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Structural and functional organization of fish assemblages in a Mediterranean shallow CO_2 vent

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Dedicated to Mariasole.

‘As the bee collects the juice of flowers
without damaging color and fragrance,
so wise dwells in the world.’

‘Come l'ape raccoglie il succo dei fiori
senza danneggiarne colore e profumo,
così il saggio dimori nel mondo.’
[*Siddhārtha Gautama Buddha*]

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CHAPTER 1: General Introduction

The anomalous increasing of surface seawater temperatures and the lowering of ocean pH are only two consequences of global changes that are causing deep damages worldwide (Doney et al. 2012, IPCC 2014). Among the other problems, the rising CO_2 emissions, caused primarily by anthropogenic combustion of fossil fuels, cement production and land use changes, are being absorbed by the ocean at faster rate since the beginning of the industrial era. This phenomenon, known as 'ocean acidification', is considered as one of the main threat for marine life (Caldeira & Wickett, 2003, Portner et al. 2014).

Over the last years, the Intergovernmental Panel on Climate Change (IPCC) has analysed a sequence of economic growth scenarios for climate projections. Examples include the "business-as-usual emission scenario" (IS92a) that assumes rapid economic and population growth peaking in mid-century. This scenario suggests that atmospheric CO_2 levels could approach 800 ppm by the end of the century. Corresponding biogeochemical models indicate that surface ocean water pH will drop from a pre-industrial value of about 8.2 to 7.8 within 2100 with consequences on seawater chemistry and marine biota (Caldeira & Wickett, 2003, Doney et al. 2009, Feely et al. 2004, 2009, Sabine et al. 2004, Tyrrell et al. 2011). Recently, ongoing projections that consider also more stringent emissions scenario (RCP2.6), consistent with the Copenhagen Accord of keeping mean global temperature under control, suggested that this seawater chemistry alteration will affect a great numbers of taxa before the end of the century, with consequences on ecosystems services (Gattuso et al. 2015). For this reason, forecasting the ecological impacts of ocean acidification is of high priority for science, management, and policy makers (IPCC, 2014).

Overall CO_2 seems to act as a resource for primary producers, representing an additional energetic cost for consumers, with consequences on species interactions and, hence, will result hard-to-predict 'winners' and 'losers' (Gaylord et al. 2015). In a recent meta-analysis study, Kroeker et al. (2010) revealed decreasing in survival, calcification, growth, development and abundance in response to acidification when the broad range of marine organisms is gathered together. However, the magnitude of these responses varies among taxonomic groups, suggesting variation in sensitivity (Kroeker et al. 2013). Moreover, in response to environmental changes animal behavior (*i.e.* recruitment, predator–prey interactions, competition, reproduction, migration and dispersal) can be affected with consequences on organisms interactions and ecological processes (Nagelkerken & Munday,

2016), and a serious risk is forecasted also in terms of biodiversity (Cheung et al. 2009, Sunday et al. 2016). Indeed, the end-Permian mass extinction has been correlated to a massive CO_2 release in the atmosphere, among other possible triggers (Knoll et al. 2007, Kump et al. 2009). Furthermore, all the consequences forecasted for marine ecosystems are particularly exacerbated in the closed seas, like the Mediterranean Sea (Fraile et al. 2016, Lacoue-Labarte et al. 2000, Lesjeunes et al. 2010). Finally, it is important to consider indirect and synergetic effects when forecasting abundance patterns from single-species laboratory experiments (Harvey et al. 2013).

In this context, shallow CO_2 vents (or seeps) provide potential analogues of forecasted acid ocean and represent a great opportunity to understand the responses of organisms exposed long-term to low pH conditions. Vents are naturally acidified environments that are used worldwide as natural laboratories to test acidification effects not only on single species, but also on whole communities, evaluating indirect effects on ecosystems (Fabricius et al. 2011, Hall-Spencer et al. 2008, Munday et al. 2014, Nagelkerken et al. 2015). However, it is necessary caution because of other confounding factors that can hide the effects of acidification on its own. Indeed, although vents release mainly carbon dioxide, they can be characterized by peculiar chemical and physical characteristics, like output of H_2S or toxic trace elements (Dando et al. 1999, Tarasov et al. 2005). Additionally, these systems are shaped by natural variability of environmental features that may be controlled in laboratory experiments. For this reason, the benefits and limitations of different research approaches must be considered carefully and an integration of laboratory, mesocosm and *in situ* experiments is important to understand the consequences of ocean acidification (Andersson et al. 2015, Fabry et al. 2010).

In the last years, a surprisingly amount of research has been focused on ocean acidification effects and the necessity to understand the long-term responses of communities and ecosystems has emerged, creating models of future projections (Riebesell & Gattuso, 2015). The ultimate goal of contemporary ocean acidification research is to project how marine ecosystems will be affected by changes in seawater carbonate chemistry in combination with other global and local stressors, including warming, deoxygenation, eutrophication, invasive species and overfishing (Riebesell & Gattuso, 2015, Russel et al. 2009). At the beginning, most ocean acidification studies have been conducted on calcifying organisms (mainly calcareous plankton, echinoderms, corals and mollusks) suggesting negative effects on growth, calcification rate, and survival (Anthony et al. 2008, Doney et al. 2009, Fabricius et

al. 2008, Kleypas et al, 2006), however also on not-calcifying ones can be altered. For instance, experiments on fish have revealed effects on physiology and behaviour, but many other aspects are still unknown (Ishimatsu et al. 2008).

Fish have been considered able to cope with extra-cellular acidosis thanks to their acid-base regulation (Melzner et al. 2009). However, both direct and indirect effects have been tested (*i.e.* olfactory and auditory functions - Dixson et al. 2010, Munday et al. 2009a, Simpson et al. 2011; reproduction – Miller et al. 2013; calcareous structure - Bignami et al. 2013; behaviour and neurosensory functions - Domenici et al. 2012, Munday et al. 2014, Nilsson et al. 2012 - habitat alteration and interaction with other species - McCormik et al. 2013, Nagelkerken et al. 2015). The impacts of climate change will vary among life stages, with larvae and juveniles expected to be more vulnerable (Baumann et al. 2012, Ishimatsu et al. 2004, Pankhurst & Munday, 2011). As an example, larvae exposed to acidified seawater (about 1000 ppm of CO_2 - pH = 7.8) lost the ability to differentiate between the chemical cues of predatory and non-predatory species (Dixson et al. 2010). Additionally, olfaction, vision and auditory senses can be negatively affected by ocean acidification and overall behavioral abnormalities have been found in response to high CO_2 levels (Munday et al. 2014, Nagelkerken et al. 2015). Considering the importance of these functions to help larvae in orientation and selection of settlement habitat, to keep them at safe distance from predators, and to escape from a predatory attack, deep effects on population dynamics and fish community structure are expected (Nagelkerken & Munday, 2015). However, studies suggest a species-specific response, with various sensibility at different CO_2 levels. For instance, Munday et al. (2011a) observed no effects on spiny damselfish otolith calcification at high CO_2 levels (850 μatm), while Munday et al. (2011b) and Checkley et al. (2009) highlighted otolith hypercalcification in white sea bass larvae exposed to 993 and 2558 μatm. To date, most information available on fish response to ocean acidification have been obtained in closed systems, and only a few studies have been carried out *in situ* (Cattano et al. 2016, Munday et al. 2014, Nagelkerken et al. 2015).

Fish are a key biological component whose monitoring is relevant not only from the ecological standpoint, but also for the economic repercussions (*i.e.* impacts on seafood - Branch et al. 2013). Effects on individual performance, trophic linkages, recruitment dynamics, connectivity between populations and other key ecosystem processes are expected on fish (Munday et al. 2008, 2009b, 2010). Here, we dealt with the assessment of the effect of high CO_2 / low pH conditions on the structural and functional organization of fish

assemblages in a Mediterranean shallow CO_2 vent. In Mediterranean, one of the most active shallow CO_2 vent is located in Levante Bay of Vulcano Island (Aeolian Archipelago, Italy – Boatta et al. 2013). Exploiting the peculiar conditions of this area, we compared the responses of a fish assemblage exposed long-term to high CO_2 emissions (Low pH site, pH = 7.8), against two assemblages living at normal pH (Controls, pH = 8.2). We hypothesized that the organization of fish assemblages in the low pH site is altered from that found in the two controls, with negative repercussions on the structure and overall trophic organization. To test our hypothesis we used several attributes and biological markers related to species composition and ecological structure.

First, by using underwater visual census techniques, we assessed the fish community structure in terms of species richness, number of individuals and size-class structure (**Chapter 2**). Then we carried out samplings to evaluate the fish assemblages in terms of trophic organization, niche and trophic levels by using stable isotopes of carbon and nitrogen (**Chapter 3**). Exploiting the geochemical composition of the vent seawater, the direct input of metals and the peculiar pH and Eh conditions, we evaluated the synergic effect of acidification and metals bio-availability by measuring mercury bioaccumulation in fish and biomagnification (**Chapter 4**). Finally, we analysed the morphological characteristics of otoliths (earbones made of aragonite) to assess the effect of acidification on fish calfified structures. Moreover, otoliths were used as natural tags to elucidate fish "habitat use" and to evaluate their reliance on the site interested by CO_2 emissions, by measuring natural variation in elemental and isotopic signatures (**Chapter 5**).

I would like to highlight that the four central chapters (from 2 to 5) are thought as a stand-alone studies and for this reason some parts (*i.e.* the study area and the introduction to the main topic – 'ocean acidification') could appear redundant. I chose this structure to give the possibility to read each chapter independently from the others. Finally, the results obtained from the single studies were summarised, with an overview and integration of the different objectives and the main goals achieved (**Chapter 6**).

CHAPTER 2: Community structure and species composition of a fish assemblage living in a naturally acidified environment

Abstract: Nowadays, CO_2 vents are used to test ecological hypotheses about the effects of ocean acidification on complex communities. Here, we dealt with the assessment of a coastal fish assemblage exposed to long-term high CO_2 emissions / low pH conditions, in a shallow CO_2 vent. In particular, by using non-destructive underwater visual census techniques, we compared the structure of a Mediterranean fish assemblage living in a *Cymodocea nodosa* meadow in a low pH site and in two controls with normal pH. Overall, a total of nineteen fish species belonging to six families was recorded. At all sites, necto-benthic fish, mainly Sparidae and Labridae dominated the fish assemblage, followed by a few species belonging to Serranidae, Mullidae, Pomacentridae and Mugilidae. Lower values were found in the Low pH site in terms of species richness (S), but not in the number of individuals (N) where the two controls differed each other. Moreover, single species abundance did not show a unique spatial trend, although different among sites, suggesting a species-specific response. Overall, the temporal variability hid the spatial one in the composition and abundance of the fish assemblage. Contrary to expectation, slight differences were found in the fish community structure and species composition in terms of direct effect of low pH, while more differences could be indirectly related to habitat modification. This study contributes to fill the knowledge gap on fish biodiversity in a naturally acidified environment in a moment of increasing interest towards the ecosystem functions in changing ocean conditions.

Keywords: Mediterranean fish assemblages, species composition, CO_2 vent, underwater visual census, ocean acidification, *Cymodocea nodosa* meadow.

2.1 Introduction

Ocean acidification is attracting growing interest worldwide due to the direct and indirect effects forecasted on marine organisms, biodiversity and ecosystem functions (Barry et al. 2011, Hoegh-Guldberg & Bruno, 2010, Kroeker et al. 2010). The increasing anthropogenic

CO_2 absorbed by the ocean is causing changes in the carbon chemistry balance, with consequences on marine life (Ishimatsu & Dissanayake, 2010, Kroeker et al. 2013). Although many studies have focused on the response of single species, more difficult is to forecast the effects of acidification on communities and ecosystems (Riebesell & Gattuso, 2015). Moreover, on the last decades, many studies have been focused on calcifying organisms (primarily calcareous plankton, molluscs and echinoderms) that are thought to be mainly affected by the altered pH and aragonite/calcite saturation state (Doney et al. 2009).

Only recently, fish have been addressed in ocean acidification studies (Ishimatsu et al. 2008). Fish have been considered able to cope with the effect of low pH, thanks to their capacity on acid-base regulation, but recent studies have highlighted both direct effects (*i.e.* on olfactory and auditory functions - Dixson et al. 2010, Munday et al. 2009, Simpson et al. 2011; on reproduction – Miller et al. 2013; on calcareous structure - Bignami et al. 2013; on behaviour and neurosensory functions - Domenici et al. 2012, Munday et al. 2014, Nilsson et al. 2012) and indirect effects (*i.e.* habitat alteration and interaction with other species - McCormik et al. 2013, Nagelkerken et al. 2015). In particular, fish seem to be most vulnerable during the early life stages and juveniles are expected to be negatively affected directly and indirectly by low pH conditions, with consequences on fish population replenishment (Ishimatsu et al. 2004, Munday et al. 2010, Rossi et al. 2016). It has been hypothesized that species biodiversity will be negative affected by ocean acidification (Baumann et al. 2011) together with other stressors, like global warming and invasive species (Cheung et al. 2009). As a consequence, impacts are expected on fisheries, and fish represent one of the most important component of catches worldwide (Branch et al. 2013).

In addition, primary producers (*i.e.* macroalgae and seagrasses), can be altered by ocean acidification. Also in this case, calcifying organisms seem to be more vulnerable to low pH conditions (Martin & Gattuso, 2009, Porzio et al. 2011, Riebesell et al. 2010), while other species take advantage. For instance, seagrasses are expected to benefit from higher concentrations of carbon dioxide in the oceans that are predicted over the coming decades (Russell et al. 2013), but results appear contradictory (Apostolaki et al. 2014). Seagrasses exposed naturally to acidified conditions have been studied in shallow CO_2 vent in Mediterranean (Apostolaki et al. 2014, Arnold et al. 2012, Hall-Spencer et al. 2008), and species-specific responses were found (*i.e.* changes in density, biomass, phenolic content, metabolism, loss of epiphytic organisms). For instance, in Vulcano Island (Aeolian Archipelago), the seagrass *Cymodocea nodosa* living near the vent area showed lower density

and biomass but appears overall stimulated through the intense metabolism and photosynthetic activity (Apostolaki et al. 2014).

C. nodosa meadows are one of the most important habitat for many coastal fish thanks to their capacity to provide shelter from predators and a greater abundance of food (mainly small invertebrates; Guidetti & Bussotti, 2000). After *Posidonia oceanica*, *C. nodosa* have a crucial role in the Mediterranean Sea, for their role in primary production, biodiversity and food web complexity (Cancemi et al. 2002). Moreover, *C. nodosa* represent an important nursery area for many coastal fish and the presence of juveniles emphasizes the potentially important function of nursery exerted by such seagrass systems during the first life stages of several species (Guidetti & Bussotti, 2000).

To date, most studies conducted in naturally acidified ecosystems have shown a loss of calcareous species and deep ecosystem shifts due to acidified conditions (Fabricius et al. 2011, Hall-Spencer et al. 2008, Kroeker et al. 2012), but only a few studies have focused on fish in these environments to test the consequences of altered behaviour of juveniles on the structure of fish communities (Munday et al. 2014). CO_2 vents are potential natural laboratory and represent a great opportunity to test the effect of global acidification not only on single species, but above all on whole community. Fish community structure is driven by biotic (*i.e.* settlement, predation, competition, spawning) and environmental factors (light and nutrient availability, depth, temperature, algal cover, habitat complexity) with variation at spatial and temporal scale (Azzurro et al. 2007, De Raedemaecker et al. 2010, Fernandez et al. 2005, La Mesa et al. 2011). Generally, stressful conditions alter the community structure, decreasing the species richness and increasing the numbers of individuals of tolerant organisms (Guidetti et al. 2002).

Here, we hypothesized that the structure of a coastal fish assemblage living in a naturally acidified site is negatively affected in terms of fish size, species richness and numbers of individuals due to the direct effect of high CO_2 on fish behaviour and early fish stages. To test our hypothesis, we compared fish assemblages exposed long-term to high CO_2 emission (low pH site) against fish assemblages living in normal pH (control sites), and we verify whether there are differences in the community structure between sites at different pH across time, in terms of species composition and number of individuals, and whether there are differences of size structure, abundance and frequency of occurrence both at species and at fish community level.

2.2 Materials and methods

2.2.1 Study area

The shallow hydrothermal system of Vulcano Island is one of the most active sites in the Aeolian Archipelago (24 km off the NE coast of Sicily, Italy). Gas composition of the main venting area located in Levante Bay, on the eastern side of the Island, is dominated by CO_2 (97-99% vol.), which generates a pH gradient (from 5.5. to 8.1) along the north shore of the bay (Boatta et al. 2013, Capaccioni et al. 2011, Italiano et al. 2009). Emissions include also small quantities of H_2S (<2.2%), which rapidly decrease with distance from the vent (Boatta et al. 2013). Water composition in terms of major elements (Cl, SO_4, Na, K, Ca and Mg) is close to that of Mediterranean surface waters, while greater variability is recorded for dissolved Fe concentrations, which showed maximum values close to the vents (Boatta et al. 2013). The area is characterized by acidic and reducing conditions, causing changes in major and trace element geochemical fluxes at the sediment-seawater interface (Vizzini et al. 2013). Seawater carbonate chemistry parameters in the Levante Bay range between 2.78 and 3.17 mmol/kg for the total alkalinity and 0.02 and 3.64 for the aragonite saturation state (for details see Boatta et al. 2013).

2.2.2 Field work

Non-destructive underwater visual census (UVCs), the most used technique for assessment of coastal fish assemblages (Harmelin-Vivien et al. 1985), were carried out in six times (September, October and November 2014, May, June and July 2015) at the following three sites: the low pH site (hereafter "Low pH", mean pH =7.80 ± 0.09) about 250 m far from the primary CO_2 vent in Levante Bay, a control site (hereafter "Ctrl 1", mean pH = 8.19 ± 0.03) 500 m far from the primary vent, and another control site in Lipari Island (hereafter "Ctrl 2", mean pH = 8.22 ± 0.02) about 6.5 km from the first two sites (**Fig. 2.1**). Controls were chosen to be similar in terms of orientation (South-East), depths (2-5 m) and vegetal coverage (*Cymodocea nodosa* meadow) to the Low pH site. At each sampling occasion, a total of six replicates were carried out between 11.00 A.M. and 3.00 P.M.

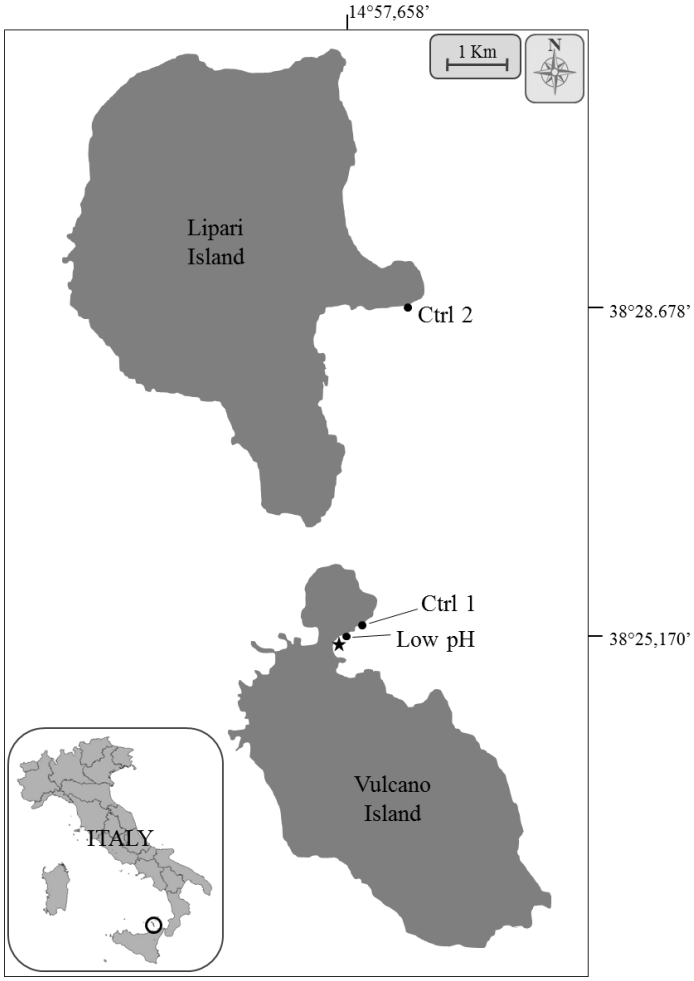

Fig. 2.1 *- Map of the study area showing the sampling sites in Vulcano (Low pH and Ctrl 1) and Lipari Islands (Ctrl 2). The primary vent is indicated by the star.*

UVCs were carried out along 10 m long × 5 m wide strip transects (total surface area 50 m^2), randomly placed in each site at a depth of 2 to 5 m within *C. nodosa* meadows. Cryptic fish (*i.e.* Blenniidae, Gobiidae, Scorpaenidae) were kept out from the surveys to avoid underestimation (Willis, 2001). Fish were identified to species level and abundance was estimated by using seven pre-established abundance classes (1, 2-5, 6-10, 11–30, 31–50, 51–100, >101 number of individuals) according to Harmelin-Vivien et al. (1985). Fish density (ind./100 m^2) was calculated by taking into account the mid-point of each abundance class. Moreover, fish were assigned to three size-classes: small (S), medium (M) and large (L), corresponding each to one-third of the maximum total length reported in the literature according to Fischer et al. (1987).

At each sampling occasion, seawater physicochemical variables (temperature, salinity, dissolved oxygen, pH and Eh) were measured through a portable multiparametric probe (Hanna Instrument, HI98194) and seagrass shoot density was estimated by counting shoots within a 20x20 cm^2 square (three replicates per transect).

2.2.3 Data analysis

Univariate analysis of variance (one-way ANOVA) was used to test for differences in *C. nodosa* seagrass shoot density between the three sampling sites.

As regards fish assemblages, the following two factors-experimental design was considered: Site (fixed with 3 levels: Low pH, Ctrl 1 and Ctrl 2) and Time (random and orthogonal to Site, with 6 levels: September, October and November 2014, May, June and July 2015). Frequency of occurrence [(number of censuses in which a species was recorded/total number of censuses) x 100], density (number of individuals / 100 m^2), number of individuals (N) and species richness (S) were calculated.

Fish community structure was analyzed by using both univariate and multivariate statistical techniques, through STATISTICA (StatSoft, 2011) and PRIMER 6 with PERMANOVA +add-on (Anderson et al. 2008) software packages, respectively. Statistical analysis was carried out only on the species whose percentage abundance was higher than 2% in at least one of the sampling sites, while species richness and number of individuals were calculated on all the species sampled (see **Annex I**).

Univariate analysis of variance (factorial ANOVA) was used at species level to test differences in frequency of occurrence and abundance and, at assemblage level, to test differences in total abundances and species richness. Cochran's test and Shapiro-Wilk's test were used to check for homogeneity of variances and normality respectively, and where ANOVA assumptions were not satisfied, data were transformed using log (x+1). Where significant differences were present, Tukey's *post hoc* tests were used for pairwise comparisons.

Fish abundance data were transformed with the log (x + 1) notation and resembled using Bray Curtis similarity matrices. Differences of fish assemblages between sites were tested at a multivariate level by using permutational multivariate analysis of variance

(PERMANOVA) both on total fish abundance and on that of each size class (S, M and L) to compare the size structure of fish community among sites. To evaluate the variation in density of fish assemblages at multivariate level in each site, we made a constrained ordination (with site as constrained factor) using a canonical analysis of principal coordinates (CAP; Anderson & Willis 2003). The similarity percentage (SIMPER) procedure was employed to identify the major fish taxa contributing to dissimilarities between sampling sites.

Moreover, species were assigned to five trophic groups (small piscivores, invertivores, detritivores, planktivores, and herbivores) following the classification by Guidetti & Sala (2007) and according to FishBase database (Froese & Pauly, 2016 - **Annex I**).

2.3 Results

2.3.1 Environmental variables and seagrass shoot density

Slight differences were found among the three sampling sites in terms of surface seawater temperature (mean of the three sites: 22.2 ± 0.4 °C), salinity (39.2 ± 0.1 psu) and dissolved oxygen (7.1 ± 0.2 mg l^{-1}). In contrast, pH showed the expected high variability between sites (pH: 7.80 ± 0.09, 8.19 ± 0.03, and 8.22 ± 0.02 in Low pH, Ctrl 1 and Ctrl 2 respectively). Coincident with lowered pH values, redox potential ranged between 79.3 ± 5.6 mV in the Low pH site, 85.6 ± 8.3 mV in Ctrl 1 and 111.6 ± 13.7 mV in Ctrl 2.

