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Assemblage structure and secondary production of mesozooplankton in shallow water volcanic CO_2 vents of the Azores

Ana Navarro Campoy

Dissertação para obtenção do grau de:

Mestrado em Biologia Marinha

Trabalho efetuado sob a orientação de:

Maria Alexandra Teodósio
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Declaro ser a autora deste trabalho, que é original e inédito. Autores e trabalhos consultados estão devidamente citados no texto e constam da listagem de referências incluída.

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Date:

Signature:

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Abstract

Atmospheric CO_2 concentrations have increased by at least 30% since pre-industrial times due to human activities. Part of this CO_2 has been absorbed by oceans, inducing ocean acidification and, therefore, several impacts in the marine biota. Natural shallow-water CO_2 vents have generated a substantial interest in recent years as *in situ* laboratories for ocean acidification studies. The present study was focused on the effects of ocean acidification on mesozooplankton communities. Two active volcanic areas with submarine CO_2 emissions, in the islands of São Miguel and Faial, were chosen and independently studied, through the choice of three different sites: Reference, Intermediate and Vent, characterized for their increase in the CO_2 degassing activity, and consequent pH decrease. Differences in the abundance, diversity and structure of mesozooplankton among sites were described, along with the characterization of the community in these areas, since previous information is scarce. Differences were found in the composition of the zooplanktonic assemblages among sites in São Miguel and among dates in Faial. Through the nMDS analyses, Radiozoa, *Paracalanus parvus* and *Evadne spinifera* appeared more related to vent conditions, while Cirripedia nauplii were closer to the reference conditions. Conditions in Faial did not allow a clear separation among sites in the nMDS analyses, since the strong currents mix the water, dissipating the effect of the gas emissions, and variation among days becomes more important and statistically significant. The RNA:DNA ratio of selected mesozooplankton populations was used, as proxies for physiological condition. RNA:DNA did not show a clear pattern of variation, copepods in São Miguel and cladocerans in Faial had an higher ratio in the reference sites, but with no gradual decrease to the Vent. Fish eggs seem to have an inverted pattern. Additional experiments conducted under natural conditions were performed to determine the egg production rate (EPR) of the dominant free-spawning copepod species, as a proxy for secondary production. In São Miguel, the EPR showed a decreasing trend along the CO_2 gradient, with no differences between the exposure times. In Faial, EPR was higher in the reference, but it did not differ significantly from the other sites. This study demonstrated the suitability of the submarine degassing sites in S. Miguel and Faial Islands for investigating the effect of future dissolved CO_2 levels in planktonic and pelagic communities of the NE Atlantic. It also provided the first *in-situ* evidence of a significant decrease of EPR of copepods under near future CO_2 levels.

Key words: ocean acidification, mesozooplankton, shallow-water CO_2 vents, copepods, egg production rate.

Resumo

Desde os tempos pre-industriais que se registou um incremento do CO_2 atmosférico, de pelo menos 30% devido a atividades humanas, principalmente pelo uso de combustíveis fósseis. Os oceanos têm absorvido parte deste CO_2 através da sua capacidade de "sumidouro de carbono", induzindo mudanças na composição química da água do mar (acidificação do oceano), com potenciais impactos nos organismos marinhos. As possíveis consequências ecológicas deste processo têm motivado um incremento no esforço de investigação nos últimos anos. A maioria dos efeitos nos organismos marinhos tem sido observada em experiências no laboratório, onde o CO_2 é manipulado em escalas temporais curtas. Não obstante, a necessidade de usar diferentes abordagens, como experiências de campo, em ambientes marinhos específicos que contenham comunidades, mais do que espécies isoladas, tornou-se evidente. Locais de desgaseificação natural de CO_2 em águas pouco profundas têm gerado um interesse substancial como laboratórios *in situ* para estudos de acidificação do oceano. Alterações significativas na estrutura da comunidade bentónica têm sido associadas com estes locais de desgaseificação, mas os efeitos no plâncton permanecem largamente desconhecidos. O presente estudo analisou os efeitos da acidificação do oceano nas comunidades de mesozooplâncton. Os objetivos especificos foram (i): o estudo observacional sobre as comunidades zooplanctónicas, para determinar se existem diferenças consistentes na abundância, diversidade e composição entre os locais afetados por emissões de CO_2 e os locais de controlo, sem emissões; e (ii) a relação entre os padrões observados no zooplâncton em relação aos resultados detetados na química da água do mar e do gás das emissões, (iii) a descrição do estado ecofisiológico de grandes grupos de zooplâncton utilizando índices derivados dos ácidos nucleicos e índices de produção de ovos e a sua relação com o possível stress causado pela acidificação. Para atingir estes objetivos foram seleccionadas duas áreas vulcânicas activas, com emanações de CO_2 submarinas, uma na ilha de São Miguel e outra na ilha de Faial, no arquipélago dos Açores. Cada ilha foi independentemente estudada, através da escolha de três lugares diferentes: *Reference*, *Intermediate* e *Vent*, ao longo de um gradiente na emissão de CO_2, e consequente diminuição de pH. O pH em São Miguel variou de 8.06 no *Reference* (pCO_2 383.80 µatm) a 7.75 no *Vent* (pCO_2 983.10 µatm), enquanto que no Faial diminuiu de 8.13 no *Reference* (pCO_2 319.37 µatm) a 7.88 no *Vent* (pCO_2 894.08 µatm). O CO_2 foi o principal constituinte das emissões de gás nas duas ilhas (acima de 98%), minimizando o efeito de outros gases como H_2S. Em São Miguel o mesozooplâncton foi amostrado usando arrastos oblíquos com uma rede WP2 de Ø60cm e malhagem de 200 µm,

entre os dias 3 e 7 de Julho de 2014, enquanto que no Faial foi usada uma rede com malhagem de 500µm e a amostragem foi feita entre os dias 10 e 15 de Julho de 2014. Foram descritas as diferenças na abundância, diversidade e estrutura do mesozooplâncton entre locais, em paralelo com a caracterização das comunidades nestas áreas, já que a informação prévia era escassa. Foram identificados organismos de nove filos, num total de 71 taxa diferentes, 45 em S. Miguel e 61 no Faial. Em São Miguel foram encontradas diferenças entre locais na abundância total e nas abundâncias de Arthropoda e Chordata, com um incremento no *Vent*, relativamente ao *Intermediate* e o *Reference*. No Faial foram apenas encontradas diferenças na diversidade dos Arthropoda. A análise PERMANOVA ilustrou as diferenças na composição do zooplâncton entre locais, em São Miguel, e entre datas, no Faial. Os principais contribuintes para estas diferenças, realçados pelas anaáises SIMPER, foram *Evadne spinifera*, *Paracalanus parvus*, nauplios de Cirripedia e Radiozoa. Através das análises nMDS, Radiozoa, *Paracalanus parvus* e *Evadne spinifera* apareceram mais relacionados às condições do *Vent*, enquanto que nauplios de Cirripedia estiveram mais perto das condições do *Reference*. No Faial, os principais contribuintes foram *Bassia bassensis*, Radiozoa e efiras de Scyphozoa. As condições nesta ilha não permitiram uma separação entre locais nas análises nMDS, dado que as fortes correntes misturaram a coluna de àgua, dissipando o efeito das emissões de gases, e a variação entre dias é aparentemente mais importante e estatisticamente significativa.

O racio RNA:DNA de populações de mesozooplâncton seleccionadas foi usado como indicador da condição fisiológica. O RNA:DNA não mostrou um padrão claro de variação: Copepoda em São Miguel e Cladocera no Faial apresentaram valores mais elevados no *Reference*, mas este não diminuiu de forma gradual ao longo do gradiente de CO_2. Os ovos de peixe parecem ter um padrão invertido. Experiências adicionais foram levados a cabo para determinar a taxa de produção de ovos (EPR) das espécies dominantes de copépodes, como indicador da produção secundária. As câmaras de incubação foram colocadas perto do fundo durante períodos de 24 e 72h em São Miguel, e 72h no Faial. Em São Miguel, a EPR mostrou uma tendência de diminuição ao longo do gradiente de CO_2, sem diferenças nos tempos de exposição. No Faial, EPR foi maior no *Reference*, mas não diferiu significativamente dos outros locais.

Este estudo demonstrou o potencial das zonas de desgaseificação submarina nas ilhas de S. Miguel e Faial para a investigação dos efeitos dos níveis futuros de CO_2 dissolvido nas

comunidades planctónicas e pelágicas do Atlântico NE. Este trabalho também permitiu registar a primeira evidência *in-situ* de uma diminuição significativa da produção secundária em copépodes sob concentrações de CO_2 previstas para o final do século.

Palavras-chave: acidificação do oceano, mesozooplâncton, locais de desgaseificação natural de CO_2 em águas pouco profundas, copépodes, taxa de produção de ovos.

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1. Introduction

1.1. Global change

Economic growth in the industrial age has brought unprecedented wealth to the planet; in the U.K., the birthplace of the industrial revolution, real GDP per capita has grown by 12 times since 1830 (Erickson, 2014). Growth in economic activity has also come at a cost, as greenhouse gases (GHG) emissions have grown since pre-industrial times (IPCC, 2007), while CO_2 has increased by approximately 30% (Carman *et al.*, 2004).

Since 1751 approximately 337 billion metric tons of carbon have been released to the atmosphere from the consumption of fossil fuels and cement production. Half of these emissions have occurred since the mid-1970s. The 2007 global fossil-fuel carbon emission estimate, 8365 million metric tons of carbon, represents an all-time high and a 1.7% increase from 2006 (Boden *et al.*, 2010). Continued emission of greenhouse gases will cause further warming and long-lasting changes in all components of the climate system, increasing the likelihood of severe, pervasive and irreversible impacts for people and ecosystems (IPCC, 2014).

Carbon dioxide represents 75% of anthropogenic GHG emissions, followed by CH_4, N_2O and others fluorinated gases (figure 1.1). The human activities responsible for the increased emissions include in first place the burning of fossil fuels use and industrial processes and, to a lesser extent, changes to land use, including deforestation.

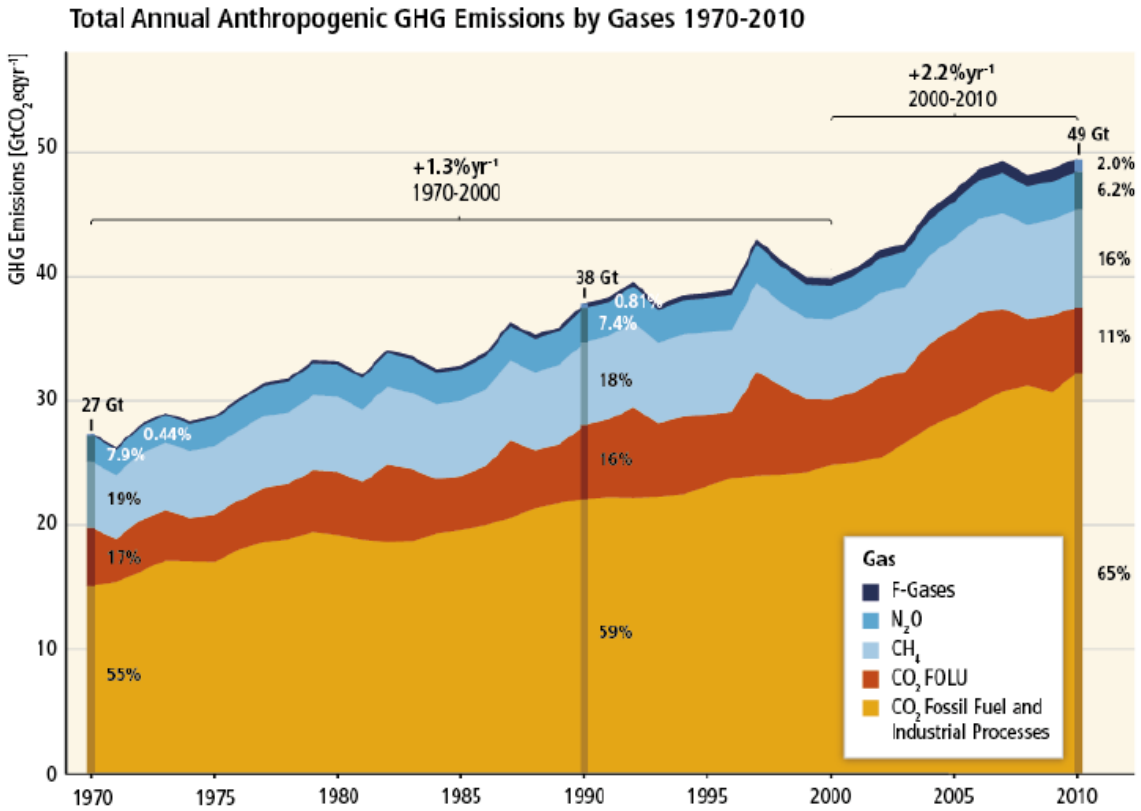

Figure 1.1. Total annual anthropogenic greenhouse gas emissions (gigatonne of CO_2-equivalent per year) for the period 1970 to 2010 by gases: CO_2 from fossil fuel combustion and industrial processes; CO_2 from Forestry and Other Land Use (FOLU); methane (CH_4); nitrous oxide (N_2O); fluorinated gases covered under the Kyoto Protocol (F-gases) (*From: ipcc, 2014*).

Measurements of atmospheric CO_2 during the last 56 years have revealed a consistent increase, from an annual average of 315,24 ppm in 1958 to 365,11 ppm in 2014 (CO2Now.org, 2014). This current level is also much higher than it has been at any time over the course of human civilization (Harrould-Kolieb & Savitz, 2009). In spite of these large CO_2 sinks, atmospheric CO_2 increased at a rate of approximately 3.3 Gt C y^{-1}, and thus atmospheric CO_2 concentrations continued to rise (Carman *et al.*, 2004). A large fraction of anthropogenic climate change resulting from CO_2 emissions is irreversible on a multi-century to millennial time scale, except in the case of a large net removal of CO_2 from the atmosphere over a sustained period (ipcc, 2014).

Besides CO_2 contribution, methane in second place is a potent greenhouse gas, and oceans seem as a supplier of vast quantities to the atmosphere (Ingall, 2008). It has been proposed by Karl *et al.* (2008) that the net efflux of methane from ocean to atmosphere is driven by aerobic methane production, fuelled by the microbial use of methylphosphonic acid (MPn) as a source of phosphorus in phosphate-stressed waters. During MPn utilization, methane is quantitatively released, whereas phosphorous is incorporated into new cell mass (Karl *et al.*, 2008). Metcalf *et al.* (2012) identified a phosphonate biosynthetic gene cluster in an abundant

marine archaeon of surface waters, reaffirming the idea of that MPn synthesis is prevalent in marine systems. Since nutrients availability in the upper ocean is largely controlled by the upwelling from the deep-ocean, and mixing between surface and deep-ocean layers is predicted to decrease, it could promote the use by marine microbes of phosphonates (Karl *et al.*, 2008) found in the low-molecular-weight fraction of dissolved organic matter (Ingall, 2008).

1.2.Ocean acidification

The oceans cover about 71% of Earth's surface to an average depth of 3700 m (Pörtner *et al.*, 2014). They play a vital role in global biogeochemical cycles, contribute enormously to the planet's biodiversity and provide a livelihood for millions of people (Raven *et al.*, 2005). Several sources indicate that oceans have the capacity to absorb part of this carbon dioxide, acting as a "carbon sink" and thus, moderating the impact of climate change on terrestrial life (Harrould-Kolieb & Savitz, 2009). Approximately one-third of the CO_2 that has entered the atmosphere over the past 100 years has been absorbed into ocean surface waters and has resulted in the elevation of partial pressure of CO_2 in seawater and reduction of seawater pH (Kurihara, 2008).

Without the oceans playing this role, the concentration of carbon dioxide in the atmosphere would be 55% higher (Fabry *et al.*, 2004). Prior to the Industrial Revolution the oceans were in relative equilibrium with the atmosphere, absorbing about the same amount of carbon dioxide each year as they released (2.15 billion metric tons of CO_2) (Harrould-Kolieb & Savitz, 2009). However, as the concentration of carbon dioxide in the atmosphere has increased, the flux of CO_2 from the atmosphere to the ocean has reduced the average pH of sea water by about 0.1 pH units over the past century, with the greatest reduction occurring at high latitudes (Cramer *et al.*, 2014), equivalent to a ca. 25 % increase in acidity (Havenhand, 2012). The oceans will continue to absorb carbon dioxide from the atmosphere as long as the concentration of carbon dioxide in the surface waters is less than that in the atmosphere (Harrould-Kolieb & Savitz, 2009).

The carbon dioxide absorption will result in an unavoidable decreasing of the pH over the next several centuries, comparing to any inferred from the geological record of the past 300 million years, with the possible exception of those resulting from rare, extreme events such as bolide impacts or catastrophic methane hydrate degassing (Caldeira & Wickett, 2003). It is predicted that by 2100 surface ocean pH could fall by 0.3/0.4 units and *p*CO2 of 750ppm

(Fitzer *et al.*, 2012; Dupont & Thorndyke, 2009; Havenhand, 2012), and according with the IPCC emissions scenarios, if there is an unrestricted burning of fossil fuels, an extreme scenario with a reduction of 0.7 units will occur until 24th century (Caldeira & Wickett, 2003).

The acid–base chemistry of the CO_2–carbonate system in the sea

The alkalinity of seawater, which expresses the buffering capacity of water or the capacity of the water to neutralize acids, is governed by the minerals dissolved, to which bicarbonates contribute largely. The pH is an indication for the acidity of a substance. It is determined by the number of free hydrogen ions (H^+). Thus, the surface waters of the open ocean vary between pH 7.9 and 8.3, while coastal waters, in contrast, routinely vary between mean pH 7.5 and 8.5 dependent on the habitat and show much larger seasonal and diel fluctuations (Kerrison, 2011). It is largely a function of the dissociation of dissolved inorganic carbon (DIC), whose relative proportions by mass are ~0.5% aqueous CO_2, ~89% bicarbonate (HCO^{3-}), and ~11% carbonate ions (CO_3^{2-}) (Waldbusser & Salisbury, 2014). According to Henry's Law, in a simple aqueous solution we would expect the concentration of CO_2 in the air to be proportional to that in the solution of carbonic acid (Raven *et al*, 2005). So changes in atmospheric pCO_2 will directly affect the carbonate system of the surface ocean, since atmosphere and surface ocean exchange CO_2 on time scales of several months (Rost *et al.*, 2008) (figure 1.2).

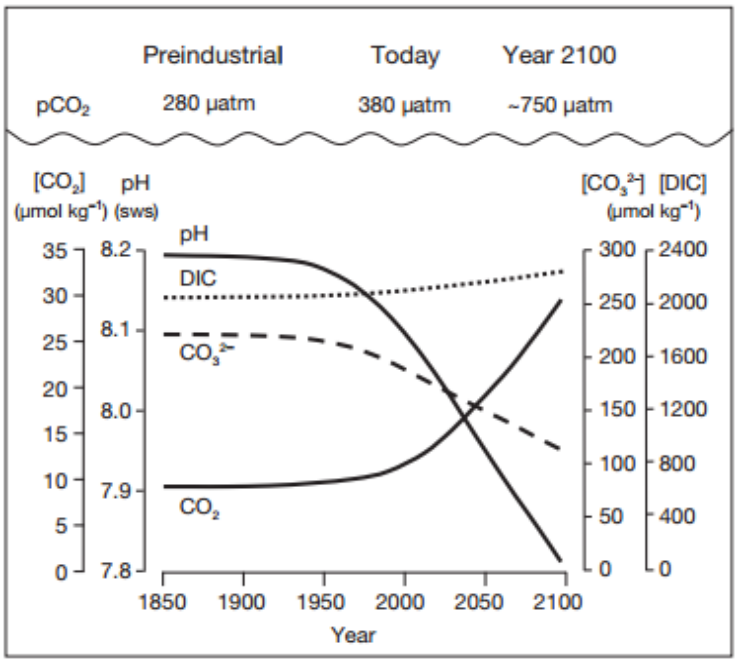

Figure 1.2. Predicted changes in the surface ocean carbonate system in response to changes in atmospheric pCO2 assuming the IS92a Scenario (*From: Rost et al, 2008*).

