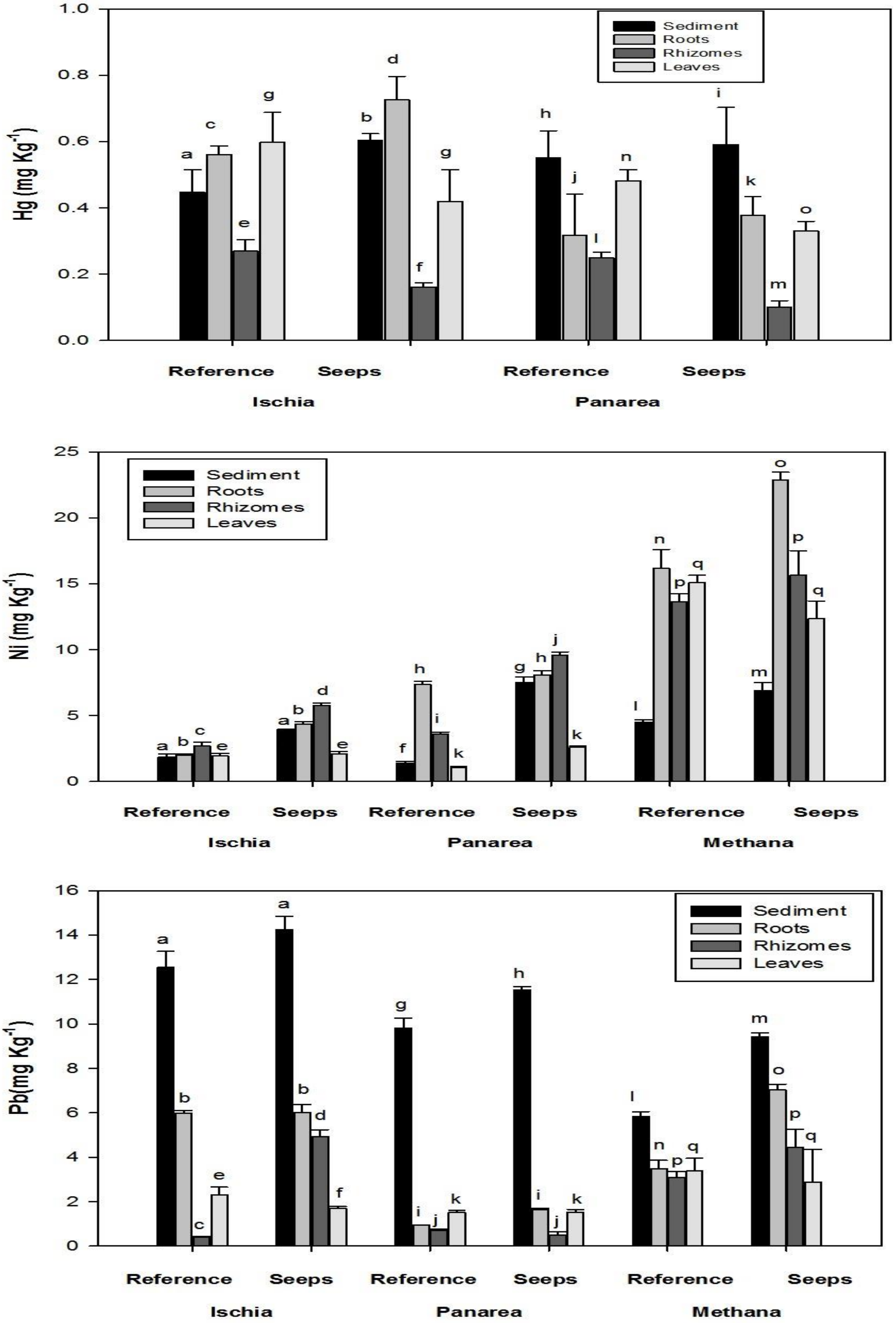


Fig. 3. continued



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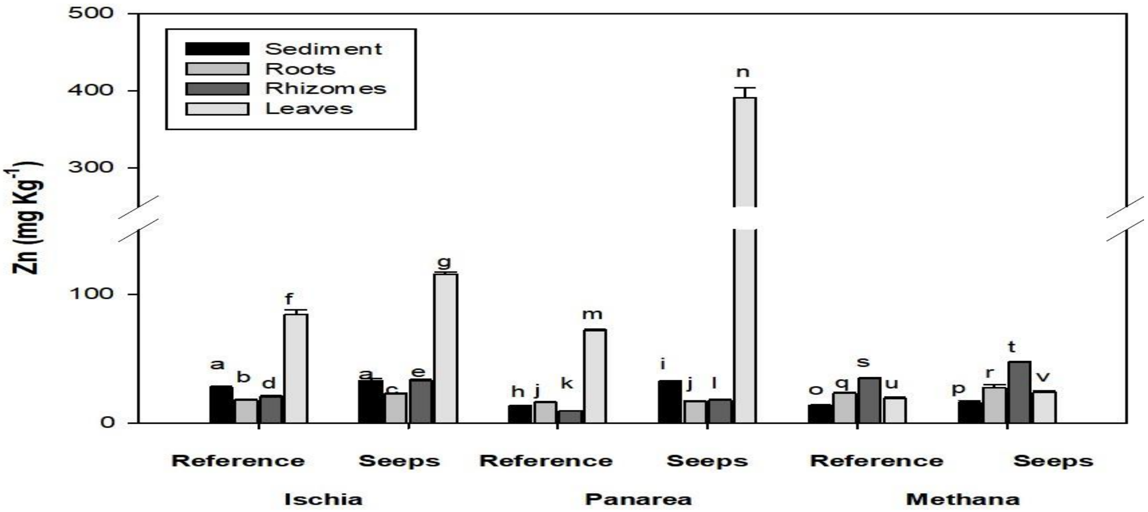
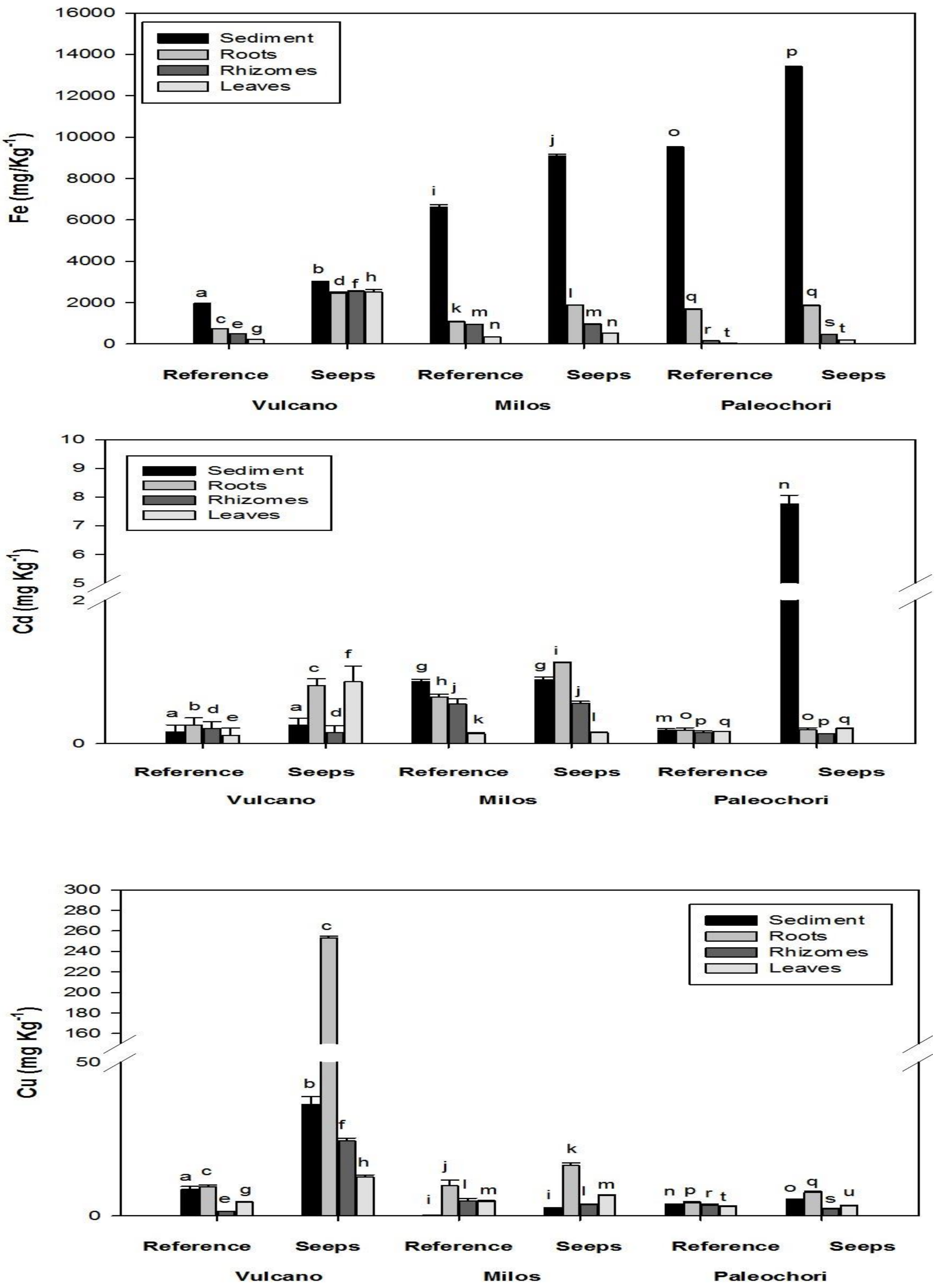