In general, *C. nodosa* shoot density was higher in the two Controls compared to Low pH (ANOVA: $F_{2, 321} = 7.702$, $p < 0.001$, *post hoc* tests: Low pH<Ctrl 1=Ctrl 2) (**Fig. 2.2**). In more detail, higher shoot densities were recorded during spring-summer months with a peak in June in Ctrl 1 (1504.2 ± 329.7) than in autumn months when the minimum was registered in October in the Low pH site (159.3 ± 46.2 shoot/m^2).

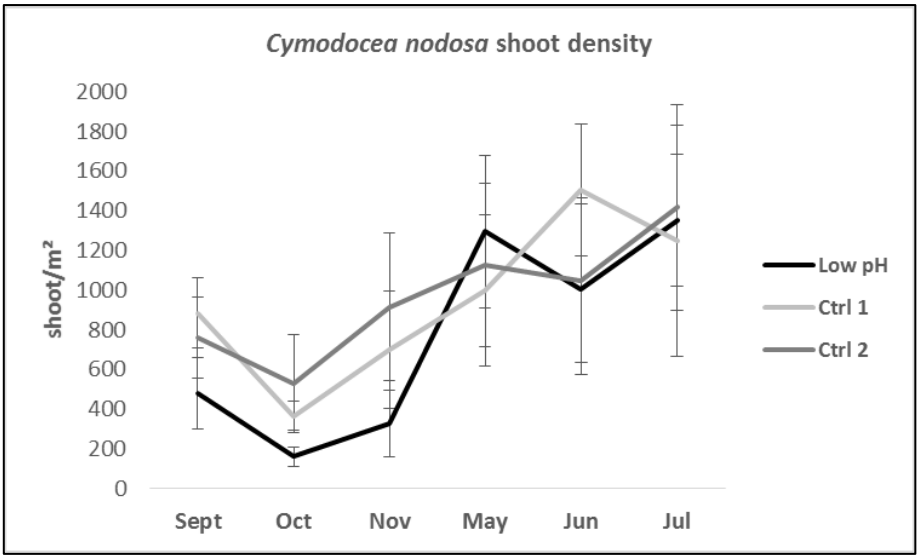

Fig. 2.2 *- Cymodocea nodosa shoot density (mean ±SD) in each site (Low pH, Ctrl 1 and Ctrl 2) during the six sampling times (from September 2014 to July 2015).*

2.3.2 Fish assemblage

Overall, nineteen fish species belonging to six different families were recorded. In general, fish assemblages were dominated by Labridae (10 species) and Sparidae (5 species), followed by a few species belonging to Pomacentridae, Mullidae, Mugilidae and Serranidae. Most species belonged to the trophic groups of invertivores with the exception of one herbivore (*Salpa sarpa*), one detritivore (*Mugil cephalus*), two planktivores (*Chromis chromis* and *Oblada melanura*) and one small piscivore (*Serranus scriba*) (for details see **Annex I**).

In general, in the three sites the most frequent species belonged to Sparidae (*i.e. S. salpa* and *Diplodus vulgaris*), Labridae (*i.e. Coris julis* and *Symphodus tinca*) and Pomacentridae (*C. chromis*) families (**Fig. 2.3a**). In particular, the most frequent species in the Low pH site was *C. julis* (97.2%), followed by *C. chromis* (66.7%) and *S. tinca* (50%). Also in Ctrl 1, the most frequent species was *C. julis* (88.9%), followed by *S. tinca* (80.6%), *C. chromis* (63.9%) and *Thalassoma pavo* (55.6%), while in Ctrl 2, the most frequent species was *D. vulgaris* (86.1%) followed by *T. pavo* (77.8%), *C. julis* (75%) and *S. tinca* (52.8%). Spatial differences were found for all the species with the exception of *S. salpa* and *Mullus surmuletus* and were species-specific with no unique trends. Similar trend was found for *C. chromis* and *D. vulgaris* whose frequencies were similar in Low pH and Ctrl 1 compared to Ctrl 2 (respectively lower in Ctrl 2 for *C. chromis* and the opposite for *D. vulgaris*). On the other

hand, lower frequencies were highlighted for *O. melanura* and *T. pavo* in the Low pH site than in both controls. Moreover, only *M. surmuletus* and *C. julis* showed significant differences for the factor Time, while *C. chromis*, *Symphodus cinereus* and *S. ocellatus* showed differences for the interaction 'Site x Time', without a unique trend for temporal variation (**Tab. 2.1**).

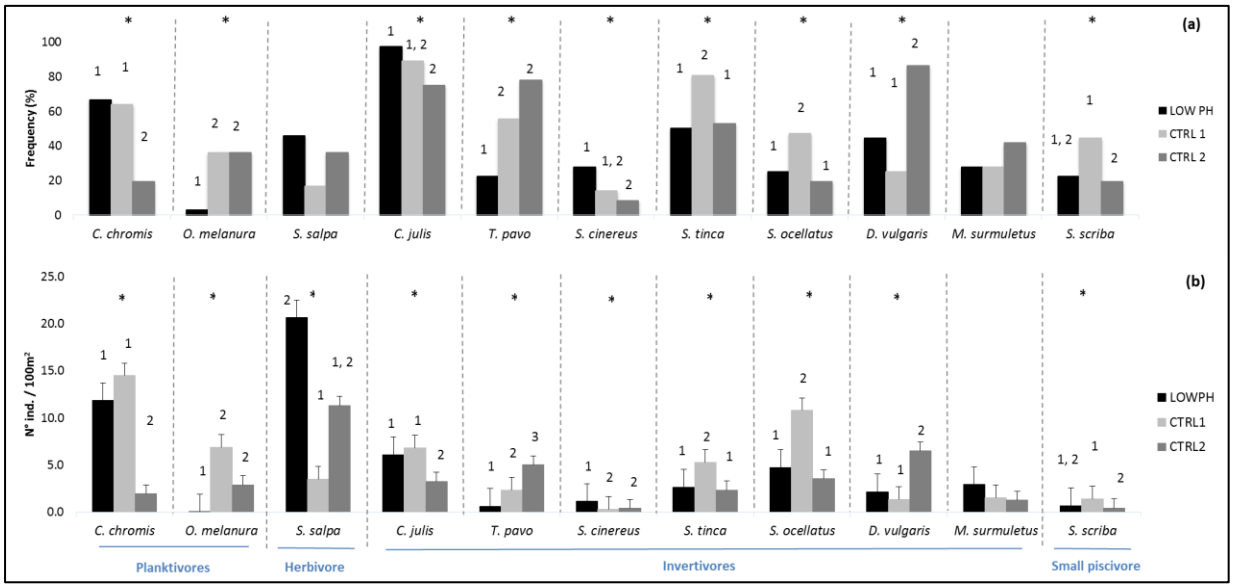

Fig. 2.3 *- Frequency of occurrence (a) and density (b - mean ±SE) of the most abundant (>2%) fish species in the three sites (Low pH, Ctrl 1 and Ctrl 2). Results of the ANOVA are showed with a star and differences among sites (post-hoc Tukey's test) are indicated by numbers above each bar, with the same number indicating no-significant differences.*

Table 2.1 *- ANOVA results testing differences in fish species frequency of occurrence for the factors site and time. Probability levels: n.s. = not significant; * p<0.05; ** p<0.01; *** p<0.001.*

Source		***C. chromis***				***O. melanura***				***S. salpa***			
	df	SS	MS	F	p	SS	MS	F	p	SS	MS	F	p
Site	2	5.06	2.53	13.93	***	2.67	1.33	8.89	***	1.59	0.79	2.73	n.s.
Time	5	1.89	0.38	2.08	n.s.	1.19	0.24	1.59	n.s.	2.79	0.56	1.92	n.s.
Site*Time	10	3.72	0.37	2.05	*	2.89	0.29	1.93	n.s.	2.00	0.20	0.69	n.s.
Residual	90	16.33	0.18			13.50	0.15			26.21	0.29		
		C. julis				***T. pavo***				***S. cinereus***			
	df	SS	MS	F	p	SS	MS	F	p	SS	MS	F	p
Site	2	0.91	0.45	5.33	**	5.63	2.81	14.34	***	0.72	0.36	3.36	*
Time	5	1.96	0.39	4.61	***	1.41	0.28	1.43	n.s.	2.00	0.40	3.72	**
Site*Time	10	1.65	0.16	1.93	n.s.	2.26	0.23	1.15	n.s.	2.61	0.26	2.43	*
Residual	90	7.67	0.09			17.67	0.20			9.67	0.11		
		S. tinca				***S. ocellatus***				***D. vulgaris***			
	df	SS	MS	F	p	SS	MS	F	p	SS	MS	F	p
Site	2	2.06	1.03	4.62	*	1.56	0.78	4.83	*	7.02	3.51	17.55	***
Time	5	2.11	0.42	1.90	n.s.	2.53	0.51	3.14	*	0.74	0.15	0.74	n.s.
Site*Time	10	1.50	0.15	0.68	n.s.	4.33	0.43	2.69	**	1.20	0.12	0.60	n.s.
Residual	90	20.00	0.22			14.50	0.16			18.00	0.20		
		M. surmuletus				***S. scriba***							
	df	SS	MS	F	p	SS	MS	F	p				
Site	2	0.46	0.23	1.29	n.s.	1.35	0.68	3.61	*				
Time	5	3.94	0.79	4.38	**	1.94	0.39	2.07	n.s.				
Site*Time	10	3.09	0.31	1.72	n.s.	1.98	0.20	1.06	n.s.				
Residual	90	16.17	0.18			16.83	0.19						

Fish density overall reflected the pattern of frequency of occurrence examined above (**Fig. 2.3b**). Seven out of eleven fish species common to the three sites showed significant among-site differences in the abundance, while five additional species showed differences also for the interaction term 'Site x Time' (**Tab. 2.2**). In general, fish did not show a clear spatial pattern, but changes were species-specific. For instance, the herbivore *S. salpa* was more abundant in Low pH (20.7 ± 18 ind/100 m^2) and Ctrl 2 (11.3 ± 15 ind/100 m^2) than in Ctrl 1 site (3.5 ± 11 ind/100 m^2). *C. chromis* and *C. julis* were more abundant in Low pH and Ctrl 1

than in Ctrl 2, while *T. pavo* and *D. vulgaris* were more abundant in Ctrl 2 than in the other sites. Tukey's *post hoc* tests highlighted a general trend for the interaction 'Site x Time', namely higher densities were found in September compared to the other sampling times for *O. melanura* in Ctrl 1 than in the other sites and for *S. ocellatus* in Low pH and Ctrl 1 than in Ctrl 2. Additionally, the density of *M. surmuletus*, *S. salpa* and *S. cinereus* was higher in the Low pH in June and September compared to the other sites and times.

Table 2.2 *- ANOVA results testing differences in fish species abundance for the factors site and time. Probability levels: n.s. = not significant; * p<0.05; ** p<0.01; *** p<0.001.*

Source		***C. chromis***				***O. melanura***				***S. salpa***			
	df	SS	MS	F	p	SS	MS	F	p	SS	MS	F	p
Site	2	27.59	13.80	12.73	***	9.10	4.55	8.89	***	12.04	6.02	5.33	**
Time	5	6.83	1.37	1.26	n.s.	3.58	0.72	1.40	n.s.	27.48	5.50	4.87	***
Site*Time	10	17.03	1.70	1.57	n.s.	13.07	1.31	2.55	**	29.79	2.98	2.64	**
Residual	90	97.52	1.08			46.06	0.51			101.57	1.13		
		C. julis				***T. pavo***				***S. cinereus***			
	df	SS	MS	F	p	SS	MS	F	p	SS	MS	F	p
Site	2	5.32	2.66	9.93	***	11.43	5.72	17.53	***	0.76	0.38	3.26	*
Time	5	13.32	2.66	9.95	***	4.32	0.86	2.65	*	3.05	0.61	5.22	***
Site*Time	10	1.72	0.17	0.64	n.s.	3.46	0.35	1.06	n.s.	2.73	0.27	2.34	*
Residual	90	24.09	0.27			29.35	0.33			10.51	0.12		
		S. tinca				***S. ocellatus***				***D. vulgaris***			
	df	SS	MS	F	p	SS	MS	F	p	SS	MS	F	p
Site	2	5.01	2,51	5.78	**	5.68	2.84	4.60	*	15.85	7.93	17.43	***
Time	5	7.05	1,41	3.25	*	24.48	4.90	7.93	***	1.67	0.33	0.73	n.s.
Site*Time	10	3.02	0,30	0.70	n.s.	32.42	3.24	5.25	***	2.88	0.29	0.63	n.s.
Residual	90	39.02	0,43			55.58	0.62			40.93	0.45		
		M. surmuletus				***S. scriba***							
	df	SS	MS	F	p	SS	MS	F	p				
Site	2	0.09	0.05	0.16	n.s.	1.07	0.54	3.42	*				
Time	5	10.34	2.07	7.25	***	1.55	0.31	1.98	n.s.				
Site*Time	10	5.78	0.58	2.03	*	1.90	0.19	1.21	n.s.				
Residual	90	25.67	0.29			14.10	0.16						

Percentage of abundance for the three size classes (S, M, L) showed a different trend for each fish species, and no general pattern was detected (**Fig. 2.4**). A few species showed an overall homogeneous size class distribution among the three sites (*i.e. C. julis, T. pavo* and *S. tinca*), while others had a different size structure. As an example, for *S. ocellatus, D. vulgaris* and *M. surmuletus* the larger class was almost absent in the Low pH site, while size distribution was more heterogeneous in the two controls. At multivariate level, abundance of all size classes was different among sites and times. In particular, the small size class showed differences among the three sites with higher values in the Low pH (PERMANOVA: pseudo-$F_{2, 60}$ = 3.11, p(perm) < 0.01) and no a unique trend was found among different times. With regard to the medium and large classes, differences were found for the interaction 'Site x Time' (pseudo-$F_{10, 83}$ = 1.67, p(perm) < 0.01) and the factor Site, respectively (pseudo-$F_{2, 65}$ = 4.18, p(perm) < 0.01; pair wise: Ctrl 2<Ctrl 1=Low pH). In accord to PERMANOVA results, the CAP ordinations showed the three sites interspersed for the small size, while showed superimposing between Low pH and Ctrl 1 for medium and large sizes and a separation for Ctrl 2 on the left side (**Fig. 2.5**).

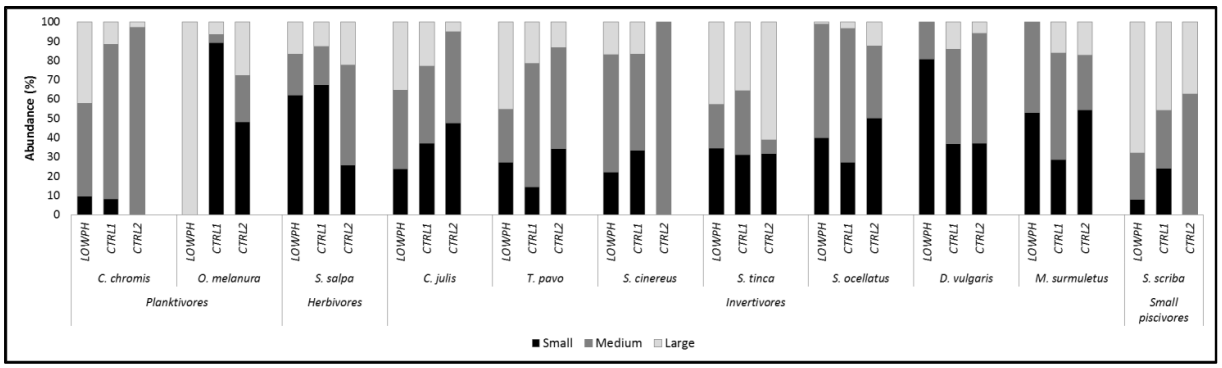

***Fig. 2.4** - Percentage abundance of the three size classes (Small, Medium, Large) of each fish species in the three sampling sites (Low pH, Ctrl 1, and Ctrl 2).*

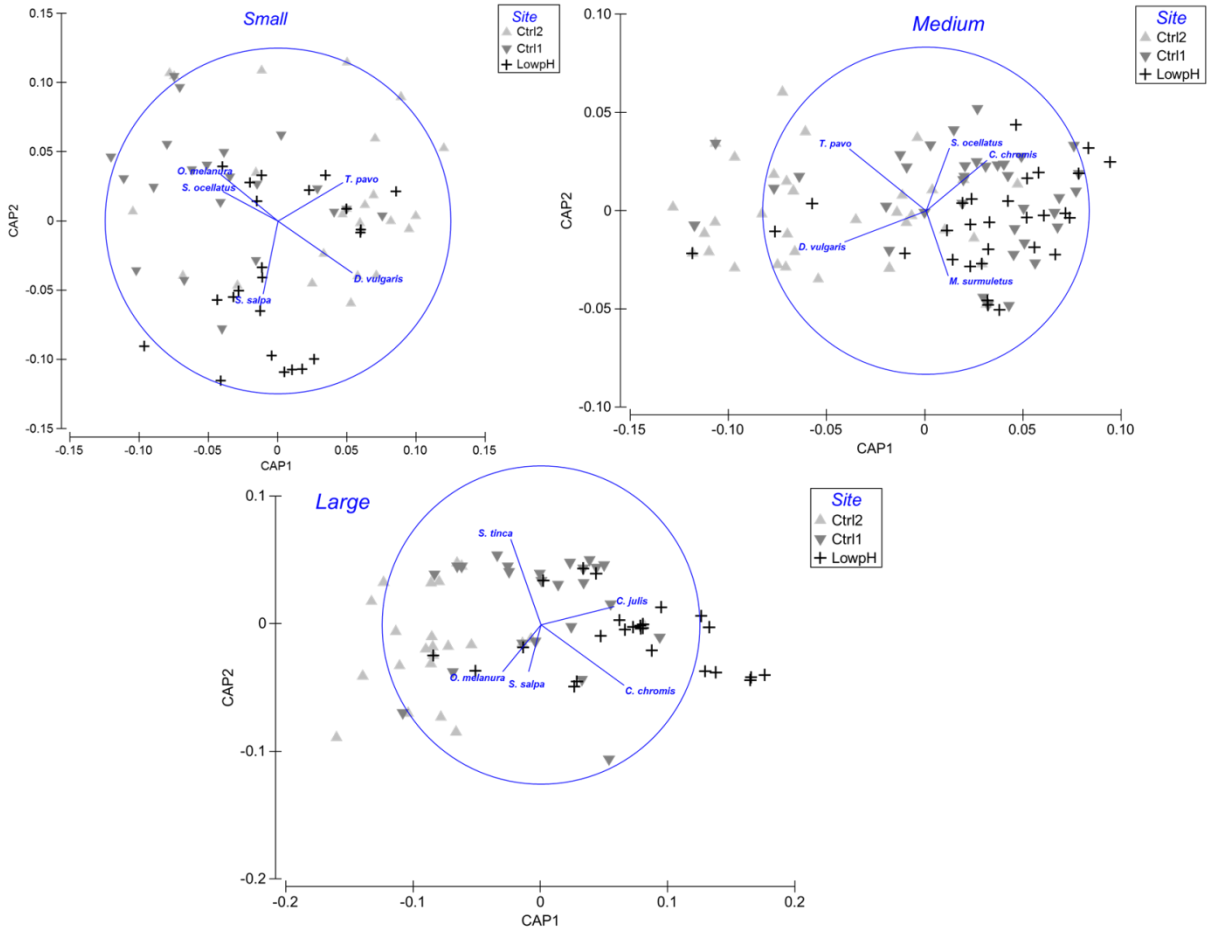

Fig. 2.5 *- Canonical analysis of principal coordinates (CAP) of the abundance of small, medium and large size classes fish in the three sampling sites (Ctrl 1, Ctrl 2 and Low pH). Vectors of the species contributing most to the ordination (Pearson correlation > 0.03) are superimposed.*

Likewise medium-sized class fish, PERMANOVA test on total fish abundance showed significant differences for the interaction between 'Site x Time' (**Tab. 3**). However, no general trend was found among sampling times but only a partial separation between sites (**Fig. 2.6**). In particular, in September and June the three sites differed significantly each other with higher fish abundance in Low pH than Ctrl 1 and Ctrl 2. In October, November and July, Ctrl 2 differed from Low pH and Ctrl 1, while no significant differences were found among sites in May.

Table 2.3 *- PERMANOVA results testing differences for the factors site and time in total fish abundance. Probability levels: * p<0.05; ** p<0.01.*

Source of variation	*df*	**MS**	**Pseudo-F**	**P (perm)**
Site	2	18064	6.4378	**
Time	5	6398.9	4.2307	**
Site x Time	10	2805.9	1.8552	*
Residual	90	1512.5		

Additionally, PERMANOVA highlighted differences in the interaction 'Site x Time', the graphical ordination of the canonical analysis of principal coordinates (CAP) explained 74.1 % of total variation and showed a certain separation among sites (Low pH on up-right side, Ctrl 1 down and Ctrl 2 on the left side), without a unique temporal trend (**Fig. 2.6**). The species that correlate most with the ordination were *C. chromis, C. julis, D. vulgaris, O. melanura, S. ocellatus, S. tinca* and *T. pavo*. Accordingly, SIMPER analysis revealed that the species that discriminate between Low pH site and Ctrl 1 (average dissimilarity = 62.5%) were *C. chromis, S. ocellatus, S. salpa* and *S. tinca,* while the highest dissimilarity was recorded between Low pH and Ctrl 2 (average dissimilarity = 68.8%) due to the species *C. chromis, S. salpa, D. vulgaris* and *T. pavo.* On the other hand, the main species that discriminate among the two controls were *C. chromis, D. vulgaris, S. ocellatus* (average dissimilarity = 66.7%), contributing each by more than 10% to the dissimilarity between sites (**Tab. 2.4**).

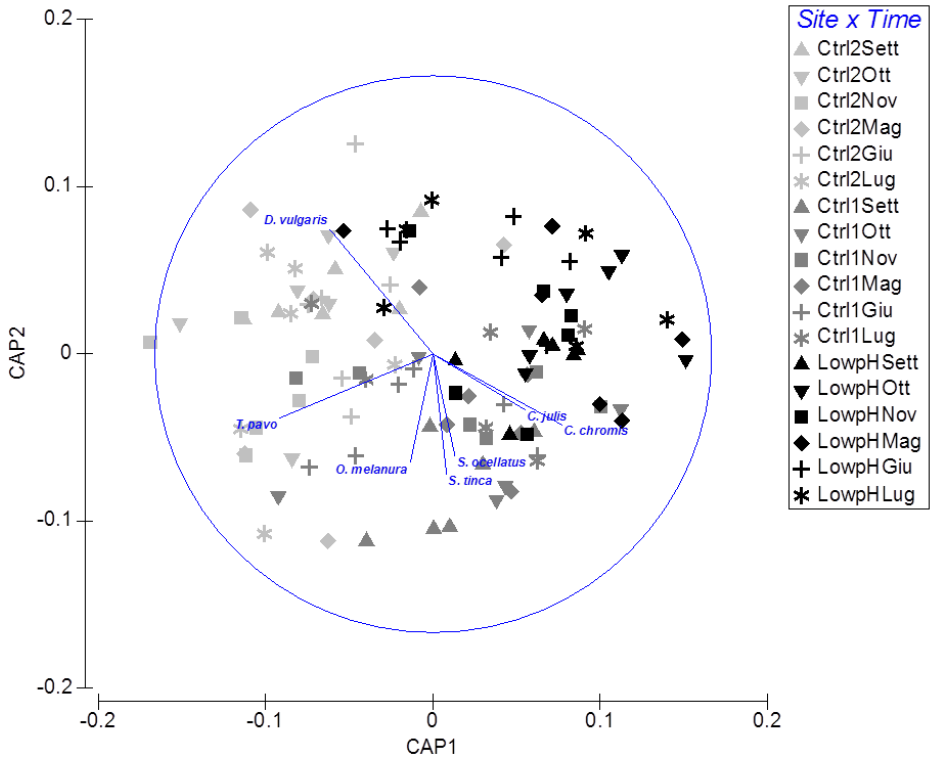

Fig. 2.6 *- Canonical analysis of principal coordinates (CAP) of total fish abundance in each time in the three sampling sites (Low pH, Ctrl 1 and Ctrl 2). Vectors of the species contributing most to the ordination (Pearson correlation > 0.03) are superimposed.*

Table 2.4 *- SIMPER analysis showing fish species contributing most to dissimilarity between sites and average abundance of each site (Low pH, Ctrl 1 and Ctrl 2). Av. Ab.: Average abundances; Contr.%: dissimilarity contribution.*

Species	**Contr.%**	**Av. Ab. Low pH**	**Av. Ab. Ctrl 1**	**Contr.%**	**Av. Ab. Low pH**	**Av. Ab. Ctrl 2**	**Contr.%**	**Av. Ab. Ctrl 1**	**Av. Ab. Ctrl 2**
C. chromis	17.19	1.35	1.33	15.46	1.35	0.27	15.05	1.33	0.27
D. vulgaris	7.31	0.49	0.30	12.71	0.49	1.19	12.63	0.30	1.19
S. ocellatus	12.42	0.47	0.92	7.72	0.47	0.40	11.59	0.92	0.40
S. tinca	10.95	0.58	1.03	8.69	0.58	0.57	9.84	1.03	0.57
C. julis	8.65	1.27	1.25	9.85	1.27	0.79	9.75	1.25	0.79
O. melanura	8.10	0.02	0.71	5.81	0.02	0.50	9.72	0.71	0.50
T. pavo	7.09	0.18	0.56	11.15	0.18	0.98	9.33	0.56	0.98
S. salpa	12.27	1.08	0.27	14.58	1.08	0.77	9.29	0.27	0.77
M. surmuletus	6.68	0.39	0.32	6.50	0.39	0.37	6.09	0.32	0.37
	Average dissimilarity = 62.46			Average dissimilarity = 66.73			Average dissimilarity = 68.85		

Differences in the structure of fish assemblages were evaluated comparing the total number of individuals (N) and the species richness (S). N was slightly higher in Ctrl 1 than in Low pH and Ctrl 2 (30.2 ± 22.9 ind/50 m^2 in Ctrl 1; 28.0 ± 29.6 ind/50 m^2 in Low pH and 20.4 ± 17.0 ind/50 m^2 in Ctrl 2), with significant differences for the interaction 'Site x Time'. In particular, Tukey's *post hoc* test revealed higher N in September in Ctrl 1 and Low pH site. In contrast, ANOVA performed on species richness S (5.00 ± 1.12 in Ctrl 1; 4.83 ± 1.40 in Ctrl 2 and 4.34 ± 1.64 in Low pH) showed significant differences for both factors Site and Time, but not for their interaction. Tukey's test highlighted a difference only between Low pH and Ctrl 1, and S values were generally higher in September than in spring-summer months **(Tab. 2.5)**.