A portion of the dissolved carbon dioxide reacts with seawater to form carbonic acid (H_2CO_3), and then it can divides in HCO^{3-} (bicarbonate ion) releasing one H^+ to the solution. When the H^+ content is low, the CO_3^{2-} (carbonate ion) increase, while a high H^+ concentration causes a reaction to the carbonate becoming HCO^{3-}. Thus, the net effect of the dissolution of CO_2 in seawater is to alter this equilibrium, increasing the concentrations of H^+, H_2CO_3 and HCO^{3-}, while decreasing concentrations of CO_3^{2-} (figure 1.3).

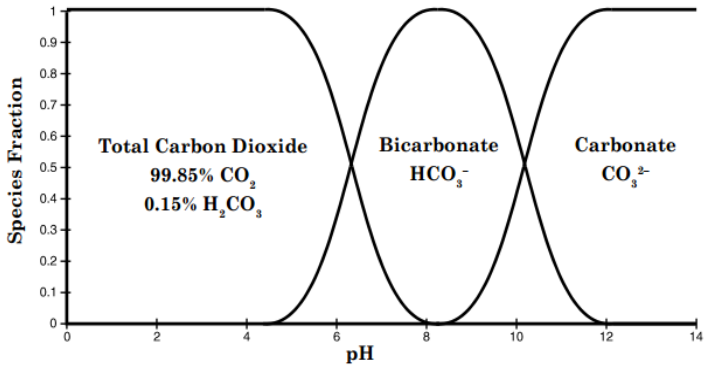

Figure 1.3. Distribution of Total Carbon Dioxide, Bicarbonate, and Carbonate in function of the seawater pH (*From: Raven et al., 2005*)

The term 'carbonate buffer' is used to describe how the dissolved inorganic carbon system in seawater acts to diminish changes in ocean H^+ concentration, and thus stabilize pH (Raven *et al.*, 2005) (figure 1.4). It occurs first for the CO_2 uptake from the atmosphere and second, for the interaction of seawater with oceanic sediments composed of $CaCO_3$, which is mainly controlled by a "biological pump". The pump may be defined as the movement of CO_2 that enters into the ocean from the atmosphere to the deep-ocean floor through biological processes, i.e. photosynthetic fixation of CO_2 by phytoplankton, passive export of organic carbon (e.g. fecal pellets of zooplankton, detritus, and dead organisms) and carbonates (e.g. shells and bones) by gravitation, or through vertical migration of zooplankton to the deep ocean (Kurihara *et al.*, 2004a).

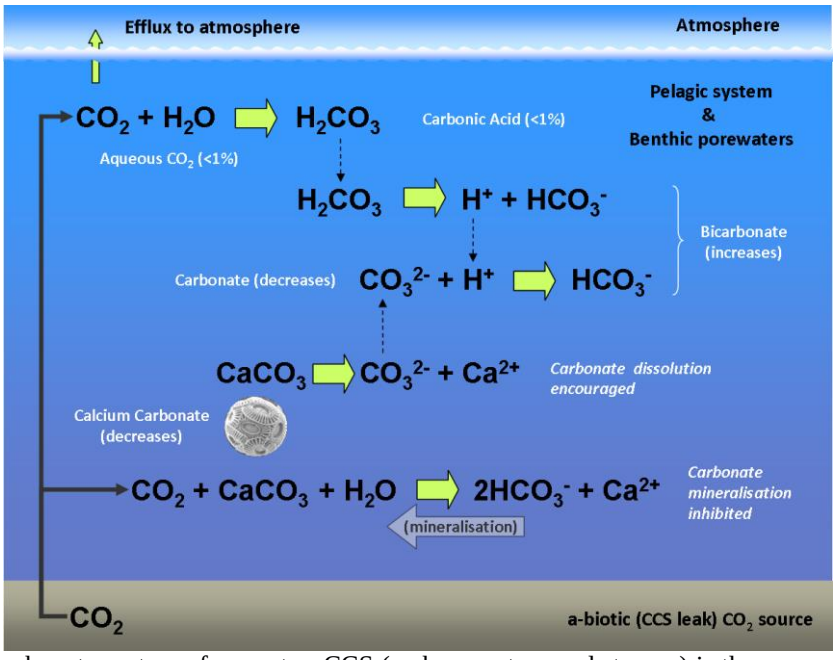

Figure 1.4. The carbonate system of seawater. CCS (carbon capture and storage) is the excess of CO_2 produced that is dissolved and stored into the ocean. It will participate dissolving the $CaCO_3$ and altering the carbonates equilibrium (*From: British Geological Survey*).

The preservation of the organisms in sea floor sediments depends on the solubility of $CaCO_3$ in seawater and on the concentration of carbonate ions. Thus, there is a critical concentration of carbonate ions in seawater (the saturation concentration) below which $CaCO_3$ will start to dissolve. Because $CaCO_3$ solubility increases with decreasing temperature and increasing pressure, the critical concentration occurs at a depth, the 'saturation horizon', below which seawater is undersaturated and $CaCO_3$ will tend to dissolve and above which seawater is super-saturated and $CaCO_3$ will tend to be preserved. Because the $CaCO_3$ mineral calcite is less soluble than the form aragonite, the aragonite saturation horizon is shallower. Because added CO_2 decreases the carbonate ion concentration, the saturation horizons will become shallower with increasing releases of human derived CO_2 to the atmosphere (Raven *et al.*, 2005). It is estimated that highlatitude surface oceans will become undersaturated with respect to aragonite by the year 2050, which may lead to the dissolution of aragonite shells (Kurihara, 2008).

Consequences for the biota

While the chemical processes underlying ocean acidification are well understood and accepted, we are just beginning to understand the wide-ranging effects acidification is likely to have on marine wildlife (Harrould-Kolieb & Savitz, 2009). Given that seawater carbonate chemistry can be highly variable, conditions that organisms are actually exposed to are difficult to measure Furthermore, the sensitivity of organisms can vary across life history

stages and in conjunction with other stressors (Waldbusser & Salisbury, 2014). The potential risks to marine systems from the current period of ocean acidification remain to be quantified; as yet, there are few robust indicators of the likely long-term biological consequences (Fitzer *et al.*, 2012). Evidence from the geological record shows that previous periods of intense ocean acidification, e.g. at the end of the Paleocene, coincided with mass extinction events (Jackson, 2010).

A major consequence of increasing ocean acidity is a reduction in the amount of carbonate available (as CO_3^{2-}) for marine biota. One of the most important uses of carbonate in the ocean is the formation of calcium carbonate or limestone structures such as corals skeletons, pearls, and the shells of coccolithophores, foraminiferans, pteropods or bivalves, i.e. the calcifiers (Raven *et al.*, 2005; Caldeira & Wickett, 2003; Kurihara, 2008; Fabry *et al.*, 2008). Calcification is only one physiological parameter affected by the ocean acidification, marine organisms can experience physiological stress not directly related to calcification due to an increase in CO_2 (hypercapnia) and/or a decrease in pH (Dupont & Thorndyke, 2009). The life cycle stages are differently susceptible, being reproductive and early life-story stages considered particularly vulnerable (Fitzer *et al.*, 2012; Kurihara, 2008; Dupont & Thorndyke, 2009). Even if increased acidity may not directly kill non-calcifying organisms, many are likely to be harmed in ways that reduce their overall fitness and ability to survive. These impacts could include decreased growth rate, reduced reproduction, disrupted respiratory and nervous system function and increased susceptibility to predators and disease, all of which could produce ripple effects through food webs and ecosystems. Ultimately, ocean acidification could transform the oceans, leaving them far less diverse and productive and making the lives and livelihoods of those who depend on them far more uncertain (Harrould-Kolieb & Savitz, 2009).

Most biota reside near the surface, where the greatest pH change would be expected to occur, but deep-ocean biota may be more sensitive to pH changes (Caldeira & Wickett, 2003), since $CaCO_3$ is abundant in sediments, so the pH of the deep oceans cannot change by large amounts over timescales of 10000 years (Raven *et al.*, 2005). However, another view has to be considered, organisms adapted to warm environments appear to be closer to thermal thresholds than cold-adapted organisms are, and plasticity in physiology may be costly to other functions, such as reproduction (Waldbusser & Salisbury, 2014). Further, extreme temperature events are principal drivers of biogeographic redistribution (Wethey *et al.*, 2011).

Consequences for zooplankton

Zooplankton is a key component of aquatic communities, and knowledge of how they cope with environmental stressors is important for understanding how the aquatic ecosystem as a whole will respond (Chan *et al.*, 2008). The holozooplanktonic $CaCO_3$ producers are the foraminifera (shells of calcite), euthecosomatous pteropods (shells of aragonite), heteropods (tropical and subtropical oceans, shells of aragonite not always present) and gymnosomes (shells of aragonite cast off at metamorphosis) (Fabry *et al.*, 2008), so it is expected that these groups will be affected under acidification conditions by reducing calcification.

Responses can be variable ranging from negative to neutral or even positive and appear to be species-specific even in closely related species (Troedsson *et al.*, 2013). Broadcast spawning invertebrates are particularly vulnerable to ocean acidification because fertilization of eggs and sperm occurs in the water column followed by development of larvae. Lecithotrophic larvae (10% of marine benthic invertebrates), may be better competitors and less affected since they spend less time in plankton, than planktotrophic larvae (60–90% of marine organisms) which feed on exogenous sources. Any sub-lethal reductions in rate of development and larval size may also have significant consequences for the survival of marine *larvae* because prolonged larval life phase and delayed settlement may lead to a concomitant increase in the likelihood of predation (Ross *et al.*, 2011). Larvae of benthic calcified organisms will respond with a reduction in calcification rate.

At date, most studies about how ocean acidification can influence the zooplankton community are short-term assays under controlled conditions focused in very restricted groups. These are not always relevant to predicted climate impacts on ecosystems (e.g. using unrealistic pH values and/or acid-based acidification without correcting carbonates and bicarbonates) or ecologically realistic (e.g. single species cultures) conditions (Dupont & Thorndyke, 2009). Since copepods are the main component in marine zooplankton, the most abundant species in each region should be the targets to elucidate responses to CO_2 changes.

Planktonic copepods typically account for about 55–95% of the biomass in pelagic zooplankton community and are the dominant herbivores (Longhurst, 1985). While heterotrophic protists in the microbial loop are considered to be the main consumers and recyclers of smaller plankton, copepods mainly transfer carbon captured at lower trophic levels to higher trophic levels (Troedsson *et al.*, 2013). The timing and the intensity of copepod reproduction is considered to be essential for survival of fish larvae, since they feed

largely on copepod eggs or nauplii during their early feeding stage. Furthermore, several pelagic fish stocks feed on copepods during their entire lifetime, and their individual growth as well as stock production is highly affected by copepod availability (Debes *et al.*, 2008).

Many studies, mostly under controlled conditions, manifest the effects of seawater acidification in different aspects of survival of copepods (annex 7.1). Most of these studies conducted until date showed no responses in copepods or low only when high CO_2 concentrations are applied. In addition, the effects of elevated *p*CO2 on the survival rates of copepods are highly species-specific. Negative responses are more related to early-life stages of copepods i.e. eggs and nauplii production and survival. As example, *Calanus finmarchicus* survival and early development is not affected by pCO_2 levels ≤2000 ppm, only changes at more than 7000ppm are observables (Pedersen *et al.*, 2013). As in others organisms, these effects can be studied at many levels, as was shown in the previous section. Li and Gao (2012) hypothesized that the increased partial pressure of CO_2 and acidity of seawater may affect the respiration of copepods to cope with the chemical changes, and hence it would mediate their feeding rate to meet the energy demand. *Centropages tenuiremis*, the dominant costal water calanoid copepod in South China Sea, was able to perceive the chemical changes in seawater (>1700 µatm, pH < 7.60) with avoidance strategy. The respiration increased at elevated CO_2 (1000 µatm) and associated acidity (pH 7.83) and its feeding rates also increased correspondingly, except for the initial acclimating period, when it fed less.

The naupliar production has been a common way to determine the reproductive response on copepods. Different assays under different controlled pH conditions have been carried out. The naupliar production of *Tisbe battagliai* (Guernsey, UK) increased significantly at pH 7.95 compared with pH 8.06 followed by a decline at pH 7.82, attributed to an initial stress response which was succeeded by a hormesis-like response at pH 7.67 (Fitzer *et al.*, 2012). Further, there was a significant growth reduction and a significant increase in the proportion of carbon relative to oxygen within the cuticle as seawater pH decreased. This strongly suggests that copepods preferentially reallocate resources towards maintaining reproductive output at the expense of somatic growth and cuticle composition. These responses may drive shifts in life history strategies that favour smaller brood sizes, females and perhaps later maturing females, with the potential to profoundly destabilise marine trophodynamics (Fitzer *et al.*, 2012). In contrast, the nauplii of *Tigriopus japonicas*, a common benthic copepod in coastal areas of the temperate zone of Japan, show a high tolerance to elevated pCO_2 environments (37,000 µatm) (Kita *et al.*, 2013). Cripps *et al* (2014) affirm in their recent

study that nauplii show the highest lethal effects of CO_2; *Acartia tonsa* nauplii mortality rates increased threefold when *p*CO2 concentrations reached 1000 µatm (year 2100 scenario) with lethal concentration 50 at 1084 µatm pCO_2. In comparison, eggs, early copepodite stages, and adult males and females were not affected lethally until pCO_2 concentrations ≥3000 µatm.

The egg production rate of *Acartia spinicauda, Calanus sinicus* and *Centropages tenuiremis* (the dominant copepods in the southern coast of China) is significantly inhibited by the increased *p*CO2 and the exposure time duration (6.92≤pH≤7.39), while *Acartia pacifica* only show inhibition with the exposure time duration (Zhang *et al.*, 2011). Calanus glacialis egg production (an Arctic shelf-water copepod) showed no effects under CO_2-induced seawater acidification. However, a reduction in pH to 6.9 significantly delayed hatching and possibly reduced overall hatching success (Weidmann *et al.*, 2012).

Acartia steueri and *Acartia erythraea* (Japan) showed sensitivity when cultured under increses pCO_2 of +10000ppm (pH 6.8) relative to control. The egg production rates of copepods decreased significantly. In addition, the nauplius mortality rate increased in *A. erythraea*. The survival rates of adult of *A. steueri* were not affected when reared under increased CO_2 for 8 days, however longer exposure times could have revealed toxic effects of elevated CO_2 concentrations (Kurihara *et al.*, 2004b). These CO_2 values are too large, since they do not reflect an expected change in the near future. It reflects the results of one of the strategies proposed to reduce anthropogenic CO_2, i.e. the direct injection of anthropogenic CO_2 into the deep ocean. The local CO_2 concentration is expected to be as high as 20,000 ppm and the pH below 5.8, what is expected to affect the water column; thus the effects on zooplankton and bacteria are of primary concern (Kurihara *et al.*, 2004a).

Acartia clausi (Mediterranean sea) under the realistic predicted values two of pH 7.83 and temperature 20°C (+4ºC) exhibited egg production rate and hatching success decreased significantly over the duration of exposure at future pH under actual and future temperature conditions (Zervoudaki *et al.*, 2013). Acidification does not have an obvious direct effect on the vital rates of the copepod, with the exception of excretion possibly. Therefore, the combination of acidification, ambient oligotrophic conditions and warming could affect the ability of the species to allocate resources for coping with multiple stressors.

1.3.A different challenge for the marine zooplankton

Natural CO_2 vents have generated a substantial interest in recent years as *in situ* laboratories for acidification studies. Field experiments, at sites with naturally-elevated CO_2 conditions, are potentially useful analogues for investigating the effect of future dissolved CO_2 levels on marine organisms and ecosystems (Calosi *et al.*, 2013, Kerrison *et al.*, 2011). Some marine CO_2 vents are at ambient seawater temperature and lack toxic sulphur compounds; such vents can prevail for years to millennia and may be used to advance our understanding (Hall-Spencer *et al.*, 2008). This opens a new door of opportunities at many levels, leaving behind mono-species studies under so restricted conditions for the zooplankton community, where the interactions within and between species and with the environment cannot be addressed.

The existence of shallow water submarine volcanic ventis is well known off volcanic islands and provinces (Cardigos *et al.*, 2005), occurring over a wide depth range, from the intertidal to the abyss. The deepest active hot vent known so far with associated fauna is the Ashadze field located at 4000–4100 m depth at 12858V N on the Mid-Atlantic Ridge (Tarasov *et al.*, 2005). Within submersed CO_2 vents, it is important to distinguish between deep-sea hydrothermal vents and shallow-water vents. At deep-sea hydrothermal vents on mid-ocean ridges there is usually a high biomass of largely endemic, but species poor, fauna that depends on chemosynthesis-based production. By contrast, at less than 200 m depth, shallow-water vents tend to have a low biomass of a more diverse fauna with few, or no, endemic species (Cardigos *et al.*, 2005). In addition, shallow water hydrothermal ecosystems are in the euphotic zone, i.e. there is primary production. Biological data have been published for approximately 55 deep-water and 21 shallow-water hydrothermal vent ecosystems (Tarasov *et al.*, 2005) (figure 1.5).