Fig. 3. Element concentrations (mean ± SE, n=5) for Fe, Cd, Cu, Hg, Ni, Pb and Zn for *Posidonia oceanica* in sediments and within plant compartments at reference and CO_2 seep sites off Italy and Greece. Error bars represent standard error. Different letters indicate significant difference between reference and CO_2 seeps site for each location.



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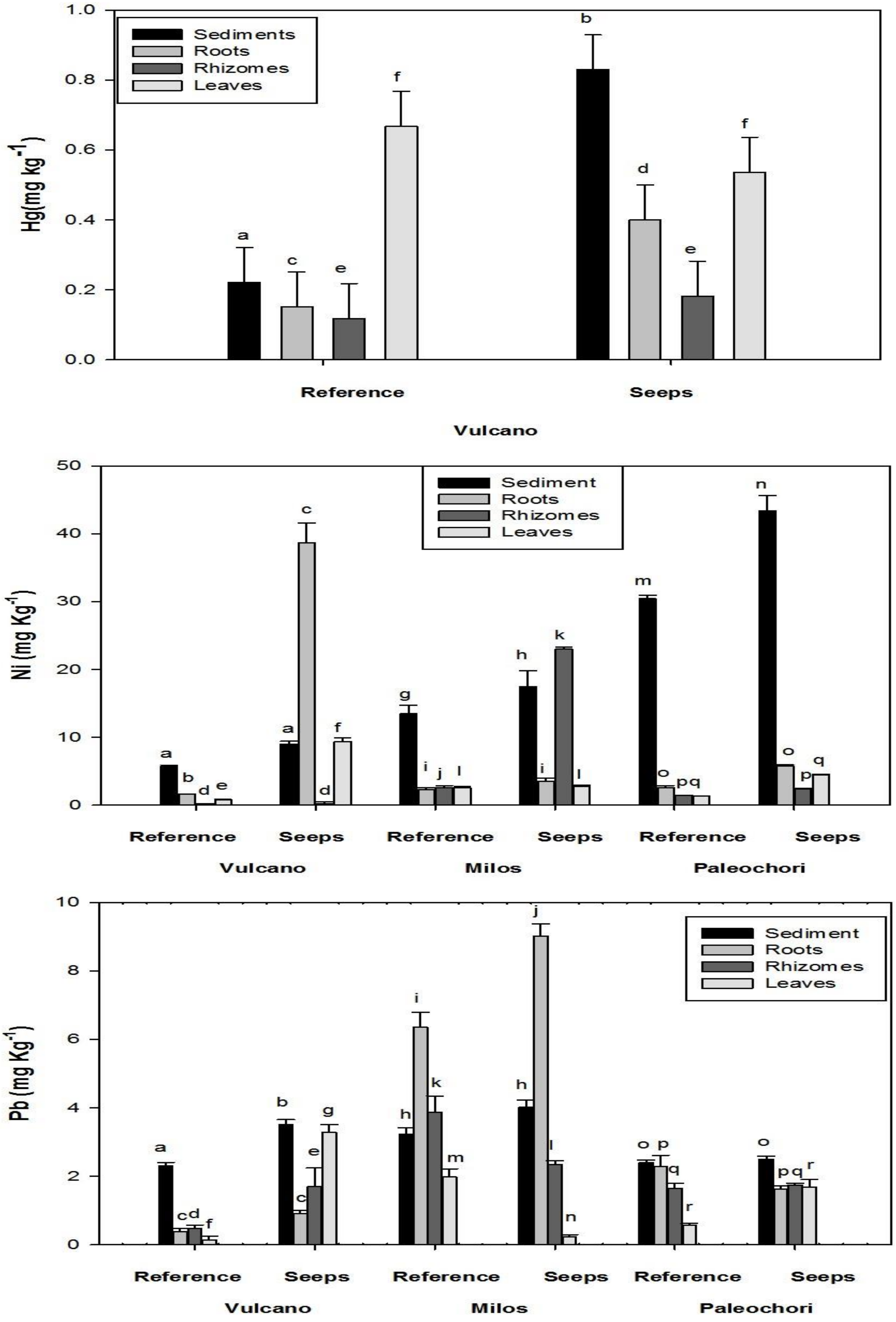