Table 2.5 *- ANOVA testing differences for the factors site and time in species richness (S) and number of individuals (N). Probability levels: n.s. = not significant; * p<0.05; ** p<0.01; *** p<0.001.*

Source		**S**				**N**			
	df	SS	MS	F	p	SS	MS	F	p
Site	2	29.56	14.78	4.13	*	0.53	0.27	3.11	*
Time	5	72.33	14.47	4.05	**	2.00	0.40	4.66	***
Site*Time	10	43.11	4.31	1.21	n.s.	2.14	0.21	2.49	**
Residual	90	321.67	3.57			7.74	0.09		

2.4 Discussion

The decrease of ocean pH (Caldeira & Wickett, 2003, IPCC, 2014) is predicted to affect negatively biodiversity due to direct and indirect effects on marine biota (Cheung et al. 2009). While calcareous organisms are considered more susceptible, also other components, like fish, can be affected directly or indirectly, with consequences on their distribution and abundance (Perry et al. 2005). Our study dealing with the assessment of fish assemblages exposed to naturally high CO_2 / low pH conditions revealed slight changes in the community structure compared to those living in two control areas. Contrarily to expectations, fish diversity and community structure differed little between Low pH and controls. In particular, although species richness showed a decrease in the acidified site compared to the closer

control site, similarities were found in the species-specific number of individuals between these two sites. Moreover, no general trend was found at fish community level, but species-specific responses were showed by the descriptive parameters of fish community (abundance, frequency of occurrence).

A reason that could explain the lack of differences is that fish can overtake the low pH conditions thanks to their capacity to move. This seems to be confirmed by the similarity of the abundance between Low pH and Ctrl 1 (closer to the impacted site than the second control) for three out of nine species that showed differences among sites (*C. chromis*, *C. julis* and *D. vulgaris*). Moreover, the analysis on size classes confirmed this similarity between the nearest sites (Low pH and Ctrl 1), compared to the further Ctrl 2, with regard to medium and large-sized individuals, highlighting the mobility of species censused. Finally, contrarily to expectations, higher abundance of small individuals was found in the Low pH compared to controls, indicating the tolerance of juveniles to low pH conditions. Also, the similarity percentage analysis highlighted the difference between Low pH and Ctrl 2 and showed that *C. chromis*, *S. salpa*, *D. vulgaris*, *S. ocellatus* and *S. tinca* contributed mainly to the dissimilarity between Low pH and controls. In addition, also at multivariate level fish assemblages showed only a partial separation among sites, and no general trend was found among times.

The heterogeneous size-distribution of fish belonging to different species in the Low pH site and the higher abundance of small-sized individuals compared to the controls, suggests also a role of *C. nodosa* seagrass meadow in providing refuge, despite the lower density of the seagrass meadow in the acidified site than controls. As found in previous studies, *C. nodosa* meadows play a paramount role as nursery areas, providing food and shelter (Guidetti & Bussotti, 2000, 2002). Moreover, fish abundance has been shown to be related to depth and structural features (*i.e.* complexity of habitat or seagrass canopy) of the seagrass beds (Guidetti, 2000, Guidetti & Bussotti, 2000, Fernández et al. 2005). Here, we found a slight decrease in fish species richness in the Low pH site compared to Ctrl 1; this result may be related to the reduction of *C. nodosa* density, and opens interesting scenarios on the indirect effects of ocean acidification accordingly to a recent study gathering data from worldwide CO_2 vents (Sunday et al. 2016). Indeed, Sunday et al. (2016) predicted the indirect effects driven by ocean acidification on fauna diversity through changes in habitat-forming species. The decline of structural complexity of biogenic habitat (*i.e.* coral reefs, mussel beds and seagrasses / macroalgal habitats) was found to negatively affect biodiversity.

Particularly interesting is the case of *S. salpa*, that is the main herbivorous fish in the Mediterranean Sea, and the only one that showed a higher abundance in the Low pH site. The greater palatability of *C. nodosa* exposed long-term to high CO_2 emissions (Apostolaki et al. 2014), and a minor content of phenolic substances (deterrent to herbivory - Arnold et al. 2012) in the *C. nodosa* leaves, could explain the major presence of *S. salpa* in the seagrass meadow. Moreover, conspicuous groups of small-sized *S. salpa* were observed feeding in the Low pH site (personal observation). Fish could be attracted by abundant prey found in the Low pH site. Indead, the higher cost to balance the acid-base regulation may be compensated by a high prey availability (mainly Amphipoda and Polychaeta) associated to macrophytes was found in the low pH site of Vulcano Island (Vizzini et al. submitted). Moreover, this result opens another question about the role that herbivores will have in the future acidified ocean, because of their role of top-down control on primary producers (Poore et al. 2012). New insights are emerging into the role of herbivores and their compensatory effect under high CO_2 conditions to maintain the resistance of communities to disturbance (Ghedini et al. 2015). Compensatory dynamics are expected, indeed, to be an important stabilising mechanism through which communities respond to environmental change. Another experimental study conducted by Mertens et al. (2015) showed that the herbivore gastropod *Turbo undulatus* can take under control primary producers until a certain temperature throughout his grazing. Beyond this threshold, however, consumption declined whilst productivity increased. There is still a lack of knowledge about the consequence of the synergetic effect of forecasted ocean acidification and warming on the grazing of herbivores fish and their metabolic rate. Many studies highlighted that most species will be more sensitive when subjected to both acidification and warming, two of the greatest threats to marine biodiversity (Kroeker et al. 2013, Nagelkerken & Connell, 2015).

In this context, attention deserves also the case of the two sympatric Labridae, the rainbow wrasse *Coris julis* and the ornate wrasse *Thalassoma pavo*, that are widespread in the entire Mediterranean Sea. Recent studies showed that the interaction of these species are potentially exacerbated by seawater warming and they are differently distributed related to water temperature and, in particular, *T. pavo* is considered the 'warm-water' wrasse and *C. julis* the 'cool-water' one (Milazzo et al. 2013, 2016). In our study the density and the frequency of occurrence of these two species were opposite: *C. julis* was found more abundant in the low pH site and the adjacent control site than in the further control site, while *T. pavo* showed an opposite trend. This finding let hypothesize that *C. julis* could be more tolerant to low pH

conditions compared to the sympatric species and open a new question regarding the synergetic effects of warming and acidification. A recent meta-analysis study on different taxonomic groups has suggested that biological responses are stronger (either positive or negative) when different stressors interact synergistically (Harvey et al. 2013).

Another reason that could explain the lack of differences in fish community living in the low pH site could be the natural variability of the site also for the pH conditions. Indeed, the Low pH site experiences normal pH according to weather conditions. Due to the geo-morphological setting of the Levante Bay, acidified water masses mostly run parallel to the northern shoreline of the bay, when predominant winds belong to north-western sectors (Boatta et al. 2013). However, the pH gradient created by the primary vent along the northern cost of the bay is deleted when wind direction is from South - East (Scirocco wind). As suggested by Kroeker et al. (2012), it is possible that the natural fluctuations in carbonate chemistry at the vents allow organisms to tolerate better or adapt, generation after generation, to these particular conditions. Indeed, laboratory studies have found impact on organisms above all when there is a sharp reduction in pH, without time of adaptation (Ishimatsu et al. 2008).

Evolutionary mechanisms could help organisms to adapt from one generation to another, but this mechanism implies genetic variation (Crozier & Hutchings, 2014). Transgenerational acclimation occurs when the environment experienced by parents influences the performance of offspring (Sunday et al. 2014). Through a field experiment in the same study area, Cattano et al. (2016) found that the ocellated wrasse *Symphodus ocellatus* offspring brooded in different CO_2 conditions had similar responses, but after transplanting portions of nests to the high CO_2 site, embryos from parents that spawned in ambient conditions had higher metabolic rates. Thus, adaptive mechanisms can have a crucial role for fish, improving their resilience to environmental conditions. However, another possibility is that replenishment of fish becomes from outside the vent area and hence from "non-acidified populations", as suggested by other authors (Kroeker et al. 2012, Munday et al. 2014). In this case, plastic responses in physiology, morphology, or behaviour could help maintaining fitness in a new "stressful" environment and take less time (days to months, or within a life stage). Moreover, a meta-analysis on the effects of ocean acidification on different taxonomic groups highlighted that highly mobile organisms (*i.e.* fish or crustaceans) with developed intracellular / extracellular pH regulatory mechanisms may be more resilient to ocean acidification (Kroeker et al. 2010).

Our study confirms previous results from another CO_2 vent in Papua New Guinea (Munday et al. 2014). Also in that study, although juvenile reef fishes at CO_2 seeps exhibit behavioral abnormalities similar to those seen in laboratory experiments, fish diversity and community structure differed little between impacted and control areas. As suggested by Munday et al. (2014), the little differences in fish community structure could be driven by the differences in habitat composition (different corals community) and not by the direct effects of high CO_2. Moreover, temporal variability can hide spatial one in the abundance of the fish assemblages.

Overall, our findings suggest that the predictions for the acidification of the near-future ocean (pH = 7.8 in the year 2100) will not affect directly fish species composition and community structure but it is more probable that indirect effects will cause greater changes in this component (*i.e.* habitat changes, prey availability or interaction with non-native species - Molnar et al. 2008). In general, fish are able to cope with ocean acidification and the majority of laboratory and mesocosm experiments, that have found negative effects, used pCO_2 levels much higher than the levels forecasted for the end of the century and CO_2 exposure periods were less than 4 days in 79% (Ishimatsu & Dissiniake, 2010). For this reason we think that *in situ* experiments and the use of naturally acidified environments are useful to evaluate the species distribution and the structure of populations exposed long-term to low pH conditions and the responses both at species-specific level and at community one.

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Annex I *- List of fish species censused in the three sampling sites (Low pH, Ctrl 1 and Ctrl 2), trophic group and abundance (%) of each species.*

Family	Species	Trophic group	Low pH	Ctrl 1	Ctrl 2
Labridae	*Coris julis*	Invertivore	11.1	11.4	8.1
	Thalassoma pavo	Invertivore	<2	3.9	12.4
	Symphodus cinereus	Invertivore	2.1	<2	<2
	Symphodus tinca	Invertivore	4.9	8.8	5.8
	Symphodus ocellatus	Invertivore	8.6	18.0	8.8
	Symphodus roissali	Invertivore	<2	<2	<2
	Symphodus rostratus	Invertivore	-	<2	<2
	Symphodus mediterraneus	Invertivore	<2	<2	<2
	Symphodus doderleini	Invertivore	<2	<2	<2
	Labrus viridis	Invertivore	<2	<2	-
Sparidae	*Oblada melanura*	Planktivore	<2	11.5	7.1
	Diplodus vulgaris	Invertivore	3.9	2.3	16.1
	Diplodus annularis	Invertivore	<2	<2	<2
	Diplodus sargus	Invertivore	<2	<2	<2
	Sarpa salpa	Herbivore	37.6	5.8	28.0
Serranidae	*Serranus scriba*	Small piscivore	<2	2.3	<2
Mullidae	*Mullus surmuletus*	Invertivore	5.4	2.6	3.2
Pomacentridae	*Chromis chromis*	Planktivore	21.6	24.2	4.8
Mugilidae	*Mugil cephalus*	Detritivore	-	<2	-

CHAPTER 3: Trophic structure and isotopic niche of a coastal fish assemblage living in a naturally acidified environment

Abstract: Fifteen coastal fish species were analysed together with sediment, zooplankton, macroalgae and seagrass. The trophic structure and the isotopic niche of a fish assemblage was investigated, by using carbon and nitrogen stable isotopes analysis. In particular, we compared the isotopic signature of fish sampled in high CO_2 / low pH conditions (Low pH site) against fish assemblages living in two normal pH sites (Controls). An overall ^{13}C and ^{15}N-depletion was detected in the Low pH site compared to the two controls. On the other hand, only a few species showed among sites differences for $\delta^{15}N$ and trophic level. Also the analysis of fish trophic groups showed a similar trend for nitrogen among sites (with the exception of invertivores), and a different signal for carbon towards the higher trophic levels. Moreover, at fish community level, a clear separation were found in carbon isotopic signature and not for nitrogen. Trophic niche and isotopic diversity indices highlighted a clear shift in the isotopic niche towards lower $\delta^{13}C$ in the Low pH. Overall, although fish community were found isotopically altered, no functional and structural changes were present both in terms of trophic diversity and trophic levels. Thanks to this study, we first gave a representation of the isotopic niche of a fish community exposed long-term to a naturally acidified environment, providing valuable insights into the predicted effects of ocean acidification on food web dynamics.

Keywords: ocean acidification, trophic structure, fish assemblage, stable isotopes, shallow CO_2 vent.

3.1 Introduction

Ocean acidification is nowadays considered one of the greater threats for marine life due to the forecasted consequences on ecosystems functioning (Nagelkerken & Connell, 2015, Kroeker et al. 2010, 2013). In this context, primary producers seem to take advantage from this seawater chemical alteration, and evidences from laboratory, mesocosm and *in situ*

experiments suggest that CO_2 can act as a resource for some of them by increasing carbon fixation rates in photosynthetic organisms (Connell et al. 2013, Russell et al. 2013). On the other hand, some organisms are considered as good acid-base regulators (*i.e.* fish), but a series of direct and indirect effects have been found on physiological and behavioral responses (Checkley et al. 2009, Ferrari et al. 2011, Nilsson et al. 2012, Simpson et al. 2011, among the others). However, most studies have been conducted in closed systems and only a few of them (Cattano et al. 2015, Milazzo et al. 2016, Munday et al. 2014, Nagelkerken et al. 2015) have exploited naturally acidified ecosystems (*i.e.* CO_2 vent).

CO_2 vents offer the opportunity to test acidification effects not only at species level, but also on whole community, due to long-term exposure of organisms to high CO_2 / low pH conditions (Fabricius et al. 2011, Hall-Spencer et al. 2008, Kroeker et al. 2011, Vizzini et al. 2013). In this context, scant information is available about ocean acidification consequences on food web interactions, although the primary importance of this issue is recognized (Rossoll et al. 2012). The shift in terms of quality, abundance and composition of the underlying basal resources in food web (*i.e.* plankton, macroalgae and seagrasses) will affect the direct and indirect consumers, with consequences on the structure of the whole food web (Connell & Russell, 2010, Connell et al., 2013). This, in turn, may affect the trophic organization of fish communities through changes at lower trophic levels (Beaugrand et al. 2003a, 2003b). Food web organization is the balance of top-down and bottom-up effects (Pinnager et al. 2000, Power, 1992, Smith et al. 2010) and fish are an important component in this context as they are higher in trophic levels and for their role in top-down control on food web dynamics (*i.e.* direct control on grazers and indirect control on fleshy macroalgae – Bonaviri et al. 2009, Galasso et al. 2015, Guidetti & Sala, 2007). Additionally, fish represent a critical natural resource for worldwide catches (FAO 2010).

Stable isotopic analysis (SIA) has become a common tool in food web ecology to study aspects of trophic structure, since its earliest application (De Niro & Epstein, 1981, Peterson & Fry, 1987). SIA is widely used also to evaluate the consequences of a stressor on the isotopic niche (*i.e.* invasive species - Alomar et al. 2016, Jackson et al. 2012; ecosystem fragmentation - Layman et al. 2007a; impact of anthropogenically derived organic matter - Vizzini & Mazzola, 2004, 2006). SIA have been used in vent areas to test the effects of acidification on organisms exposed long-term to high CO_2 emissions, to study changes in the food web and in the trophic organization of macroinvertebrates (Ricevuto et al. 2015, Vizzini et al. submitted). For instance, a study conducted in the vent located in Ischia Island by using

SIA, showed that high pCO_2 / low pH conditions can have a direct effect on the isotopic signatures of organic matter sources with no dramatic consequences on three herbivorous Polychaeta species, which showed trophic plasticity and did not modify their food habits (Ricevuto et al. 2015). Moreover, Vizzini et al. (submitted) observed that the effect of CO_2 enrichment combines with the resource-effect to drive lower diversity of motile invertebrates with dominance of tolerant species (non-carnivorous consumers) and simplified the food web structure with lower trophic diversity and length.

Here, we tested the hypothesis that, as a consequence of stressful environmental conditions created by the vent, fish assemblage will be negative affected in terms of trophic diversity, with a decrease in the trophic levels and an overall simplification of the isotopic niche. To assess this hypothesis, we compared the trophic organization and the isotopic niche of a fish assemblage living in a low pH site (pH = 7.8) with two assemblages living in control areas (pH = 8.2). In particular, we analyzed and compared among sampling sites:

1. the isotopic signature ($\delta^{13}C$ and $\delta^{15}N$) and the nutritional quality (C:N ratio) of basal food sources (sediment, zooplankton, macroalgae and seagrasses);
2. the isotopic signature ($\delta^{13}C$ and $\delta^{15}N$) and the trophic level of 15 fish species, both at species and trophic group levels;
3. the response of fish community in terms of trophic niche features.

3.2 Materials and Methods

3.2.1 Study area

The shallow hydrothermal system of Vulcano Island is one of the most active sites in the Aeolian Archipelago (24 km off the NE coast of Sicily, Italy). Gas composition of the main venting area located in Levante Bay, on the eastern side of the Island, is dominated by CO_2 (97-99% vol.), which generates a pH gradient (from 5.5. to 8.1) along the north shore of the bay (Boatta et al. 2013, Capaccioni et al. 2011, Italiano et al. 2009). Emissions include also small quantities of H_2S (<2.2%), which rapidly decrease with distance from the vent (Boatta et al. 2013). Water composition in terms of major elements (Cl, SO_4, Na, K, Ca and Mg) is close to that of Mediterranean surface waters, while greater variability is recorded for dissolved Fe concentrations, which showed maximum values close to the vents (Boatta et al.

2013). The area is characterized by acidic and reducing conditions, causing changes in major and trace element geochemical fluxes at the sediment-seawater interface (Vizzini et al. 2013). Seawater carbonate chemistry parameters in the Levante Bay range between 2.78 and 3.17 mmol/kg for the total alkalinity and 0.02 and 3.64 for the aragonite saturation state (for details see Boatta et al. 2013).

The following three sites were chosen: the low pH site (hereafter "Low pH", mean pH = 7.80 ±0.09) about 250 m far from the primary CO_2 vent in Levante Bay, a control site (hereafter "Ctrl 1", mean pH = 8.19 ±0.03) 500 m far from the primary vent, and another control site in Lipari Island (hereafter "Ctrl 2", mean pH = 8.22 ±0.02) about 6.5 km from the first two sites (**Fig. 3.1**). Controls were chosen to be similar in terms of orientation (South-East), depths (2-5 m) and vegetal coverage (*Cymodocea nodosa* meadow) to the Low pH site.

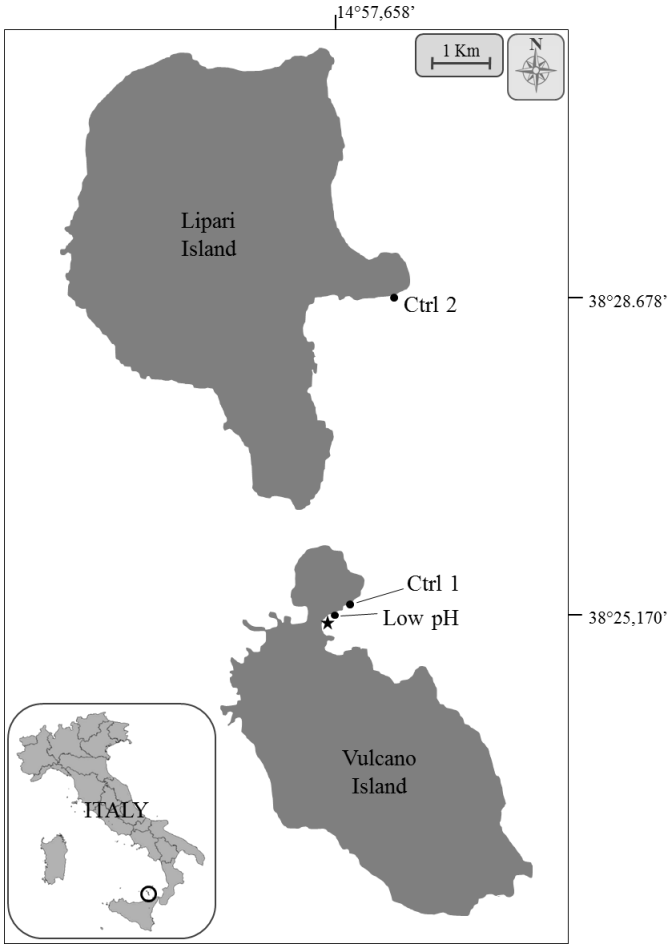

Fig. 3.1 *- Map of the study area showing the sampling sites in Vulcano (Low pH and Ctrl 1) and Lipari Islands (Ctrl 2). The primary vent is indicated by the star.*

3.2.2 Sample collection and laboratory analysis

Sampling was carried out from September to November 2014. Fish were collected by using small trammel nets, fish traps, hook and line. Surface sediment was also collected using a PVC hand-corer (Ø 4 cm, 0-1 cm, five replicates per site); zooplankton was sampled by towing a net (mesh size: 200 μm) for approximately 30 min (three replicates for Lipari and three replicates for Vulcano Island); macroalgae and seagrasses (three replicates per site for each species) were collected by hand from scuba divers. All the samples were stored at -20 °C and transferred to the laboratory until chemical analyses were performed.

In the laboratory, macroalgae [*Cystoseira spp*., *Halopteris scoparia*, and *Dictyota dichotoma*] and the seagrass *C. nodosa* were rinsed with distilled water and epiphytes were removed by surface scraping. Fish were identified at species level and classified into trophic groups following the classification by Guidetti & Sala (2007) and FishBase database (Froese & Pauly, 2016). The trophic groups considered were: planktivores, herbivores, invertivores and small piscivores. Total length was measured to the nearest 0.1 mm and then dorsal muscle was processed for analysis (**Tab. 3.1**).