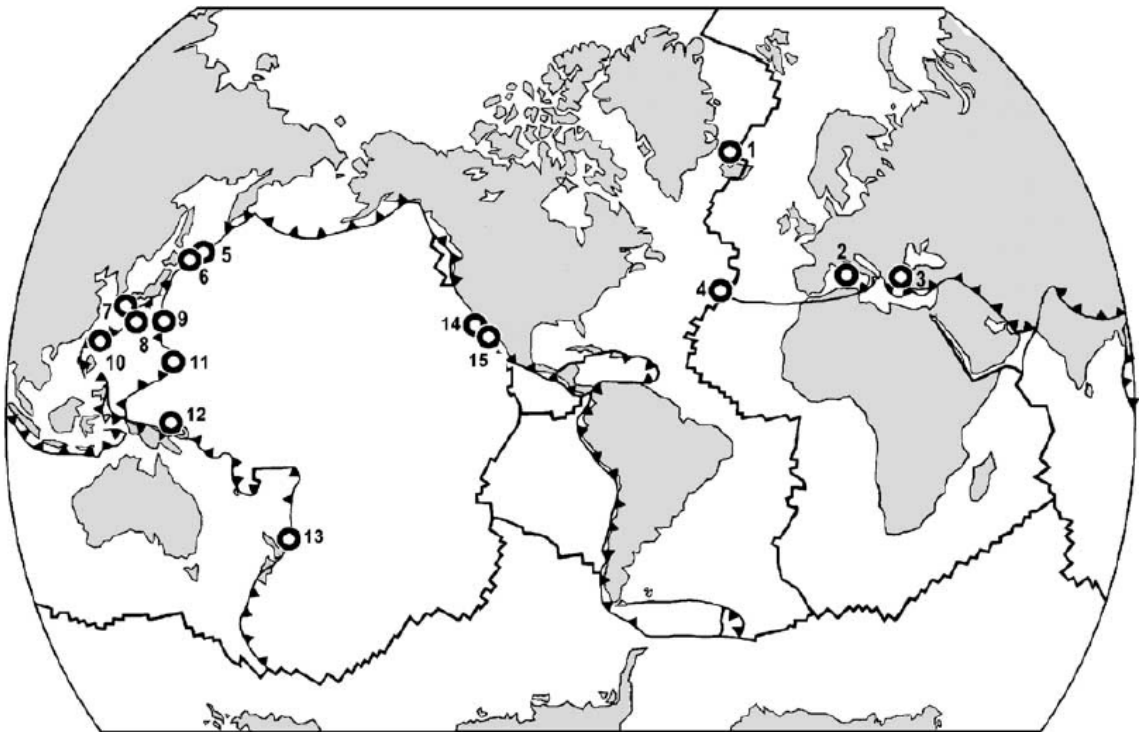

Figure 1.5. Areas of shallow-water (<200 m) hydrothermal venting with known data on biota (in several cases one symbol shows more than one closely located areas). 1.Kolbeinsey, 2.Tyrrhenian Sea (Capes Palinuro and Messino, Bahia Pozzuoli, Panarea Island), 3.Aegean Sea (Islands Santorini and Milos), 4.D. João de Castro Bank, Azores, 5.Kraternaya Bight, Ushishir Island, Kuril Islands, 6.Kunashir Island, Kuril Islands, 7.Kagoshima Bay, 8.Tokora and Iwo Islands (Kita-Iwo-jima and Akuseki-jima), 9.Nishino-shima Sintoh, Ogasawara Islands, 10.Kueishan Is., Taiwan, 11.Esmeralda Bank, Mariana Islands, 12.Matupi Harbour, New Britain Island and Tutum Bay, Ambitle Island, Papua New Guinea, 13.Bay of Plenty, New Zealand, 14.White Point, Palos Verdes, California, 15.Punta Banda and Punta Mita, Baja California (*From: Tarasov et al., 2005*).

Any study performed in these environments requires a previous description of the physical-chemical characteristics of the vent site, to stablish properly the perimeter with high CO_2 emissions and where its activity ceases. Daily-variations can appear around the main points (Kerrison *et al.*, 2011) as well as other gases and sulfides. Accordingly, analyses of gas composition, temperature, salinity and alkalinity acquire special relevance.

Previous studies on shallow-water vents consider a great diversity of groups, as bacteria (Cardigos *et al.*, 2005; Brinkhoff *et al.*, 1999, Kerfahi *et al.*, 2014), benthic invertebrates (Cardigos *et al.*, 2005; Calosi *et al.*, 2013; Hall-Spencer *et al.*, 2008; Gamenick *et al.*, 1998; Pettit *et al.*, 2013), algae (Cardigos *et al.*, 2005; Hall-Spencer *et al.*, 2008), fishes (Cardigos *et al.*, 2005) or seagrass (Hall-Spencer *et al.*, 2008; Arnold *et al.*, 2012), but only a recent study in the coast of Normanby Island (Papua New Guinea) take in count these environments to study possible effects on zooplankton communities. Smith *et al.* (2014) show that zooplankton abundance appears severely reduced in low pH waters compared to control sites. As result of night samplings, zooplankton abundance appeared highest and the difference between pH sites was greater. Additionally, certain taxonomic groups were also reduced in

abundance in low pH waters. These results indicate that CO_2 vents are optimal tools to manifest acidification effects on these communities, and similar studies in others regions of the world with implications to the trophic web assessments are of priority concern.

1.4.Determining physiological status: RNA:DNA ratio

Stablish how environmental stressors affect marine organisms through determining its physiological state has been among of the main aims of several studies. Within this framework, nucleic acid derived indices have been applied with success in microbial communities, invertebrates and fishes (Chícharo & Chícharo, 2008), and RNA:DNA ratio (R:D) has particularly excelled.

The technique is based in the changes produced over the cellular concentration of RNA, which is highly dependent on growth rate, and indirectly on environmental conditions. On an individual basis, DNA per somatic cell is assumed to be constant in sexually mature adults so that the R:D ratio can be related to the magnitude of RNA transcription, protein synthesis and hence growth condition (Pommier *et al.*, 2012). It was firstly proposed by Sutcliffe (1965), followed by Holm-Hansen *et al.* (1968), who suggest that the concentration of DNA would be a good measure of living carbon in phytoplankton.

Temperature is the dominant factor influencing copepod growth under adequate food supplies; conversely, when the temperature range is narrow, food becomes the predominant determining factor for growth (Chícharo & Chícharo, 2008). Nevertheless, R:D interpretation is not always simple and direct. High R:D has been attributed in copepods to some type of stress that led to produce proteins to cope with stress-induced denaturation of other proteins (Vehma *et al.*, 2012) and Pommier *et al.* (2012) found that the R:D of copepods was not correlated with DNA concentration, which suggested a spatial variability likely related to variable growth conditions of the copepod population. R:D has not been considered a good indicator of somatic growth, since DNA is growth-dependent and there are confounding factors related to the moult process; after a phase of hyperplasia (DNA proliferation) a phase of hypertrophy (protein assimilation) follows and hence the coupling of both processes depends on the moulting rate (Yebra *et al.*, 2011). For this reason, R:D appear as a good indicator of nutritional condition only in adult individuals.

This variability in the results suggests that even if R:D is a great methodology to assess physiological state of the organisms, results have to be considered with precaution.

1.5.Aims

The present study is part of the project MOFETA (MOFETA - EXPL/MAR-EST/0604/2013), which general objective is investigating the ecological effects of ocean acidification in shallow water volcanic CO_2 vents of the Azores. This multidisciplinary project is structured around the following objectives: 1. composition and quantification of the gases emitted in the submarine vents; 2. spatial variability of physical-chemical characteristics of seawater; 3. observational study on planktonic assemblages; 4. observational study on macrobenthic assemblages; and 5. short-term manipulative field experiments with copepod assemblages. Only the tasks related to zooplankton (3 and 5) will be developed here as follow:

1. Observational study on zooplanktonic assemblages

Determine if there are consistent differences in the abundance, diversity and composition of zooplankton assemblages between sites affected by CO_2 emissions and control sites, without emissions.

Relate the observed patterns in zooplankton assemblages in relation to the results of seawater chemistry and gas emissions.

Describe biochemical condition of major zooplanktonic groups using nucleic acids (growth and biomass indicators) and relate their ecophysiological status (RNA:DNA ratio) to possible stress caused by CO_2.

2. Short-term manipulative field experiments with copepod assemblages

Compare the Egg Production Rates (EPR) of the most abundant copepod free-spawning species between sites affected by CO_2 emissions and control sites, without emissions, to assess the possible effect of elevated CO_2 concentrations on the EPR.

2. Methodology

2.1.Study site

The study was conducted in the Azores archipelago, composed of nine volcanic islands localized in the mid-Atlantic ridge. Two active volcanic areas with submarine CO_2 emissions, one in the island of São Miguel and one in the island of Faial, were chosen for the purpose of this study (figure 2.1). São Miguel and Faial were selected within the archipelago because of the previous knowledge about the existence of submersed shallow-water CO_2 vents in two specific locations of these islands. Three sampling sites were selected within each island, two locations along the gradient of CO_2 emissions (Intermediate and Vent) and one Reference site, with similar characteristics, but no degassing. Test dives and pH measurements with a CTD equipped with a pH probe (YSI6600) were the bases to select the Vent site (were the emissions were stronger) and two subsequent locations (Reference site and Intermediate gradient site) at a reasonable distance and with similar bottom geology and depth conditions.

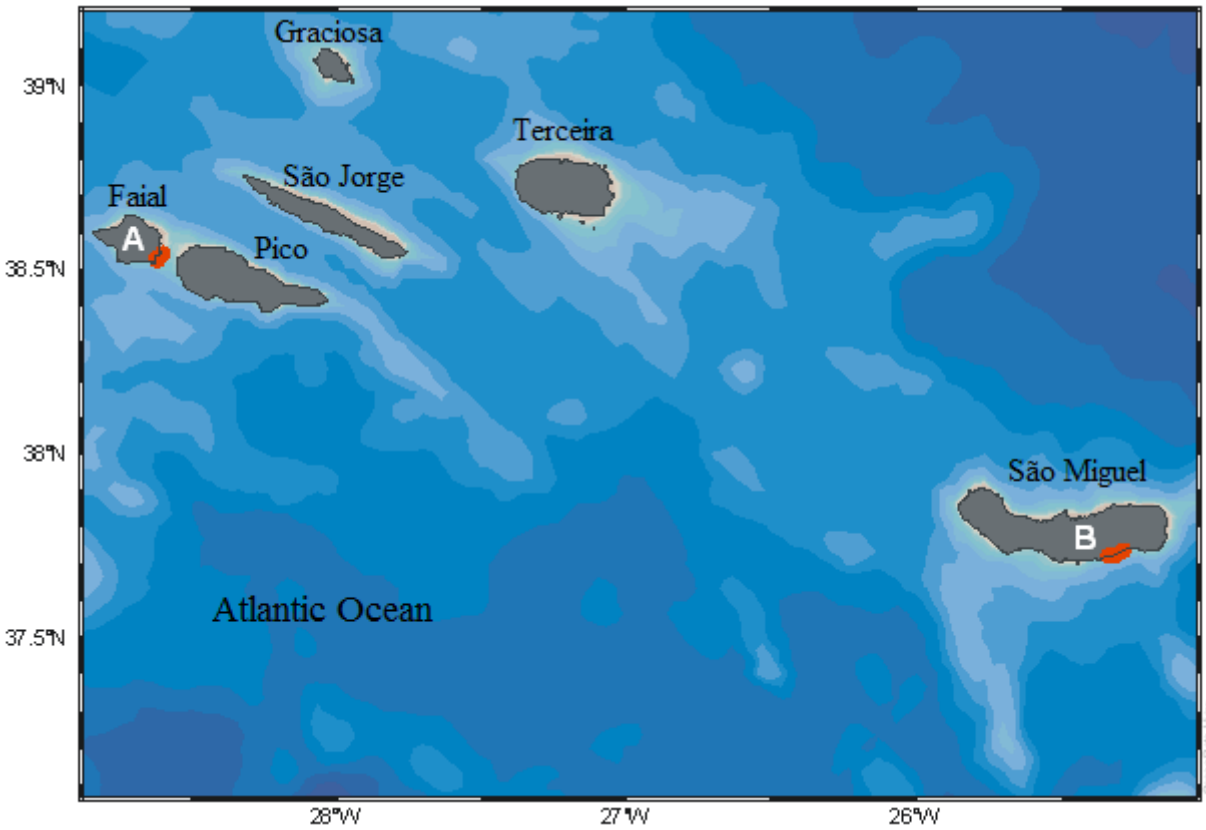

Figure 2.1. Sampling locations in the islands of Faial (A - Ponta da Espalamaca) and São Miguel (B - Ribeira Quente).

In São Miguel the Vent site (Ponta da Lobeira) was located in the south coast, close to the village of Ribeira Quente, in the flank of Furnas Volcano, between the Reference at Garajau and Intermediate site at Covões (figure 2.2). In Faial, the sampling sites were located in the

southeast of the Island; the Reference site at the exit of the Horta port, while the Vent and Intermediate sites were in front of Ponta da Espalamaca (figure 2.3). Conditions between both islands were very different, with the maximum depth of 10 meters at São Miguel, while at Faial it was 37 meters. Given their proximity to the Faial-Pico channel, the sites in Faial were strongly exposed to tidal currents. The "vent sites" in both islands also differed in terms of their geological characteristics, since some of the emissions in São Miguel were warm while in Faial they were always cold. According to these characteristic they can been denominated as hot vents and cold seeps (Tarasov *et al.*, 2005).

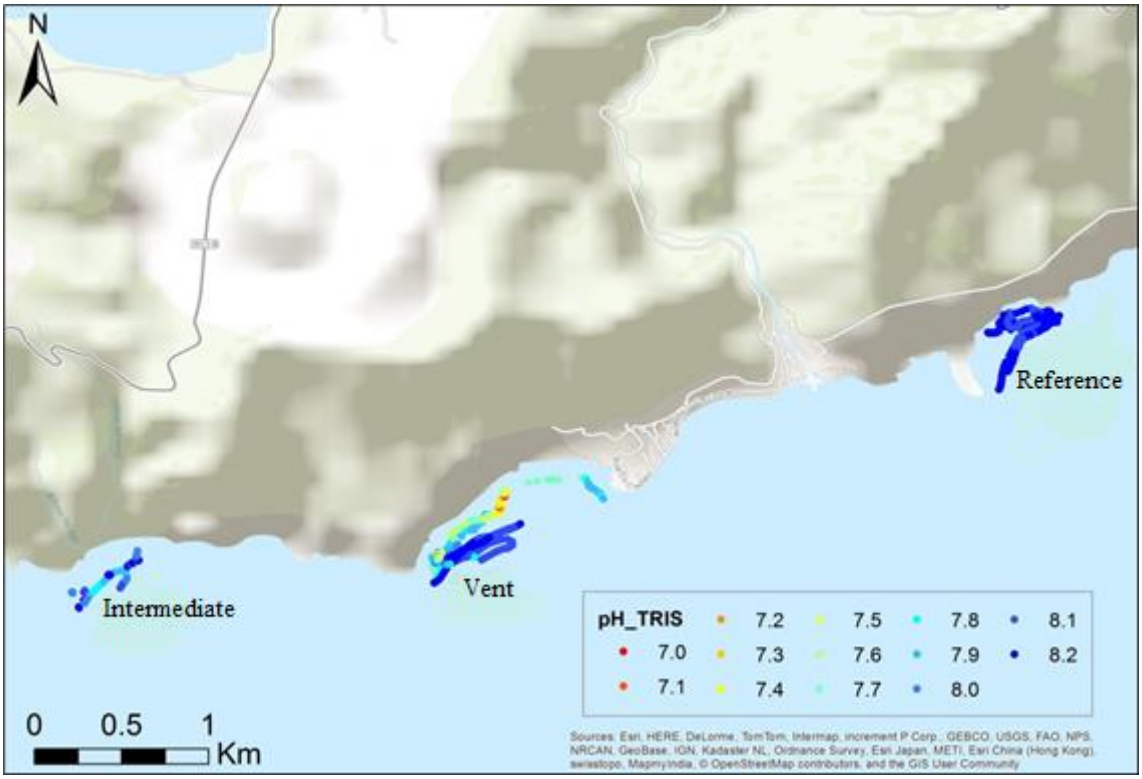

Figure 2.2. Map with *in situ* measurements of surface pH (total scale) for the 3 locations sampled during the field campaign in S. Miguel (03/07/2014-08/07/2014): Reference (37º43'39.097"N 25º18'41.292"W), Intermediate ((37º43'34.565"N 25º19'41.635"W) and Vent (37º43'28.736"N 25º19'28.596"W).

Figure 2.3. Map with *in situ* measurements of surface pH (total scale) for the 3 locations sampled during the field campaign in Faial (10/07/2014-15/07/2014): Reference (38º32'20.184"N 28º36'12.816"W), Intermediate ((38º32'31.74"N 28º35'58.272"W) and Vent (38º32'3.433"N 28º36'56.855"W).

2.2.Seawater chemistry and gas composition

On each day of the sampling campaign, the physicochemical characteristics of the seawater (temperature, salinity and pH) were measured in situ, using a YSI6000 multiprobe. In addition to the *in situ* measurements, three water samples were collected at the surface (0-2m) and near the bottom (6-10m in São Miguel and 15-38m in Faial) each site to be analyzed in the laboratory and obtain the seawater carbonate chemistry variables. pH was measured in the laboratory using a glass electrode (WTW, pH 340i) calibrated with a TRIS seawater buffer. Total alkalinity was obtained through potentiometric titration, following Dickson et al. (2003), and using a Metrohm Titrino Plus 848 equipped with a 869 Compact Sample Changer, and calibrated with certified Reference material supplied by A. Dickson. Seawater samples were filtered through a 0.2 µm membrane and measured within 48h.

Gas sampling was done in three patches of CO_2 flow in each venting site (Ribeira Quente and Ponta da Espalamaca), using Giggenbach bottles (bottles filled with NaOH 4N and under vaccum). Thus, acid gases dissolve in the basic solution and the more inert gases (non absorbed) remain in the headspace of the bottle. Then, samples were analyzed in the laboratories of CVARG (*Centro de Vulcanologia e Avaliação de Riscos Geológicos*,

University of Azores) through gas chromatography, potentiometric titration and colorimetric titration techniques. Three replicates were collected in each area.

2.3.Zooplankton assemblages

Mesozooplankton was sampled using oblique tows with a WP2 net Ø60cm and 200 μm mesh, equipped with a flow meter to determining the amount of water passing through the plankton net, during 10 minutes and at approximately 2 knots. Five tows were effectuated at São Miguel at each station between days 3 and 7 of July, 2014. In Faial, a net with 500μm mesh was used instead of the 200μm mesh used in São Miguel, since during the first sampling, the smaller one was lost. Three tows in each station of Faial were conducted between days 10 and 15 of July, 2014. Immediately after sampling, mesozooplankton was preserved in 4% borax buffered formaldehyde. Another oblique tow with the same procedure was effectuated at each location to preserve mesozooplankton partially in RNA later for biochemical determinations (RNA:DNA ratio) and partially was kept alive for manipulative experiments (egg production rates). These tows were conducted with a modified cod end, without a mesh in order to collect the organisms without much damage.

The total biovolume of each sample was determined by the method of displacement volume. Sub-samples were obtained using a Folsom splitter and 300-500 organisms per sample were counted and identified under a binocular microscope. The level of taxonomic resolution was different among phyla. Inside Arthropoda, all organisms found in the samples belong to the subphylum Crustacea, and resolution was higher than in the others phyla. Different life stages of Cirripedia (cyprid and nauplii), Copepoda (Nauplii), Euphausiacea (caliptopis, furcilia and nauplii), fish (eggs and larvae) and Coleoidea (eggs and larvae) were considered as different taxa for every analysis. *Pelagia noctiluca*, despite not to be quantified and included in the analysis since the sampling was not directed at these organisms which do not belong to mesozooplankton, appeared in some tows on the venting sites of São Miguel and Faial.

A different approach was considered for a better description of the community and comparison with other areas. As described in other studies (Shi *et al.*, 2015, Sun *et al.*, 2010), classification of zooplankton functional groups can be based on the size of the zooplankton, food preferences, trophic functionality, interactions between one another or relationships with higher trophic levels. In this study, it was considered to separate Crustacean according with its size (small, big and giant crustacean) since it was a dominant group in terms of abundance in São Miguel. Cnidaria, Mollusca, fish, Radiozoa e Tunicata were differentiated as independent

functional groups, while Annelida, Chaetognatha, Echinodermata and Foraminifera were pooled as "Others" since its abundance was too low in both islands.

2.4.RNA:DNA ratio

The most abundant and well preserved taxa were used for the RNA:DNA analysis in each site of both São Miguel and Faial islands. Nucleic acids were obtained using a method based in the microplate fluorescent assay (MFA) of Ikeda *et al.* (2007), which is a modification of the sequential fluorometric method of Bentle *et al.* (1981). Bentle *et al.* (1981) method is based on the use of an ethidium bromide fluorometric technique, where the nucleic acids are sequentially degraded by nucleases (RNase and DNase). Wagner *et al.* (1998) modified the sequential fluorometric method to the MFA with 96-well microtiter plates by adopting a sarcosyl extraction technique and eliminating the DNase step, allowing the measurement of nucleic acids of several samples at the same time.