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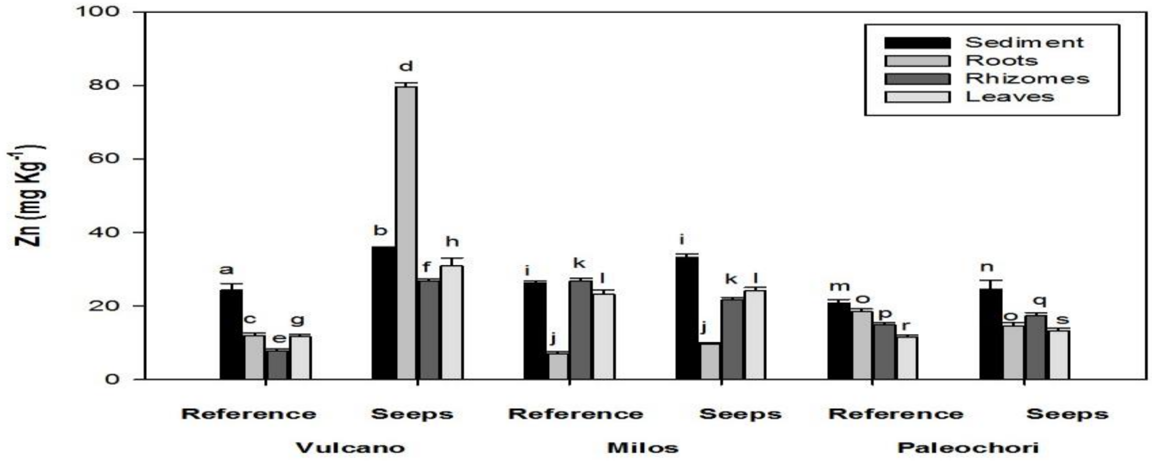
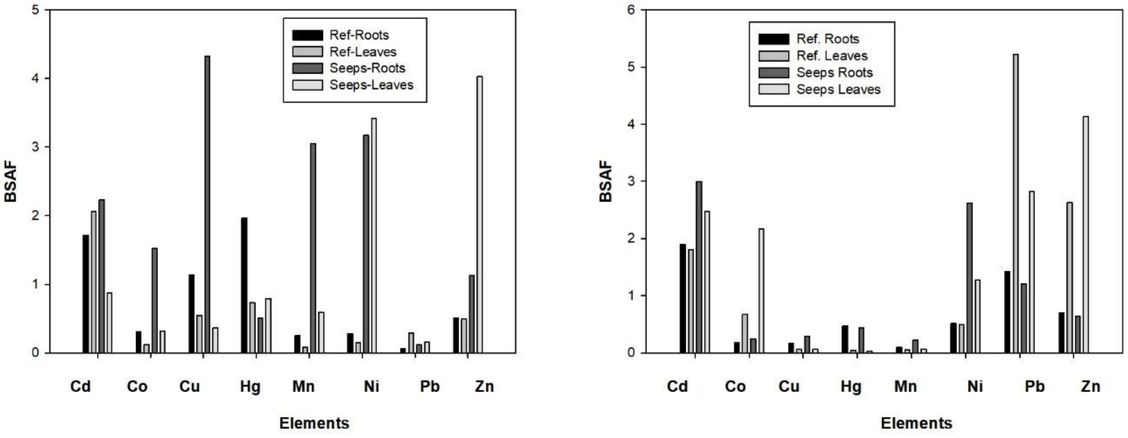
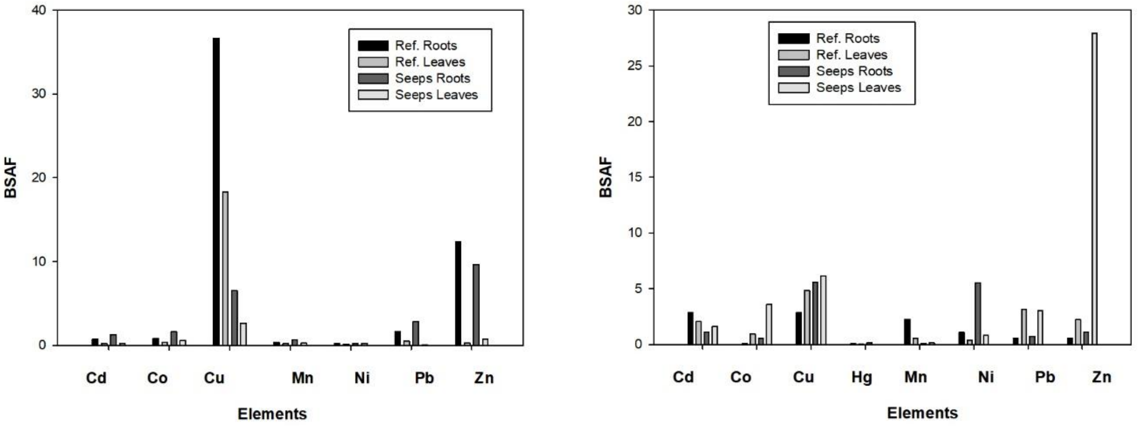


Fig. 4. Element concentration (mean ± SE, n=5) for Fe, Cd, Cu, Hg, Ni, Pb and Zn for *Cymodocea nodosa* in sediments and within plant compartments at reference and CO_2 seeps off Italy and Greece. Error bars represent standard error. Different letters indicate significant difference between reference and CO_2 seep sites for each location.



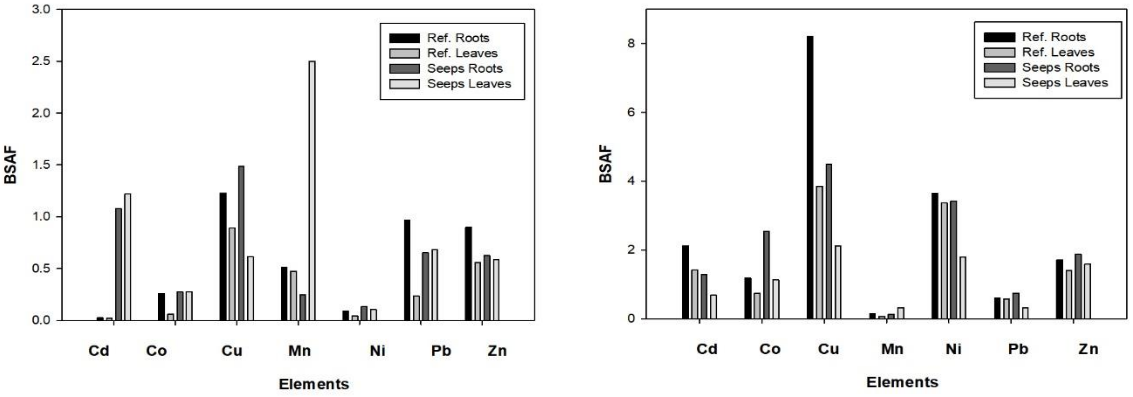
a. Vulcano

d. Ischia



b. Milos

e. Panarea



c. Paleochori

f. Methana

Fig. 5. Mean values of Bio-Sediment Accumulation Factor of trace elements for *Cymodocea nodosa* at reference and CO_2 seeps at Vulcano (a), Milos (b) and Paleochori (c) and for *Posidonia oceanica* at Ischia (d), Panarea (e) and Methana (f) at Italy and Greece respectively. Reference sites are represented as Ref. and CO_2 seeps as seeps for each location.