All samples (both abiotic and biotic) were freeze-dried (ALPHA 1-4 LDplus, Martin-Christ) and ground to a fine powder using a ball mill (MM 200 Retsch). Stable nitrogen and carbon isotopes was analysed in all samples through an Isotope Ratio Mass Spectrometer (Thermo Scientific Delta Plus XP) connected to an Elemental Analyser (Thermo Scientific Flash EA 1112). Carbon and nitrogen isotopic ratios were expressed in conventional δ unit notation as parts per mil deviations from the international standards, Vienna Pee Dee Belemnite and atmospheric nitrogen (N_2), following the formula:

$$\delta X = [(R_{sample}/R_{standard}) - 1] \times 10^3$$

where X is ^{13}C or ^{15}N and R is the relative $^{13}C/^{12}C$ or $^{15}N/^{14}N$ ratio. Analytical precision based on the standard deviation of replicates of internal standards was 0.2‰.

3.2.3 Data analysis

Univariate analysis (one-way ANOVA) was carried out to evaluate differences among sampling sites in terms of $\delta^{15}N$, $\delta^{13}C$ and C:N ratio (used as index of nutritional quality for

basal resources) of sediment, macroalgae, seagrasses and fish (at species level and trophic groups). Cochran's test and Shapiro-Wilk's test were used to check for homogeneity of variances and normality, respectively. Where significant differences were present, Tukey's *post hoc* test was used. Macroalgae data were analysed without species distinction, due to the low number of species in common in the three sites. For the zooplankton the t-test analysis was used to test the differences between Lipari and Vulcano area. Univariate analysis was carried out by using STATISTICA software package (StatSoft, 2011).

To estimate the trophic level of fish (hereafter "TL"), the following equation was used:

$$TLf = [(\delta^{15}Nf - \delta^{15}Nref) / f] + TLref$$

where $\delta^{15}N_f$, $\delta^{15}N_{ref}$ and TL_{ref} are the stable nitrogen isotope signature of the fish, the stable nitrogen isotope signature species and the trophic level of the baseline, respectively; while f is the expected $\delta^{15}N$ isotopic fractionation per TL (3.4 according to Post, 2002). In this case, zooplankton was used as baseline, and we used the mean of the two areas of Lipari and Vulcano due the lack of difference in terms of nitrogen isotopic values of zooplankton among the two areas.

To test differences in isotopic signature at fish community level, multivariate analyses were performed considering the fish species common in the three sampling sites (*Chromis chromis, Coris julis, Diplodus vulgaris, Gobius bucchichi, Oblada melanura, Sarpa salpa, Serranus scrib*a, *Symphodus roissali, Symphodus tinca, Scorpaena porcus* and *Thalassoma pavo*). Moreover, *Diplodus annularis* was taken out from the analysis due to the low number of replicates. Fish species sampled only in two out of the three sites (*Labrus viridis, Symphodus ocellatus* and *Symphodus mediterraneus*) were took out from the multivariate analysis.

$\delta^{13}C$ and $\delta^{15}N$ data were normalized and resembled using Euclidean distance similarity matrices. Differences between sites were tested at a multivariate level by using the permutational multivariate analysis of variance (PERMANOVA). Differences in the trophic structure of fish assemblages were graphically represented in a two-dimensional ordination plot by non-metric multidimensional scaling (n-MDS). Analyses were carried out by using PRIMER 6 with PERMANOVA +add-on software package (Anderson et al. 2008).

Moreover, in order to characterize the isotopic niche at each sampling site, stable isotope signatures were used to estimate the community-wide metrics (Layman et al. 2007b), by

using the SIBER package (Stable Isotope Bayesian Ellipses; Jackson et al. 2011) through the statistical R Programming Environment (v.3.3.1; R Studio Team, 2015). The following metrics were used to describe the trophic structure in terms of trophic diversity and redudancy: a) $\delta^{15}N$ Range (NR) is the difference between the most enriched and most depleted $\delta^{15}N$ values and provides information on the trophic length; b) $\delta^{13}C$ Range (CR) is the difference between the most enriched and the most depleted $\delta^{13}C$ values and estimates the diversity of basal resources exploited; c) Total Area (TA) is the convex hull that encompasses the data points in the isotopic bi-plot space and indicates the width of the trophic niche; d) mean Distance to Centroid (CD) is the average Euclidean distance of each species to the centroid $\delta^{13}C$-$\delta^{15}N$ and represents the trophic diversity and species spacing within the isotopic space; e) mean Nearest Neighbour Distance (NND) is expressed as the Euclidean distance of each species to the nearest neighbour and measures species density and packing within the community, given by the proximity of each species to another within the same isotopic space (trophic redundancy); and f) Standard Deviation of the Nearest Neighbour Distance (SDNND) provides information on the evenness of species packing. In addition, according to Jackson et al. (2011), corrected standard ellipse area (SEAc) was estimated by Bayesian inference, to gain a more accurate measure of the isotopic niche and avoid any bias induced by sampling size.

3.3 Results

Overall, sediment, primary producers (macroalgae, seagrasses) and fish presented ^{13}C- and ^{15}N-depleted signatures in the Low pH site compared to the two controls (**Fig. 3.2**). Ranges of $\delta^{13}C$ were between -22.9 ‰ and -13.5 ‰ (mean ± SD: -18.8 ± 2.1 ‰) in the Low pH site, -21.2 ‰ and -8.8 ‰ (-16.9 ± 2.9 ‰) in the Ctrl 1, and -21.7 ‰ and -9.0 ‰ (-16.5 ± 2.8 ‰) in the Ctrl 2, while those of $\delta^{15}N$ were between -0.9 ‰ and 8.1 ‰ (5.4 ± 2.9 ‰) in the Low pH site, 1.3 ‰ and 9.1 ‰ (6.1 ± 2.5 ‰) in the Ctrl 1, 2.0 ‰ and 7.9 ‰ (6.1 ± 2.1 ‰) in the Ctrl 2.

In particular, the most depleted isotopic values were recorded for sediment in every site, with among-sites significant differences only for $\delta^{15}N$ (ANOVA: $F_{2, 12} = 35.177$, $p < 0.001$; Low pH<Ctrl 1=Ctrl 2) and C:N ratio ($F_{2, 12} = 13.151$, $p < 0.001$; Low pH<Ctrl 1=Ctrl 2; mean ± SD: 5.8 ± 1.1 Low pH, 10.2 ± 2.3 Ctrl 1, 10.6 ± 1.3 Ctrl 2). Macroalgae showed the same

trend, with lower values of $\delta^{15}N$ ($F_{2, 24} = 5.96$, $p < 0.01$) and lower C:N ratio ($F_{2, 24} = 10.73$, $p < 0.01$) in the Low pH compared to the controls, while no differences were found for $\delta^{13}C$ values ($F_{2, 24} = 2.70$, $p > 0.05$). On the other hand, the seagrass *C. nodosa* showed lower values for $\delta^{13}C$ in the Low pH than in the two controls ($F_{2, 6} = 52.24$, $p < 0.001$), while $\delta^{15}N$ was higher in Ctrl 2 than in the other sites ($F_{2, 6} = 14.29$, $p < 0.01$) and no differences were found in C:N ratio. The only exception was presented by the zooplankton, that did not show differences in $\delta^{15}N$ values and C:N ratio, while more depleted values of $\delta^{13}C$ were found in Lipari compared to the Vulcano area (df = 4, t-value = -2.82, $p < 0.05$).

A total of 15 fish species belonging to six families (Gobiidae, Labridae, Pomacentridae, Scorpaenidae, Serranidae, Sparidae) was sampled and analysed to describe the food web structure and the trophic organization of the fish community in each site (for details see **Tab. 3.1**). Three out of fifteen species were sampled only in two sites (*L. viridis* and *S. mediterraneus* in the Low pH site and Ctrl 2; *S. ocellatus* in the Low pH site and Ctrl 1), while *Diplodus annularis* was taken out from the analysis due to the low number of specimens sampled.

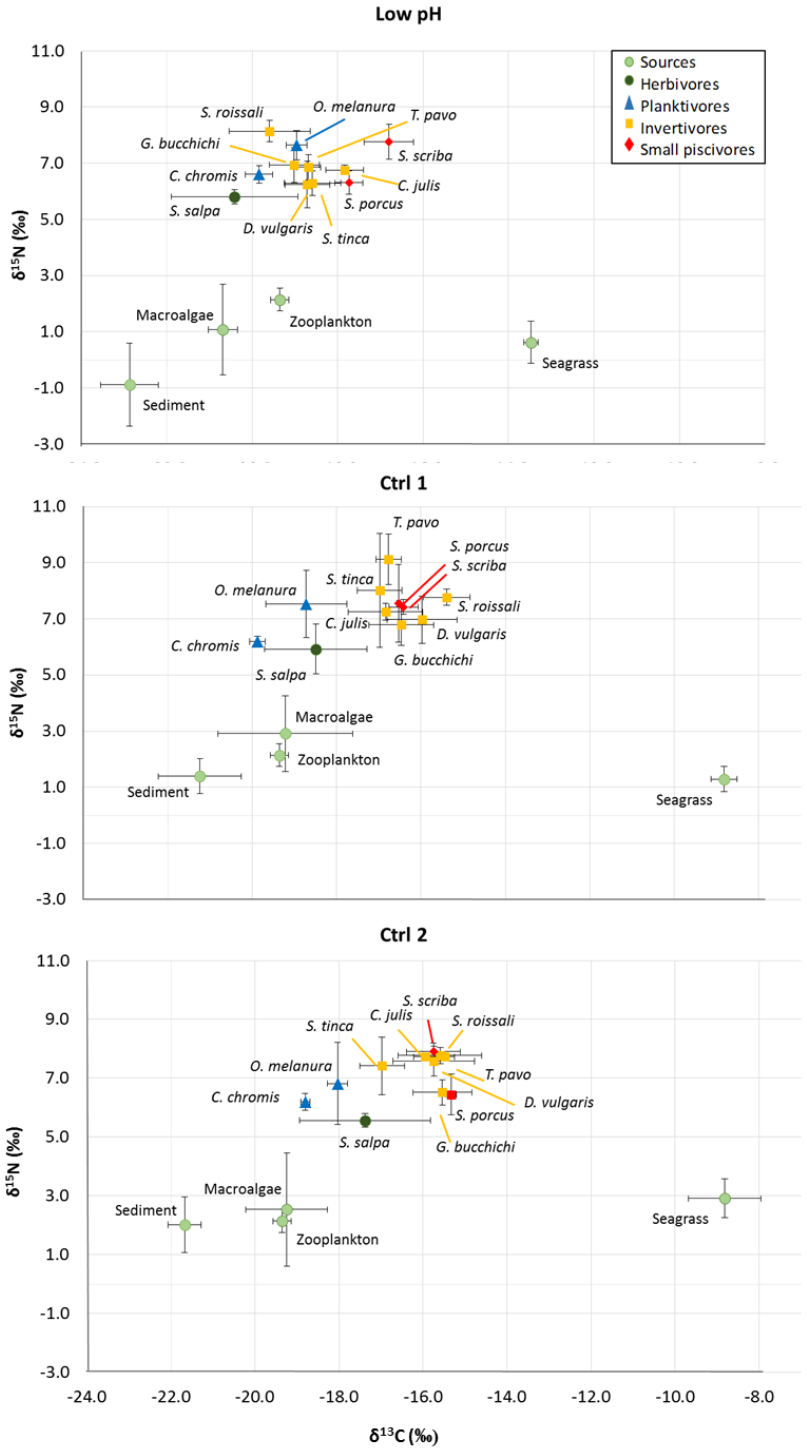

Fig. 3.2 *- Bi-plot of $\delta^{13}C$ (‰) vs $\delta^{15}N$ (‰) of sediment, zooplankton, macroalgae, seagrasses and fish common in the three sampling sites (Low pH, Ctrl 1 and Ctrl 2). Fish are grouped into trophic groups: herbivores (S. salpa), planktivores (C. chromis and O. melanura), invertivores (C. julis, D. vulgaris, G. bucchichi, S. roissali, S. tinca and T. pavo), and small piscivores (S. scriba and S. porcus). Each point represents the mean of single species and the relative standard deviation is reported.*

Table 3.1 *– Family, trophic groups, total length - TL in cm: mean (SD) - and number of individuals (N) for fish species analysed in each sampling site (Low pH, Ctrl 1 and Ctrl 2).*

			Low pH		Ctrl 1		Ctrl 2	
Family	**Species**	**Trophic group**	**TL**	**N**	**TL**	**N**	**TL**	**N**
Pomacentridae	*Chromis chromis*	Planktivore	10.2 (0.5)	5	9.1 (0.8)	5	8.5 (0.8)	5
Gobiidae	*Gobius bucchichi*	Invertivore	7.8 (0.3)	5	8.5 (1.6)	5	7.9 (1.3)	4
Sparidae	*Diplodus annularis*	Invertivore	13.6 (1.5)	4	18.0 (-)	1	13.1 (-)	1
	Diplodus vulgaris	Invertivore	8.6 (2.4)	4	9.0 (1.0)	5	8.9 (0.1)	5
	Oblada melanura	Planktivore	10.6 (0.7)	3	10.0 (2.8)	3	8.1 (1.7)	4
	Sarpa salpa	Herbivore	18.5 (1.4)	5	22.8 (1.0)	5	25.1 (2.2)	4
Labridae	*Coris julis*	Invertivore	12.3 (1.8)	5	13.1 (2.1)	5	11.7 (2.2)	5
	Thalassoma pavo	Invertivore	11.3 (0.1)	2	10.7 (1.4)	5	11.6 (0.9)	5
	Labrus viridis	Invertivore	11.6 (1.0)	3	-	0	10.5 (1.2)	3
	Symphodus ocellatus	Invertivore	6.4 (0.1)	5	5.9 (0.36)	5	-	0
	Symphodus mediterraneus	Invertivore	6.0 (1.4)	4	-	0	9.4 (0.07)	3
	Symphodus roissali	Invertivore	8.9 (1.0)	5	8.5 (1.2)	4	8.8 (0.7)	5
	Symphodus tinca	Invertivore	14.4 (1.3)	5	11.4 (1.2)	4	13.7 (3.1)	5
Scorpaenidae	*Scorpaena porcus*	Small piscivore	11.0 (2.1)	3	13.7 (1.8)	3	13.0 (2.8)	5
Serranidae	*Serranus scriba*	Small piscivore	14.2 (1.6)	5	9.8 (3.7)	5	13.7 (2.5)	5

Univariate PERMANOVA revealed, overall, a general trend for fish $\delta^{13}C$ with more depleted values in the Low pH site than controls. Eleven out of fourteen species showed among-sites differences for $\delta^{13}C$, with the exception of *O. melanura*, *L. viridis* and *S. mediterraneus*. Overall, these species showed isotopic carbon values lower at the Low pH site than at least in one of the two controls. The only exception was *C. chromis* that showed a carbon isotopic signature lower in Ctrl 2 and no differences between Low pH and Ctrl 1 (**Tab. 3.2**).

Table 3.2 *- PERMANOVA results testing for differences in fish species carbon isotopic signature among sites. Probability levels: n.s. = not significant; * p<0.05; ** p<0.01.*

$\delta^{13}C$		***S. salpa***				***C. chromis***				***O. melanura***			
	df	MS	Pseudo - F	p	*df*	MS	Pseudo - F	p	*df*	MS	Pseudo - F	p
Site	2	11.74	6.52	*	2	1.85	21.3	**	2	0.68	2.97	n.s.
Residual	11	0.06			12	0.09			5	0.23		
post hoc		Low pH = Ctrl 1 < Ctrl 2				Ctrl 2 < Low pH = Ctrl 1						
		D. vulgaris				***G. bucchichi***				***C. julis***		
	df	MS	Pseudo - F	p	*df*	MS	Pseudo - F	p	*df*	MS	Pseudo - F	p
Site	2	14.94	23.13	**	2	15.11	32.05	**	2	4.45	12.76	**
Residual	12	0.63			11	0.47			12	0.35		
post hoc		Low pH < Ctrl 1 = Ctrl 2				Low pH < Ctrl 1 = Ctrl 2				Low pH = Ctrl 2< Ctrl 1		
		L. viridis				***S. mediterraneus***				***S. ocellatus***		
	df	MS	Pseudo - F	p	*df*	MS	Pseudo - F	p	*df*	MS	Pseudo - F	p
Site	1	2.41	21.42	n.s.	1	12.77	71.62	n.s.	1	16.32	61.31	*
Residual	3	0.11			4	0.18			8	0.27		
post hoc		no differences				no differences				Low pH < Ctrl 1		
		S. roissali				***S. tinca***				***T. pavo***		
	df	MS	Pseudo - F	p	*df*	MS	Pseudo - F	p	*df*	MS	Pseudo - F	p
Site	2	22.73	31.35	**	2	14.08	32.73	**	2	7.52	100.03	**
Residual	12	0.72			12	0.43			9	0.07		
post hoc		Low pH < Ctrl 1 < Ctrl 2				Low pH < Ctrl 1 < Ctrl 2				Low pH = Ctrl 1< Ctrl 2		
		S. porcus				***S. scriba***						
	df	MS	Pseudo - F	p	*df*	MS	Pseudo - F	p				
Site	2	5.51	136.87	**	2	1.14	4.91	*				
Residual	7	0.04			12	0.29						
post hoc		Low pH < Ctrl 1 < Ctrl 2				Low pH = Ctrl 1 < Ctrl 2						

Moreover, a few species showed differences in terms of $\delta^{15}N$ and no general trend was found among sites. For instance, *L. viridis* (pseudo-$F_{1, 3}$ = 11.62, p(MC) < 0.05) and *C. julis* (pseudo-$F_{2, 12}$ = 28.04, p(MC) < 0.01) showed higher $\delta^{15}N$ values in Ctrl 2, while *T. pavo* (pseudo-$F_{2, 9}$ = 8.63, p(MC) < 0.05) exhibited higher values in Ctrl 1. On the other hand, *C.*

chromis showed higher $\delta^{15}N$ in the Low pH than in controls (pseudo-$F_{2, 12} = 4.07$, p(MC) < 0.05).

Based on the method adopted for the TL calculation, a few differences were found among sites as well as for $\delta^{15}N$. *C. julis* and *L. viridis* showed lower TL values in Low pH than controls, while *T. pavo* showed higher TL in Ctrl 1 and *C. chromis* in Low pH site (**Fig. 3.3**). Overall, no general trend was found among the sites, and although afferent to different trophic groups, almost all the species analysed showed a similar trophic level ranging between 3.1 and 3.8 in Low pH, 3.1 and 4.0 in Ctrl 1 and 3.0 and 3.8 in Ctrl 2. As an example, the only herbivorous fish analysed (*S. salpa*) showed similar values as invertivores in every site, while small piscivores (*S. scriba* and *S. porcus*) showed similar trophic levels as those invertivores.

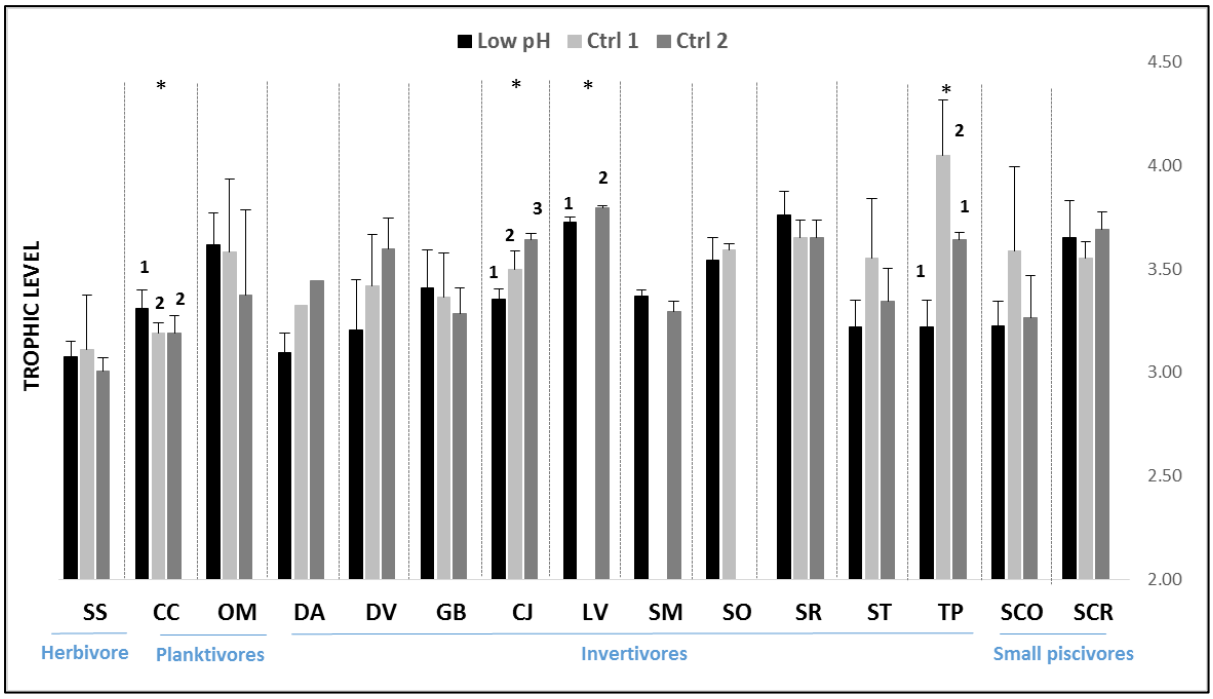

Fig. 3.3 *- Mean trophic level of fish sampled in the three sites (Low pH, Ctrl 1 and Ctrl 2). Fish are classified in trophic groups. Fish label: SS, Sarpa salpa; CC, Chromis chromis; OM, Oblada melanura; DA, Diplodus annularis; DV, Diplodus vulgaris; GB, Gobius bucchichi; CJ, Coris julis; LV, Labrus viridis; SM, S. mediterraneus; SO, Symphodus ocellatus; SR, S. roissali; ST, S. tinca; TP, Thalassoma pavo; SCO, Scorpaena porcus; SCR, Serranus scriba. Numbers indicate significant differences amnong sites.*

Also, a clear ^{13}C- depletion was found for each trophic group with the exception of planktivores, that showed similar mean values in Low pH site and Ctrl 1. With regard to $\delta^{15}N$, instead, the Low pH site did not show differences for each trophic groups among the three sites with the exception of invertivores that were more depleted in the Low pH than in both controls (**Fig. 3.4** and **Tab. 3.3**).

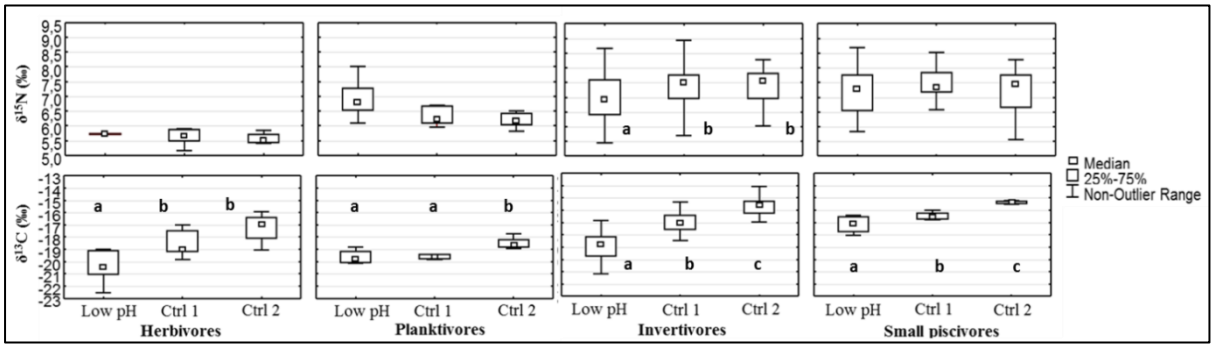

Fig. 3.4 *- Mean of $\delta^{15}N$ and $\delta^{13}C$ (‰) in fish trophic groups of the three sampling sites (Low pH, Ctrl 1 and Ctrl 2). Letters indicate significant differences among sampling sites.*

Table 3.3 *- PERMANOVA results testing for differences in fish trophic groups for carbon and nitrogen isotopic signature among sites. Probability levels: n.s. = not significant; * p<0.05; ** p<0.01; *** p<0.001.*

$\delta^{13}C$		***Herbivores***				***Planktivores***		
	df	MS	Pseudo - F	p	*df*	MS	Pseudo - F	p
Site	2	11.74	6.52	*	2	11.74	10.26	**
Residual	11	1.8			20	1.8		
$\delta^{15}N$								
	df	MS	Pseudo - F	p	*df*	MS	Pseudo - F	p
Site	2	0.14	0.44	n.s.	2	0.14	0.57	n.s.
Residual	11	0.33			20	0.67		
$\delta^{13}C$		***Invertivores***				***Small piscivores***		
	df	MS	Pseudo - F	p	*df*	MS	Pseudo - F	p
Site	2	11.74	116.93	**	2	11.74	22.11	**
Residual	111	1.8			22	1.8		
$\delta^{15}N$								
	df	MS	Pseudo - F	p	*df*	MS	Pseudo - F	p
Site	2	0.14	5.68	**	2	0.14	0.26	n.s.
Residual	111	0.66			22	0.71		

In addition, at fish community level multivariate analysis showed differences among all the sites for $\delta^{13}C$ (PERMANOVA: pseudo-$F_{2, 12} = 20.29$, p(MC) = 0.001), while the Low pH site was different from both controls for $\delta^{15}N$ (pseudo-$F_{2, 12} = 3.53$, p(MC) = 0.001). Accordingly, n-MDS ordination showed a clear separation between sites for $\delta^{13}C$ (**Fig. 3.5a**), that was less evident for $\delta^{15}N$ (**Fig. 3.5b**).