Prior to the assay, a batch of 3 to 30 organisms were sorted depending on the taxa. Zooplankton organisms were homogenized by sonication (3 pulses 50 A during 1 min) with cold sarcosyl extraction buffer. The volume of extraction buffer was between 100 and 200 µl (0.5%). Then all samples were shaken for 30 minutes at room temperature using a vortex mixer equipped with a multiple-vial head. Afterwards, they were centrifuged (12 000 r.p.m, 0-4ºC) for 15 min to sediment any organism remain particle. The samples were diluted 1:10 with Tris buffer to reduce the sarcosyl concentration to 0.05%. In each run, duplicate 20-40 µl aliquots of supernatants of the samples and duplicates of 0, 1.1, 1.7 and 2.3 µg ml^{-1} DNA standard solutions (λ-phagus 0.25 mg ml^{-1} from Roche) and 0, 7.3, 14.6 and 21.9 µg ml^{-1} RNA standard solutions (16s-23s *E. coli* 4 µg $µl^{-1}$ from Roche) were transferred to Nunclon 96-well, black, round-bottom microplates. The ratio of DNA and RNA slopes was 8, which can be used to compare RNA/DNA ratio results determined by other protocols (Caldarone *et al.*, 2006). Gel Red solution (15 µl) was added to each well, and the plates were shaken gently at room temperature. The fluorescence was then scanned after addition of the fluorescent dye on a microplate reader (Biotek synergy HT model SIAFRTD) with 360 nm (excitation) and 590 nm (emission) (first scan- total fluorescence RNA and DNA). Following the first scan, RNase solution (15 µl, 0.12 µg ml-1) was added to each well and incubated at 37ºC for 30 minutes. The concentration of DNA was calculated directly by the standard curve. The concentration of RNA was determined indirectly by subtraction of DNA fluorescence (second scan) from total fluorescence (first scan). The fluorescent dye used was Gel red solution.

Many protocols based upon this methodology used ethidium bromide (EB), which exhibits fluorescence when bound to nucleic acids (which gives the total nucleic acid concentration in one reading). However in recent years EB has been largely replaced with alternatives like Gel Red, which are safer to use and exhibit the desired fluorescence when exposed to nucleic acids.

2.5.Copepod Egg Production Rates (EPR)

Oblique tows, as described above were effectuated, but using a modified cod end, without mesh, in order to minimize damage to the organisms. As fast as was possible, the collected material was introduced in the incubation chambers for that location (figure 2.4), and placed by divers 1 m above the sea bottom.A total of 18 chambers were placed at each Island, and the time of incubation in São Miguel lasted 24h (3 replicates per site) and 72h (3 replicates per site) while in Faial the six replicates per site were incubated for 72h. When removed, the content was preserved in lugol. Each PVC chamber had 38cm high and 10cm of diameter, divided inside with a 500µm mesh in a superior part with 30cm and an inferior part with 8cm, in order to allow eggs fall down to avoid predation. Around the chambers there was a mesh with 50 µm, to maintain the eggs inside but allow exchange of seawater and smaller food particles. For determining the Egg Production Rates (EPR) of copepods, eggs and nauplii were considered over the total adult females. In São Miguel all individuals inside the chambers were identified as Paracalanus sp. and Clausocalanus sp., while in Faial calanoids as Centropages sp. and other harpacticoids species were identified.

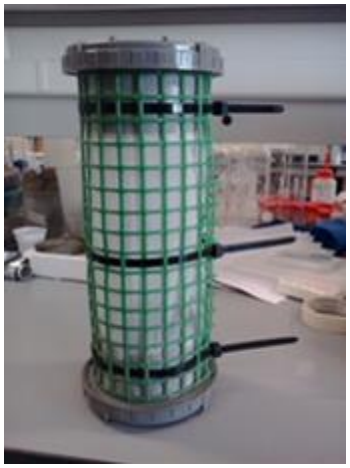

Figure 2.4. Incubation chambers for copepod EPR quantification.

2.6.Data analyses

Patterns of variation in zooplanktonic assemblage structure along the CO_2 gradient in each island were analyzed by multivariate statistical methods. Each Island (São Miguel and Faial)

was considered independently, and two orthogonal factors were considered in the analyses: Site was considered a fixed factor with three levels corresponding to areas with different volcanic CO_2 emissions (Intermediate and Vent) and control areas, without emissions (Reference); Date was considered a random factor with 5 levels in São Miguel and 3 in Faial. Statistical differences among assemblages were tested using Permutational multivariate analysis of variance (PERMANOVA) on Bray-Curtis similarities with untransformed abundances. The similarity percentages routine (SIMPER) was used to examine the contribution of each taxon to average dissimilarities between sample groups. Ordination by non-metric multidimensional scaling (nMDS) was used to visualize patterns in the biological dataset.

Abundance (N), nº of taxa (S) and diversity (d) were calculated for the entire assemblage and for the dominant phyla using RStudio version 3.1.2. (R Core Team, 2013), while eveness (J´) was obtained with the DIVERSE routine on the PRIMER 6 statistical (PRIMER- E. Plymouth Marine Laboratory). Abundances and relative abundances of functional groups were as well calculated using RStudio. Univariate analyses of variance (ANOVA) were used to test for statistical differences in the taxa highlighted by the SIMPER routine, abundances, nº of taxa, diversity indices, secondary production (egg production rate) and biovolume of samples. Differences between means have been considered statistically significant for $p < 0.05$. Homogeneity of variances was previously tested with the Bartlett test, and pair-wise tests for group means were done *a posteriori* on significant effects. Statistical analyses and data representation were done using RStudio.

3. Results

3.1.Seawater chemistry and gas composition

pH varied along the water column according with figure 3.1. In São Miguel, pH decreased under 7.0 in the Vent site near to the bottom and in the surface, while in the Intermediate and Reference sites, lowest values appeared nearest to the surface, about 7.6 and 8.0 respectively. In Faial, pH varied similarly in Vent and Intermediate sites, being under 7.0 in the bottom, while in the Reference it oscillated from 7.85 to 8.15 along the water column. The water column in São Miguel varied from 10 m in the Reference and Intermediate sites to 7m in the Vent, while in Faial it was almost 40 m in the Intermediate and Vent, and 30 m in the Reference site.

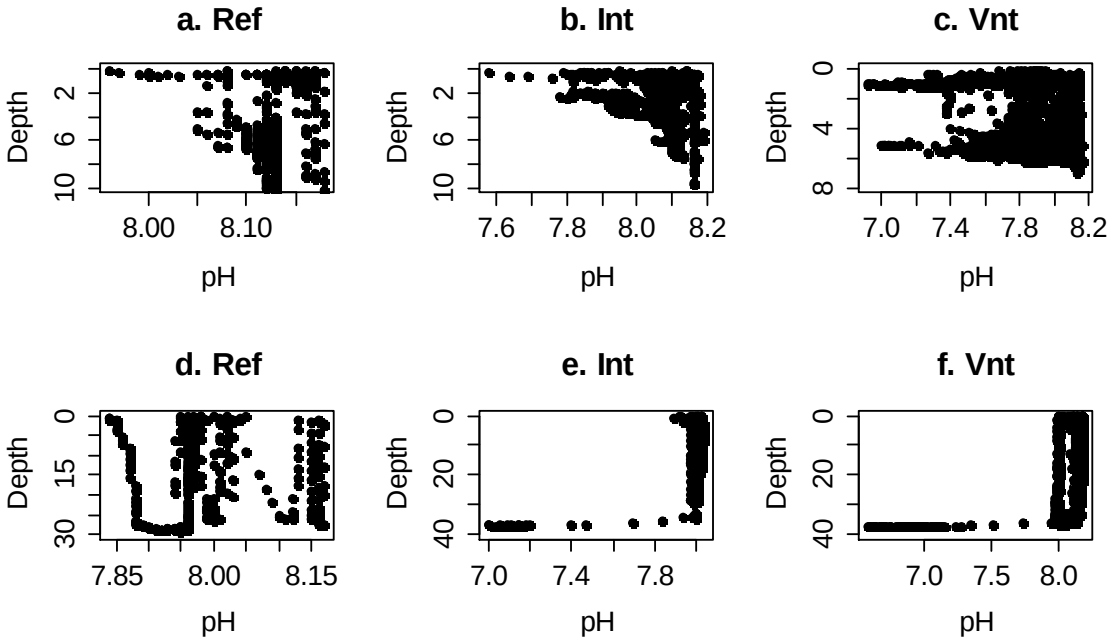

Figure 3.1. pH variation along the water column in three sites: Reference (Ref), Intermediate (Int) and Vent (Vnt) of São Miguel (a, b, c) and Faial (d, e, f).

Figure 3.2 shows the pH and temperature values frequency in São Miguel (11a-b) and Faial (11c-d). In both islands, highest values of pH were constant through the three sites, while lowest values appeared only in the venting site, and Intermediate was between both. Temperature scarcely varied. In São Miguel, lowest values increased from Reference to Vent, while in Faial this variation was not constant.

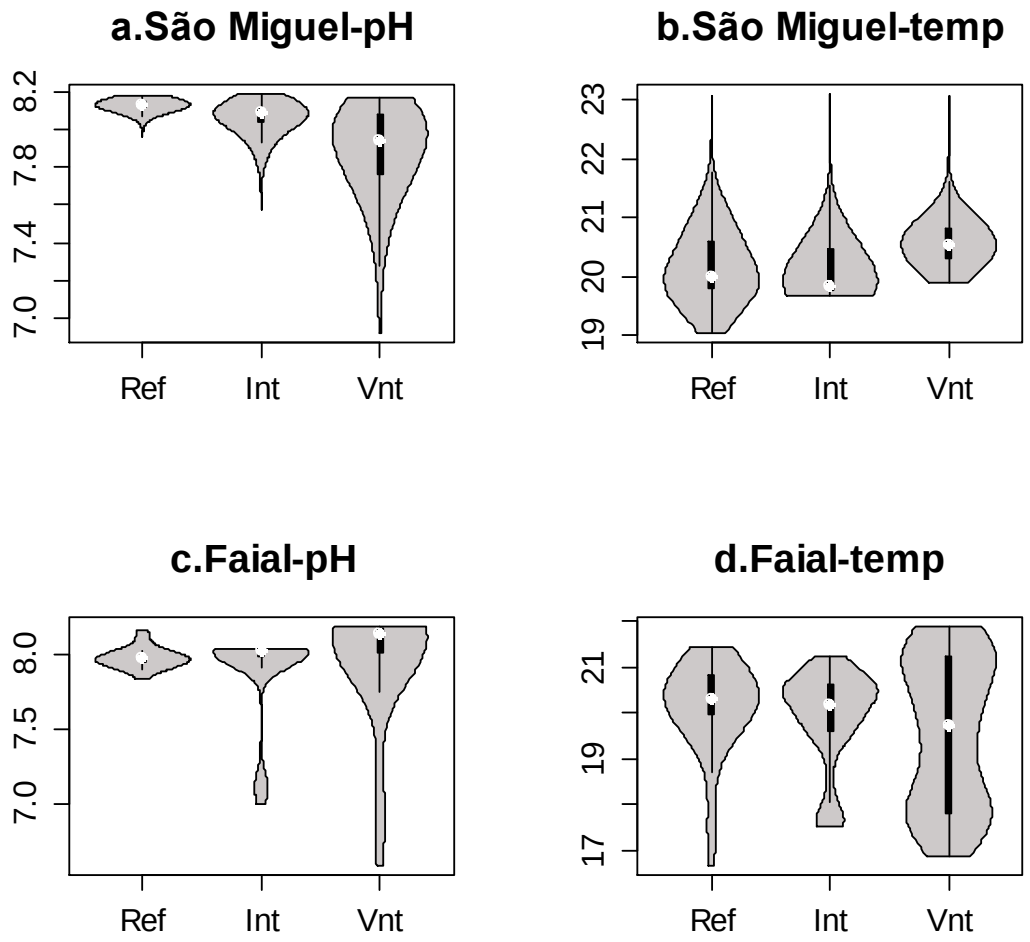

Figure 3.2. Violin plots for pH (a) and temperature (b) values distribution in the three sites (Ref-Reference, Int-Intermediate, Vnt-Vent) of São Miguel. White points are the median of the data and black boxes indicate the interquartile range.

Seawater carbonate chemistry variables (daily means) for each of the three sites sampled in both São Miguel and Faial are summarized in tables 3.1 and 3.2. The water column was sampled at the surface (0-2m) and bottom (10m in São Miguel and 38m in Faial). In São Miguel island, temperature and salinity did not vary significantly from bottom to surface and among sites (19.79 to 20.67ºC, 36.78 to 36.92ppt), only minimum temperature in the bottom increased minimally from Reference to Vent. pH decreased gradually from the Reference to the Vent site in the surface, while in the bottom, the Intermediate value did not follow this pattern. In Faial, salinity only increased in the surface 0.21 ppt from the Reference to the Vent, and temperature did not vary between sites; there were some grades of difference from bottom to surface due to a thermocline (ref-1.53ºC, int-1.4ºC, vnt-2.39ºC). Measurements of pH in table 3.2 show no variation in the pH at the surface, while at the bottom it dropped from

8.13 in the Reference to 8.02 in the Intermediate and 7.88 in the Vent. Total alkalinity did not vary substantially among sites or between both islands.

Table 3.1. Seawater carbonate chemistry variables (means±SE, n=3) for each site (Ref-Reference, Int-Intermediate, Vnt-Vent) on São Miguel. The parameters represented are: temperature (Temp), salinity (Sal), pH (total scale), total alkalinity (TA), CO_2 (total scale), CO_2 pressure, bicarbonate (HCO_3), carbonate (CO_3^{2-}), CO_2, ion hydroxide (OH), saturation of calcite (ΩCa) and aragonite (ΩAr).

Site	Depth layer	Temp (ºC)	Sal (ppt)	pH	TA (µmol/kgSW)	TCO_2 (µmol/kgSW)	pCO_2 (µatm)	HCO_3 (µmol/kgSW)	CO_3 (µmol/kgSW)	OH (µmol/kgSW)	ΩCa	ΩAr
Ref	Surface (0-2m)	20.67±0.28	36.79±0.07	8.06±0.02	2366.33±8.70	2075.43±14.80	388.67±19.33	1856.97±19.38	206.33±5.93	4.83±0.18	4.87±0.13	3.20±0.10
	Bottom (5-10m)	19.79±0.20	36.83±0.07	8.07±0.02	2375.23±0.59	382.53±12.85	383.80±26.41	2086.70±12.85	382.53±26.35	4.53±0.2	4.83±0.35	3.18±0.12
Int	Surface (0-2m)	20.56±0.33	36.85±0.02	7.99±0.04	2385.57±2.37	2133.20±23.21	483.37±54.96	1935.37±36.22	182.60±14.94	4.13±0.47	4.30±0.35	2.80±0.25
	Bottom (5-10m)	20.16±0.22	36.92±0.06	8.11±0.01	2376.27±1.56	2376.27±11.18	337.90±14.56	2057.50±11.18	336.73±14.49	5.2±0.25	5.3±0.15	3.13±0.12
Vnt	Surface (0-2m)	20.54±0.05	36.78±0.08	7.75±0.11	2412.20±2.23	2268.67±48.70	983.10±277.61	2118.03±65.89	119.67±25.79	2.50±0.61	2.83±0.61	1.83±0.38
	Bottom (5-10m)	20.28±0.07	36.83±0.08	7.87±0.13	2385.33±6.74	2385.33±59.31	742.50±273.57	2188.80±59.31	740.00±272.67	3.23±0.84	3.57±0.8	2.3±0.51

Table 3.2. Seawater carbonate chemistry variables (means±SE, n=3) for each site (Ref-Reference, Int-Intermediate, Vnt-Vent) on Faial. The parameters represented are: temperature (Temp), salinity (Sal), pH (total scale), total alkalinity (TA), CO_2 (total scale), CO_2 pressure, bicarbonate (HCO_3), carbonate (CO_3^{2-}), CO_2, ion hydroxide (OH), saturation of calcite (ΩCa) and aragonite (ΩAr).

Site	Depth layer	Temp (ºC)	Sal (ppt)	pH	TA (µmol/kgSW)	TCO_2 (µmol/kgSW)	pCO_2 (µatm)	HCO_3 (µmol/kgSW)	CO_3 (µmol/kgSW)	OH (µmol/kgSW)	ΩCa	ΩAr
Ref	Surface (0-2m)	20.75±0.19	37.87±0.13	8.12±0.01	2376.17±1.52	2042.13±8.47	331.07±13.43	1798.63±13.43	233.13±6.64	5.63±0.24	5.46±0.15	3.56±0.1
	Bottom (15-38m)	19.22±0.81	37.44±0.03	8.13±0.03	2367.23±7.74	2042.63±5.89	319.37±21.32	1805.7±9.79	226.53±5.85	4.97±0.12	3.43±0.09	5.33±0.15
Int	Surface (0-2m)	20.57±0.30	37.90±0.14	8.13±0.01	2330.20±26.27	1996.07±28.62	315.30±14.04	1754.93±30.33	231.17±6.64	5.63±0.3	5.42±0.17	3.54±0.11
	Bottom (15-38m)	19.17±0.21	37.44±0.15	8.02±0.11	2360.20±5.83	2092.07±58.26	467.73±153.90	1885.17±88.96	191.6±35.92	4.13±0.97	2.91±0.54	4.5±0.8
Vnt	Surface (0-2m)	20.60±0.54	38.08±0.26	8.14±0.02	2342.50±30.12	1996.80±30.53	305.47±16.60	1748.9±29.5	238.33±3.9	5.87±0.09	5.57±0.09	3.64±0.06
	Bottom (15-38m)	18.21±0.22	37.46±0.06	7.88±0.21	2365.73±5.38	2127.93±90.00	894.08±589.23	1916.85±117.25	180.9±47.2	3.78±0.73	2.78±0.73	4.24±1.1

Three gas samples (replicates A, B, C) were taken in the venting sites of São Miguel and Faial to obtain the gas composition (table 3.3). CO_2 was always above 99.3%, while N_2 and O_2 with Ar appeared in low content. Under 0.01% were He, H_2S, CH_4 and H_2, these last three below detection limit in Faial.

Table 3.3. Gas emissions and composition in São Miguel and Faial venting sites (b.d.l.-below detection limit).

Submarine vent	Replicate	CO_2 (molar%)	H_2S (molar%)	CH_4 (molar%)	H_2 (molar%)	He (molar%)	N_2 (molar%)	O_2 + Ar (molar%)
São Miguel	A	99.49	b.d.l.	1.14E-02	1.39E-04	1.01E-03	0.37	0.13
	B	99.86	9.66E-04	1.25E-02	4.00E-05	9.95E-04	0.11	0.02
	C	99.58	8.97E-02	1.39E-02	3.97E-02	1.06E-03	0.23	0.05
Faial	A	98.61	b.d.l.	b.d.l.	b.d.l.	6.32E-03	1.27	0.12
	B	98.91	b.d.l.	b.d.l.	b.d.l.	3.41E-03	0.94	0.15
	C	99.35	b.d.l.	b.d.l.	b.d.l.	2.24E-03	0.56	0.09

3.2.Zooplankton assemblages

Organisms from nine different phyla were identified in São Miguel and Faial, with a total of 71 different taxa, 45 in S. Miguel and 61 in Faial (annex 7.2). *Pelagia noctiluca* was collected in the venting sites of both islands, one individual on 6th in São Miguel and three in Faial, two on 12th and one on 15th. Crustacea (Arthropoda) was the most abundant group in São Miguel (figure 3.3), with significantly greater abundance in the venting site, relative to the Intermediate and Reference sites (F value=17.81, p=0.0003). Significant differences among sites were also observed in the abundance of Chordata (F value=9.98, p=0.003) and in the total abundance (F value=22.94, p=0), when considering all taxa (annex 7.6).