Bio Sediment Accumulation Factor index indicated that both *C. nodosa* and *P. oceanica* roots and leaves accumulated higher trace element concentrations than those found in sediments at CO_2 seeps, though different element was accumulated by each seagrass. For *P. oceanica* mean BSAF>1 was observed in roots and leaves at Panarea and Methana and in leaves at Ischia (Fig.5). In *P. oceanica* Cd, Cu, Hg, Ni, Pb and Zn were observed with BSAF>1 in the roots or leaves at all three CO_2 seeps. For *C. nodosa* BSAF>1 were observed for roots at Milos and Paleochori and for leaves at Vulcano. Trace elements (Cd, Cu, Ni and Zn) were observed with BSAF >1 in the roots and leaves of *C. nodosa* at all three CO_2 seeps (Fig.5). Most elements (4/9) with BSAF>1 were found at CO_2 seeps off Vulcano and Panarea for *C. nodosa* and *P. oceanica* respectively (Fig.5).

Correlations between metal content in sediments and those recorded in *P. oceanica* compartments were significant and positive for Zn and Ni in rhizomes at Ischia and Panarea seeps respectively (Table 5). Correlations were negative for Ni and Cd in rhizomes at Methana and Panarea seeps respectively (Table 5). The correlation for element content between any two organs of *P. oceanica* were positive and significant for Cd and negative for Cu at Ischia CO_2 seeps (Table 5).

Correlations of Fe content in sediment and those found in rhizomes of *C. nodosa* at Vulcano were significant and positive, whereas significant and negative correlation were observed for Zn content found between sediment and rhizomes and leaves (Table 6). For any two plant organs Cd was found with positive co-relation at Vulcano, where significant and negative correlation was observed for Cu and Hg at Vulcano and Fe and Cu at Milos (Table 6).

Correlation between elements and plant compartments of both seagrass indicate an absence of common trend for these elements between the roots, rhizomes and leaves of seagrasses at CO_2 seeps.

Table 5. Correlation between trace elements in sediments versus *P. oceanica* roots, rhizomes and leaves and plant compartments at Mediterranean CO_2 seeps. The correlation co-efficient (r) and significance level (p) are presented. Numbers in bold indicate significant correlation, only trace elements with significant correlations are shown

		Sediment-roots		**Sediment-rhizomes**		**Sediment-Leaves**	
Location	**Element**	r	p	r	p	r	p
Ischia	Zn	-0.234	0.704	0.870	**0.048**	0.321	0.598
Panarea	Cd	0.841	**0.014**	-0.910	**0.032**	-0.064	0.918
	Ni	-0.358	0.554	0.884	**0.046**	-0.787	0.114
		Seagrass compartments					
		Roots-Rhizomes		Roots-Leaves		Rhizomes-Leaves	
Ischia	Cd	0.300	0.683	0.975	**0.016**	0.359	0.517
	Cu	-0.273	0.657	0.877	**0.049**	-0.577	0.308

Table 6. Correlation between trace elements in sediment versus C. *nodosa* roots, rhizomes and leaves and between plant compartments at Mediterranean CO_2 seeps. The correlation co-efficient (r) and significance (p) level are presented. Numbers in bold indicate significant correlations, only trace elements with significant co-relation are shown

		Sediment-roots		**Sediment-rhizomes**		**Sediment-Leaves**	
Location	**Element**	r	p	r	p	r	p
Vulcano	Fe	0.437	0.462	0.992	**0.000**	-0.836	0.078
		Seagrass Compartments					
		Roots-Rhizomes		Rhizomes-Leaves		Roots-Leaves	
Vulcano	Cd	1.000	**0.016**	0.158	0.783	0.158	0.783
	Cu	-0.986	**0.002**	0.534	0.354	-0.620	0.265
	Hg	-0.135	0.783	0.216	0.683	-0.947	**0.016**
Milos	Fe	-0.975	**0.016**	-0.300	0.683	0.205	0.683
	Cu	-0.872	0.083	-1.000	**0.016**	0.872	0.083

4.4. Discussion

The six volcanic CO_2 seeps that we surveyed in Greece and Italy, all had higher trace element concentrations in sediments than at reference sites. This is to be expected, as volcanic seeps emit fluids often rich in CO_2, H_2S and precipitates of trace metals that accumulate in sediments (Aiuppa et al., 2000; Sternbeck et al., 2001). Levels of metals at volcanic seeps can be harmful to marine life (Vizzini et al., 2013), so most work using seeps as analogues for ocean acidification has been carried out on hard substrata away from harmful levels of H_2S and metals (Fabricius et al.,2014; Milazzo et al., 2016). As seagrass colonise sediments we aimed to find out why they thrive at certain CO_2 seeps but not at others, as this may relate to the effects of pH on the bioavailability of metals that can be toxic.

In shallow marine CO_2 seeps off Greece (Milos, Paleochori and Methana) and Italy (Vulcano, Ischia and Panarea) redox and pH conditions are altered by the emission of CO_2 and H_2S (Sedwick et al., 1996; Boatta et al., 2013). This alters adsorption/desorption phenomena of elements in sediments and water column (Prokop et al., 2003). Our surveys showed clear enrichment of Fe and all trace element (Cd, Cu, Hg, Ni, Pb and Zn) levels in sediments, which we expected as volcanic seeps are usually enriched with trace elements (Monia-Renzi et al., 2011). Our Italian CO_2 seep sediment samples had higher trace element concentrations than those in Greece. The concentrations that we found in the sediments at the Vulcano seeps were in the same range as those found by Vizzini et al., (2013). At Panarea, Pb concentrations were lower than those recorded by Monia-Renzi et al., (2011), which is probably because metal concentrations are heterogeneous around seeps and depend on highly variable redox and pH conditions. Mercury was found at all three seep locations off Italy, but was not detected at the Greek seeps confirming that not all seeps emit mercury (Stoffers et al., 1999).

Comparing trace element concentrations of sediments from the six CO_2 vent locations of Greece and Italy with SQGs (Long et al., 1998; MacDonald et al., 2000), the obtained values were higher than TEL and ERL values and remained lower or equal to PEL and ERM values. Based on SQGs, element such as Cd at Paleochori, Hg at Vulcano and Cu at Ischia were found at levels that are likely to have adverse biological impacts, whereas at Methana and Panarea none of the trace elements were at high enough levels to be likely to have adverse biological impacts. Our results have similar values for Ni from Vulcano as found by Vizzini et al., (2013) from SQGs indices. The moderate or adverse biological impacts of the sediments depends on the concentration of element in the sediments and the metal speciation form they are present.

Higher number of elements with adverse impacts at the CO_2 seeps than reference sites observed in our results indicates that metal speciation is affected by low pH resulting in increased availability along with the metal enrichment at CO_2 seeps.