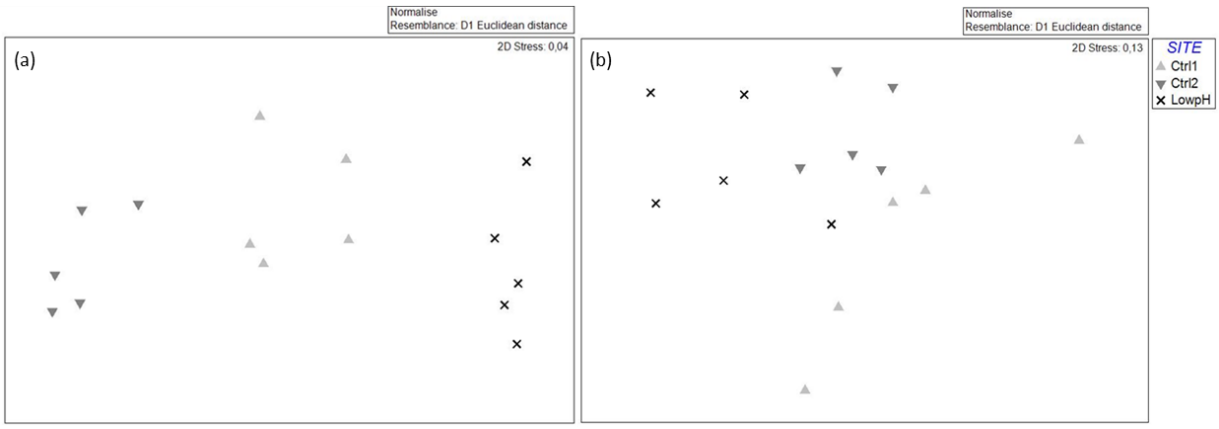

***Fig. 3.5** - Non-metric multidimensional scaling (n-MDS) for differences in fish $\delta^{13}C$ (‰ - a) and $\delta^{15}N$ (‰ - b) values between sampling sites.*

A general look at the species distribution of the fish community within the isotopic space revealed a clear shift in the isotopic niche towards lower $\delta^{13}C$ in the Low pH site compared to controls, while lower differences were found in $\delta^{15}N$ (**Fig. 3.6**). Additionally, niche width was larger in Ctrl 1 than in Low pH and Ctrl 2, as reflected by NR and CR metrics. Also TA and SEAc changed in width, position and shape, showing a separation between sites and above all among the Low pH and Ctrl 2 in terms of position, while similar metrics were found between these two sites. Community CD, NND and SDNND, providing measure of trophic diversity and species packing, did not show noticeable variations among sampling sites, with the exception of SDNND that was lower in the Low pH.

	NR	CR	TA	SEAC	CD	NND	SDNND	TL
Low pH	2.33	3.59	5.96	3.21	1.20	0.54	0.25	3.08 – 3.76
Ctrl 1	4.06	3.90	6.97	6.22	1.33	0.62	0.43	3.11 – 4.05
Ctrl 2	2.70	3.57	5.23	3.26	1.21	0.50	0.44	3.01 – 3.80

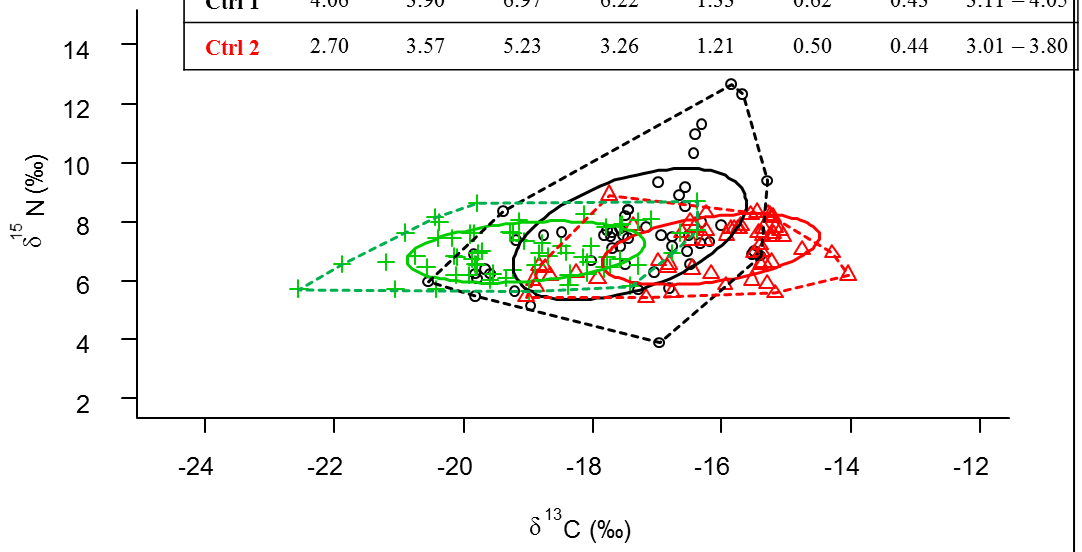

Fig. 3.6 *- Fish distribution in the isotopic space at Low pH (green), Ctrl 1 (black) and Ctrl 2 (red) sites. Solid lines enclose the standard ellipse area (SEA), dotted lines encompass convex hull areas (corresponding to Layman's Total Area - TA) of fish communities. In the table, results of Layman's metrics: nitrogen range (NR), carbon range (CR), mean distance to centroid (CD), mean nearest neighbour distance (NND), and standard deviation of the nearest neighbour distance (SDNND). The range of trophic level (TL) are reported.*

3.4. Discussion

We tested the hypothesis that high CO_2 / low pH conditions affect negatively the trophic structure of a fish assemblage living in a naturally acidified environment. Overall, we found a greater difference in carbon than nitrogen isotopic signature of fish among sites (both at species, trophic group and community level), but this isotopic alteration was not reflected in an alteration of fish assemblage trophic structure. Additionally, although a few exceptions, slight differences were found in the trophic levels. The total extent of trophic niche, described estimating the total area occupied by species in the isotope space and measuring their overall density, did not show dramatic changes among sites.

This shift in carbon isotopic signature of fish community is related to the use of ^{13}C-depleted basal food sources. Indeed, macroalgae and seagrasses exploit volcanic isotopic depleted

nutrients and increase photosynthetic enzyme discrimination against the heavy carbon isotope as reported in previous studies under high CO_2 conditions (Apostolaky et al. 2014, Vizzini et al. 2010). Moreover, our findings highlight a change in the basal resources and confirm the enhanced nutritional quality (in terms of C:N ratio) in the Low pH site compared to the controls, as found by Vizzini et al. (submitted). Probably *S. salpa* is attracted by the more palatability of macrophytes (Apostolaky et al. 2014, Arnold et al. 2012), explaining the higher abundance and frequency of occurrence found in the Low pH (see Chapter 2), that is the main herbivorous fish in the Mediterranean Sea. Noticeable is the trophic level measured for this species in the three sampling sites, that is comparable to that found in higher trophic level consumers (invertivorous and small piscivorous fish), suggesting an omnivorous diet instead of a strictly herbivorous one (Whitehead et al. 1986). On the other hand, species like *S. roissali* and *S. porcus* showed the same trophic level among sites. The comparable TL found among different trophic groups may be explained considering that specimes analysed had small and similar sizes (with the exception of *S. salpa*) and a quite homogeneous diet based on small benthic invertebrates (Labropoulou et al. 1997, Stergiou & Fourtouni, 1991). This may have resulted in similar positions within the trophic jerarchy, while a higher trophic dissimilarity cannot be ruled out in advanced life stages due to ontogenetic diet variation.

Overall, almost all the fish analyzed belonged to a trophic level ranging between 3.0 and 4.0 in the three sites, with a mean trophic level that fell into the range of omnivorous with a preference of animal material (TL range: 2.9 – 3.7 as defined by Stergiou & Karpouzi, 2002) and TL was slightly lower in the Low pH site for a few species (*C. julis*, *T. pavo* and *L. viridis*), with the only exception of *C. chromis* (higher in Low pH than in Ctrl 1 and Ctrl 2. As showed by Vizzini et al. (submitted), most invertebrates associated to seagrasses in the low pH area are herbivores and detritivores, with a predominance of Amphipoda and Polychaeta. It is feasible that the high abundance of these groups have caused a trophic shift in fish and a decreasing in TL (*i.e.* the invertivores like *C. julis*, *T. pavo* and *L. viridis*), due to their plasticity in feeding habits strategy to respond to site-specific changes in food availability (plasticity was found also by Ricevuto et al. 2015 for Polychaeta *spp*. in Ischia vent area).

Although the slight difference in the fish trophic level has been found for a few species, overall fish community at the Low pH site showed a packing of species as showed by NR and SDNND values of Layman's metrics, while controls appear more extended. Slitghly lower trophic levels, the narrower nitrogen range, higher species packing might change food web

features possibly caused by a massive consumption by invertivorous fish of hervivorous / detritivorous invertebrates, dominating in the Low pH area. Moreover, Layman's metrics were overall lower in the Low pH and Ctrl 2, while Ctrl 1 showed higher values suggesting a greater degree of trophic diversity. Fish exploit the vent area for feeding, but their mobility allows them to 'escape' to close sites when conditions are more stressful. In this context, Ctrl 1 (the nearest control) could represent a "buffer zone", were a few meter away there is a recovery of normal environmental conditions. Although at community level species richness was lower in the Low pH than controls (Chapter 2), some species show a greater tolerance to these conditions than other species. As an example, *G. bucchichi* is a highly sedentary species that showed a 3-fold higher abundance in low pH than control sites (Nagelkerken et al. 2015).

The effect of high CO_2 / low pH conditions did not cause dramatic alteration on fish trophic organization, but rather some variations were found at species level. This study confirms the importance to use natural acidified environments to test hypothesis about ocean acidification on food webs and trophic structure of whole communities, challenging in laboratory experiments. However, ocean acidification is unlikely to act alone, but in concert with other stressors (*i.e.* nutrient pollution, warming, invasive species, over-fishing). Differences in the basal resources of food web, together with local threats, indeed, could alter the native trophic structure with consequences on ecosystem function and stability (Alomar et al. 2016, Jackson et al. 2012). Moreover, ocean acidification can cause a deterioration of the nutritional quality of basal sources with consequences on the food chain relationships (*i.e.* diatom-copepod – Rossoll et al. 2012), and reducing synthesis of omega-3 polyunsaturated fatty acids in phytoplankton, which are essential nutrients for normal human growth and development and have many beneficial effects on human health (Kang et al. 2011).

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CHAPTER 4: Mercury accumulation in fish exposed long-term to high CO_2 emissions in a naturally acidified environment

Abstract: The problem known as ocean acidification is not an isolated threat for marine organisms, as it often acts in synergism with other problems (*i.e.* heavy metals pollution). Here, exploiting the leakage of trace elements from the shallow CO_2 vent of Vulcano Island (Aeolian Archipelago, Italy), we evaluated the bioaccumulation of total mercury (THg) in 14 coastal fish species typical of a Mediterranean rocky reef assemblage, dwelling in a *Cymodocea nodosa* meadow. In particular, we compared fish sampled in a high CO_2 / low pH site (pH = 7.8), against fish living in two control areas (pH = 8.2). Moreover, levels of THg were measured in sediment, macroalgae and seagrass in the same sampling sites. In general, we found higher mercury concentrations in the low pH site in all the matrices (abiotic and biotic) analysed, and significant differences were highlighted among sites both at the fish species level and at the community one. Moreover, biomagnification power and trophic magnification factor (TFM) were calculated for each site through the relationships between log[Hg] and $\delta^{15}N$ (used as a proxy of trophic level). Although trophic transfer values were found positive in each sites, no differences in the biomagnification rates were found among sites. This study highlights the importance to evaluate synergetic effect of ocean acidification with other stressors and the need to take care in the choice of sites to test ecological hypothesis in field experiments.

Keywords: Mediterranean fish assemblage, shallow CO_2 vent, trophic transfer, mercury bioaccumulation.

4.1 Introduction

Among climate changes, ocean acidification is one of the major threat forecasted for marine biota and ecosystem functioning (Gaylord et al. 2015, IPCC, 2014, Kroeker et al. 2013). However, acidification is not an isolated threat as it acts in synergism with other stressors (*i.e.* warming, invasive species, heavy metals pollution, etc.). For instance, the decreasing of

ocean pH, the rising of anthropogenic pollutants (*i.e.* heavy metals) and the consequent interaction with marine organisms has been studied in a number of recent works (Basallote et al. 2014, Lacoue-Labarthe et al. 2009, 2011, Roberts et al. 2013, Zeng et al. 2015 for a review). Heavy metals, such as mercury and lead, are the most common types of coastal contaminants detected in relatively high concentrations in waters and sediments of many coastal and estuarine systems (Doney et al. 2009). Trace elements have seawater speciation schemes that are strongly influenced by pH (Byrne et al. 2002), and as pH decrease, elements solubility increase (*i.e.* a decrease of pH from 8.1 to 7.4 increases the solubility of iron of 40% - Millero et al. 2009). The form or speciation of a metal in natural waters can change its properties, such as the solubility and the strength to form complex with inorganic and organic ligands. As a consequence, elements become differently bioavailable for organisms. For instance, copper in the ionic form is toxic to phytoplankton while it is not dangerous complexed to organic ligands (Millero & Pierrot, 2002).

Among trace elements, mercury is one of the most harmful and toxic at low concentration, and it has both anthropogenic and natural sources (Valavanidis & Vlachogianni, 2010). It can accumulate in the inorganic form in the sediments, where is subject to biological methylation (Ullrich et al. 2001). Methylmercury, the most toxic form of Hg, is available for organisms and is highly subject to biomagnification along food web (Campbell et al. 2005, Nfon et al. 2009, Power et al. 2002). Indeed, diet is the main way to accumulate Hg and it is well known that fish food seems to constitute the main route of heavy metals uptake for humans, producing a wide range of health risks (Brambilla et al. 2013, Castro-González & Méndez-Armenta, 2008). For this reason, numerous recent studies have been conducted in anthropogenic polluted areas to evaluate the risk for humans consumption (Bonsignore et al. 2013, Sprovieri et al. 2011). In addition, several studies have shown that the use of $\delta^{15}N$ can be useful to trace bio-contaminant transfer in food webs (Cabana & Rasmussen, 1994, Nfon et al. 2009, Power et al. 2002, Vizzini et al. 2013b).

Volcanic vents (both deep and shallow) are characterized by the leakage of major and trace elements together with gases (*i.e.* CO_2, H_2S, H_2, CH_4). Moreover, the waters surrounding the vent area are acidic and reducing (Dando et al. 1999, Tarasov et al. 2005), and these particular conditions change the solubility, the speciation and the bioavailability of trace elements in sediments close to the vent (Kadar et al. 2007, Roberts et al. 2013). Previous studies on organisms exposed to high CO_2 emissions in deep vents showed that these environments can constitute a main route for intake of trace metals for a range of organisms,

from krill to fish species (Kadar et al. 2005a, 2005b, 2007, Martins et al. 2006, Price & Pichler, 2005, Ruelas-Inzunza et al. 2003, Tovar-Sanchez et al. 2009). A recent study in the shallow CO_2 vent located in Levante Bay (Vulcano Island, Aeolian Archipelago), conducted on sediments and seagrass trace metals levels, concluded that the bay is affected by a low contamination of trace elements in the sediments with potential adverse biological impacts (Vizzini et al. 2013a). Potentially harmful trace elements in sediments, that are more soluble and labile in the low pH area, are sequestered mainly by primary producers with controversial effects. For example, phytoplankton can be negatively affected by the higher solubility of free ionic copper and positively influenced by the higher availability of iron (Millero et al. 2009).

CO_2 vents represent the opportunity to study, in a natural laboratory, the synergetic effect between ocean acidification and toxic elements, and to investigate the possible consequences on whole communities. In this context, we hypothesized that the fish community exposed long-term to volcanic emissions that generate particular conditions in terms of pH, altered potential redox and emissions of trace elements, is negatively affected by mercury accumulation, with consequences on biomagnification rate along the higher trophic levels. In particular, to test our hypothesis, we measured and compared in an area interested by volcanic emissions (low pH site) against two controls:

- the levels of THg in sediment and primary producers (macroalgae and seagrass);
- the levels of THg in fish both at species and at community level;
- the mercury biomagnification along the fish trophic levels by using the trophic magnification factor (TMF).

4.2 Materials and Methods

4.2.1 Study area and experimental design

The Aeolian Archipelago (Southern Tyrrhenian Sea, North-Eastern Sicily) is one of the most active volcanic area in the Mediterranean Sea, characterized by several submarine seeps that have been extensively studied (Boatta et al. 2013, Capaccioni et al. 2011, Italiano et al. 2009). In the last years, a growing interest is born towards the CO_2 vent of Vulcano Island (located in Levante Bay), used as a natural laboratory to test the effects of ocean acidification

on marine biota (Apostolaki et al. 2014, Arnold et al. 2012, Calosi et al. 2013, among others). The area is characterized by acidic and reducing conditions, causing changes in major and trace element geochemical fluxes at the sediment-seawater interface (Vizzini et al. 2013a). Water composition in terms of the major elements (Na, K, Ca and Mg) is close to that of Mediterranean surface waters, while greater variability is recorded for dissolved Fe concentrations and other trace elements, which showed maximum values close to the vents (Boatta et al. 2013, Horwitz et al. 2014, Kadar et al. 2012).

Three sampling sites were selected to be similar in terms of orientation (South-East), depths (2-5 m) and habitat (*Cymodocea nodosa* meadow). In particular, we chose a low pH site in Vulcano Island, close the primary CO_2 vent in the Levante Bay, about 250 meters far away from the vent (hereafter "Low pH", mean pH = 7.80 ±0.09); a control site is located in the same Bay in Vulcano Island (about 500 meters far away from the Low pH site, hereafter "Ctrl 1", mean pH = 8.19 ±0.03) and a second control site in Lipari Island (hereafter "Ctrl 2", mean pH = 8.22 ±0.02) (**Fig. 4.1**).

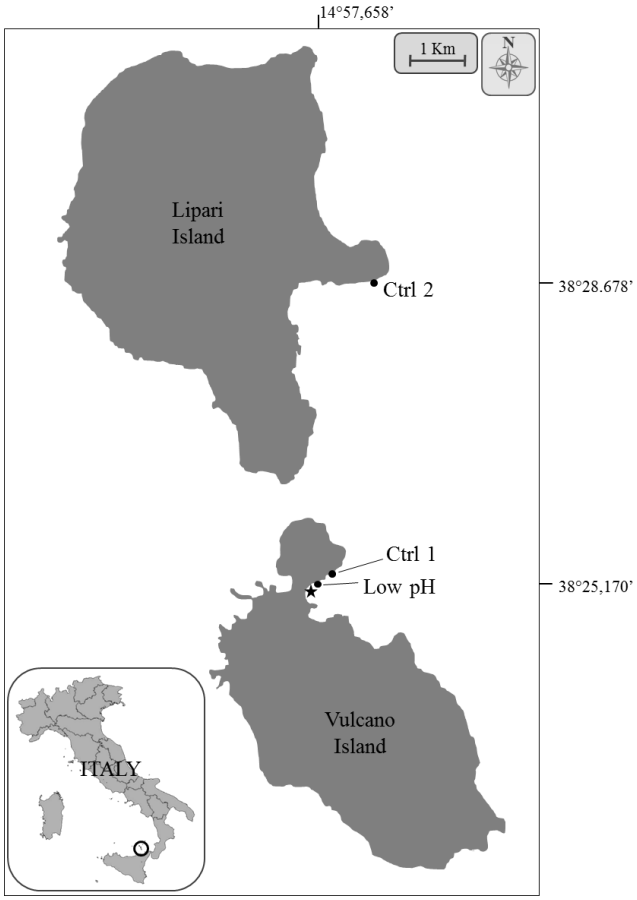

Fig. 4.1 *- Map of the study area showing the sampling sites in Vulcano (Low pH and Ctrl 1) and Lipari Islands (Ctrl 2). The primary vent is indicated by the star.*

4.2.2 Samples collection and laboratory analysis

Sampling was carried out from September to November 2014. Fish were collected by using small trammel nets, fish traps, hook and line (for details see **Tab. 4.1**). Surface sediment was also collected using a PVC hand-corer (Ø 4 cm, 0-1 cm, six replicates per site); macroalgae and seagrasses (three replicates per site for each species) were collected by hand from scuba divers. All the samples were stored at -20 °C and transferred to the laboratory until chemical analyses were performed.

In the laboratory, macroalgae [*Cystoseira spp*., *Caulerpa prolifera*, *Halopteris scoparia* and *Flabellia petiolata*] and the seagrass *Cymodocea nodosa* were rinsed with distilled water and epiphytes were removed by surface scraping. Fish were identified at species level and classified into trophic groups following the classification by Guidetti & Sala (2007) and using the available information about diet in the FishBase database (Froese & Pauly, 2016). The trophic groups considered were: planktivores, herbivores, invertivores and small piscivores (**Tab. 4.1**). Total length was measured to the nearest 0.1 mm and then dorsal muscle was processed for analysis.

All samples (abiotic and biotic) were frozen at -80°C, then freeze-dried (ALPHA 1-4 LDplus, Martin-Christ) and ground to a fine powder using a ball mill (MM 200 Retsch). An Inductively Coupled Plasma-Optical Emission Spectrometer (ICP-OES, Optima 8000, PerkinElmer) with an hydride-generation system (linked to ICP-OES) was used to analyse THg in samples digested in a microwave system (MARS CEM). Dried samples were mineralized in Teflon digestion vessels. Sediments were mineralised with 9 ml HNO_3 67–70%,0.5 ml H_2O_2 30%, 3 ml HF 30% and 2.5 ml Milli-Q deionized water (Method 3052, United States Environmental Protection Agency 1996), while biological tissues by using 5 ml HNO_3 67–70%, 1 ml H_2O_2 30% and 4 ml Milli-Q deionized water (following the method used by Vizzini et al. 2010). For each cycle of mineralization one analytical blank was prepared. The analytical procedure was checked using a standard reference material (recovery comprised between 87 and 98%), provided by the National Research Council of Canada: dogfish muscle DORM-4 (National Research Council of Canada) for fish, *Lagarosiphon major* BCR-060 (Institute for Reference Materials and Measurements) for primary producers and marine sediment NIST 2702 (National Institute of Standards and Technology) for

sediments. THg concentration was expressed as mg/kg of dry weight (dw). To compare Hg values found in other studies and with European Union Maximum Residue Limits, we converted dry weight into wet weight according to Magalhães et al. (2007).

Stable nitrogen isotope ratio was analysed in all samples through an Isotope Ratio Mass Spectrometer (Thermo Scientific Delta Plus XP) connected to an Elemental Analyser (Thermo Scientific Flash EA 1112). Isotopic values were expressed in conventional δ unit notation as parts per mil deviations from the international standards, atmospheric nitrogen (N_2), following the formula $\delta X = [(R_{sample}/R_{standard}) - 1] \times 10^3$, where X is ^{15}N and R is the corresponding $^{15}N/^{14}N$ ratio of sample and standard. Analytical precision based on the standard deviation of replicates of internal standards was 0.2‰.

4.2.3 Data analysis

To test the difference among sampling sites in the levels of total mercury in sediment and primary producers (macroalgae and seagrasses), the bioaccumulation in the fish species and the transfer along trophic levels of the fish community, we considered a one-factor experimental design (factor 'Site', fixed at three levels: Low pH, Ctrl 1 and Ctrl 2). Univariate and multivariate statistical tests were performed by using STATISTICA v.10 (StatSoft) and PRIMER v.6.0 + PERMANOVA (Anderson et al. 2008) software packages, respectively.