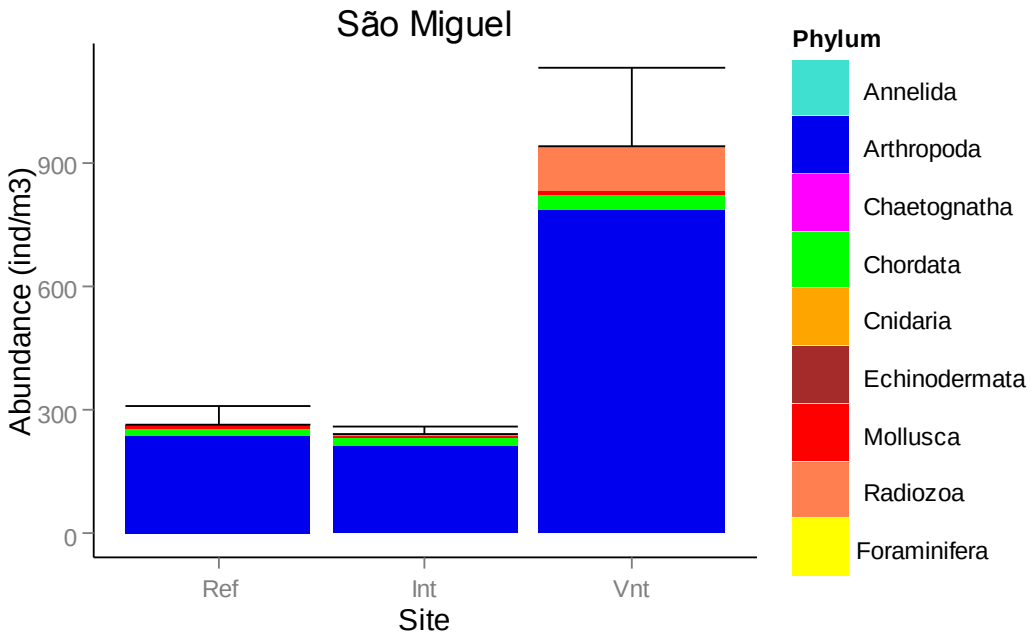

Figure 3.3. Abundances per zooplankton phylum (ind/m^3, mean±SE) for the three sampling sites in São Miguel (Ref-Reference, Int-Intermediate, Vnt-Vent).

Patterns in terms of nº of taxa (S), diversity (H) and eveness (J´) among sites were analyzed (annexes 7.5 and 7.6). When all taxa were considered, all these indices varied inversely to the CO_2 gradient, although no significant differences were observed for particular phyla or the total.

Radiozoa dominated the assemblages in all sites of Faial, and in contrast with S. Miguel, the smallest values of total abundance were found in the Vent site (figure 3.4). No significant differences were found in the abundance of particular phyla or the total taxa (annex 7.7).

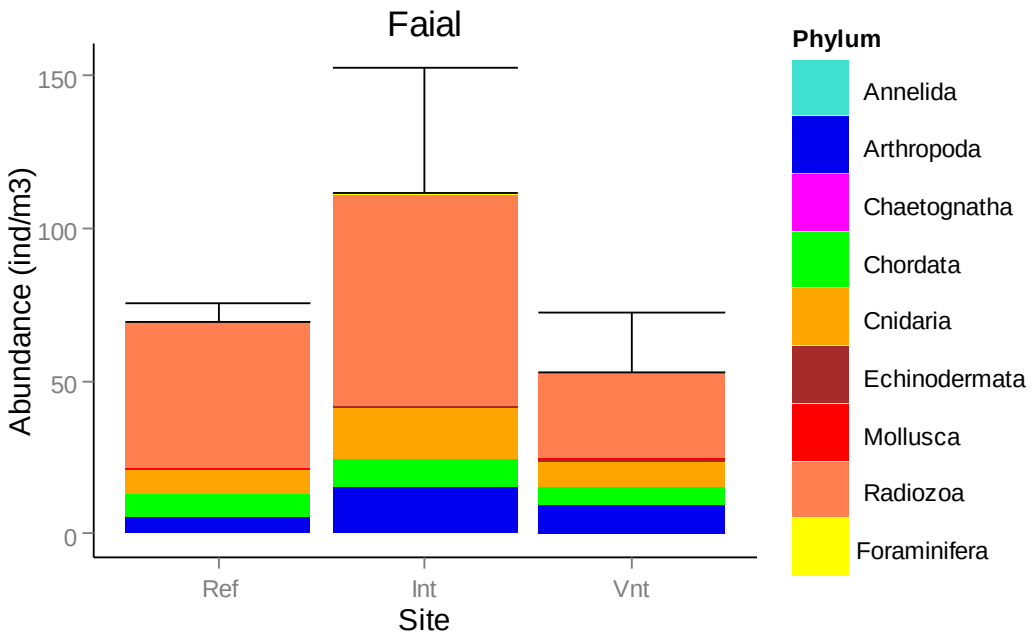

Figure 3.4. Abundances per zooplankton phylum (ind/m^3, mean±SE) for the three sampling sites in Faial (Ref-Reference, Int-Intermediate, Vnt-Vent).

In general, there were not significant differences in the nº of taxa, diversity and eveness patterns analyzed among sites of Faial. The only exception was the diversity of Arthropoda (F value=8.35, p=0.02) (annex 7.7). It decreased along the CO_2 gradient (annex 7.5).

According with the zooplankton functional groups approach, the abundances distribution among sites is represented in annex 7.3 and relative abundance of each group is in annex 7.4. In São Miguel, small crustacean dominate over big and giant crustacean, being representative of the community (Reference-86.07%, Intermediate-86.49%, Vent-82.20%). In Faial, the distribution of relative abundances among groups is more balanced. The most abundance group is Radiozoa (Reference-69.33%, Intermediate-62.10%, Vent-53.47%), while Cnidaria has a good representation compared to São Miguel (Reference-11.56%, Intermediate-15.04%, Vent-15.08%). ANOVA analyses on the abundances of functional groups showed significant differences among sites for small crustacean (F value=16.4, p=0.00037) and Tunicata (F value=10.47, p=0.00233) in São Miguel only.

PERMANOVA on São Miguel data showed that composition of the zooplanktonic assemblages differed significantly between the three sites considered, while the differences among sampling days were not significant (Table 3.4).

Table 3.4. PERMANOVA on Bray-Curtis similarities for untransformed abundances in São Miguel. Site is fixed and Date is a random factor; unrestricted permutations (9999) of data were used. Df-degrees of freedom, Sum Sq-Sum of squares, Mean Sq-Mean of squares.

	Df	Sum Sq	Mean Sq	F Model	R^2	*P*
Site	2	0.9324	0.4662	7.2187	0.5034	0.001
Date	4	0.4032	0.1008	1.5606	0.2177	0.138
Residuals	8	0.5167	0.0646		0.2789	
Total	14	1.8522			1.0000	

Non-metric multi-dimensional scaling (nMDS-Bray-Curtis similarities) analyses was done to illustrate the distribution of the main species highlighted by the SIMPER routine. The main contributors (>5%) to the differences among sites were the cladoceran *Evadne spinifera* (37.90% Ref-Int, 50.23% Ref-Vnt, 44.25% Int-Vnt), the calanoid copepod *Paracalanus parvus* (30.83% Ref-Int, 22.93% Ref-Vnt, 29.66% Int-Vnt), Cirripedia nauplii (7.92% Ref-Int) and Radiozoa (10.22% Ref-Vnt, 10.22% Int-Vnt). The nMDS (figure 3.5) shows the distribution of the samples sites and data. Dimension 1 on the nMDS separated the samples of the Vent site from the Reference and Intermediate sites, with the exception of one sample of Reference and Vent collected on July 7. Dimension 2 divided the Reference and Intermediate samples, except for a samples collected on July 5 in the Intermediate site. Sample on July 3 in the Reference appears in the limit of dimension 2. Radiozoa, *Paracalanus parvus* and *Evadne spinifera* seem more related to Vent conditions, while Cirripedia nauplii is closer to the Reference conditions.

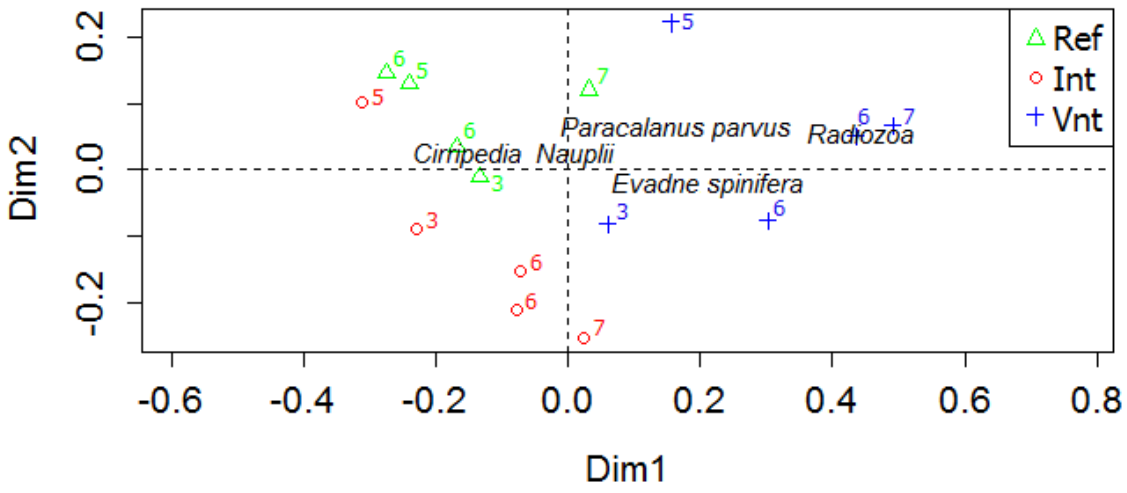

Figure 3.5. Non-metric multi-dimensional scaling (nMDS-Bray-Curtis similarities) analyses of zooplankton assemblages in São Miguel.

Univariate analysis of variance for the abundance of the species highlighted by the SIMPER routine showed significant differences in the abundance of *Evadne spinifera* among sites (F value=7.562, p=0.0075), while for *P. parvus*, Cirripedia nauplii and Radiozoa there were not significant differences in their abundance (annex 7.8).

PERMANOVA was done on Faial data, showing that composition of the zooplanktonic assemblages did not differ between sites, but it was significant among sampling days (Table 3.5).

Table 3.5. PERMANOVA on Bray-Curtis similarities for untransformed abundances in Faial. Site is fixed and Date is a random factor; unrestricted permutations (9999) of data were used. Df-degrees of freedom, Sum Sq-Sum of squares, Mean Sq-Mean of squares.

	Df	Sum Sq	Mean Sq	F Model	R^2	*p*
Site	2	0.1957	0.0978	0.9353	0.1701	0.502
Date	2	0.5362	0.2681	2.5630	0.4661	0.036
Residuals	4	0.4184	0.1046		0.3638	
Total	8	1.1503			1.0000	

Results of the SIMPER routine, showed that the main contributors (>5%) to the differences among sites were Radiozoa (38.75% Ref-Int, 39.45% Ref-Vnt, 40.42% Int-Vnt), the Siphonophoran *Bassia bassensis* (14.43% Ref-Int, 7.02% Ref-Vnt, 13.41% Int-Vnt), and Scyphozoa ephyra (6.68% Ref-Vnt). The non-metric multi-dimensional scaling (nMDS-Bray-Curtis similarities) shows the distribution of the samples sites and data (figure 3.6).

Dimension 2 on the nMDS separated samplings on 15 July from samplings on 14 July, while 12 July appears in the left side of dimension 1, and in both sides of dimension 2. No differentiation can be made among sites. *Bassia bassensis* is correlated with samples on 14 July, while Radiozoa and Scyphozoa ephyra are more related to 12 July and 15 July respectively.

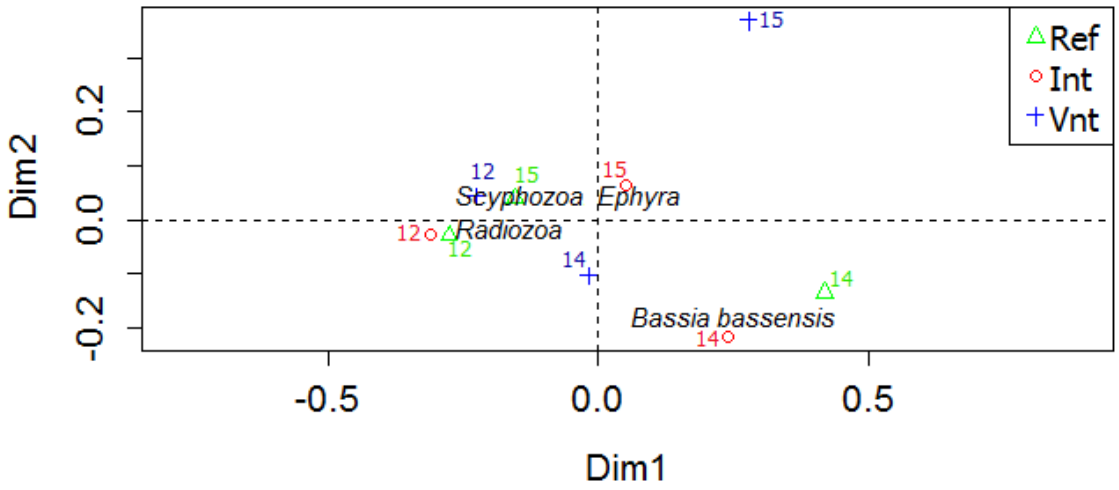

Figure 3.6. Non-metric multi-dimensional scaling (nMDS-Bray-Curtis similarities) analyses of zooplankton assemblages in Faial.

No significant differences were found in the univariate analysis of variance for the abundance of the species highlighted by the SIMPER routine (annex 7.8).

In both islands, the biovolume of zooplankton samples was generally smaller in the Reference site, relative to the sites with CO_2 degassing (figure 3.7). Nevertheless, this pattern was not significant (F value=0.19, p=0.83 in S.Miguel; F value=0.44, p=0.67 in Faial), as illustrated in table 3.6.

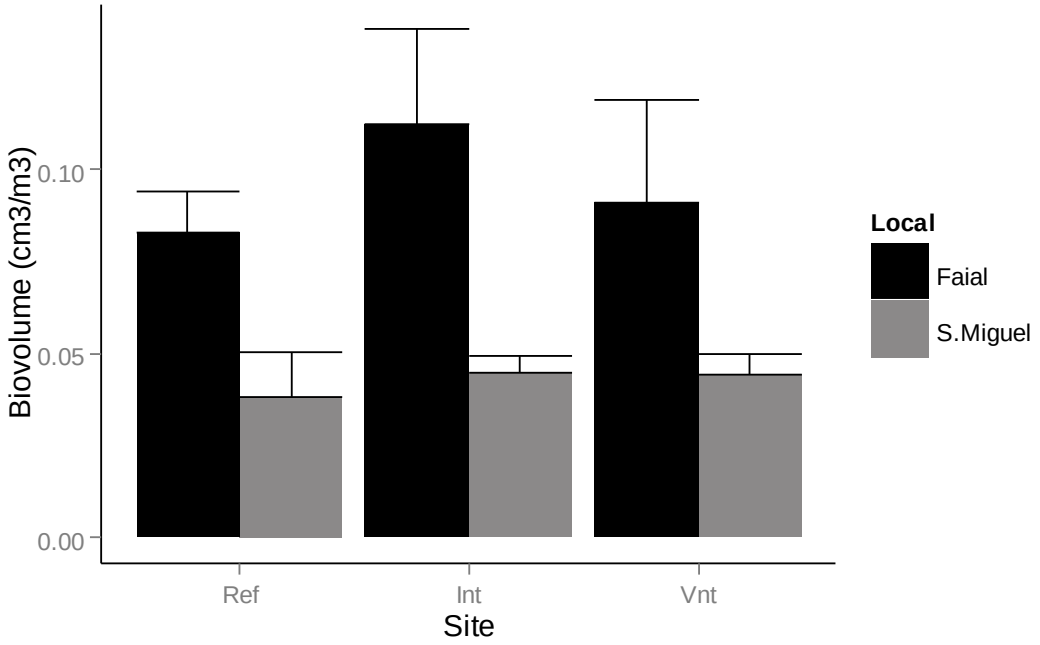

Figure 3.7. Samples biovolume (cm^3/m^3) of each site (Ref-Reference, Int-Intermediate, Vnt-Vent) of São Miguel and Faial (means±SE).

Table 3.6. ANOVA on biovolume. Df-degrees of freedom, Sum Sq-Sum of squares, Mean Sq-Mean of squares (n=5 in São Miguel and n=3 in Faial).

	Df	Sum Sq	Mean Sq	F value	*p*
Site	2	0.00	6.36e-05	0.19	0.83
Residuals	12	0.00	3.35e-04		
Site	2	0.00	0.00	0.44	0.67
Residuals	6	0.01	0.00		

3.3.RNA:DNA ratio

In São Miguel, only copepods and fish eggs were analyzed independently from other groups, while the rest of organisms were pooled. The RNA:DNA values in São Miguel ranged from 0.02 to 2.98, with no consistent pattern of variation among sites or groups of organisms (figure 3.8). The RNA:DNA ratio of fish eggs consistently showed larger values in sites affected by CO_2 relative to Controls, while copepods copepods showed the opposite trend and no pattern was discernible for other groups. In Faial, the RNA:DNA ratio of fish eggs consistently increased along the CO_2 gradient and cladocerans showed a similar pattern to copepods in São Miguel, while other groups showed no clear trend.

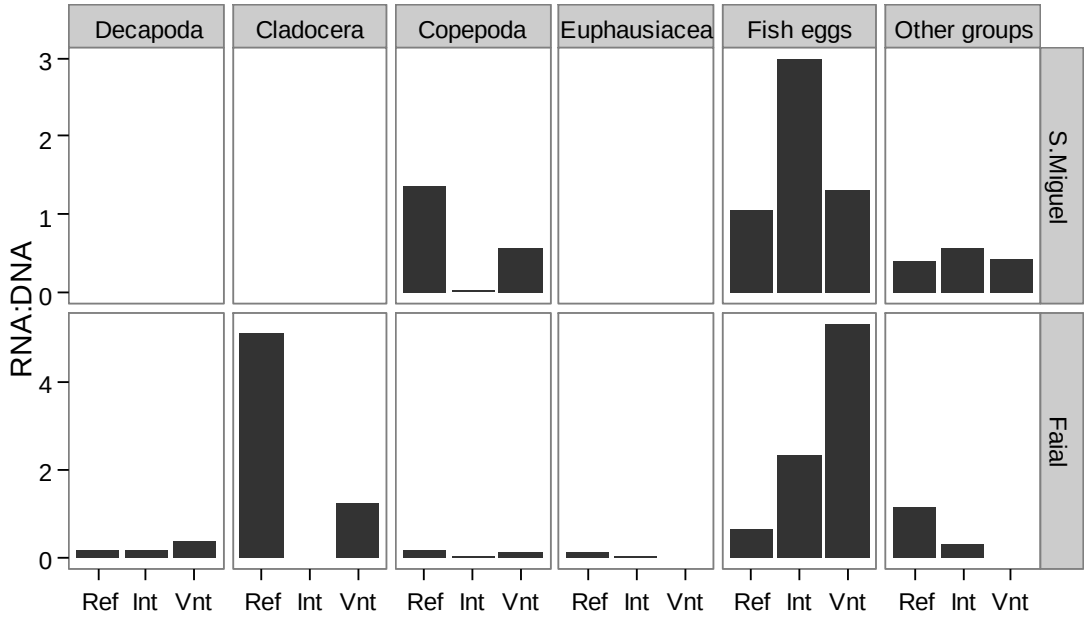

Figure 3.8. RNA:DNA of the main groups found at each site (Ref-Reference, Int-Intermediate, Vnt-Vent) of São Miguel and Faial Islands. Decapoda, cladocera and euphausiacea were groups only analyzed in Faial.