To understand if sediments enriched with trace elements were also available for uptake, the upper 5 cm of the sediments were sampled where the root/rhizome matte system of both *C. nodosa* and *P. oceanica* are present that takes active part in metal uptake and thus keeps this layer of sediment oxygenated for various redox reactions (Kristensen, 2000). Secondly trace metals that are easily available for uptake are dominant in this upper layer (Pekey et al., 2004; Atkinson et al., 2007) increasing the bioavailability of these elements both in sediment pore water and water column (Atkinson et al., 2007). The uptake of various elements in both seagrass compartments from sediments were reflected from bio-sediment accumulation factor index. BSAF which indicates higher/lower element concentration in plant compartments than observed in sediments, showed BSAF>1 for different trace element (Cd, Cu, Ni, Pb and Zn) for *P. oceanica* and for *C. nodosa* (Cd, Cu and Ni) within sediment-roots and sediment-leaves at all six CO_2 seeps. Higher variation in accumulation were observed in P. *oceanica* than *C. nodosa* indicating that *P. oceanica* have greater capability than *C. nodosa* for accumulation of these trace elements (Lafabrie et al., 2007; Bonanno et al., 2016 and references there in) from sediments at the CO_2 seeps.

Considering the capability of both seagrasses to accumulate elements from CO_2 seep sediments, the principal uptake rate of elements in both seagrasses were the sediment-root and leaf-roots pathways. The extent to which these uptake methods are active or passive depends on the physiological regulation of the seagrass and the element concentration in the surrounding to which seagrass are exposed to (Lafabrie et al., 2007; Bonanno et al., 2016 and references there in) which was observed in our studies, higher Zn concentration in sediments were reflected in the leaves of *C. nodosa* at Vulcano CO_2 seeps. The high accumulation patterns of trace elements in the roots and leaves of *P. oceanica* and *C. nodosa* suggests a mixed tolerance strategy, based on root accumulation and loosing leaves. Root accumulation are common in rooted species, both terrestrial and aquatic, where bulk of the trace elements were stored for protection against the adverse effects on photosynthetic process (Bonanno &Di Martino,2016 and references therein). The other mechanism involves accumulation of toxic elements in rhizomes, which doesn’t participate actively in photosynthetic processes, similar pattern of accumulation was observed for Ni in *C. nodosa* rhizomes at Milos and *P. oceanica* rhizomes at Panarea seeps (Figs. 3 and 4). Malea and Haritinoids, (1999) and Bonanno & Di Martino, (2016) observed this possible tolerance mechanism in *C. nodosa*, whereas Di Leo et al., (2013) and Richir &Gobert, (2016) observed it in *P. oceanica*. They pointed out that active mobilization of metals like Cd, Ni and Pb from roots to shoots in the seagrass, facilitated metal loss in both seagrasses due to the high turnover rate of leaves. Uptake kinetics and passive absorption properties of leaves also differ from roots and element absorbed can be internally redistributed through active or passive transport mechanisms (Bonanno & Di Martino, 2016; Richir &Gobert, 2016).

Though the element concentration were higher in the sediments at CO_2 seeps, all elements are not preferably accumulated from sediments through the sediment-root pathways suggesting element specific uptake by seagrass. We found there was no positive correlation for Cd in the sediment-roots, sediment-rhizomes and sediment-leaves for *P. oceanica* (Table 5) except for *P. oceanica* at Panarea CO_2 seeps, indicating that Cd is not transferred from sediments into the *P. oceanica* compartments (Lafabrie et al., 2007; Di Leo et al., 2013). However, positive correlation for Cd in leaves-roots for Ischia (Table 5) and roots-rhizomes for Vulcano, indicate the preferable route for Cd transfer in *P. oceanica* and *C. nodosa* is by water column through leaves and then are translocated to roots (Lafabrie et al., 2007; Di Leo et al., 2013). This indicates efficient distribution pattern of Cd at CO_2 seeps from roots-rhizomes-leaves as found by Malea et al., (1994) in *P. oceanica* off Greece. The negative correlation for Cu, Ni and Zn found in the *P. oceanica* compartments (Table 5) indicate that though these elements are present in higher concentrations in the sediment but they do not get transferred between the root-rhizome-leaves pathway. However, these elements are essential elements and required for *P. oceanica* in biochemical pathways indicating absorption from the water column at the CO_2 seeps (Di Leo et al., 2013). This explains higher concentration of these trace elements in the leaves of *P. oceanica* (Fig.2) and involvement of *P. oceanica* in cycling of these elements (Lafabrie et al., 2007; Di Leo et al., 2013).

Significant positive correlation was observed in *C. nodosa* for Fe in sediment-rhizomes (Table 6). This indicates mobility of Fe from sediment to the *C. nodosa* compartments as observed previously for Fe by Malea et al., (1999). Positive or negative correlation between *Cymodocea nodosa* compartments indicate that, *C. nodosa* have effective active uptake kinetics to translocate trace elements to entire plant system. This was observed by Malea et al., (1999) for Cd, Cu and Pb and by Ahmad et al., (2015) for Cd and Cu in *C. serrulata.* Mobility of Zn within *C. nodosa* compartments have a positive correlation, which agrees with the findings from Malea et al., (1999) for Zn. Hg was found with negative correlation in *C. nodosa* compartments at Vulcano, indicating Hg being toxic is not allowed for translocation within the seagrass compartments, similar results were found for *C. nodosa* by Sanchiz et al., (2001).

Correlation data between seagrass compartments are different because element accumulation patterns in seagrass are governed by multitudes of factors (Llagostera et al., 2011) as compared to land plants where metal uptake happens by two phases, one from sediments to roots and secondly from roots to other plant parts (Kvesitadze et al., 2009). So, in our studies at CO_2 seeps increased levels of elements in seagrass roots, rhizomes and leaves indicate the capacity of seagrass to absorb element simultaneously from sediments and water, as they are always submerged. This also indicates seagrass at these CO_2 seeps have adapted to the variation in element concentrations found in sediments at these CO_2 seeps with an effective mechanism of survival. Based on element concentrations, *P. oceanica* and *C. nodosa* can regulate their gene expression for metal toxicity which was observed for *P. oceanica* by Lauritano et al., (2015) and *C. nodosa* by Olive et al., (2017).

At CO_2 seeps the sediments receive a higher input of elements from the emission of seep fluids and thus there is a possibility of increase of element concentration in the water column too. Generally dissolved metal concentrations in overlying waters are low due to low solubility of metals in seawater and absorption on sediments. But this is going to change in near future due to ocean acidification and low pH as metal speciation is influenced by pH variation and low pH favours release of metals from pore waters (Simpson et al., 2004: Atkinson et al., 2007) that are generally not bioavailable. Once emitted from the seeps metals are absorbed on sediments where biological and chemical process results in redox stratification of metal bound particles with depth (Eggleton et al., 2004; Basallote et al., 2014), until resuspension of these particles happens due to physical processes and bioturbation. Resuspension of these metal bound particles with oxygenated overlying waters results in metal speciation in the dissolved phase (Simpson et al., 2002), making the metals bioavailable in pore waters (Simpson and Batley, 2003). Once released from pore waters into water column, these metals are bioavailable to aquatic organisms till precipitation of these metals are initiated by the fine fraction (<63 micron) of the sediments suspended in water column (Zoumis et al., 2001; Fan et al., 2002).