Analysis of variance (one-way ANOVA) was carried out to evaluate differences among sampling sites in mercury concentrations of sediment, macroalgae, seagrass and fish (both at species and trophic group levels). Cochran's and Shapiro-Wilk's tests were used prior to analyses to verify the homogeneity of variances and the normality, respectively. Where significant differences were present, Tukey's *post hoc* test was used. Macroalgae data were transformed using log (x + 1) to meet the assumptions for the use of parametric test and analysed without species distinction, due to the low number of species common in the three sites.

Moreover, to test the differences in mercury at fish community level, a multivariate analysis was performed only on species in common in the three sites (analysis was performed on a subsampling of three replicates for each species per site). Euclidean distances resemblance

matrix was calculated on normalized data of mercury levels and then permutational analysis of variance (PERMANOVA) and multivariate principal coordinates analysis (PCO) were carried out. The projection bi-plots of the variables that best correlated to the PCO axes were superimposed to visualize the variables most responsible for the patterns of multivariate dispersion. The similarity percentage (SIMPER) procedure was employed to identify the major fish taxa contributing to dissimilarities between sampling sites.

To evaluate mercury transfer along the trophic levels, simple linear regressions were performed for each site using $\delta^{15}N$ as independent variable and log-transformed THg concentration as dependent variable, using only the common species in the three sites. The slope (b) of the regression $\log[Hg] = a + b (\delta^{15}N)$ is the biomagnification power of the trace element and represents the change in concentration per unit change in $\delta^{15}N$ over the food chain. A positive slope indicates biomagnification, whereas a negative slope indicates trophic dilution. Trophic magnification factors (TMFs) values were calculated from the slope according to the formula: $TMF = 10^b$ (Nfon et al. 2009). TMF > 1 indicates accumulation of the trace element in the food chain, whereas a value < 1 suggests its dilution.

4.3 Results

Mercury concentration in sediments and primary producers showed a similar trend among sites, with higher levels of THg in Low pH compared to controls. In particular, sediments showed mean values of 0.03 ±0.01 mg/kg in Ctrl 1, 0.04 ±0.02 mg/Kg in Ctrl 2 and 0.08 ±0.01 mg/Kg in the Low pH, with differences between Low pH and the two controls (ANOVA: $F_{2, 15} = 22.69$, $p < 0.001$). Seagrasses showed similar Hg levels in the two control sites (0.04 ±0.008 mg/Kg, 0.01 ±0.001 mg/Kg in Ctrl 1 and Ctrl 2, respectively), while higher values were found in the Low pH (0.12 ±0.02 mg/Kg - $F_{2, 6} = 81.31$, $p < 0.001$). Additionally, macroalgae showed THg values higher in the Low pH (0.08 ±0.04 mg/Kg), than in both controls (0.03 ±0.01 mg/kg and 0.02 ±0.01 mg/kg in Ctrl 1 and Ctrl 2 respectively - $F_{2, 24} = 16.61$, $p < 0.001$) (**Fig. 4.2**).

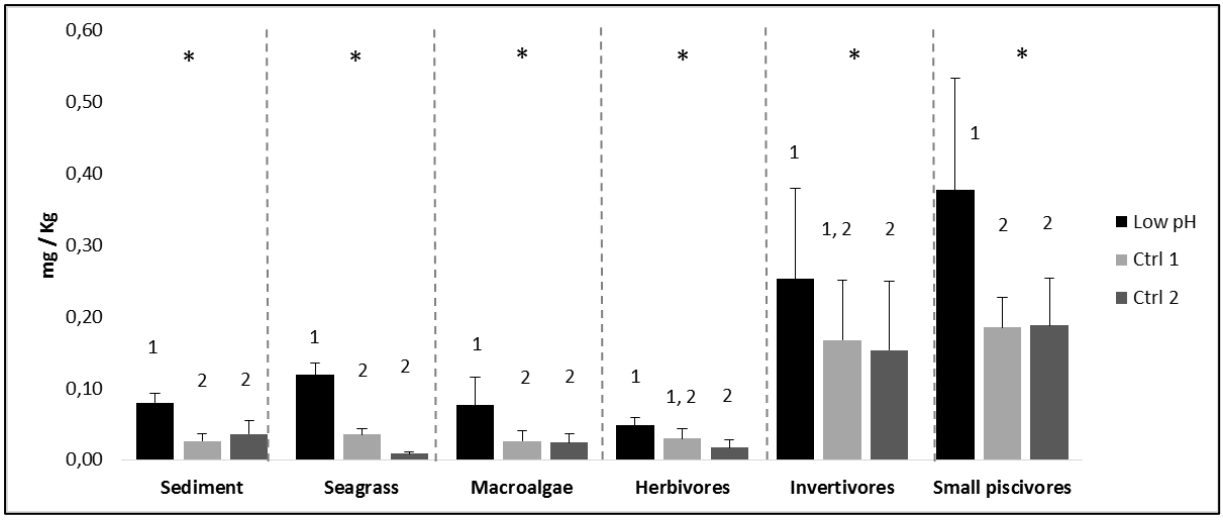

Fig. 4.2 *- Mean concentration (+SD) of total mercury (mg/Kg) in sediment, primary producers (macroalgae and seagrass), and fish (grouped in trophic groups) in the three sampling site (Low pH, Ctrl 1 and Ctrl 2). Asterisks indicate significant differences among sampling sites and numbers indicate results from post hoc tests (significance level: α = 0.05).*

Fish trophic groups reflect the same trend found in sediments and basal sources (higher in Low pH than controls). Overall, considering all the matrices analysed, the two controls ranged between 0.02 and 0.03 mg/Kg of THg in Ctrl 1, 0.02 and 0.04 mg/Kg in Ctrl 2 and 0.04 and 0.08 mg/Kg in Low pH. Generally herbivores (*Sarpa salpa* and *Parablennius sanguinolentus* - $F_{2, 9} = 7.04$, $p < 0.05$) showed lower levels than all the other groups, while the highest value was found in the small piscivores (*Serranus scriba* and *Scorpaena porcus* - $F_{2, 15} = 7.13$, $p < 0.01$). The most conspicuous group was represented by invertivores and showed a gradient between Low pH and Ctrl 2 ($F_{2, 81} = 3.87$, $p < 0.05$). Tukey's *post hoc* tests showed greater differences between Low pH and the second control, and only small piscivore group sampled in the impact site were higher than the two controls (**Fig. 4.2**).

At fish species level, although species-specific responses were found, a general trend showed higher THg level in the impact site than controls. Fish analysed belonged to six families (Labridae, Sparidae, Gobiidae, Blenniidae, Scorpenidae, Serranidae - for details see **Tab. 4.1**) and almost all were in common among the three samplings sites, with the exception of *Labrus viridis*, *P. sanguinolentus*, *Symphodus ocellatus* and *Symphodus mediterraneus*, collected only in Low pH and one control. Overall, the species analysed showed greater THg levels in the Low pH compared to the controls with significant among-site differences in 7 out of 14 species. In particular, *S. scriba* showed the highest value among the species

analyzed and Tukey's *post hoc* test highlighted higher mercury concentration in the Low pH than the two controls. On the other hand, *S. mediterraneus*, *Symphodus roissali* and *S. salpa* showed higher THg values in the Low pH than Ctrl 2 and intermediate values in Ctrl 1, while *Coris julis* showed lower THg value in Ctrl 2 compared to the other sites (**Fig. 4.3**).

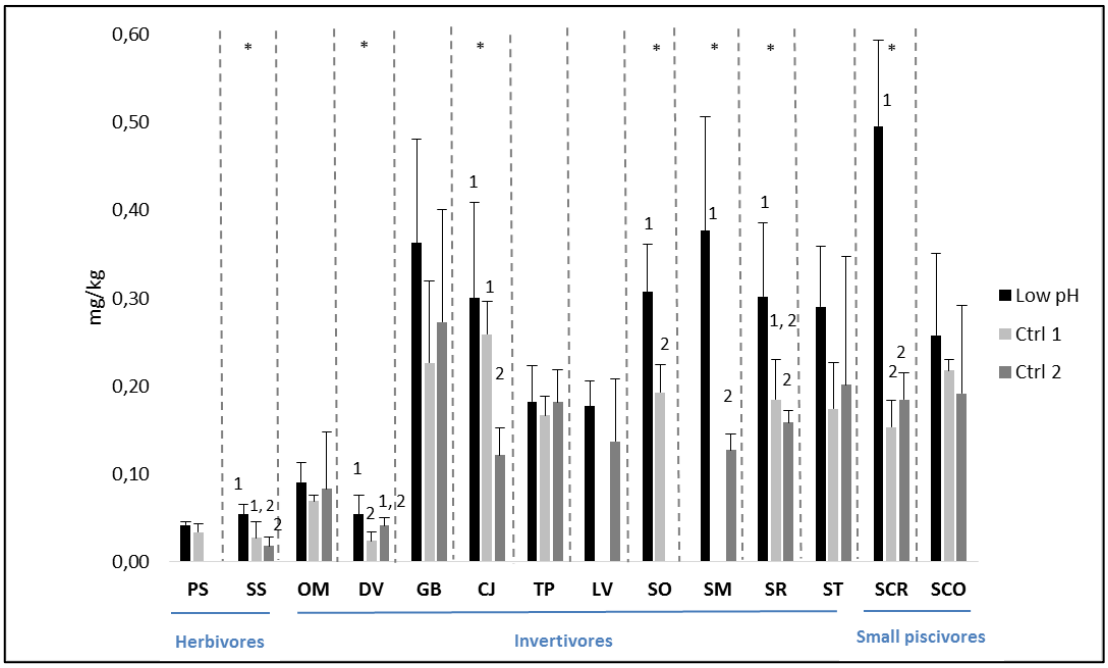

Fig. 4.3 *- Mean concentration (+SD) of total mercury (mg/Kg) in muscle of fish analyzed in each sampling site (Low pH, Ctrl 1 and Ctrl 2). Species are grouped in trophic groups. Fish label: PS, Parablennius sanguinolentus; SS, Sarpa salpa; OM, Oblada melanura; DV, Diplodus vulgaris; GB, Gobius bucchichi; CJ, Coris julis; TP, Thalassoma pavo; LV, Labrus viridis; SO, Symphodus ocellatus; SM, S. mediterraneus; SR, S. roissali; ST, S. tinca SCR, Serranus scriba; SCO, Scorpaena porcus. Asterisks indicate significant differences among sites and numbers indicate results post hoc tests.*

Table 4.1 *- Summary of fish species, family, trophic group, number of individuals (N), and range of total length (cm – TL) analyzed per species in each sampling sites (Low pH, Ctrl 1 and Ctrl 2).*

			Low pH		Ctrl 1		Ctrl 2	
Family	**Species**	**Trophic group**	**N**	**TL**	**N**	**TL**	**N**	**TL**
Labridae	*Coris julis*	Invertivore	4	10.2 – 18.1	4	11 – 15.8	4	10.2 – 16.6
	Thalassoma pavo	Invertivore	5	10 – 11.4	5	10.5 - 14.6	5	11.5 – 14.5
	Labrus viridis	Invertivore	3	10.3 – 12.2	0	/	3	9.7 – 11.4
	Symphodus ocellatus	Invertivore	6	6.2 – 7.5	6	5.3 – 7.5	0	/
	Symphodus mediterraneus	Invertivore	4	8.2 – 10.3	0	/	4	9.4 - 11
	Symphodus roissali	Invertivore	3	8.3 – 10.5	3	6.8 – 8.7	3	8 – 9.7
	Symphodus tinca	Invertivore	4	9.7 - 16	4	8.1 – 13.5	4	9.8 – 18.7
Sparidae	*Diplodus vulgaris*	Invertivore	4	8.8 – 14.8	4	7.4 - 13	4	8.7 - 9
	Oblada melanura	Invertivore	3	10 – 11.1	3	8 - 12	3	7.3 – 10.7
	Sarpa salpa	Herbivore	4	16.7 - 18	4	21.5 – 24.5	4	15.8 – 27.5
Gobiidae	*Gobius bucchichi*	Invertivore	5	7.3 – 8.1	5	7.1 – 11.3	5	6.8 – 9.7
Blenniidae	*Parablennius sanguinolentus*	Herbivore	3	8.5 – 12.9	3	7.9 – 8.9	0	/
Scorpenidae	*Scorpaena porcus*	Small piscivore	3	9.2 - 14	3	12.4 - 15	3	10.2 – 16.2
Serranidae	*Serranus scriba*	Small piscivore	3	14.2 – 16.2	3	10.3 – 11.9	3	11.4 – 17.1

At multivariate level, PERMANOVA showed differences in THg levels of fish assemblages among the three sites (Pseudo – $F_{2, 6} = 2.66$, $p(MC) < 0.05$), with higher values in Low pH compared to Ctrl 1 and no differences with Ctrl 2 (pair wise *post hoc* test: Low pH > Ctrl 2 = Ctrl 1). The graphical ordination of principal coordinate analysis showed a separation among Low pH (in the right side) and the two controls (in the left side), and the first two axis (PCO1 and PCO2) explained 67.6% of total variation (**Fig. 4.4**). SIMPER analysis showed that the species that mainly contributed to discriminate among sites and that accumulated greater THg were *S. scriba*, *D. vulgaris* and *S. roissali* among Low pH and Ctrl 1, *C. julis*, *S. roissali* and

S porcus among Low pH and Ctrl 2, and *C. julis*, *O. melanura* and *S. tinca* among the controls.

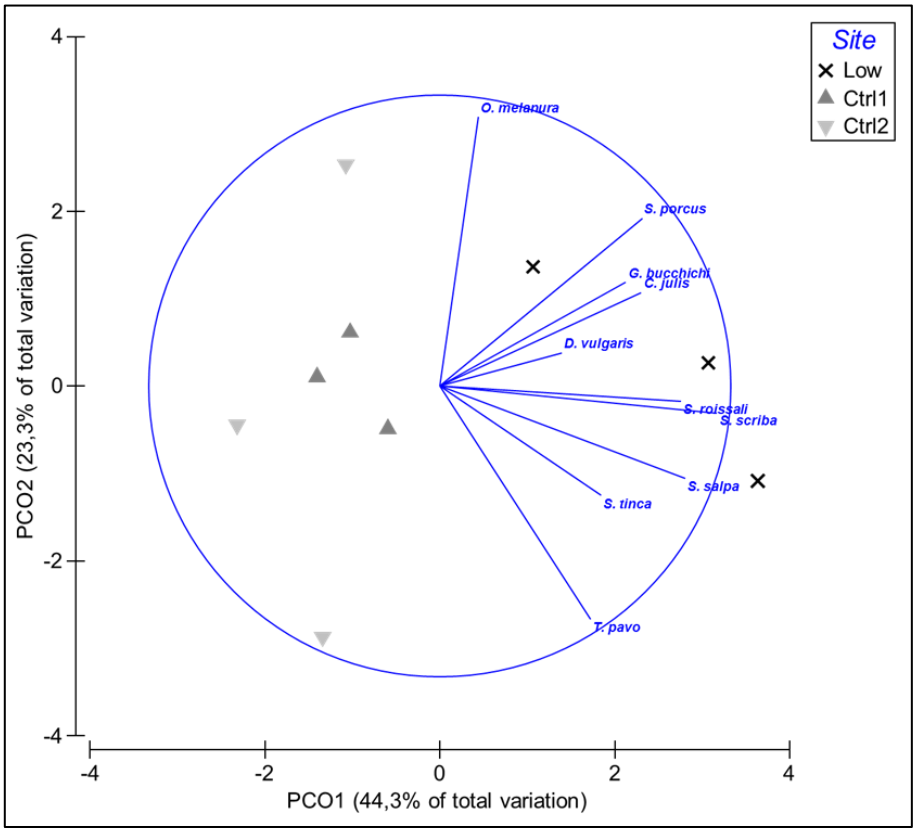

Fig. 4.4 *- Principal coordinates analysis (PCO) ordination based on normalized Euclidean distance of mercury concentrations of fish common in the three sampling site (Low pH, Ctrl 1 and Ctrl 2). Fish species vectors are superimposed. The direction of vectors indicates the correlation and the length is proportional to the correlation value.*

Linear regression analysis revealed significant correlations between $\delta^{15}N$ and the log-transformed Hg concentrations of fish (**Fig. 4.5**). Although the correlation coefficients were low, slopes of the regressions were positive and significant for the three sites: Low pH ($p = 0.0054$, $b = 0.1865$, $r^2 = 0.20$), Ctrl 1 ($p = 0.0077$, $b = 0.1561$, $r^2 = 0.18$), and Ctrl 2 ($p = 0.0015$, $b = 0.2303$, $r^2 = 0.25$). Trophic magnification factor (TMF) was calculated for each site and showed values greater than 1 (TMF = 1.54, 1.43 and 1.70 for Low pH, Ctrl 1, and Ctrl 2 site, respectively).

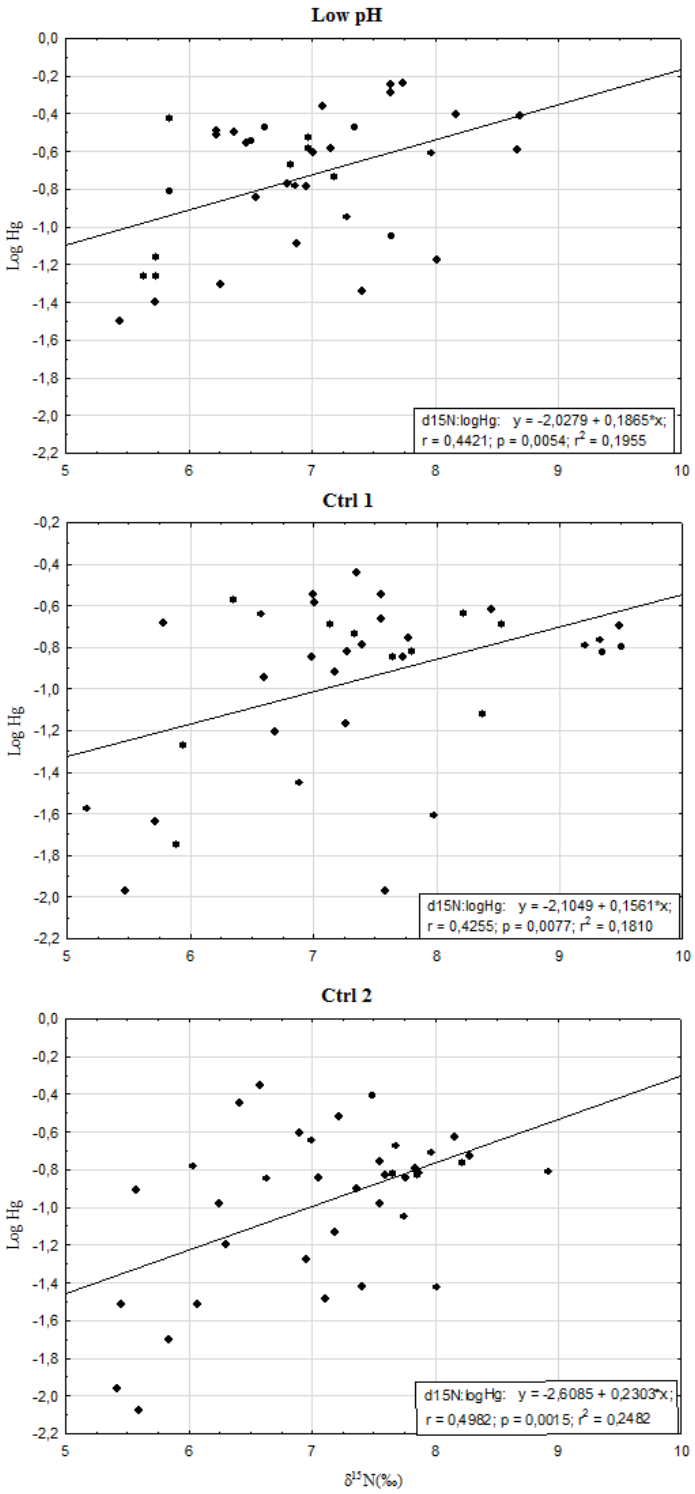

Fig. 4.5 *- Relation between trophic level, determined by $\delta^{15}N$, and logarithm of mercury (log [Hg]) concentration in fish species in each sampling site.*

4.4 Discussion

The forecasted decrease of ocean pH in the next centuries together with the introduction of trace metals in the ocean (both of natural and anthropogenic origin) will affect their availability and accumulation in the marine biota, with consequences on ecosystem functions (Zeng et al. 2015). To date CO_2 vents are exploited to test the effect of acidification on organisms exposed long-term to low pH conditions. Moreover, these sites can offer the possibility to test the synergistic effect of acidification with other stressors (Kadar et al. 2005a, 2005b, 2007, Martins et al. 2006). Here, we focused the attention on total mercury levels of fourteen coastal fish species from a Mediterranean CO_2 vent and we found that although fish reflected the higher mercury concentrations found in sediments and basal resources, a weak biomagnification characterized sites with different pH conditions.

Our findings showed a general increase in mercury bioaccumulation in the Low pH site than the two controls, confirming our hypothesis. Almost all the species investigated showed higher THg levels in the Low pH site, but a few fish showed similarities with Ctrl 1. Moreover, fish showed an increase in THg along the trophic levels (from herbivores to invertivores, followed by small piscivores), but this trend did not translate in higher biomagnification rates in Low pH site than in controls. Biomagnification was highlighted through the linear regression determined by $\delta^{15}N$ and log [Hg] in the three sampling sites, and the trophic magnification factor (TFM) showed similar values in the three sites. The slopes of the regression of log[HgT] against $\delta^{15}N$ were 0.20, 0.18 and 0.25 (for Low pH, Ctrl 1, and Ctrl 2, respectively), showing values within the range observed for Hg biomagnification in food webs from different geographic locations (range: 0.16 - 0.29; Nfon et al. 2009). Finally, at fish community level, the principal component analysis (PCO) highlighted a separation between the Low pH and the two controls, but PERMANOVA analysis showed a difference only between Low pH and Ctrl 1.

In the same area of Vulcano, previous studies focused on other compartments and organisms, and found higher levels of metals close to the vent, confirming the impact of the volcanic emissions. For instance, Vizzini et al. (2013a) determined the levels of trace elements in sediments, in the leaves of *Cymodocea nodosa* seagrass and its epiphytes across a spatial pH gradient in the vicinity of the primary vent. These results indicated that concentrations of some elements (As, Ba, Hg, Mo, Ni, Pb, and Zn) were generally greatest in the locations close to the primary CO_2 vent, and their concentration reached lower values at about 350 m

away from the primary vent, thus our Ctrl 1 (located in Levante Bay) should not be impacted by the vent. However, the similarity for a few species between the Low pH and the nearest control could be explained by the greater mobility of some species and the confirm that fish use this area to feed (Chapter 2). Moreover, McClintock et al. (2014) reported similar results on the accumulation of trace elements (As, Cd, Co, Cr, Hg, Mo, Ni, Pb and V) in shells of four different species of gastropods, which were collected from three sites in Levante Bay with different pH values. On the other hand, Horwitz et al. (2014) found changes in trace elements accumulation in the sea anemone *Anemonia viridis* between impacted and control sites, although no apparent signs of stress were detected (Suggett et al. 2012).

Particularly interesting is the case of *Gobius bucchichi* - Steindachner 1870, a small site-attached fish, that lives together with *A. viridis* and is abundant in the acidified area of Vulcano Island (Nagelkerken et al. 2015). We found high levels of THg, similar to those of fish belonging to higher trophic levels such as the small piscivore *Scorpaena porcus*. The Bucchich's goby is a highly sedentary species that seems to be the most contaminated by mercury due to its direct intake from the sediment. Indeed, fish can accumulate metals not only through ingestion, but also through skin and gills, and this is probably correlated above all with the habitat use of the species (Desta et al. 2008). However, the high density of this species let hypothesize that it developed efficient mechanisms of excretion / detoxification, as a consequence of the longtime exposition to these particular conditions (Kádár et al. 2005a). Moreover, fish species of ecological or economic importance such as *D. vulgaris* and *S. salpa*, showed significantly higher Hg levels in the low pH site, although they were overall low (maximum 0.05 ppm dw) compared to other polluted sites (*i.e.* Bonsignore et al. 2013, Copat et al. 2012).

Another study conducted in Panarea Island (close to Vulcano Island), measured the levels of harmful elements following an episode of volcanic activity and showed how fast can be the recovery of different compartments (biotic and abiotic) after an intense perturbation (Andoloro et al. 2012). Each species, however, can have a different response because bioaccumulation of metals in fish depends mainly on diet, but also on age or length of fish (Zhang & Wong, 2007). Moreover, fish absorb heavy metals from the surrounding environment depending on a variety of biotic factors (such as the characteristics of the species under consideration, the exposure period, the concentration of the element) and abiotic factors (such as temperature, salinity, pH and seasonal changes – Copat et al. 2012). Comparison with values found in deep vent in the Mid-Atlantic Ridge hydrothermal vent

fields (Martins et al 2006) showed Hg values lower in our study, but confirm the role exerted by the vent systems in keep available heavy metals toxic like mercury.