3.4.Copepod Egg Production Rates (EPR)

For determining the Egg Production Rates (EPR) of copepods, eggs and nauplii were considered over the total adult females. All individuals inside the chambers were identified as *Paracalanus sp.* and *Clausocalanus sp.* in São Miguel, and calanoids as *Centropages sp.* and others harpacticoids species in Faial.

In São Miguel, the EPR showed a decreasing trend along the CO_2 gradient (figure 3.9), with significant differences among sites (F value=4.45, p=0.0384) (table 3.7). The effect of exposure time (24 or 72h) was also tested in S. Miguel, with no differences in the egg production rate (F value=0.6176, p=0.6176) (table 3.7).

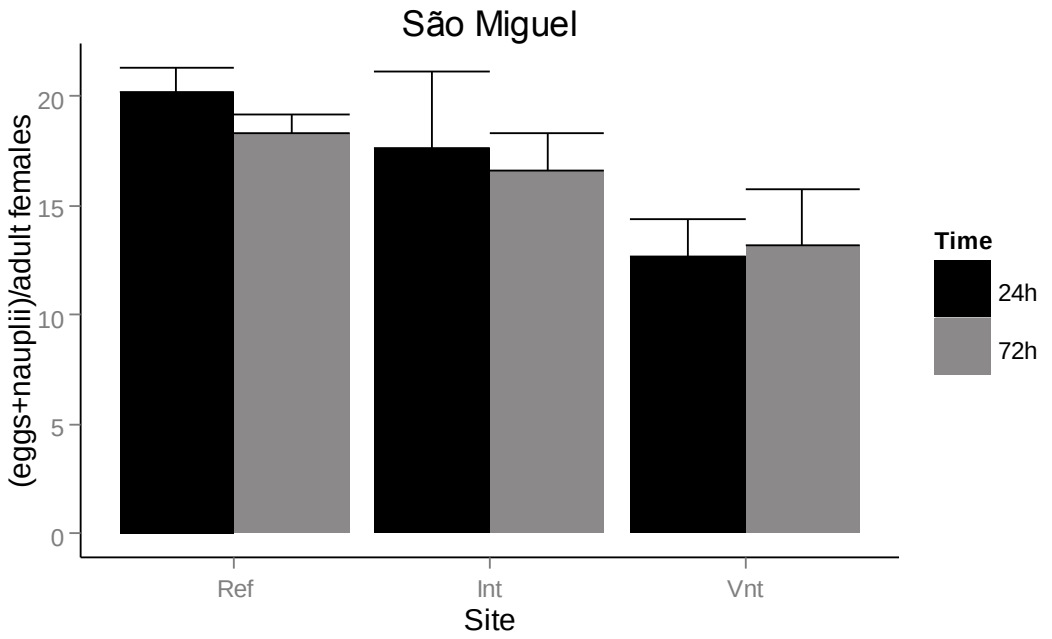

Figure 3.9. Egg production rate ((eggs+nauplii)/adult females) at each site (Ref-Reference, Int-Intermediate, Vnt-Vent) of São Miguel (means±SE). Incubation chambers were placed for periods of 24 and 72h.

Table 3.7. ANOVA on São Miguel egg production rate (24 and 72h incubation). Df-degrees of freedom, Sum Sq-Sum of squares, Mean Sq-Mean of squares (n=3).

	Df	Sum Sq	Mean Sq	F value	*p*
Site	2	111.41	55.71	4.45	0.0384
Time	1	3.30	3.30	0.264	0.6176
Site:Time	2	3.62	1.81	0.145	0.8670
Residuals	11	137.72	12.52		

Incubation chambers were all placed in Faial for 72h (figure 3.10). As in S. Miguel, the production was higher in the Reference site, but there were not significant differences among the three sampling sites (F value=4.37, p=0.07) (table 3.8).

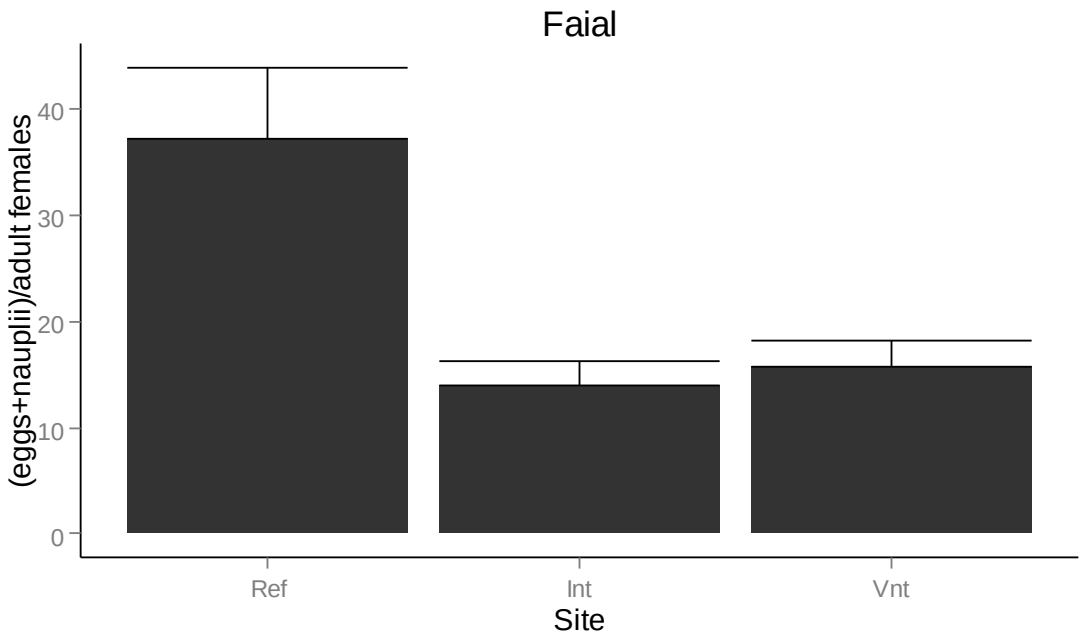

Figure 3.10. Egg production rate ((eggs+nauplii)/adult females) at each site (Ref-Reference, Int-Intermediate, Vnt-Vent) of Faial (means±SE, n=3). Incubation chambers were deployed during 72h.

Table 3.8. ANOVA on Faial egg production rate (24h incubation). Df-degrees of freedom, Sum Sq-Sum of squares, Mean Sq-Mean of squares (n=3).

	Df	Sum Sq	Mean Sq	F value	*p*
Site	2	1001.7	500.9	4.37	0.07
Residuals	6	687.8	114.6		

4. Discussion

4.1.Seawater chemistry and gas composition

Carbon dioxide was the dominant gas in the submarine degassing areas of Espalamaca (Faial) and Ponta da Lobeira (S. Miguel) with values consistently exceeding 98%. The low concentrations of toxic gases such as hydrogen sulfide (H_2S) entails an additional advantage to the, since they would be confounding factors in the interpretation of the results. Accordingly, from the perspective of the composition of gas emissions, both these sites can be considered suitable for long-term studies on the effects of ocean acidification.

São Miguel and Faial where the two islands considered in our study as clear example of response to ocean acidification, but despite of their proximity, differences in their physical characteristics prevented the direct comparison of the zooplankton communities found in them. Even more important, their comparison in this study was not possible due to mesh size of the nets used in the sampling (200 µm in S. Miguel and 500 µm in Faial).

In São Miguel island, salinity did not vary among sites and the small increase in bottom temperature from Reference to Vent is probably due to the smaller deep in the Vent site (figures 10 and 11). Thus, main differences between sites are due to changes in CO_2 and pH levels. Punctual measurements in table 3.1 generally show higher values of CO_2 near the bottom, relative to the surface and this trend is more pronounced at the Vent site. As showed in figure 1.2, actual values of CO_2 pressure at the ocean surface are estimated in 380 µatm. In São Miguel, 388.67 µatm was considered the Reference value since there was no bubbling where it was measured. It increases to 483.37 in the Intermediate and to 983.10 in the Vent, showing a gradient within the range of the Representative Concentration Pathways in the latest IPCC assessment report (AR5), varying between 421 (RCP2.6) and 936 (RCP8.5) ppm CO_2. Accordingly results at the Vent site should be considered as representatives of the more extreme scenario at the end of this century. This corresponded to pH decrease from 8.06 to 7.75 (0.31 units), which is also within the range predicted by 2100 (0.3/0.4 units) (Fitzer *et al.*, 2012; Dupont & Thorndyke, 2009; Havenhand, 2012).

The CO_2 is bubbled from the bottom and dissolved in the waters around the venting sites and probably expelled to the air, resulting in chemical changes that are within a range expected according to current projections of future atmospheric CO_2 levels. The pH values observed at the Vent site are definitively more realistic than those used in some of the previous

manipulative experiments carried out in controlled conditions (annex 7.1). As presented in figure 3.2, median and minimum values of pH decrease to the Vent site, but higher values are represented in the three sites. pH values are vertically distributed along the water column (figure 3.1), with the lower values being generally observed in the surface, except at the Vent site where very low values were also observed near the bottom. This suggests a natural gradient could exist if CO_2 is bubbled and concentrated in the surface before to be released to the atmosphere where the concentration is generally lower. In fact, pCO_2 is greatest at the surface in all sites, however, most of the pH measurements were done near the bottom or at the surface, which may have biased the results for the Intermediate levels of the water column, which were less represented.

Faial shows totally different characteristics from São Miguel. The water column goes to 38m and the strong currents between Faial and Pico islands (figure 2.1) make the selected areas do not have the same insulation and stability than in São Miguel. It was already reported by Caramanna *et al.* (2010) that the volume of the water affected by chemical variations is influenced by local conditions, such as the presence of thermocline and currents. This led to a lower water residence time and faster dissipation of the CO_2 gradient (Agostini *et al.*, 2015). It has been proposed by Agostini *et al.* (2015) that a mapping of pH levels and a longer pH monitoring would led to a better delimitation of the areas affected for the venting activity. What was appreciated from the data recorded with the probe during dives was a fast dissipation of the pH gradient not only vertically, but also horizontally, making difficult the assessment of the effects of the venting activity on moving animals. As it can be clearly appreciated in figure 3.1, CO_2 bubbles were totally dissolved in the water column before they reach the surface (which did not occur in São Miguel), where the values are normal. pH at the Reference site varies along the water column, within a range of higher values (7.85-8.15). Values of pH lower than 7 were registered in the Vent and until 7 in the Intermediate, forming a gradient in the selected areas (figure 3.2). Values on table 3.2 show a pH decreasing in the bottom, while surface pH is constant across sites. Bottom pCO_2 increased to the Vent, and the dissipation in surrounding waters led to similar values in the surface than in the bottom of the Reference site. This is because the water column exerts a great influence on the dissipation of the gradient despite the pCO_2 values in the bottom being stronger than in São Miguel (894.08 µmol/kgSW in Faial vs 742.50 in São Miguel). This needs to be considered for the interpretation of the results in this and future studies. In fact, the intensity and extent of the CO_2 gradient in Faial are strongly influenced by the hydrological conditions (depth, currents,

thermocline), so it is more likely to affect benthic communities than the planktonic and pelagic communities.

4.2.Zooplankton assemblages

Copepods were the most abundant component of zooplankton in São Miguel, being calanoids as *Paracalanus parvus* or *Centropages typicus* the main species (annex 7.2). Along with cladocerans as *Evadne spinifera*, they lead to the dominance of the phylum Arthropoda (figure 3.3). Athropoda and Chordata present a significantly greater abundance at the venting site in São Miguel. The functional groups approach allowed discern between small, big and giant crustacean all the taxa inside Arthropoda and Tunicata and fish inside Chordata. Thus, analyses identified small crustacean and Tunicata as the only groups with significant increased abundance in the Vent site. Oikopleuridae was the main representative of tunicates, and its increased abundance goes according with previous studies concerning gelatinous zooplanktonic components. This positive correlation of appendicularians with acidification conditions was already manifested in a mesocosm-scale study on the effects of temperature, pCO_2 and bloom structures on the appendicularian *Oikopleura dioica* (Troedsson *et al.*, 2013), with an increase on populations, either for a positive regulation of a low pH or for a negative effect of an increased pH. Abundance was positively correlated with increased pCO_2, temperature and nutrient levels, consistent with hypotheses concerning gelatinous zooplankton in future oceans. Thus, results in this study suggest that appendicularians will play more important roles in marine pelagic communities and vertical carbon transport under projected ocean acidification and elevated temperature scenarios. These species ingest smaller-sized particles than copepods, by-passing the microbial loop by directly transferring bacteria and nanoplankton to higher trophic levels, and they are vectors of global vertical carbon flux through trapping of prey in their frequently discarded houses (Troedsson *et al.*, 2013). *Pelagia noctiluca* was not included in the analysis but its occurrence only in the Vent sites on both islands does not seem like causality. Future sampling campaigns in these areas including a task leaded to these macrozooplankton components would bring more specific and useful information.

On the other hand, some species of arthropods as the cladocera *Evadne spinifera*, or copepods as *Paracalanus parvus* increase their abundance drastically in the Vent site, while richness, diversity and eveness are not significantly different among sites (annex 7.6). These species dominate in all sites, therefore the environmental differences in the Intermediate and Vent

sites are benefiting the zooplanktonic community. In fact, PERMANOVA analysis reflects these differences among sites and highlights the stability of the study sites since there are not significant differences among collection dates. Differences in the species composition were reported in other studies. Cooke *et al.* (2006) reported higher abundance of calanoid adults, cyclopoids, and Conochilus spp., a planktonic rotifer in the acidic and oligotrophic freshwater lake Giles in northeastern Pennsylvania. *Aglaodiaptomus spatulocrenatus* egg ratios, *Cyclops scutifer* egg ratios, and nauplii were also significantly higher at the high pH, whereas *Daphnia catawba* egg ratios were significantly higher at the low pH. These results are not conclusive to pH responses because they are combining the effects of DOM, UV and pH. However, it leads them to hypothesize that against changes in the seawater chemical composition, some species will be more benefit than others. Thus, in an open system with acidified and non-acidified areas, some differences in the species composition could be expected. Nevertheless, our results showed no differences in the species composition, only the reported increase in the abundances of some taxa. Results on nº of species, diversity and evenness among sites in both islands have to be considered along with the different resolution levels in the taxonomy, since for example Arthropoda has been more developed than Cnidaria, increasing the probabilities to find statistical differences in these indexes.

The nMDS in São Miguel shows a clear clustering of sites, with only some dates overlapped. Reference and Intermediate sites seem to have characteristics more like each other than with Vent. The nMDS shows the affinity of *P.parvus*, *E.spinifera* and Radiozoa for the venting conditions, while Cirripedia nauplii show preference for areas not acidified. Previous studies focused on the larval stage of barnacles do not report this effect. For example, *Amphibalanus amphitrite* showed no affection in the early life phases under a pH of 7.4 (McDonald *et al.*, 2009); and a population model of *Semibalanus balanoides* using empirical data showed that a decreasing on pH from 8.2 to 7.8 only get a significant affect below a critical temperature (Findlay *et al.*, 2010). Cirripedia together with *P.parvus* and *E.spinifera* are characterized for a calcium carbonate external body, being more likely affected by acidification, but since the early developmental stages of many marine species are suspected to be most sensitive (Dupont & Thorndyke, 2009; Kroeker *et al*., 2010; Cripps *et al*., 2014), Cirripedia on its planktonic life stage could have lower resistance. *Paracalanus parvus* and *Evadne spinifera*, as representative species of the community, have highlighted their apparent resilience under high CO_2 conditions. Most of the previous studies in copepods (annex 7.1), showed no effect on the survival of these organisms under controlled conditions of decreased pH. Detrimental

effects of CO_2 were only detected during the early stages, as the egg production and the hatching success seem to be affected. Havenhand (2012), on his study on the Baltic Sea key functional groups, said that it is expected that copepods will be resilient to near-future ocean acidification (≤1000 μatm CO_2). He explains that the extreme pCO_2 levels used in experiments elicited little or no response, and that the relatively rapid generation times of copepods confers a high potential for adaptability. Nevertheless, as Mayor *et al.* (2012) concluded, there is a context-dependency that highlights the need for cautious interpretation and application of data from individual climate-change studies. Copepods, as a group, may be well equipped to deal with the chemical changes associated with ocean acidification, however, long-term exposures examining the synergistic effects of ocean acidification with other climate stressors, particularly warming on population viability and success, have yet to be conducted (Weidmann *et al.*, 2012).

On the other hand, Radiozoa was the only taxa with low representation in the Reference and Intermediate sites, and a peak of abundance in the Vent, showing high tolerance to low pH and thus a fitness advantage over less resistant organisms. Increased abundance of the overall zooplanktonic community in the venting site, were pH conditions are suboptimal, entails an adaptation capacity of many predominant groups, and regression of rarer species. Thereby, the most abundant species in the community are the main contributors to the differences among sites and have higher abundance in the venting sites, while *Cirripedia* nauplii decrease under acidification conditions, as others groups with low occurrence. *Radiozoa* was not a dominant group in the Reference and Intermediate sites, but it was the third group most abundant in the Vent. It can be a direct link between the high CO_2 concentration in the water and the chlorophyll content in the Radiozoa cells. As them, phytoplankton would be benefited by the venting emissions, and it could explain the higher zooplankton abundance. Only organisms without resistance for these conditions would not proliferate in these environments.

In Faial, Radiozoa acquires more importance in terms of abundance (figure 3.4), while gelatinous components as siphonophorous are responsible for the high biovolume in the samples (figure 3.7). There is not a tendency in the abundance of the organisms with the CO_2 gradient; most of the taxa increase in the Intermediate site, where the total abundance is higher. Only Arthropoda diversity decreases significantly with the CO_2 concentration. In fact, the PERMANOVA analysis shows how there are no significant differences between sites, but there are differences between sampling dates. Thus, the nMDS relates each of the taxa highlighted in the SIMPER analysis with a different date. It reveals the unstable conditions in

Faial, which are manifested among the different sampling days. Since the CO_2 gradient is completely dissolved in the vicinity of the emissions, and its effect is not discernible in the remainder water column, this area is led suitable to test the acidification effect on the planktonic and pelagic communities than the Lobeira site in S. Miguel. In the same line, the differences in the diversity of arthropods among sites cannot be justified with the CO_2 levels considering that these organisms are distributed along the water column.