Concentration of Cu, Ni, Cd, Pb, Zn, increases with low pH as these metals are released from sediments at low pH (Atkinson et al., 2007; Basallote et al., 2014). Low pH also favours the release of elements like Hg from the sediment surface layers bound to sulphides and free sulphides are oxidised in the sediment upper layer by oxygen provided by seagrass matte system to release Hg. The chemical form in which the metal is present (bound to organic or inorganic compounds) in the sediment is a key issue determining its bioavailability. Metals like Cu which can be associated with minerals in low pH conditions are not likely to be released into water column, since they are trapped within the crystal structure of minerals, remaining unavailable to aquatic system (De Orte et al., 2014), whereas acidification of seawater near the CO_2 seeps tends to release the metals that are less strongly associated with sediments increasing their potential bioavailability (Riba et al., 2004). Thus, acidification due to low pH can increase the concentration of dissolved metals, which could affect the toxicity of the sediment to the exposed organism i.e., by increasing metal (Cu, Cd and Zn) bioavailability (Basallote et al., 2014).

In our research, all the six CO_2 seeps had low pH (7.4-7.9) conditions which increased the availability of Fe and Cd, Cu, Ni, Pb and Zn in their free ion forms and hence more availability and toxicity depending on the organisms (Roberts et al., 2013). However, Fe and trace elements (Cd, Cu Hg, Ni, Pb and Zn) toxicity have never been studied on *P. oceanica*, but a few studies have been conducted on *Cymodocea serrulata* (Prange and Dennison. 2000), *Halophila ovalis* and *H. spinulosa* (Prange and Dennison, 2000; Ambo-Rappe et al., 2011). Many of the concentrations found in our research for Cu and Pb are significantly higher than the toxic range previously reported for various seagrasses, affecting their photosynthesis as well as root and leaf structures (Ambo -Rapee et al., 2011; Prange and Dennison. 2000). In our studies toxicity tests were not performed, but the levels of trace elements that we found suggest possible toxic effects on seagrasses at CO_2 seeps (Richir, 2016). This certainly helps explain why seagrasses grow well and can take advantage of elevated CO_2 levels at some seeps but not at others.

Tu sum up: ocean acidification will affect trace metal chemistry, speciation and their bio-availability (Millero et al., 2009; Zeng et al., 2015) and may impact seagrass ecosystem in areas with contaminated sediments.

Natural CO_2 seeps are now widely used in global change studies, but our studies show CO_2 seeps off Italy and Greece have moderate to high metal levels that seagrasses tend to accumulate. Organisms that eat seagrass may accumulate and transfer trace elements resulting in toxic effects (Bouchon et al.,2016). Therefore, while considering CO_2 seeps as field laboratories for global change studies, the effect of trace element contamination should be kept in mind (Vizzini et al., 2013).

We have shown that Mediterranean CO_2 seep sites consistently have elevated levels of trace elements in sediments and that these can be used to study the interactions between ocean acidification, metal bioavailability and accumulation within seagrasses. We affirm warnings by Bary et al., (2011), Vizzini et al., (2013), Lauritano et al., (2015) and Olive et al., (2017) that care is needed when using volcanic CO_2 seeps as analogues for the effects of ocean acidification as there can be areas of H_2S emissions and toxic levels of metals that may be confounding factors. In some cases, such as Ischia, we found that high levels of trace elements such as copper were not bioavailable to seagrass. In other cases, such as Vulcano, we found that elevated levels of Zn were bioavailable and became concentrated in seagrass roots and rhizomes. Our findings are relevant to those agencies responsible for monitoring the effects of trace metal contamination in the marine environment, since increased CO_2 levels can increase metal availability and toxicity.

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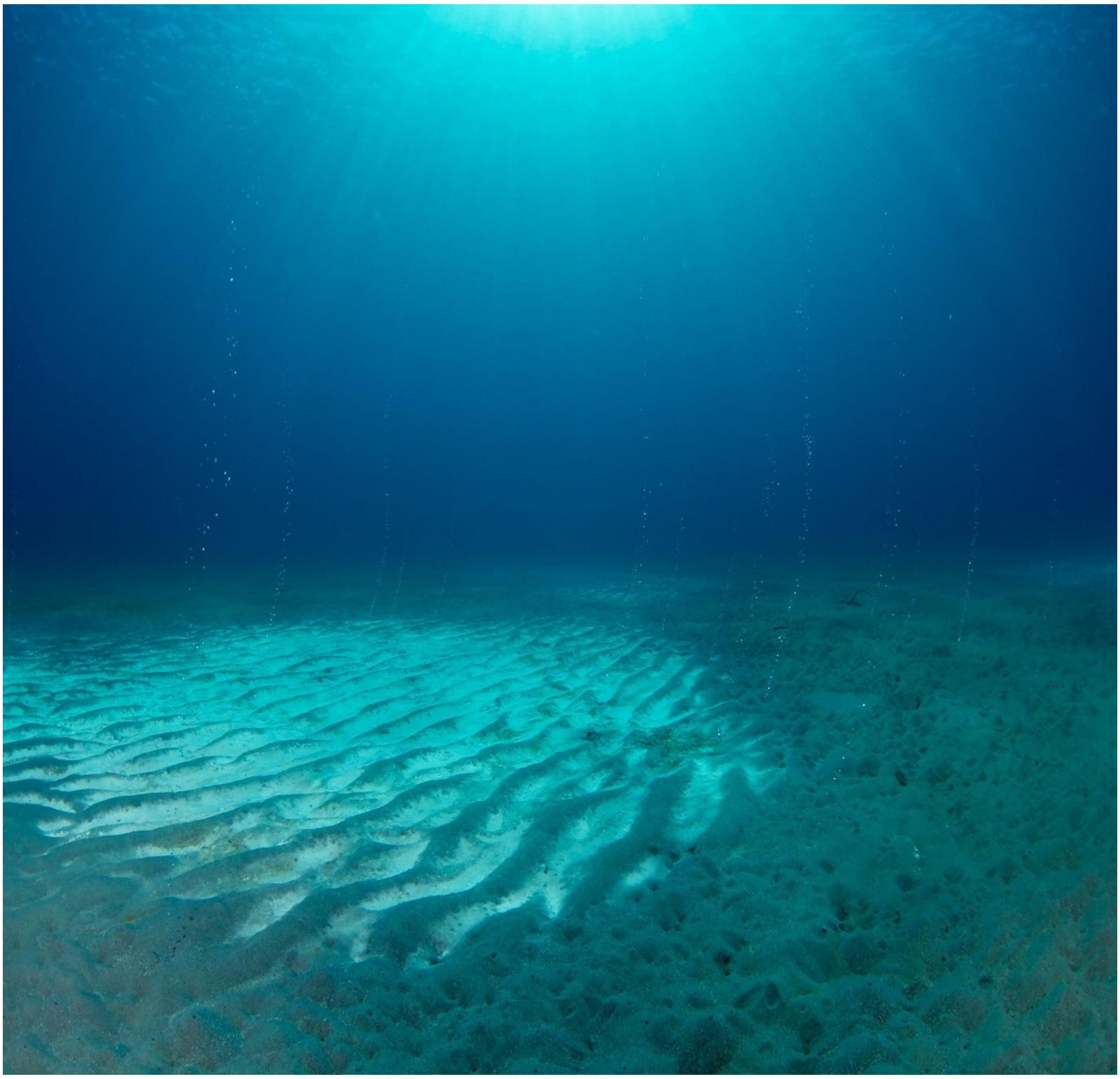
General Discussion