In addition, comparisons with European Union Maximum Residue Limits after wet-weight conversion of Hg concentration (Magalhaes et al. 2007). For Hg, the maximum content allowed is 0.5 mg/Kg wet weight of in the edible part of fish for humans. Mean Hg values found in Levante Bay in the 14 species analyzed did not exceed the limit set by the European regulation. Although mercury levels in fish, primary producers and sediments from the low pH site were always higher than controls, the weak biomagnification rate along the fish trophic levels indicates no particular problems for human consumption.

This study confirms a general decrease of contamination at a few hundreds of meters from the primary vent (Ctrl 1 is only at 500 m from the primary vent), as found by Vizzini et al. (2013a). As a consequence, particular attention deserves the choice of sampling sites when we test the effect of acidification in field experiments near CO_2 vent, due to the possible confounding effect coming from other stressors, above all for species with limited movements.

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CHAPTER 5: The influence of high CO_2 / low pH conditions on otolith shape and composition of six coastal fish species at a Mediterranean CO_2 vent

Abstract: Naturally acidified environments, such as CO_2 vents, are important sites to evaluate the potential effects and impacts of increased ocean acidification on marine ecosystems and biota. Here we assessed the effect of high CO_2 / low pH on otoliths of six coastal fish species (*Chromis chromis*, *Coris julis*, *Diplodus vulgaris*, *Gobius bucchichi*, *Sarpa salpa*, *Symphodus ocellatus*) in a Mediterranean shallow CO_2 vent. Taking into consideration the major and trace elements that are found near the vent and the gradient of dissolved inorganic carbon, we compared the otolith chemical signatures of fish exposed long-term to elevated CO_2 emissions and reduced pH (pH=7.8) against fish living in two control sites (pH=8.2). A number of element:Ca ratios (Li:Ca, B:Ca, Na:Ca, Mg:Ca, Mn:Ca, Cu:Ca, Zn:Ca, Sr:Ca, Ba:Ca and Pb:Ca), along with $\delta^{13}C$ and $\delta^{18}O$ isotopic values, were measured in otoliths and water samples. Additionally, we combined chemical signatures with otolith shape analysis (morphometric and outline shape) to evaluate the effect of high CO_2/low pH on this structure. We observed species-specific responses with regards to shape and chemical analysis. Significant differences between sites were found in otolith shape (elliptical Fourier descriptors) of *G. bucchichi* and *D. vulgaris*. Comparison of elemental and isotopic signatures indicated that there were significant differences for *D. vulgaris*, but differences were not found for the other species. Ultimately, besides improving our knowledge of the effects of high CO_2 / low pH conditions on otoliths, the present results contribute to our understanding on the use of otoliths as natural tags.

Keywords: otolith chemistry, isotopic composition, shape analysis, ocean acidification, Mediterranean fish, CO_2 vent.

5.1 Introduction

Ocean acidification is one of the most prominent challenges that scientists and policy makers are currently facing, together with other consequences related to climate change (IPCC,

2014). Increased atmospheric CO_2 has resulted in an increase in pCO_2, and a reduction in pH and carbonate saturation indices in ocean waters (Doney et al. 2009), with implications on biota and the entire marine ecosystem (Kroker et al. 2012). Worldwide, multiple studies have focused on calcifying species with major effects forecasted for calcareous phytoplankton, corals and echinoderms (Orr et al. 2005). In contrast, effects of ocean acidification on non-calcifying species, like fish, are still less well known (Ishimatsu et al. 2008), despite the increase in studies in recent years (*i.e.* Checkley et al. 2009, Munday et al. 2014, Nilsson et al. 2012).

Ocean acidification may impact fish populations both directly through physiological and behavioral consequences (Nagelkerken & Munday, 2016, Nilsson et al. 2012) and indirectly, through habitat modification (Nagelkerken et al. 2015). In particular, larval and juveniles stages are particularly vulnerable to ocean acidification with consequences for population recruitment and connectivity (Rossi et al. 2016). Experiments on larval fish raised under pH levels projected for the end of the century have produced species-specific results (Munday et al. 2011a), however, negative effects on fish are generally observed and it has been hypothesized that there will likely be consequences to fish abundances under future acidified oceans (Baumann et al. 2011). Moreover, the central role of fish to worldwide seafood catches makes it necessary to understand the potential effects of ocean acidification on fisheries (Branch et al. 2013).

Otoliths are calcified structures of fish which can be used to estimate age and growth (Campana & Neilson, 1985). They are sensory organs involved in balance, orientation, sound detection, and therefore are extremely important for fish survival. Otoliths are metabolically inert, grow continuously and incorporate micro and trace elements from the environment into the aragonite protein layers (Campana, 1999, Doubleday et al. 2014). These characteristics have enabled otoliths to be used as natural tags to unravel fish movement, habitat use and reconstruct environmental life-histories (Di Franco et al. 2012, Elsdon et al. 2008, Izzo et al. 2015, Reis-Santos et al. 2013a, Rooker et al. 2016, Sturrock et al. 2012). In particular, otoliths, along with other calcified structures such as shells or fish skeletons, precipitate in equilibrium with seawater, reflecting seawater chemistry and other environmental conditions (*i.e.* salinity or temperature – Barnes & Gillanders, 2013; Miller, 2011, Reis-Santos et al. 2013b, Tanner et al. 2013). Therefore, the chemical composition of otoliths is linked to ambient seawater conditions during precipitation and is a promising tool in the context of climate change studies (Fraile et al. 2016).

Several studies have investigated otolith formation under acidified conditions (*i.e.* Bignami et al. 2013, Munday et al. 2011b, Réveillac et al. 2015). Checkley et al. (2009) first demonstrated that otoliths in eggs and larvae reared in seawater with elevated CO_2 were larger than those of fish reared in seawater with normal CO_2. Both area and otolith mass were greater for fish under future projections of atmospheric CO_2 (993 and 2558 µatm; Caldeira & Wickett, 2003). Nonetheless, most studies to date have been conducted in laboratory conditions, and it is not known whether similar patterns are seen for field studies in naturally acidified environments.

Shallow CO_2 vents provide a great opportunity to test the effect of long-term ocean acidification on marine biota and ecosystems (Fabricius et al. 2011, Hall-Spencer et al. 2008). For instance, Nagelkerken et al. (2015) demonstrated that escape speed and behavioral responses of fish exposed to long-term high CO_2 levels were reduced compared to natural conditions. Another experiment, conducted at natural CO_2 vents has also found that juvenile reef fish at seeps exhibited behavioral abnormalities similar to those seen in laboratory experiments (Munday et al. 2014). Moreover, CO_2 vents are characterized by the emission of major and minor elements (Dando et al. 1999, Sedwick & Stüben, 1996), that may accumulate in sediments and biota (Vizzini et al. 2013) and therefore could also be incorporated in the otolith matrix, providing unique information to characterize movements and site fidelity of fish in naturally acidified environments. Ultimately, natural CO_2 vents can be important sites to evaluate how ocean acidification may simultaneously affect otolith shape and chemistry, as well as the potential of otoliths as natural tags in changing ocean conditions.

Here, we studied *in situ* the effects of ocean acidification on morphological and chemical features of otoliths of six coastal Mediterranean fish species (*Chromis chromis*, *Coris julis*, *Diplodus vulgaris*, *Gobius bucchichi*, *Sarpa salpa*, *Symphodus ocellatus*) in a shallow CO_2 vent off Vulcano Island (Aeolian Archipelago, Italy). In particular, the aims of the present study were to test if: 1) otolith shape in fish exposed to high CO_2 levels is morphologically altered compared to fish living at normal pH sites; 2) otolith elemental signatures (major and trace elements, carbon and oxygen stable isotopes) provide information on the fidelity of fish to low pH sites by distinguishing individuals of a natural acidified environment from control sites.

5.2 Materials and methods

5.2.1 Study area and sample collection

The shallow hydrothermal system of Vulcano Island is one of the most active sites in the Aeolian Archipelago (24 km off the NE coast of Sicily, Italy). The main CO_2 vent is in Levante Bay, on the eastern side of the island where volcanic emissions have been extensively investigated (Boatta et al. 2013, Capaccioni et al. 2011, Italiano et al. 2009). Gas composition is dominated by CO_2 (97-99% vol.), which generates a pH gradient (from 5.5. to 8.1) along the northern shore of the bay. Emissions also include small quantities of H_2S (<2.2%), which rapidly decrease with distance from the vent (Boatta et al. 2013). Water composition in terms of the major elements (Cl, SO_4, Na, K, Ca and Mg) is close to that of Mediterranean surface waters, while greater variability is recorded for dissolved Fe concentrations, with maximum values recorded close to the vent (Boatta et al. 2013). Many elements also leak from the vent, adding dissolved Si, K, Li, Rb, Mg, Ca to surrounding seawater (Sedwick & Stüben, 1996). The area is characterized by acidic and reducing conditions, causing changes in major and trace element geochemical fluxes at the sediment-seawater interface (Vizzini et al. 2013). Seawater carbonate chemistry in the Levante Bay area ranged between 2.78 and 3.17 mmol/kg for total alkalinity and 0.02 and 3.64 for aragonite saturation state (for details see Boatta et al. 2013).

Fish samples were collected between September and December 2014 at three sites: a low pH site in Vulcano Island (hereafter "Low pH", mean pH = 7.80 ±0.09 SE) about 250 m from the primary vent, a control site in Vulcano Island (hereafter "Ctrl 1", mean pH = 8.19 ±0.03) 500 m from the primary vent, and another control site in neighboring Lipari Island (hereafter "Ctrl 2", mean pH = 8.22 ±0.02) about 6.5 km from the first two sites (**Fig. 5.1**). Control sites were chosen to be similar in terms of orientation (South-East), depths (2-5 m) and habitat (*Cymodocea nodosa* meadow) to the Low pH site. Fish were collected by nets, fish traps, hook and line and stored at -20 °C prior to being transferred to the laboratory.

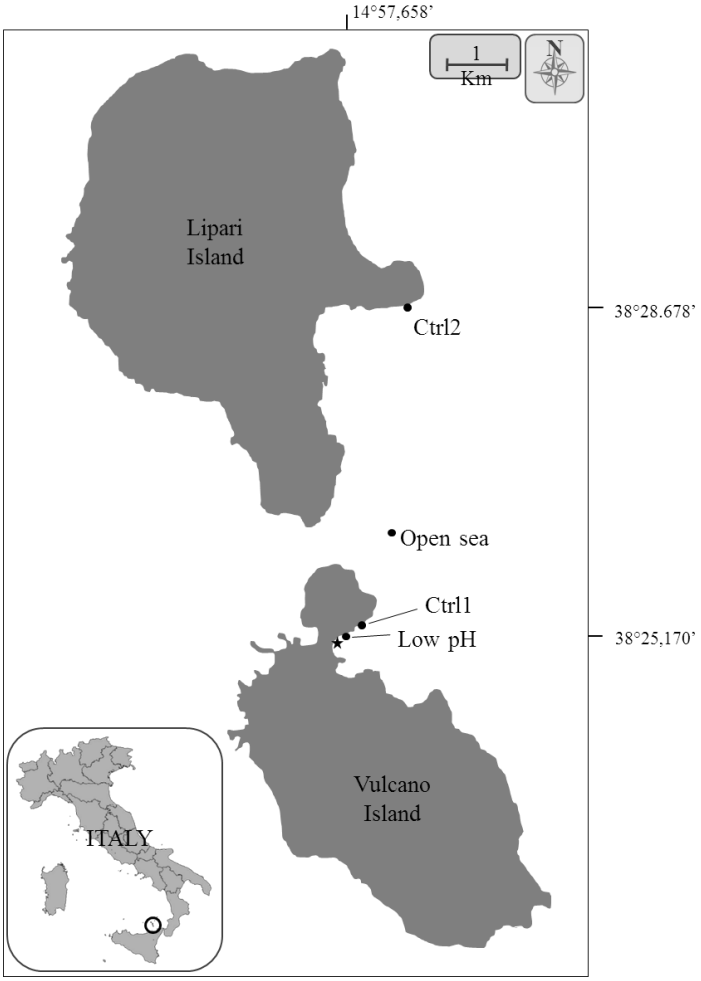

Fig. 5.1 *- Map showing the location of sampling sites in Vulcano (Low pH and Ctrl 1) and Lipari Islands (Ctrl 2). The Open sea site is indicated in the channel between the two Islands and the primary vent is indicated by the star. Italy and Aeolian Archipelago are showed in the insert.*

Seawater samples for chemical analysis were collected on four dates (December 2014 and April, June and October 2015) to evaluate temporal variability at the three fish sampling sites, as well as at the primary vent (hereafter "Vent") to characterize the volcanic emissions and at an offshore site (hereafter "Open Sea") as a marine control. We collected three replicates for dissolved inorganic carbon stable isotope analysis ($\delta^{13}C_{DIC}$) and three replicates for major (Ba, Ca, Mg, Mn, Sr) and trace element (Li and Zn) analysis. Upon collection, shallow water samples were filtered using 0.45-mm filters, acidified with 2% HNO_3 and stored in glass sample jars. All samples were stored refrigerated at 4 °C until analysis. All glass and plastic materials used in this study were previously acid washed in a 10% nitric acid bath. All reagents were Suprapur grade.

5.2.2 Seawater analysis

Dissolved Ba, Ca, Mg, Mn, Sr, Li and Zn were measured with an Inductively Coupled Plasma-Optical Emission Spectrometer (ICP-OES, Optima 8000, PerkinElmer). The analytical procedure was checked using a standard reference material (Nearshore seawater CASS-5, National Research Council of Canada). The recovery ranged from 79% to 94%. The detection limit was estimated as <1 µg l^{-1}.

Values of dissolved inorganic carbon ($\delta^{13}C_{DIC}$) were quantified following methods outlined by Capasso et al. (2005). Water samples were stored in glass bottles (50 cc) and quickly sealed using an aluminium crimp cap and gas-tight rubber/Teflon plugs, taking care that no air bubbles were present. In the laboratory, two syringes were used per water sample. The first syringe was filled with ultra-pure He gas and the second one was kept empty and used to withdraw the aqueous phase from the storage tube - 'water sampling' syringe. By inserting the two syringe needles through the septum of the storage bottles, an aliquot of water (0.2 ml) was withdrawn with the 'water sampling' syringe while ultrapure He gas was injected. The aliquot of water was then injected through the rubber septum of a screw-capped glass vial. In order to reduce any air contamination, the vials were previously flushed with ultrapure He using an automated Carbonate Preparation Device (Thermo Scientific GasBench II). This device consists of an auto sampler tray kept in a thermostatic rack. A fixed amount (150-200 µl) of 100% H_3PO_4 was automatically dispensed into the vials by means of a valve less pump. The reaction took place at 70°C. After 18 h, temperature of the thermostatic rack was lowered to 25°C and CO_2 produced by acidification was analysed in terms of carbon isotope composition by a Thermo Scientific Delta V Advantage continuous flow isotope ratio mass spectrometer. All $\delta^{13}C_{DIC}$ values were reported relative to Vienna Pee Dee Belemnite (VPDB) and the analytical precision was ±0.1‰.

5.2.3 Fish analysis

In the laboratory, fish were identified to species level and six of them, representative of a typical coastal Mediterranean fish assemblage and widespread in the entire basin were selected for otolith analysis. The damselfish *Chromis chromis* (Linnaeus, 1758) is a gregarious planktivorous species found in the column water and relatively site-attached. The labrids Mediterranean rainbow wrasse *Coris julis* (Linnaeus, 1758) and ocellated wrasse

Symphodus ocellatus (Linnaeus, 1758) are commonly found in seagrass beds and are classified as necto-benthic and sedentary fish. The sparids common two-banded seabream *Diplodus vulgaris* (Geoffroy Saint-Hilaire, 1817) and Salema *Sarpa salpa* (Linnaeus, 1758) are species of economic value and are classified as necto-benthic. The Bucchich's goby *Gobius bucchichi* Steindachner, 1870 is a small cryptic benthic and highly sedentary species. Fish standard length was measured to the nearest 0.1 mm (see **Tab. 5.1**), and sagittal otoliths were removed using forceps, cleaned in Milli-Q water, and stored dry individually in labelled plastic Eppendorf tubes.

Table 5.1 *- Number of individuals analysed for each species per sampling site (Low pH, Ctrl 1 and Ctrl 2), range of fish standard length (SL, cm) and otolith weight (OW, mg).*

	Low pH			**Ctrl 1**			**Ctrl 2**		
	N	**SL**	**OW**	**N**	**SL**	**OW**	**N**	**SL**	**OW**
C. chromis	12	6.9 - 8.7	7.9 - 17.2	11	5.1 - 8.0	3.4 - 16.3	12	5.8 - 8.3	6.2 - 16.1
C. julis	12	7.0 - 13.2	0.9 - 2.5	11	7.8 - 13.4	0.9 - 2.9	14	7.8 - 12.7	0.9 - 2.6
D. vulgaris	11	3.9 - 12.0	1.9 - 22.1	6	5.5 - 10.6	4.9 - 13.7	10	6.3 - 7.1	5.5 - 7.3
G. bucchichi	7	3.0 - 6.7	0.8 - 4.0	10	5.0 - 9.4	2.2 - 11.4	4	5.7 - 8.0	2.7 - 9.8
S. ocellatus	13	5.3 - 8.2	0.5 - 1.4	11	5.4 - 7.1	0.5 - 1.1	0	No samples	No samples
S. salpa	10	13.5 - 16.5	10.7 - 14.6	11	14 - 20	9.3 - 18.9	4	15.0 - 23.0	13.1 - 26.2

5.2.3.1 Otolith shape and morphometric analysis

Whole otoliths were digitized using Leica software QWIN W3 and a Leica DFC295 camera mounted to a Leica MZ16 stereo microscope at 1.25× magnification. Images were taken using reflected light and a black background to ensure a clear outline of the otolith. Otoliths were oriented so that the sulcus was face down (distal orientation) and the rostrum horizontally aligned. Each otolith was described using (i) elliptical Fourier descriptors (EFDs), and (ii) morphometric measurements (Fergusson et al. 2008). The coefficients of EFDs for each otolith were estimated using the program Shape v. 1.3 (Iwata & Ukai, 2002), which describes the outline of the otolith using harmonics. The Fourier power (FP) spectrum was used to determine the number of Fourier descriptors required to adequately describe the

otolith outline (Crampton, 1995). Twenty elliptical Fourier harmonics were calculated for each sample.

Otolith length, defined as the maximum length parallel to the sulcus, was measured using the software ImageJ (Rasband, 1997) and an electronic balance was used to weigh the otoliths with an accuracy of 0.01 mg (**Tab. 5.1**). All measurements were undertaken on the right otolith. Otolith relative length (ORL) was computed as the otolith length (OL) as a percent of fish standard length (SL), using the formula:

$$ORL = 100 * (OL/SL)$$

5.2.3.2 Otolith preparation and chemical analysis

After image acquisition and measurements, otoliths were prepared for elemental and isotopic analyses. The right otolith of each individual was embedded in epoxy resin (Epofix, Struers) spiked with 30 ppm of indium (^{115}In) and cut transversely through the nucleus (200−300 μm thick) using a low speed diamond saw (Isomet, Buehler). Indium was used so that the epoxy resin could be distinguished from the otolith material during analysis. Sections were polished using several grades of lapping film before being fixed onto a glass microscope slide using indium-spiked thermo-plastic glue (CrystalBond™509). Slides were then cleaned, sonicated and analysed on a New Wave 213 nm UV high performance (Nd:YAG) laser microprobe coupled to an Agilent 7500cs inductively coupled plasma mass spectrometer (LA ICP-MS). Otoliths were analysed in random order and elemental data collected from the same region for each otolith at both core (representing the larval/juvenile stage of life) and edge positions (representing recently occupied habitat) using a 30 μm spot. Each run generally consisted of 90 s acquisition: 30 s blank, to correct for background counts which were subtracted from each sample, 30 s ablation (laser fluency 5 J/cm^2) and 30 s for washout. The following elements were analysed: ^{7}Li, ^{11}B, ^{23}Na, ^{24}Mg, ^{43}Ca, ^{55}Mn, ^{63}Cu, ^{66}Zn, ^{88}Sr, ^{138}Ba and ^{208}Pb. The values of Li and B were consistently below the detection limits and therefore excluded. To measure instrument drift and precision a reference glass standard, NIST612, was analysed approximately every 10 samples, and a carbonate standard, MACS-3 (United States Geological Survey), was analysed at the beginning and end of each session. The mean CVs of repeated measures of the standards for all elements ranged between 3.0% (Mg) and 7.3% (Zn) for NIST612, and 7.1% (Cu) and 12.9% (Ba) for MACS-3. Data reduction, including background corrections, and mass count data conversion to concentrations (ppm) were

performed using Glitter (GEMOC, Macquarie University, Sydney, Australia). All otolith elemental concentration data were then converted to molar concentrations and standardized to calcium (element:Ca).

Otoliths of *C. julis*, *D. vulgaris*, *G. bucchichi* and *S. salpa* were analyzed for $\delta^{13}C$ and $\delta^{18}O$ using isotope ratio mass spectrometry (IRMS). Two regions (core and edge) were analysed in each otolith similar to that of elemental analysis. Material was milled from otoliths using a New Wave Micromill; this material was subsequently crushed and the resulting powder transferred to acid washed vials and flushed with ultrapure He, in order to reduce any air contamination. A fixed amount (150-200 µl) of 100% H_3PO_4 was automatically dispensed by an automated Carbonate Preparation Device (Thermo Scientific GasBench II) kept at 50°C. The carbonate isotopic composition of the CO_2 produced was measured by Thermo Scientific Delta V Advantage continuous flow isotope ratio mass spectrometer. Results were expressed in delta (δ) notation. The value relative to VPDB was calculated from triplicate analyses of an internal calibrated standard measured within the same batch. Precision of the carbon and oxygen isotope ratios was better than 0.1 and 0.2‰, respectively.

5.2.4 Data analyses

Univariate and multivariate statistical techniques were used to test the potential difference among sampling sites in terms of individual elements and multi-element composition for both seawater and otoliths.

Analysis of seawater data were done using univariate PERMANOVA for single elements and $\delta^{13}C_{DIC}$, and multi-elemental analysis, with the factors Site ('Si', fixed with five levels: Low pH, Ctrl 1, Ctrl 2, Vent, Open sea) and Date ('Da', random with four levels: winter, spring, summer, fall). Permanova+B20 package (Plymouth Marine Laboratory) was used to perform all the statistical analyses (Anderson et al. 2008).

Elemental otolith composition of each fish species was analysed separately using both a one-way analysis of variance (ANOVA) and a one-way permutational analysis of variance (PERMANOVA). Site ('Si') was treated as a fixed factor with three levels (Low pH, Ctrl 1 and Ctrl 2). Data were ln (x+1) transformed prior to analyses and a Euclidean distance dissimilarity matrix was used. If significant differences occurred, pair-wise *post hoc* tests were used to assess which sites differed. Non-metric multidimensional scaling (MDS) and canonical analysis of principal coordinates (CAP) were used to compare otolith elemental

signatures between acidified and control sites. In particular, MDS was used to identify sites of fish origin (core region analysis) and CAP analysis was used to distinguish among sampling sites (edge region chemical composition). For all CAP and PERMANOVA tests, we used 999 unrestricted random permutations of the raw data. A cross-validation (or leave-one-out) procedure was used to assess how accurately samples were assigned to sites in the canonical space (Anderson and Willis, 2003). In addition, PERMANOVA was used to test statistical differences in stable isotope composition of otoliths of each species between sampling sites.

Furthermore, differences in otolith shape (using elliptical Fourier descriptors) of each species among sites were also tested using PERMANOVA and CAP analysis, using the same experimental design as that used for the elemental otolith composition. Otolith relative length (otolith/fish size ratio) was analysed using ANOVA to test differences in terms of morphometric variables.

5.3 Results

5.3.1 Water chemical analysis

Chemical seawater composition showed a greater temporal variability than spatial one. Univariate PERMANOVA showed significant differences for all the elements analyzed for the interaction 'Site x Date', and only Li showed significant differences for the factor Date. Pair wise *post hoc* tests did not show a general trend for the elements analyzed (**Tab. 5.2**). In addition, analysis on dissolved inorganic carbon ($\delta^{13}C_{DIC}$), showed significant differences between dates, with pair wise tests showing the main difference between winter and all other dates (see **Tab. 5.2** and **Fig. 5.2**).