4.3.RNA:DNA ratio

The physiological state of some organisms was tested through the RNA:DNA technique to evaluate if the increased CO_2 was affecting the main groups found at each site. Since the pattern was not consistent among the groups analyzed, there was not measurable effect on the physiological or nutritional state of the communities. A visible effect on fish larvae could be expected as described by Franke & Clemmesen (2011), which found a negative relation between pCO_2 and RNA:DNA ratio in hatched herrings. Nevertheless, they considered as control a pCO_2 of 480 µatm and a treatment pCO_2 of 4635 µatm, which are values much higher than in this study and exceeding the forecasts for the end of the century. All samples were collected with little time of difference on the same day, so the daily variation of the index exposed by Chicharo & Chicharo (2008) for different species (ex. *Sardina pichardus*, *Sciaenops ocellatus*, *Ruditapes decussatus*, *Ceratoderma edule*) could not be the factor of variation here. However, it could be due to the non-selection of only adult organisms and from the same sex. RNA per unit dry weigth can be greater in females and DNA per unit dry weight greater in males, due to sexual dimorphism or physiological and behavioural differences (Chicharo *et al.*, 2007). Catarino *et al.* (2012) also found a higher RNA/DNA ratio in females than males of the sea urchin *Paracentrotus lividus*, which is attributed to ovaries having a higher energetic demand than testis. This effect could be avoided if only individuals from the same sex were selected or having a balanced same sex ratio in the samples. Increasing the number of replicates, by performing more tows per site during different days could also contribute to consolidate the results.

4.4.Copepod Egg Production Rates (EPR)

Previous studies calculated the egg hatching rate of different copepod species at *in situ* conditions (Andersen & Nielsen, 1997). Escaravage & Soetaert (1993) define hatching as the moment when at least 50% of the eggs have hatched, and Andersen & Nielsen (1997) consider a 100% hatching criterion. Here, we define hatching rate as the number of eggs

released and nauplii hatched in relation to the number of females in the sample. The nauplii produced were taken into consideration because the hatching of eggs can be produced during the time of the experiment and otherwise, the egg production would be underestimated (Andersen & Nielsen, 1997). In other methodologies, the capture and transport of copepods, as well as the change of the natural conditions as temperature or feeding, may cause a variation in the natural rates. This is the reason why incubation chambers were prepared and the experiment was carried out in the field with freshly caught copepods.

As in many experiments under controlled conditions, the egg production rate of copepods decreased under acidification conditions in São Miguel, but not with the different exposure times. Bottom pH levels in table 3.1 are quite similar with the conditions of the incubation chambers (8.07, 8.11 and 7.87 in São Miguel; 8.13, 8.02 and 7.88 in Faial). In previous studies (annex 7.1), the egg production rate and/or hatching rate were tested under controlled conditions and there was an effect of decreasing pH. Most of them were obtained for pH under 7, only Cripps *et al.* (2014) found a decreasing on *Acartia tonsa* for a pH of 7.8 and Thor & Dupont (2015) on *Pseudocalanus acuspes* for a pH of 7.75. Conditions of higher pH showed no significant effect. Considering that the lower pH level measured at the bottom was 7.75 (table 3.1), this is one of these few studies that reports a significant decreasing under realistic near future conditions, and the first one that report it in a natural environment and is not species-specific. In addition, values in table 3.1 do not correspond exactly with the location of the chambers, first because measurements and chambers placement were not done at the same time and second because chambers were accompanied by a buoy that kept them floating vertically approximately one meter above the substrate. It may have led to an even higher pH since as showed in figure 9, pH tends to increase away from the bottom until the surface, where decrease again only in São Miguel.

Generally, chambers were a good methodology to evaluate secondary production in the natural environment, as their design was in line with their functionality. After 24 or 72h underwater, and despite the strong currents in Faial, only one chamber at the Vent site in São Miguel was lost during the 72h period. Nevertheless, the decreasing in the EPR in Faial was not significant. This was probably due to the highest mesh size used in this island, since copepods were not a component of zooplankton as abundant as in São Miguel. It has to be a consideration for future experiments, as well as control the quantity of copepods introduced in the chambers to improve the results. Thus, from the six chambers used at each site in Faial, three of them did not contain copepods, having been missed. Furthermore, the chambers with

copepods had 1.44±3.94 (mean±SD) individuals, while in São Miguel they were more abundant (3.94±4.35 (mean±SD) individuals). This loss of replicates was reflected in the analysis, as the degrees of freedom differ from 11 residuals in S. Miguel (table 7) to only 6 in Faial (table 3.8). Keeping the same methodology, it would be easily solved in future experiments increasing the volume of inoculum introduced into the chambers.

The methodology used was intended to minimize the stress produced in the individuals that would affect the results, so the content of the tows was directly introduced in the chambers, with no previous selection of a fix number of females. Given the low abundances used, the species inside the chambers do not represent the complete zooplanktonic communities found in these sites. Andersen & Nielsen (1997) performed their experiments with 3-6 females in 600ml bottles, which is much more concentrated than in our case. Thus, it should avoid cannibalism and predation during the short time experiment but allow hatching of the eggs. Nevertheless, the fact of these factors be present would reduce the egg hatching rates, but it would be constant over sites. Also, the semi-permeable chambers allow the exchange of dissolved materials (including oxygen and CO_2), while allowing the exclusion of larger predators.

5. Conclusions

In the present study, two new natural shallow-water CO_2 vents in São Miguel and Faial islands were characterized as *in situ* laboratories for ocean acidification investigations. It was demonstrated their suitability for investigating the effect of future dissolved CO_2 levels in the communities of the NE Atlantic.

Differences in the total abundance of mesozooplankton among sites in São Miguel were reported, as well as in the abundances of small crustaceans and Tunicata. Only differences in the diversity of Arthropoda were found in Faial. PERMANOVA illustrated the differences in the composition of the zooplanktonic assemblages among sites in São Miguel and among dates in Faial. The main contributors to these differences in the first case highlighted by the SIMPER routine, were *Evadne spinifera*, *Paracalanus parvus*, Cirripedia nauplii and Radiozoa. Through the nMDS analysesm Radiozoa, *Paracalanus parvus* and *Evadne spinifera* appeared more related to vent conditions, while Cirripedia nauplii was closer to the reference conditions. In Faial, the main contributors were *Bassia bassensis*, Radiozoa and Scyphozoa ephyra. Conditions in this island did not allow a division among sites in the nMDS analyses, since the strong currents mix the water, dissipating the effect of the gas emissions, and variation among days becomes more important and statistically observable.

RNA:DNA did not show a clear pattern of variation, only copepods in São Miguel and cladocerans in Faial had an higher ratio in the Reference, but with no gradual decrease to the Vent. Fish eggs seem to have an inverted pattern. Egg Production Rate showed a decreasing trend along the CO_2 gradient in São Miguel, with no differences in the exposure time. In Faial, it was higher in the Reference, but it did not differ significantly.

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7. Annexes

Annex 7.1. Summary of the different studies on copepod species until data under increased CO_2 conditions. Response is presented as Un (unchanged), Inc (Increased), or Dec (Decreased) relative to the control. pCO_2 and pH values include control (first one) and treatment(s).

Copepod species	Location	Type of study	Factor(s)	Response	pCO_2 (µatm)	pH	Temperature (ºC)	Exposure time	Reference
Acartia bifilosa	Baltic sea	Lab	Antioxidant capacity Egg production rate Egg viability Nauplii development	In / Dec Un Un Un	?	8-7.6	17/	24h	Vehmaa *et al.* (2013)
Acartia clausi	Saronikos Gulf, Mediterranean Sea	Lab	Hatching rate Egg production rate Respiration rate Excretion rate	Un Un Un Dec	463-824	8.09-7.83	16, 20	5 days	Zervoudaki *et al.* (2013)
Acartia erythraea	Tanabe Bay, Japan	Lab	Females Survival Egg production rate Hatching rate Nauplii survival	Un Dec-Dec Un-Un-Dec Un-Dec-Dec	370-5,370-10,370	8.20-7.02-6.86	27	24 h	Kurihara *et al.* (2004a)
					360-2,360-5,360-10,360	8.09-7.31-7.00-6.82			
Acartia pacifica	China	Lab	Survival rate	Un	380-800-2,000-5,000-10,000	8.17-7.84-7.39-7.19-6.92	18	8 days	Zhang *et al.* (2011)
			Egg production rate	Un	380-2,000-10,000	8.17-7.39-6.92			
Acartia spinicauda	China	Lab	Survival rate	Un-Un-Un-Dec	380-800-2,000-5,000-10,000	8.17-7.84-7.39-7.19-6.92	24	8 days	Zhang *et al.* (2011)
			Egg production rate	Un-Dec	380-2,000-10,000	8.17-7.39-6.92			
Acartia steueri	Tanabe Bay, Japan	Lab	Females survival Egg production rate	Un Un-Dec	370-2,370-10,370	8.14-7.40-6.84	24	8 days	Kurihara *et al.* (2004a) Kurihara *et al.* (2004b)

Copepod species	Location	Type of study	Factor(s)	Response	pCO_2 (µatm)	pH	Temperature (ºC)	Exposure time	Reference
Acartia tonsa	Orkney, UK	Lab	Egg production rate Egg hatching success Egg volume Nauplii production	MFE[2] Un-Un-Dec-Un, FE[2] Un-Un-Dec-Un FE Un, MFE Dec-Dec-Dec-Dec, NE[2] Un-Un-Dec-Dec MFE Un-Un-Dec-Dec, FE Un MFE/FE Dec-Dec-Dec-Dec	385-1,000-2,000-3,000-6,000	8.2-7.8-7.6-7.4-7.2	23.90	96h	Cripps *et al.* (2014a)
			Eggs mr[3] Nauplii mr Copepodites mr Females mr Males mr Egg production rate Eggs C content Hatching success Nauplii recruitment	Un-Un-In-In In-In- In-In Un-Un-In-In Un-Un-In-In Un-Un-In-In Dec-Un-Dec-Dec Un-Un-Dec-Dec Dec-Dec-Dec-Dec Dec-Dec-Dec-Dec			24.4		Cripps *et al.* (2014b)
Acartia tsuensis	Japan	Lab	Survival Sex ratio Body size Hatching rate Egg production rate	Un Un Un Dec Un	380-2,380	8.17-7.32	25	30 days	Kurihara & Ishimatsu (2008)
Calanus finmarchicus	Stonehaven, Scotland	Lab	C and N content Hatching rate Egg production rate	Un Dec Un	?-8000	8.23-6.95	8.8	5 days	Mayor *et al.* (2007)
			Survival Developmental time Body size Lipid content	Un-Dec-Dec Un-Inc-Inc Inc-Dec-Un Inc-Un-Un	390-3300-7300-9700	7.31-6.97-6.85	10	28 days	Pedersen *et al.* (2013)
Calanus glacialis	Isfjorden, Arctic Ocean	Lab	Survival Time until hatching Hatching rate	Un Un-Dec Un-Dec	?	8.2-7.6-6.9	-1	9 days	Weidmann *et al.* (2012)

Copepod species	Location	Type of study	Factor(s)	Response	pCO_2 (µatm)	pH	Temperature (ºC)	Exposure time	Reference
			Egg production rate	Un					
	Fram strait	Lab	Respiration rate Body mass Gonad development Mortality	Un Un Un Un	390-3,000	6.865-9.180	0	62 days	Hildebrandt *et al.* (2015)
Calanus glacialis + *Calanus hyperboreus*	Deer Bay, high Canadian Arctic	Lab	Survival Nauplii survival	Un-Un Dec-Un	370-700-1,000	8.04-7.80-7.60	-1.6	7 days	Lewis *et al.* (2013)
Calanus helgolandicus	Stonehaven, Scotland	Lab	Hatching rate	Un	?-1,000	8.06-7.77	8-10-12	72 h	Mayor *et al.* (2012)
Calanus hyperboreus	Fram strait	Lab	Respiration rate Body mass Gonad development Mortality	Un / In / In Un / Dec / Un Un Un / Un /Un	390-3,000	8.1-7.2	0/5/10	86 days	Hildebrandt *et al.* (2015)
Calanus sinicus	China	Lab	Survival rate	Un	380-800-2,000-5,000-10,000	8.17-7.84-7.39-7.19-6.92	16	8 days	Zhang *et al.* (2011)
			Egg production rate	Un-Dec	380-2,000-10,000	8.17-7.39-6.92			
Centropages tenuiremis	Xiamen Bay, Japan	Lab	Clearance	Un-Inc-Inc		8.15-7.80-7.60-7.00	20	6 min	Li & Gao (2012)
			Feeding rate Clearance	Dec-Un-Un[1] Dec-Un-Un[1]	390-1,000	8.18-7.83		24, 36, 90 h	
			Respiration rate	Inc-Inc-Inc[1]				24, 48, 72 h	
	China		Survival rate	Un-Un-Un-Dec	380-800-2,000-5,000-10,000	8.17-7.84-7.39-7.19-6.92	21	8 days	Zhang *et al.* (2011)
			Egg production rate	Un-Dec	380-2,000-10,000	8.17-7.39-6.92			
Centropages typicus	English Channel	Lab	Egg production rate Hatching success	Un-Un-Un-Dec Un-Un-Un-Dec	385-480-620-750-9,830	8.04-7.79-7.85-7.78-6.71	14.7	4 days	McConville *et al.* (2013)
Oithona similis	Deer Bay, high Canadian Arctic	Lab	Survival Nauplii survival	Dec-Dec Dec-Dec	370-700-1,000	8.04-7.80-7.60	-1.6	7 days	Lewis *et al.* (2013)
Paracalanus parvus	São Miguel, Azores	Field	Presence	Un-In	386.24-410.64-862.8	8.07-8.05-7.81	20.33		This study

Copepod species	Location	Type of study	Factor(s)	Response	pCO_2 (µatm)	pH	Temperature (ºC)	Exposure time	Reference
Paracalanus sp. *Clausocalanus sp.*	São Miguel, Azores	Field	Egg production rate	Dec-Dec	386.24-410.64-862.8	8.07-8.05-7.81	20.33	24, 72 h	This study
Pseudocalanus acuspes	Gullmar Fjord, Skagerrak	Lab	Egg production rate Egg clutch size Respiration rate	Dec-Dec Dec-Dec In-Dec	400-900-1,550	8-7.75-7.54	5	Two generations	Thor & Dupont (2015)
Temora longicornis	English Channel	Lab	Egg production rate Hatching success	Un-Un-Un-Un Un-Un-Un-Un	385-480-620-750-9,830	8.04-7.79-7.85-7.78-6.71	14.7	4 days	McConville *et al.* (2013)
Tigriopus japonicus (benthic)	Niigata, Japan	Lab	Nauplii survival Ratio of mobile nauplii	Un Un	13,000-39,000	6.26-5.74	20	24 h	Kita *et al.* (2013)
			Hatching rate Growth rate Sex ratio	Un-Dec Un-Dec Un	5,800-37,000-110,000	7.11-6.31-5.85	23	21days	
Tisbe battagliai	Guernsey, UK	Lab	Nauplii production Growth Cuticule C:O ratio	Inc-Dec-Inc Un-Dec Inc	222, 300, 421, 585	8.06-7.95-7.82-7.67	19.25	60 days	Fitzer *et al.* (2012)

[1]Response related to the exposure time; [2]MFE: male and female exposure, FE: sole female exposure, NE: no parental exposure; [3]mortality rate

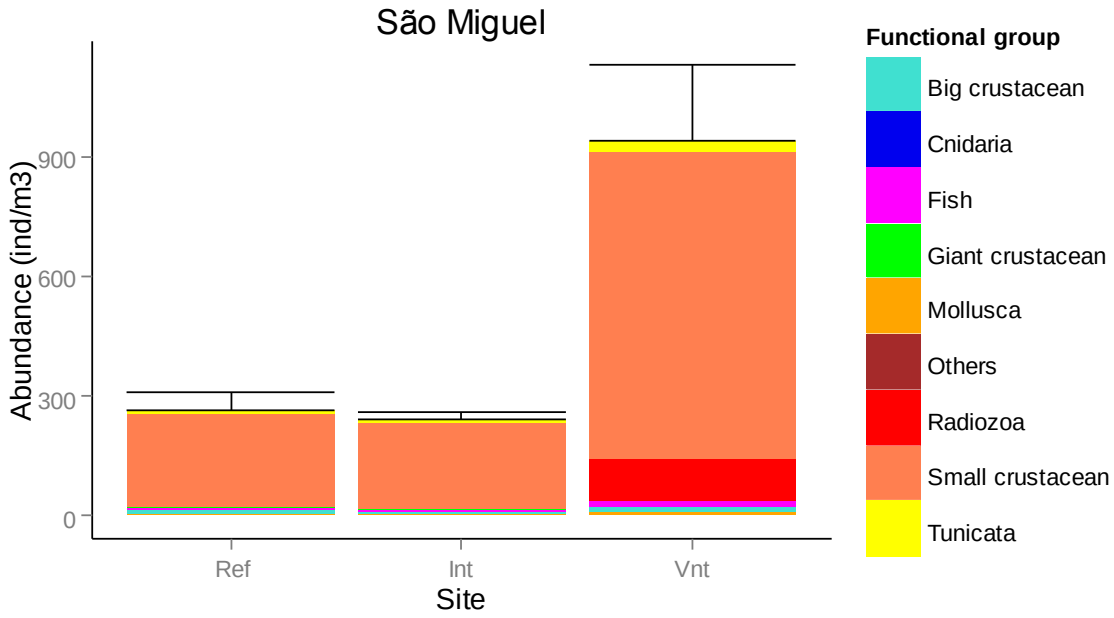
Annex 7.2. Taxa abundances (ind/m^3, mean±SE) of Reference (Ref), Intermediate (Int) and Vent (Vnt) sites in São Miguel and Faial.