General Discussion

5.1. Main findings and implications for seagrass ecosystem

Seagrasses are one of the dominant coastal ecosystems characterized by high primary production rates, nutrient concentrations and biodiversity (Thom, 2001). These habitats are important for the ocean's biogeochemical cycles, and they play a crucial role in providing ecosystem services (Gattuso et al., 1998; Erwin et al., 2009). Information on response of seagrass community and individual plants to high CO_2 scenarios are important to predict the state of seagrass community production and survival in future conditions and therefore needed to frame possible adaptive measures to keep in flow the ecosystem services they provide. This thesis contributed to assess seagrass responses to future high CO_2 levels and the main findings are as follows

- Short term CO_2 enrichment incubations on intertidal *Z. noltii* community indicated high CO_2 (750 ppm) levels projected for the end of this century will increase the net community production and decrease the community respiration during emersion periods. Light requirements of plants were reduced under CO_2 enrichment conditions (Chapter 2)
- At Mediterranean CO_2 seeps of Greece and Italy, *C. nodosa* population showed positive response to long term exposure of elevated CO_2 through higher growth, density, biomass and morphometry. Higher number of younger plants (1 and 2 years older) were found at the CO_2 seeps, whereas the number of older plants (more than 3 years) were lower near the CO_2 seeps at all three locations (Chapter 3).
- Fe and trace element (Co, Cu, Hg, Ni, Mn, Pb and Zn) concentrations were higher under high CO_2 and low pH conditions in the sediments and in *C. nodosa* and *P. oceanica* roots, rhizomes and leaves. Bioavailability of trace elements increased with increase in CO_2 concentration and low pH. Higher concentration in sediments resulted in higher accumulation of trace elements in the seagrass compartments. Trace element concentrations in sediments and plant compartments were found at levels that can be toxic to seagrass at CO_2 seeps of Greece and Italy. CO_2 seeps of Italy (Vulcano, Ischia and Panarea) were observed with higher concentration of elements than CO_2 seeps of Greece (Milos, Paleochori and Methana) (Chapter 4).

Thus, seagrass ecosystem and individual plants can get benefitted from the increased CO_2 levels in the atmosphere, whereas increase in bioavailability of elements and possible toxic effects due to ocean acidification and low pH can hinder this positive influence of high CO_2 in future scenarios.

Our results indicate positive response of seagrass to elevated levels (future projections of 750 ppm) of CO_2. This positive response of seagrass to higher CO_2 concentrations was noticed on both the short-term experiments on *Z. noltii* community and at the CO_2 seeps of Greece and Italy, where the seagrass (*C. nodosa*) have been exposed to long term elevated CO_2 levels (750 ppm and higher). Positive response of *Z. noltii* through increased community production in (Chapter 2) indicates that the plants are carbon limited in the current carbon dioxide concentration in atmosphere and gradually as CO_2 concentration increases the plants will adapt and increase their carbon utilization capacity, which can result in higher density, biomass and higher production rates as observed for *C. nodosa* at CO_2 seeps exposed to long term elevated CO_2 levels (Chapter 3). Similar response of other seagrass (*Z. marina* and *T. hemprichii*) species have been observed with a positive response to high CO_2 levels (Zimmerman et al., 1997; Jiang et al., 2010).

The positive response of seagrass to increased CO_2 and to overcome the current carbon limitation in future oceans, a lot will depend on how seagrass to utilize the dissolved inorganic carbon (DIC) available to them in high CO_2 oceans. The amount of DIC (1% $CO_{2\ (aq)}$, 90% HCO_3^- and 9% CO_3^{2-}) in the current oceans is going to get higher (Campbell et al., 2013) with an overall increase of CO_2 (>250%) compared to bicarbonate (24%, HCO_3^-) under the projected pH decrease by 2100 (Koch et al., 2013)

Seagrasses will require to utilize the abundant HCO_3^- ions to increase their photosynthetic production efficiency by extracellular dehydration of into $CO_{2(aq)}$ via membrane bound carbonic anhydrase enzymes (Beer and Rehnberg, 1997; Bjork et al., 1997) or by proton extrusion into an unstirred boundary layer adjacent to the leaf surface facilitating carbonic anhydrase activity (Uku et al., 2005). Extracellular carbonic anhydrase dehydrates HCO_3^- to CO_2, allowing CO_2 to diffuse into the cell. In general, active utilization of H^+ ions at the leaf boundary layer for HCO_3^- utilization is energetically costly compared to passive CO_2 uptake and could be limited by low light levels (Burnell et al., 2014), but the seagrass ecosystems in shallow habitats with higher light penetration can favour the use of HCO_3^- as substrate.

Positive response of *Z. noltii* production to CO_2 enrichment (Chapter 2) and increase in density and biomass of *C. nodosa* in the CO_2 seeps of Greece and Italy (Chapter 3) suggests that seagrasses here have adapted to the utilization of DIC (CO_2 and HCO_3^-) as *Z. noltii* is intertidal receiving high light intensities during emersion and the *C. nodosa* meadows at the CO_2 seeps of Greece were in shallow waters receiving higher light. Both seagrasses can prefer the use of CO_2 when available in higher concentration than HCO_3^-, as utilizing CO_2 requires less energy than HCO_3^-. Though the degree of adaptation to utilize HCO_3^- is species specific, the availability of light also plays a major role in seagrass utilizing HCO_3^-, observed in seagrass species, such as *C. serrulata* (Uku et al., 2005; Ow et al., 2016), *C. rotundata* (Schwarz et al., 2000) and *Z. marina* (Invers et al., 2001) which showed increase production due to increased DIC and light, whereas *Halophila uninervis* productivity was not affected by increased DIC but only by light (Ow et al., 2016). This implies in future oceans, increased DIC concentrations and increased production (Invers et al., 2002; Hall-Spencer et al., 2008).

The adaptation to use DIC will also depend on the available concentration of DIC in ecosystem and habitat of the seagrass. The seagrasses which will remain submerged all the time will have better capacity to adapt and utilize HCO_3^-, than intertidal species which are both submerged and air exposed (Campbell et al.,2013, Ow et al., 2016). This differences can favour one seagrass in a mixed meadow to improve and increase its carbon balance, thus increasing its competitive ability to utilize high CO_2, while other species can remain less beneficial. Similar, results have been reported for *Thalassia testudinum* and *Halophila wrightii* (Campbell et al., 2013). Increase in DIC concentration as observed at the CO_2 seeps of Greece and Italy (Chapter 3&4) will favour the increase in photosynthetic capacity of seagrass by providing more carbon for fixation and simultaneously lowering photorespiration rates (Long et al., 2004), whereas increase in light intensities will also increase the maximum photosynthetic capacity by boosting production and reducing intermediates for the carbon reduction cycle (Hall et al., 1994). As both factors have independent mode of actions, their effect on seagrass productivity will be synergistic and beneficial to the seagrasses.

DIC enrichment can also reduce the light saturation point for seagrasses and increase the production capacity of seagrasses, as observed for *Z. noltii* in our research (Chapter 1), similar results were observed for *C. serrulata* (Ow et al., 2016) and *T. hemprichii* (Jiang et al., 2010) and *Z. marina* (Zimmerman et al., 1997). This suggests seagrass can increase their light saturated photosynthetic capacity under increased CO_2 concentration to reduce the daily photoperiod required for positive carbon balance (Zimmerman et al., 1997, Ow et al., 2016) and increase their migration into greater depths.

Under future ocean acidification scenarios, seagrasses can benefit from the increased DIC concentration and CO_2 levels (Hall-Spencer et al., 2008; Fabricius et al., 2011). While short term and long term studies on seagrass have documented varying degrees of DIC limitation in seagrass, physiological processes that could regulate responses to increased DIC are less explored for seagrass. Other than DIC limitation, other factors that will regulate seagrass growth and production will be nutrient availability (Stitt and Krapp, 1999) and water temperature (Touchette and Burkholder, 2000), which are also going to increase in the future oceans due to anthropogenic pollution and climate change. Saying that, seagrass leaves will also have to compete with the leaf epiphytes (mostly algae) for the same resources of increased DIC concentrations. This competition with epiphytic algae will determine the composition, biomass and turnover rates of seagrass leaves (Campbell et al., 2013). Finally, light availability, due to anthropogenic pollution, sedimentation and epiphytic algal growth can negate the positive effects of high CO_2 on seagrass productivity in future oceans (Burnell et al., 2014).

Increase in CO_2 concentration will favour the increase in seagrass production, but productions of seagrass ecosystem will be under considerable stress from various factors such as metal contamination. Metal levels is going to increase in the world oceans due to anthropogenic impacts from coastal developments and agricultural runoff (Ivanina et al., 2015; Zeng et al., 2015). Ocean acidification and low pH will alter the speciation and availability of elements in seawater and sediments resulting in potential toxicity to the marine biota (Millero et al., 2009; Roberts et al., 2013). Higher availability of element levels in the sediments can result in possible contamination of marine sediments and higher accumulation in the seagrass compartments as observed in our results (Chapter 4).

Generally marine sediments have significant higher magnitude of metal concentrations than in water column making the sediments important reservoir of these metals (Clark, 2002). These metal levels are going to increase from anthropogenic pollution and a combination of ocean acidification and low pH in the water column (Roberts et al., 2013). Presence of metal levels in higher concentration may inhibit the primary production in marine ecosystems and decrease the efficiency of marine carbon sink (Zeng et al., 2015). Seagrasses have metal binding proteins that can help them regulate metal toxicity on their metabolic activity and physiology under normal conditions, but under ocean acidification and low pH conditions, it have been observed in *P. oceanica* (Lauritano et al., 2015) and *C. nodosa* (Olive et al., 2017) downregulation of these metal binding proteins occurs in seagrass. This downregulation of metal binding proteins suggests, seagrasses my not be able to tolerate the metal toxicity levels that is going to occur in by end of this century. This also indicates that metal levels we found in our results at Ischia and Panarea CO_2 seeps in *P. oceanica* compartments and at Vulcano in *C. nodosa* compartments (Chapter 4) are already at higher levels affecting their metabolic activity and physiology. Low longevity of *C. nodosa* at CO_2 seeps off Greece and Italy than reference sites (Chapter 3) may be an effect of metal toxicity too due to similar levels of pH and metal concentration in sediments at CO_2 seeps off Italy and Greece (Chapters 3&4).

However, accumulation patterns of metals in seagrass will determine their concentration in the roots, rhizomes and leaves of seagrass. This suggests presence of metals in higher concentration in water or sediments may not necessarily have toxic effects on seagrass as they have an exclusive storage capacity of metals in rhizomes and exclusion mechanism by leaves.

Similarly, environmental factors other than carbon dioxide will determine favourable conditions for seagrass ecosystem. These factors include the natural biophysical parameters that regulate the physiological activity and morphology of seagrasses (such as temperature, salinity, waves, currents, depth, substrate, day length, light, nutrients, water currents, wave action, epiphytes and diseases), the availability of seeds and vegetative fragments and the anthropogenic inputs that impact plant resources (such as excess nutrients and sediment loading). Combinations of these parameters will permit, encourage or prevent seagrass meadows thriving (Cullen-Unsworth et al., 2014). Climate change is a significant long-term threat to seagrass. Managing seagrasses for future resilience to climate change is about understanding current stressors and how they may change and about knowledge of temperature and ocean chemistry including developing greater knowledge of distribution limits, understanding ecosystem recovery and defining clear physical thresholds (Cullen-Unsworth et al., 2014).

5.2. Summary and Main Conclusion

Global changes in terms of elevated CO_2 levels and low pH have the potential to change the seawater carbonate chemistry and influence the marine ecosystem (Hall-Spencer et al., 2008; Porzio et al., 2011). This thesis contributes to the growing knowledge of effects of high CO_2 concentrations on seagrass ecosystem in both short term and long term enrichment. It is clear from the findings that will increase in CO_2 coentrations will have a positive impact on the seagrass productivity (Alexandre et al. 2012; Russell et al. 2013; Apostolaki et al., 2014) in both short and long term and will also increase seagrass community productivity and decrease current carbon limitation of seagrass (Zimmerman et al., 1997; Invers et al., 2001). This increase in production will also be affected by various physical factors (light, temperature) and the surrounding environment where the coastal seagrass population are found (Khan et al., 2016; Takahashi et al., 2016). This thesis observes that response of seagrass to high CO_2 concentrations will be species-specific and won't be straight forward and impact of trace metal contamination can over shadow the positive effect of CO_2 enrichment on the seagrass population and community production (Vizzini et al., 2013; Apostolaki et al., 2014).

Main Conclusions:

1. Short term CO_2 enrichment on *Z. noltii* community and sediment community indicated increased community production under CO_2 enrichment than natural conditions.
2. Effect of light and seasons had a clear impact on the net community production of Z. noltii and sediment community
3. Short term incubation methods can be used as a reliable to understand intertidal community production using CO_2 enrichment.
4. *C. nodosa* growth, density, biomass, morphometry showed an increased fitness under long term elevated CO_2 concentrations at high CO_2 seeps than control sites of Greece and Italy
5. *C. nodosa* population showed increased recruitment rate and production but longevity of plants decreased under elevated CO_2 concentrations.
6. Reconstruction techniques provide a successful tool to interpret and predict seagrass population dynamics under future high CO_2 scenarios.
7. Trace elements concentration increased under low pH conditions with continuous input from the CO_2 seeps into the sediments
8. Higher concentration in sediments was reflected in the trace element concentration in the seagrass *Cymodocea nodosa* and *Posidonia oceanica* roots, rhizomes and leaves with higher concentrations that control sites.
9. Trace element concentrations in the sediments were higher enough to have moderate or adverse biological impacts on the seagrass ecosystem
10. All the six Mediterranean CO_2 seeps of Greece and Italy was found with higher level of metal contamination than the control sites

11. Seagrass can overcome their current carbon limitation and increase their production and carbon metabolism under future high CO_2 projections and can act as a source of global carbon sink.

5.3. References

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