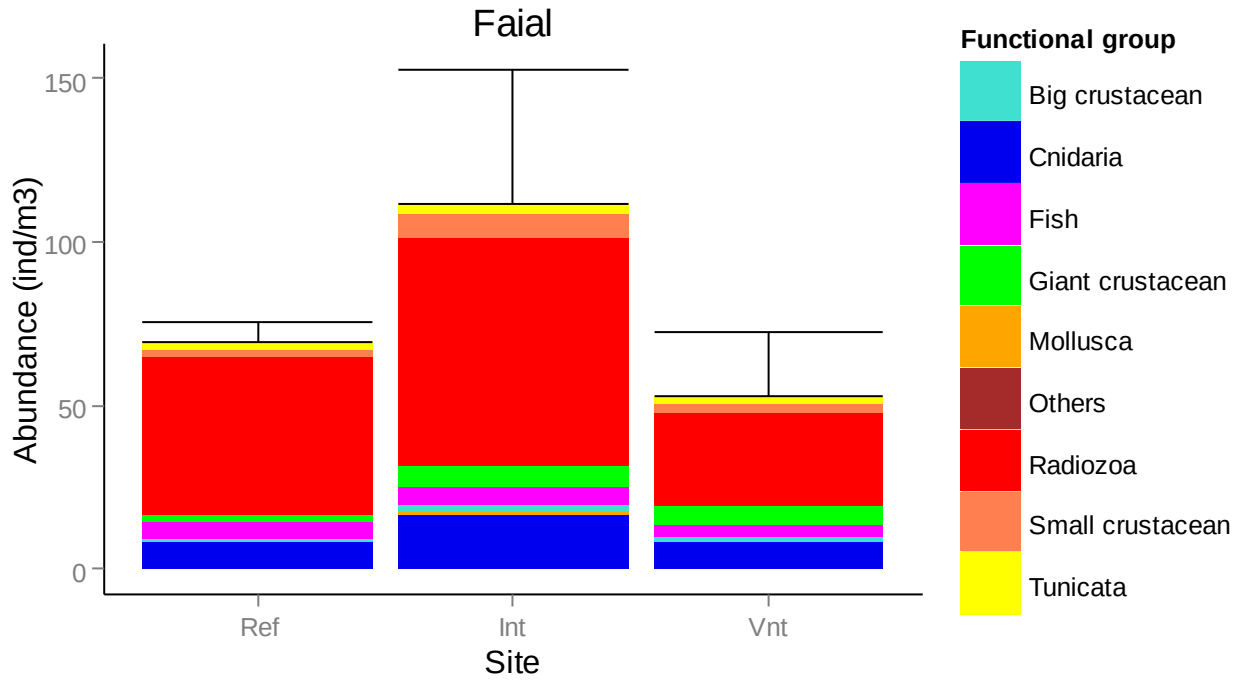
	São Miguel			Faial		
	Ref	Int	Vnt	Ref	Int	Vnt
Annelida	**0**	**0**	**0**	**0**	**0**	**0.01±0.01**
Errantia (Larvae)	0	0	0	0	0	0.01±0.01
Arthropoda	**239.45±42.63**	**216.26±20.57**	**786.86±123.83**	**5.71±1.51**	**15.57±4.83**	**9.35±3.98**
Amphipoda	0.13±0.13	0.10±0.10	0	0.04±0.04	0.26±0.26	0.02±0.02
Brachyura	0.30±0.15	0.28±0.18	0.51±0.32	0.50±0.24	1.13±0.30	1.17±0.63
Callianassidae	0	0	0	0.40±0.32	0.40±0.17	0.55±0.44
Caridea	0	0	0	0.04±0.02	0.09±0.08	0.13±0.07
Cirripedia (Cyprid)	2.13±0.83	0.33±0.17	1.67±0.60	0	0	0
Cirripedia (Nauplii)	23.44±6.73	11.03±4.66	13.15±5.97	0.67±0.55	2.82±1.21	1.00±0.54
Copepoda (Nauplii)	0.12±0.12	0	0.16±0.16	0.02±0.02	0	0.04±0.04
Cumacea	0	0.00±0.00	0	0	0	0
Euphausiacea (Caliptopis)	0.33±0.11	0.07±0.07	0.39±0.39	0.73±0.22	1.53±0.67	1.13±0.68
Euphausiacea (Furcilia)	0.12±0.07	0.07±0.07	0	0.12±0.03	1.03±0.65	0.86±0.60
Euphausiacea (Nauplii)	0.15±0.09	0.12±0.08	0	0.02±0.02	0.07±0.03	0.08±0.08
Isopoda	0	0	0	0	0.01±0.01	0.01±0.01
Monstrillidae	0.12±0.12	0.12±0.08	0	0	0	0
Stomatopoda	0	0	0	0.02±0.02	0.03±0.03	0.05±0.03
Acartia danae	0.11±0.11	0.74±0.60	0.78±0.78	0.04±0.04	0.09±0.02	0
Acartia negligens	1.18±0.54	0.87±0.50	2.18±1.99	0.04±0.04	0.17±0.11	0.01±0.01
Alpheus	0	0	0	0.11±0.11	0.09±0.09	0.34±0.28
Anapagurus	0.11±0.11	0	0	0.23±0.12	1.27±1.00	0.57±0.57
Calcinus tubularis	0	0	0	0.06±0.04	0.05±0.04	0.34±0.34
Candacia	0	0	0	0.06±0.00	0.17±0.07	0.02±0.01
Centropages typicus	8.89±3.87	6.47±4.37	13.39±6.25	0.40±0.11	0.54±0.27	0.31±0.14
Centropages violaceus	0	0	0	0.02±0.02	0.01±0.01	0.01±0.01
Clausocalanus	4.54±1.18	4.08±1.48	26.35±12.74	0.02±0.02	0.03±0.03	0.05±0.03
Corycaeus	0	0	0.78±0.78	0.02±0.02	0	0
Dardanus arrosor	0	0	0	0.02±0.01	0	0.04±0.04
Diogenes pugilator	0	0	0.26±0.06	0	0	0
Eucalanus monachus	0	0	0	0.08±0.03	0.19±0.03	0.02±0.02
Euchaeta	0	0	0	0.04±0.04	0.07±0.03	0.04±0.04
Euterpina acutifrons	0.84±0.27	0.45±0.21	0.31±0.19	0	0	0
Evadne spinifera	70.36±19.21	126.51±31.43	427.83±115.34	0.20±0.12	0.83±0.71	0.53±0.51
Galathea intermedia	0	0	0	0.02±0.02	0.02±0.02	0.04±0.04
Galathea squamifera	0	0	0	0.02±0.02	0.02±0.02	0.04±0.04
Lubbockia	0.06±0.02	0	0	0	0	0
Mecynocera clausii	0	0	0.26±0.07	0	0	0

	São Miguel			Faial		
	Ref	Int	Vnt	Ref	Int	Vnt
Microsetella	0.04±0.04	0	0.15±0.15	0	0	0
Munida	0	0	0	0	0.02±0.02	0
Nanocalanus minor	0	0.03±0.03	0	0.30±0.15	0.27±0.12	0.22±0.15
Neocalanus gracilis	0	0	0.15±0.15	0.02±0.02	0.12±0.06	0.02±0.02
Oithona	6.33±1.39	13.36±2.89	19.93±5.98	0.10±0.10	0.06±0.03	0
Oncaea	0.79±0.23	0.71±0.38	3.34±1.71	0	0	0
Paracalanus parvus	119.20±25.66	50.75±6.39	275.13±70.27	0.06±0.00	2.00±1.97	0.08±0.08
Philocheras	0.07±0.07	0	0	0.02±0.02	0.04±0.04	0
Philocheras bispinosus	0	0	0	0.02±0.02	0.08±0.08	0.10±0.10
Philocheras trispinosus	0	0	0	0.02±0.02	0.04±0.04	0
Plesionika	0	0	0	0.04±0.04	0.06±0.03	0.08±0.04
Pleuromamma robusta	0	0	0	0.11±0.11	0.02±0.02	0.04±0.04
Podon	0	0	0.26±0.07	0.02±0.01	0	0
Processa	0	0	0	0.16±0.08	0.32±0.19	0.38±0.19
Pseudocalanus	0	0	0.15±0.15	0	0	0
Pseudoevadne tergestina	0	0	0	0.53±0.37	0.64±0.26	0.38±0.07
Rhincalanus	0	0	0	0.02±0.02	0.02±0.02	0.00±0.00
Sapphirina	0	0	0	0.10±0.05	0.12±0.06	0.04±0.02
Sergestes	0	0.07±0.07	0	0.26±0.23	0.67±0.45	0.45±0.23
Chaetognatha	**0**	**0**	**0**	**0.10±0.06**	**0.21±0.16**	**0.13±0.13**
Sagittoidea	0	0	0	0.10±0.06	0.21±0.16	0.13±0.13
Chordata	**16.3±1.83**	**17.71±2.45**	**34.68±4.70**	**6.95±3.33**	**8.87±3.63**	**6.23±1.91**
Doliolida	0	0.03±0.03	0	0.99±0.42	1.40±0.63	1.43±1.11
Fritillariidae	1.30±0.58	0.78±0.22	0.31±0.19	0.10±0.06	0.34±0.05	0.30±0.17
Oikopleuridae	9.54±0.75	10.17±2.66	23.90±2.85	1.35±0.99	1.46±0.73	0.71±0.39
Fish eggs	5.42±1.68	6.70±1.64	10.39±2.35	4.39±1.92	5.52±2.51	3.69±0.38
Fish larvae	0.05±0.01	0.00±0.00	0.07±0.01	0.11±0.03	0.12±0.04	0.08±0.03
Cnidaria	**0.48±0.13**	**0.28±0.15**	**1.13±0.72**	**8.03±2.72**	**16.75±9.72**	**7.98±2.66**
Hydrozoa	0.12±0.12	0	0	0.04±0.04	0.02±0.02	0.04±0.04
Scyphozoa (Ephyra)	0.15±0.09	0.15±0.15	0.15±0.15	3.97±2.84	5.42±0.77	3.57±1.18
Siphonophorae	0.15±0.09	0.03±0.03	0.81±0.42	0.57±0.36	1.38±1.02	0.71±0.56
Bassia bassensis	0.03±0.038	0.08±0.05	0.15±0.15	3.45±3.05	9.90±9.35	3.64±3.26
Echinodermata	**0**	**0.17±0.10**	**0**	**0.19±0.10**	**0.14±0.14**	**0.40±0.39**
Echinodermata	0	0.17±0.10	0	0.19±0.10	0.14±0.14	0.40±0.38
Mollusca	**6.17±0.76**	**3.73±1.43**	**10.20±4.06**	**0.35±0.14**	**0.51±0.28**	**0.32±0.14**
Bivalvia (veliger)	0.88±0.47	0.11±0.07	0.19±0.19	0.12±0.09	0.07±0.03	0.08±0.08
Coleoidea (Egg)	0	0	0	0	0.00±0.00	0
Coleoidea (Larvae)	0	0	0	0.00±0.00	0	0.00±0.00

	São Miguel			Faial		
	Ref	Int	Vnt	Ref	Int	Vnt
Gastropoda (veliger)	5.29±0.63	3.61±1.47	10.00±3.87	0.23±0.06	0.43±0.25	0.23±0.14
Foraminifera	**0.08±0.05**	**0.04±0.04**	**0**	**0.06±0.06**	**0.34±0.30**	**0.22±0.22**
Foraminifera	0.08±0.05	0.04±0.04	0	0.06±0.06	0.34±0.30	0.22±0.22
Radiozoa	**3.90±3.81**	**3.49±3.49**	**105.83±104.66**	**48.15±1.58**	**69.13±25.39**	**28.28±13.79**
Radiozoa	3.90±3.81	3.49±3.49	105.83±104.66	48.15±1.58	69.13±25.39	28.28±13.79
Total abundance	266.42±44.03	241.68±18.18	938.70±192.45	69.55±5.79	111.52±41.10	52.91±19.59

Annex 7.3. Total abundances (ind/m^3, mean±SE) for the three sampling sites in São Miguel and Faial (Ref-Reference, Int-Intermediate, Vnt-Vent).





Annex 7.4. Relative abundances (%) of each functional group presented at each site (Ref-Reference, Int-Intermediate, Vnt-Vent) of São Miguel and Faial.

	São Miguel			Faial		
	Ref	Int	Vnt	Ref	Int	Vnt
Small crustacean	86.07	86.49	82.20	2.93	6.42	4.65
Big crustacean	3.37	2.71	1.51	1.60	1.89	2.63
Giant crustacean	0.44	0.28	0.11	3.54	5.51	10.34
Cnidaria	0.18	0.12	0.12	11.56	15.04	15.08
Mollusca	2.32	1.55	1.09	0.51	0.46	0.61
Fish	2.06	22.78	1.11	6.48	5.08	7.15
Radiozoa	1.46	1.45	11.27	69.33	62.10	53.47
Tunicata	4.07	4.55	2.58	3.53	2.89	4.63
Others	0.03	0.09	0	0.52	0.62	1.43

Annex 7.5. Number of taxa (S), Shannon diversity index (H´) and eveness (J´) (mean±SE) of each phylum presented at each site (Ref-Reference, Int-Intermediate, Vnt-Vent) of São Miguel and Faial.

		São Miguel			Faial		
		Ref	Int	Vnt	Ref	Int	Vnt
Arthropoda	S	14.2±1.24	12.8±0.73	22±0.95	24±1.15	27±1.15	22±2.52
	H´	1.53±0.05	1.38±0.09	1.32±0.12	2.03±0.17	1.37±0.24	0.69±0.27
	J´	0.52±0.02	0.49±0.07	0.43±0.05	0.83±0.03	0.74±0.04	0.82±0.03
Chordata	S	4.8±0.2	4.8±0.37	4.4±0.24	5.67±0.33	6±0	6±0
	H´	0.52±0.04	0.42±0.04	0.52±0.04	0.93±0.19	0.62±0.18	0.37±0.10
	J´	0.61±0.04	0.53±0.06	0.54±0.03	0.56±0.10	0.61±0.08	0.54±0.16
Cnidaria	S	2.2±0.37	1.8±0.37	2±0.55	3.67±0.67	4.33±0.33	4±0.58
	H´	0.03±0.01	0.01±0.01	0.03±0.02	0.73±0.12	0.66±0.17	0.38±0.09
	J´	0.38±0.24	0.2±0.2	0.17±0.17	0.39±0.06	0.47±0.09	0.47±0.11
Mollusca	S	2.6±0.24	2.2±0.2	2.2±0.2	3±0	3±0.58	2.67±0.67
	H´	0.22±0.02	0.10±0.03	0.18±0.5	0.09±0.03	0.06±0.03	0.03±0.01
	J´	0.42±0.18	0.06±0.06	0.05±0.05	0.72±0.23	0.45±0.24	0.23±0.23
Others phyla	S	0.8±0.2	0.8±0.37	0.6±0.24	2.67±0.67	3±0	2.67±0.88
	H´	0	0.13±0.13	0	0.05±0.02	0.07±0.03	0.14±0.09
	J´	-	0.91	-	0.05±0.01	0.06±0.03	0.17±0.05
Total S		20.60±1.63	18.40±1.78	17.20±1.16	35.00±0.48	39.33±0.21	33.33±0.76
Total H´		1.66±0.09	1.51±0.18	1.31±0.17	1.28±0.06	1.63±0.06	1.88±0.06
Total J´		0.55±0.03	0.53±0.07	0.46±0.05	0.36±0.07	0.44±0.07	0.54±0.07

Annex 7.6. Univariate analyses of variance for abundance, number of taxa, diversity and eveness of each phylum in São Miguel. Df-degrees of freedom, Sum Sq-Sum of squares, Mean Sq-Mean of squares, (*) - significant differences for α=0.05 (n=5).

Arthropoda

		Abundance (*)			Number of taxa			Diversity			Eveness		
	df	Mean Sq	F value	p	Mean Sq	F value	p	Mean Sq	F value	p	Mean Sq	F value	p
Site	2	521480	17.81	0.0003	6.2	1.25	0.32	0.06	1.46	0.27	0.01	0.72	0.51
Residuals	12	29289			4.97			0.04			0.01		

Chordata

		Abundance (*)			Number of taxa			Diversity			Eveness		
	df	Mean Sq	F value	P	Mean Sq	F value	p	Mean Sq	F value	p	Mean Sq	F value	p
Site	2	522.2	9.98	0.003	0.27	0.67	0.53	0.02	2.1	0.17	0.01	0.79	0.48
Residuals	12	52.34			0.4			0.01			0.01		

Cnidaria

		Abundance			Number of taxa			Diversity			Eveness		
	df	Mean Sq	F value	p	Mean Sq	F value	p	Mean Sq	F value	p	Mean Sq	F value	p
Site	2	0.99	1.07	0.37	0.2	0.21	0.81	0	0.71	0.51	0.07	0.32	0.74
Residuals	12	0.93			0.97			0			0.21		

Mollusca

		Abundance			Number of taxa			Diversity			Eveness		
	df	Mean Sq	F value	p	Mean Sq	F value	p	Mean Sq	F value	p	Mean Sq	F value	p
Site	2	53.36	1.68	0.23	0.27	1.14	0.35	0.02	2.28	0.15	0.23	3.5	0.06
Residuals	12	31.82			0.23			0.01			0.07		

Others phyla

		Abundance			Number of taxa			Diversity			Eveness		
	df	Mean Sq	F value	p	Mean Sq	F value	p	Mean Sq	F value	p	Mean Sq	F value	p
Site	2	17335	0.95	0.42	0.07	0.17	0.85	0.03	1	0.40			
Residuals	12	18301			0.40			0.03					

All taxa

		Abundance (*)			Number of taxa			Diversity			Eveness		
	df	Mean Sq	F value	p	Mean Sq	F value	p	Mean Sq	F value	p	Mean Sq	F value	p
Site	2	782006	22.94	0	14.87	1.25	0.32	0.15	1.3	0.31	0.01	0.93	0.42
Residuals	12	65512			11.93			0.12			0.01		

Annex 7.7. Univariate analyses of variance for abundance, number of taxa, diversity and eveness of each phylum in Faial. Df-degrees of freedom, Sum Sq-Sum of squares, Mean Sq-Mean of squares, (*)-significant differences for α=0.05 (n=3).

Arthropoda

		Abundance			Number of taxa			Diversity (*)			Eveness		
	df	Mean Sq	F value	p	Mean Sq	F value	p	Mean Sq	F value	P	Mean Sq	F value	p
Site	2	74.65	1.8	0.24	19	2.11	0.2	1.34	8.35	0.02	0.01	2.5	0.16
Residuals	6	41.43			9			0.16			0		

Chordata

		Abundance			Number of taxa			Diversity			Eveness		
	df	Mean Sq	F value	p	Mean Sq	F value	p	Mean Sq	F value	p	Mean Sq	F value	p
Site	2	5.55	0.2	0.82	0.11	1	0.42	0.23	3.08	0.12	0	0.09	0.91
Residuals	6	27.89			0.11			0.08			0.04		

Cnidaria

		Abundance			Number of taxa			Diversity			Eveness		
	df	Mean Sq	F value	p	Mean Sq	F value	p	Mean Sq	F value	p	Mean Sq	F value	p
Site	2	76.49	0.7	0.53	0.33	0.38	0.7	0.1	1.98	0.22	0.01	0.25	0.79
Residuals	6	108.99			0.89			0.05			0.02		

Mollusca

		Abundance			Number of taxa			Diversity			Eveness		
	df	Mean Sq	F value	p	Mean Sq	F value	p	Mean Sq	F value	p	Mean Sq	F value	p
Site	2	0.03	0.25	0.79	0.11	0.33	0.73	0	1.81	0.24	0.19	1.16	0.38
Residuals	6	0.12			0.33			0			0.16		

Others phyla

		Abundance			Number of taxa			Diversity			Eveness		
	df	Mean Sq	F value	p	Mean Sq	F value	p	Mean Sq	F value	p	Mean Sq	F value	p
Site	2	1248.4	1.46	0.30	0.11	0.09	0.91	0.01	0.90	0.46	0.01	5.03	0.06
Residuals	6	853.7			1.22			0.01			0.00		

All taxa

		Abundance			Number of taxa			Diversity			Eveness		
	df	Mean Sq	F value	p	Mean Sq	F value	p	Mean Sq	F value	p	Mean Sq	F value	p
Site	2	2735.8	1.3	0.34	28.78	1.89	0.23	0.28	1.52	0.29	0.02	1.85	0.24
Residuals	6	2106.7			15.22			0.18			0.01		

Annex 7.8. Univariate analyses of variance (ANOVA) for the taxa highlighted by the SIMPER routine. Df-degrees of freedom, Sum Sq-Sum of squares, Mean Sq-Mean of squares, (*)-significant differences for α=0.05 (n=3).

São Miguel

	Evadne spinifera (*)				*Paracalanus parvus*			Cirripedia nauplii			Radiozoa		
	df	Mean Sq	F value	p	Mean Sq	F value	p	Mean Sq	F value	p	Mean Sq	F value	p
Site	2	184782	7.562	0.0075	66125	7.037	0.095	220.5	1.288	0.311	17385	0.95	0.414
Residuals	12	24436			9397			171.3			18302		

Faial

	Radiozoa				*Bassia bassensis*			Scyphozoa ephyra		
	df	Mean Sq	F value	p	Mean Sq	F value	p	Mean Sq	F value	p
Site	2	1252.1	1.496	0.297	40.51	0.377	0.701	2.864	0.284	0.762
Residuals	6	837.1			107.52			10.084		