Table 5.2 *- ANOVA results for water elemental composition and $\delta^{13}C_{DIC}$ comparing among sampling sites and dates. Probability levels: n.s. = not significant; * = p < 0.05; ** = p < 0.01.*

Source		Ca			Mg			Sr			Li		
	df	**MS**	**Pseudo-F**	**p**	**MS**	**Pseudo-F**	**p**	**MS**	**Pseudo-F**	**p**	**MS**	**Pseudo-F**	**p**
Site	4	3.24e-2	0.91	n.s.	3.13e-4	0.30	n.s.	1.20e-3	0.37	n.s.	2.66e-3	2.02	n.s.
Date	3	1.14	730.55	**	0.11	358.3	**	0.16	153.46	**	0.11	68.58	**
Site x Date	12	0.44	23.35	**	1.07e-3	3.34	**	3.31e-3	3.17	**	1.31e-3	0.79	n.s.
Residual	35	1.56e-3			3.20e-4			1.04e-3			1.65e-3		
		Ba			**Mn**			**Zn**			**$\delta^{13}C_{DIC}$**		
	df	**MS**	**Pseudo-F**	**p**	**MS**	**Pseudo-F**	**p**	**MS**	**Pseudo-F**	**p**	**MS**	**Pseudo-F**	**p**
Site	4	0.26	1.34	n.s.	13.08	9.16	**	9.45e-2	0.21	n.s.	16.05	43.78	n.s.
Date	3	4.90	420.9	**	0.16	4.15	**	7.21	97.49	**	3.50	9.55	**
Site x Date	12	0.20	17.28	**	1.46	37.59	**	0.45	6.09	**	1.58	4.30	n.s.
Residual	35	1.16e-2			3.88e-2			7.40e-2			0.37		

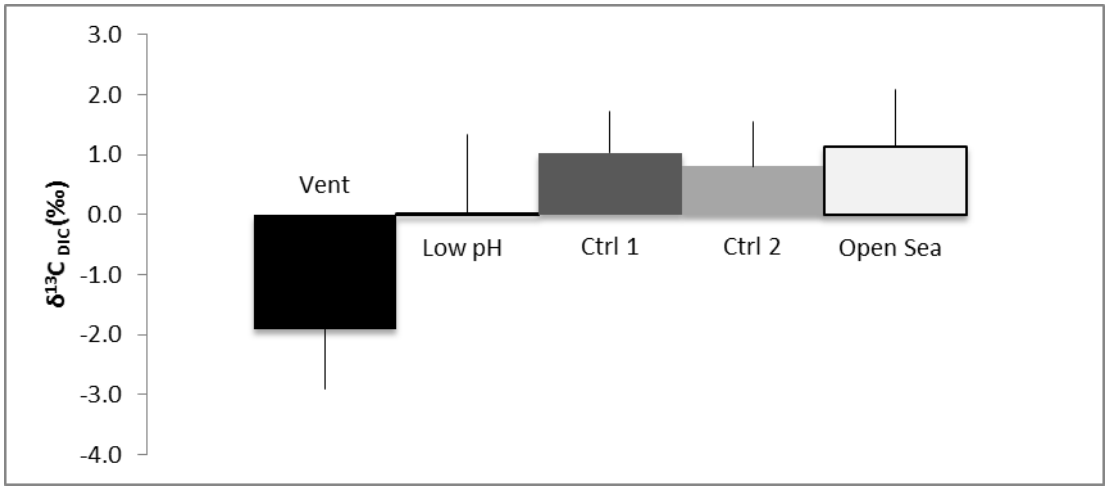

Fig. 5.2 *- Seawater dissolved inorganic carbon ($\delta^{13}C_{DIC}$, mean ±SD) in the Vent, Low pH, Ctrl 1, Ctrl 2 and Open Sea sites.*

Also multi-elemental analysis showed significant differences for the interaction 'Site x Date' (**Tab. 5.3)**. Generally, pair-wise *post hoc* tests showed that Vent and Low pH differed from the other sites in winter and fall, while this trend was not true in summer and spring when all

the sites showed differences. Consistent with PERMANOVA, CAP analysis showed a similar pattern for the Vent and the Low pH sites, compared to Ctrl 1, Ctrl 2 and Open Sea (**Fig. 5.3**).

Table 5.3 *- PERMANOVA results for the multi-elemental water composition comparing among sampling sites and dates.*

Source	*df*	**MS**	**Pseudo -F**	**P (MC)**
Site	4	14.81	121.17	0.001
Date	3	16.73	136.90	0.001
Site x Date	12	2.34	19.16	0.001
Residual	40	0.12		

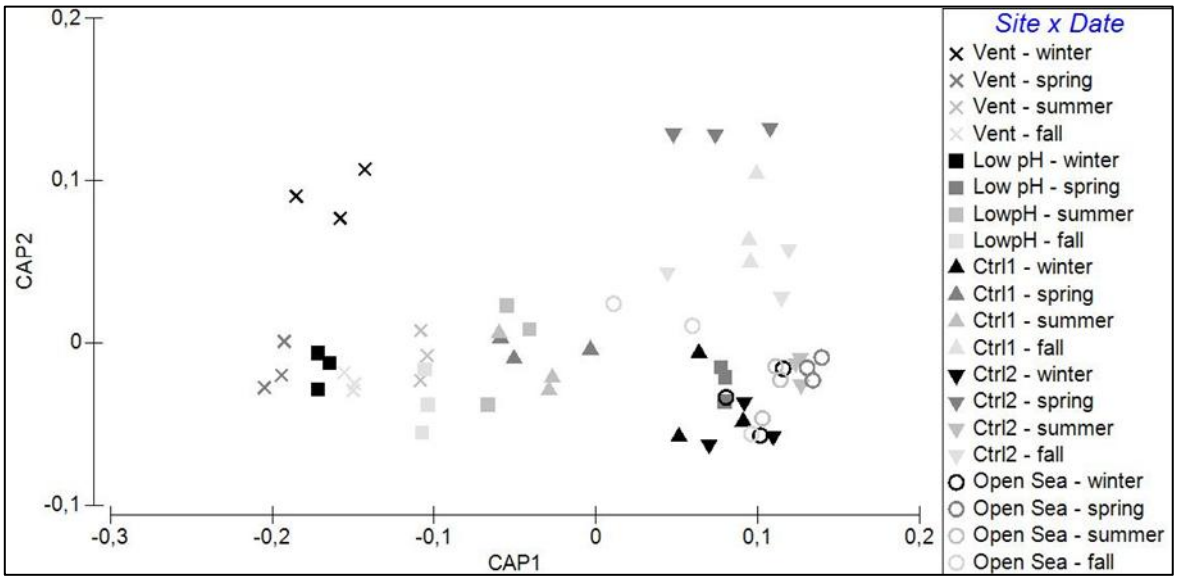

Fig. 5.3 *- CAP on multi-elemental seawater analysis for the four dates (winter, spring, summer and fall) at the Vent, Low pH, Ctrl 1, Ctrl 2 and Open sea sites.*

5.3.2 Otolith shape and morphometric analysis

Otolith shape (EFDs) differed significantly between sites for *G. bucchichi* (PERMANOVA: Pseudo-$F_{2, 21}$= 3.25, p(MC) < 0.01) and *D. vulgaris* (Pseudo-$F_{2, 23}$ = 1.95, p(MC) < 0.05).

Pair-wise *post hoc* tests confirmed significant differences between Low pH and the two controls for *G. bucchichi*, and a significant difference between Low pH and Ctrl 2 for *D. vulgaris*. These results were supported by the CAP analysis and cross validated results of 67% and 65% for *G. bucchichi* and *D. vulgaris*, respectively (**Fig. 5.4**). For the other species, no significant differences were found between sites (*C. chromis*: Pseudo-$F_{2, 33} = 1.17$, $p > 0.05$; *C. julis*: Pseudo-$F_{2, 32} = 1.52$, $p > 0.05$; *S. salpa*: Pseudo-$F_{2, 18} = 1.34$, $p > 0.05$; *S. ocellatus*: Pseudo-$F_{1, 20} = 0.34$, $p > 0.05$). However, *C. julis* and *S. salpa* in the CAP analysis showed a similar pattern for the two controls compared to the Low pH, with a cross-validation result of 63% and 62%, respectively, whereas *C. chromis* and *S. ocellatus* showed lower levels of correct classification (44% and 59%, respectively).

Otolith relative length (ORL) showed significant differences for *D. vulgaris* between sites (ANOVA: $F_{2,23} = 16.20$, $p < 0.001$) and Tukey's *post-hoc* test confirmed that the Low pH site differed from the two controls. No significant differences in ORL were found for the other species between sites ($p > 0.05$).

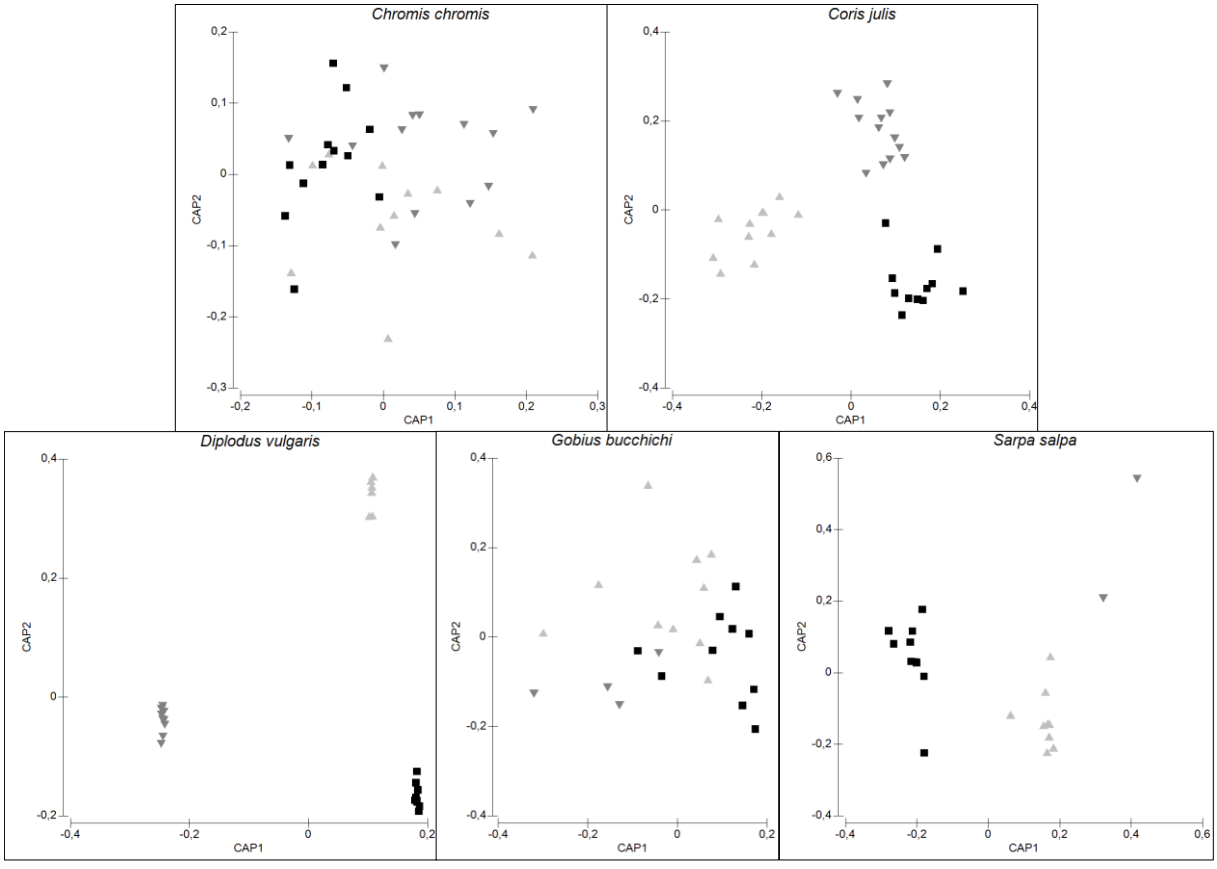

Fig. 5.4 *- CAP analysis of otolith shape (elliptical Fourier descriptors) for Chromis chromis, Coris julis, Diplodus vulgaris, Gobius bucchichi, and Sarpa salpa from Low pH (■), Ctrl 1(▲) and Ctrl 2 (▼). S. ocellatus not shown, as species was only present in two sampling sites.*

5.3.3 Otolith chemical analysis

The concentration of single elements varied significantly for some species, but no common patterns in the element:Ca ratios could be observed either among species or sites, both in the edge and core regions (**Fig. 5.5** and **Fig. 5.6**, respectively). In particular, analyses of variance revealed significant differences in the concentration of Sr in otolith edge of *C. julis* (ANOVA: $F_{2, 22} = 5.88$; $p < 0.01$), of Ba in *D. vulgaris* ($F_{2, 22} = 7.76$; $p < 0.01$), of Cu in *S. ocellatus* ($F_{1, 22} = 6.49$; $p < 0.05$) and Pb in *S. salpa* ($F_{2, 22} = 4.02$; $p < 0.05$). Tukey's *post hoc* tests revealed that the Low pH was different compared to Ctrl 2 for *C. julis* and *S. salpa*; and low pH site was different compared to the two control sites for *D. vulgaris*. Analysis of variance on the concentration of single elements (Na, Mg, Ca, Mn, Cu, Zn, Sr, Ba and Pb) in the core region did not show any significant differences for *C. julis*, *D. vulgaris*, *G. bucchichi* and *S. salpa*. For *C. chromis,* significant differences in Sr were found (ANOVA: $F_{2, 27} = 4.51$; $p < 0.05$), and Tukey's *post hoc* tests revealed that Ctrl 1 was significantly different to Low pH and Ctrl 2. Significant differences between Low pH and Ctrl 1 were also found for Na in *S. ocellatus* ($F_{2, 18} = 4.51$; $p < 0.05$), Cu ($F_{2, 18} = 4.51$; $p < 0.05$) and Pb ($F_{2, 18} = 4.51$; $p < 0.05$).

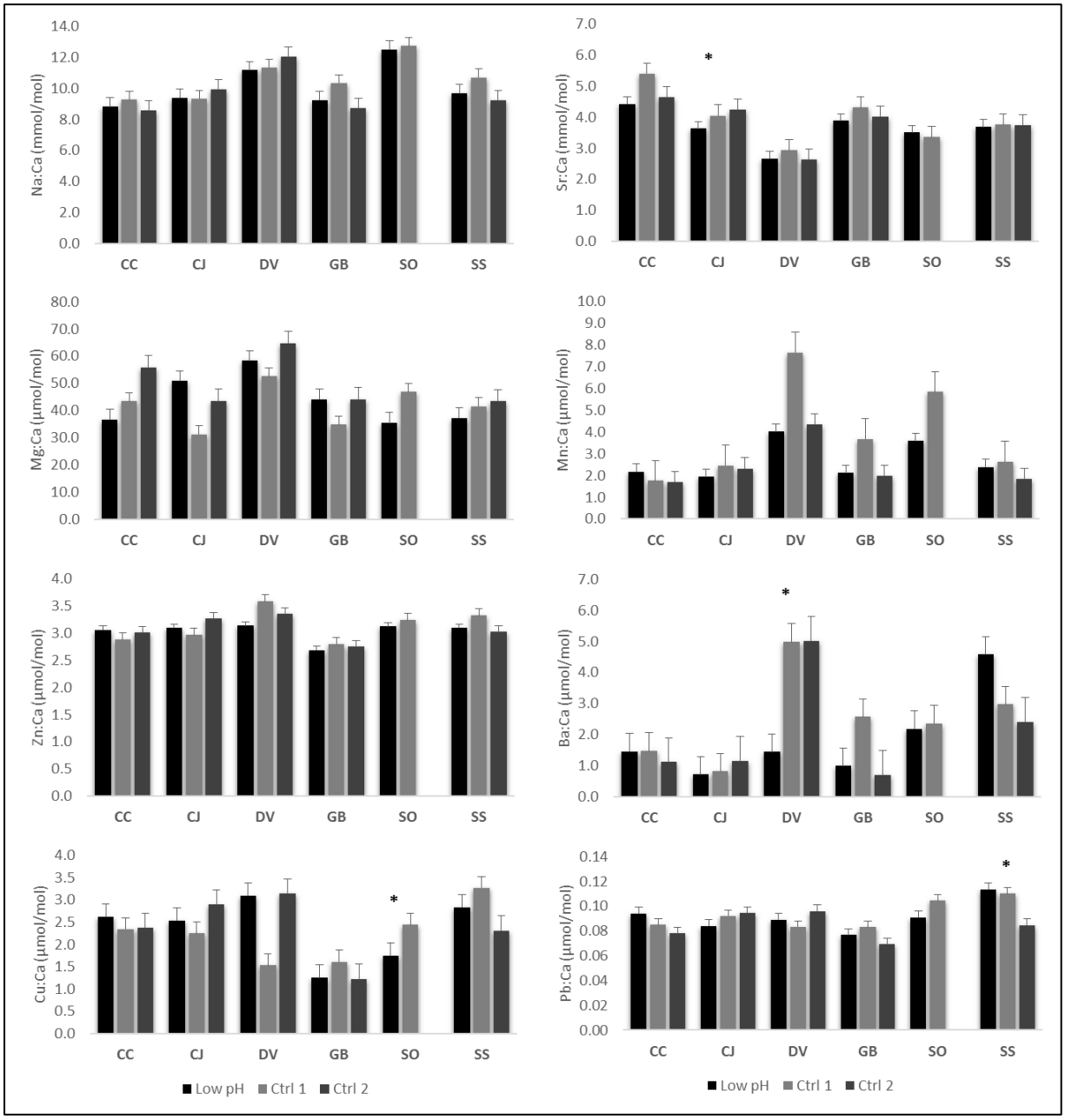

Fig. 5.5 *- Otolith edge concentrations (mean + SE) of Na, Sr, Mg, Mn, Zn, Ba, Cu, and Pb for Chromis chromis (CC), Coris julis (CJ), Diplodus vulgaris (DV), Gobius bucchichi (GB), Symphodus ocellatus (SO), Sarpa salpa (SS) in the three sampling sites (Low pH, Ctrl 1 and Ctrl 2). Significant differences among sites ($p < 0.05$) per species are showed by asterisks.*

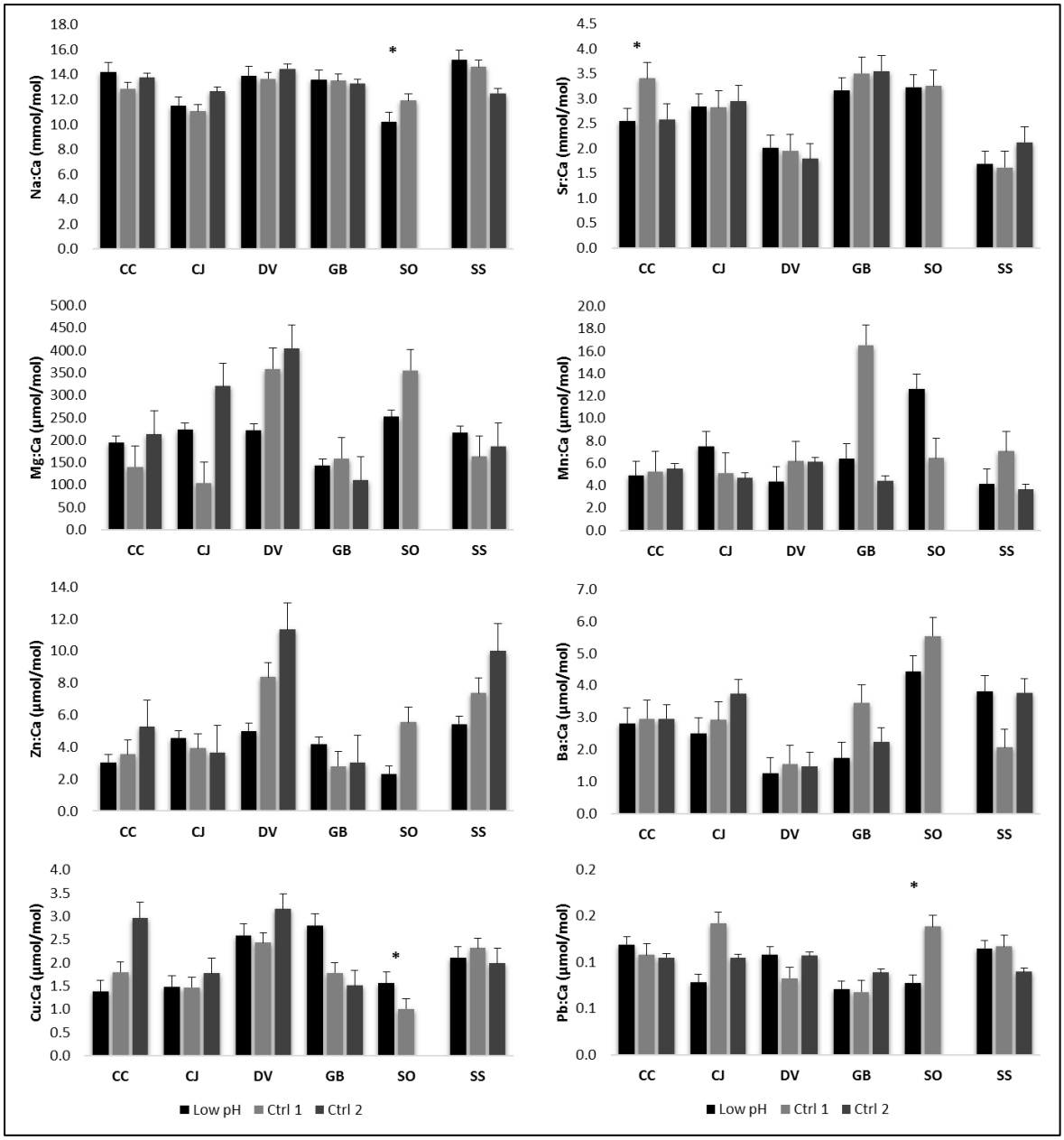

Fig. 5.6 *- Otolith core concentrations (mean + SE) of Na, Sr, Mg, Mn, Zn, Ba, Cu, and Pb for Chromis chromis (CC), Coris julis (CJ), Diplodus vulgaris (DV), Gobius bucchichi (GB), Symphodus ocellatus (SO), Sarpa salpa (SS) in the three sampling sites (Low pH, Ctrl 1 and Ctrl 2). Significant differences among sites (p < 0.05) per species are showed by asterisks.*

The multi-element compositions differed between the two otolith regions (core and edge). No significant differences between sites were found for the core region for all the species analyzed (PERMANOVA, $p > 0.05$) and also ordination via MDS did not show patterns among sites. In contrast, edge elemental analysis showed differences between sites for *D. vulgaris* (PERMANOVA: Pseudo-$F_{2, 22} = 4.0123$; $p < 0.01$). Pair wise *post hoc* tests revealed that the low pH site was significantly different compared to Ctrl 1 and 2. No significant

differences were found in multi-elemental concentration for *C. chromis*, *C. julis*, *G. bucchichi* and *S. salpa*. For these species, although PERMANOVA on edge was not significant, CAP analysis generally successfully classified fish sampled in the Low pH site and the two control sites, namely *S. ocellatus* (83 %) and *C. chromis* (76 %) (**Tab. 5.4**).

Table 5.4 *- Cross-validation results from CAP analysis on otolith edge multi-elemental analysis. Results are given as percentage of the total fish classified for each site.*

	C. julis	*C. chromis*	*D. vulgaris*	*G. bucchichi*	*S. salpa*
Low pH	75	77.8	81.8	28.6	60
Ctrl 1	66.7	70	33.3	70	63.6
Ctrl 2	63.6	81.8	62.5	75	25

Analysis of otolith isotopic composition ($\delta^{13}C$ and $\delta^{18}O$) did not show significant differences among sampling sites for *C. julis*, *G. bucchichi* and *S. salpa*, with the exception of *D. vulgaris* (PERMANOVA core: Pseudo-$F_{2, 16} = 10.70$; $p < 0.01$; edge: Pseudo-$F_{2, 22} = 7.94$; $p < 0.01$). Pair-wise *post hoc* tests confirmed significant differences between low pH and the two control sites for the core region, while for the edge low pH and control 1 showed significant differences with regards to control 2. In general, isotopic results were similar between sites, with most otoliths from controls with more depleted values than the Low pH site, above all for the edge region (with the exception of *S. salpa*). Overall, isotopic values were more depleted in the core region than in otolith edges for all species (**Fig. 5.7**).

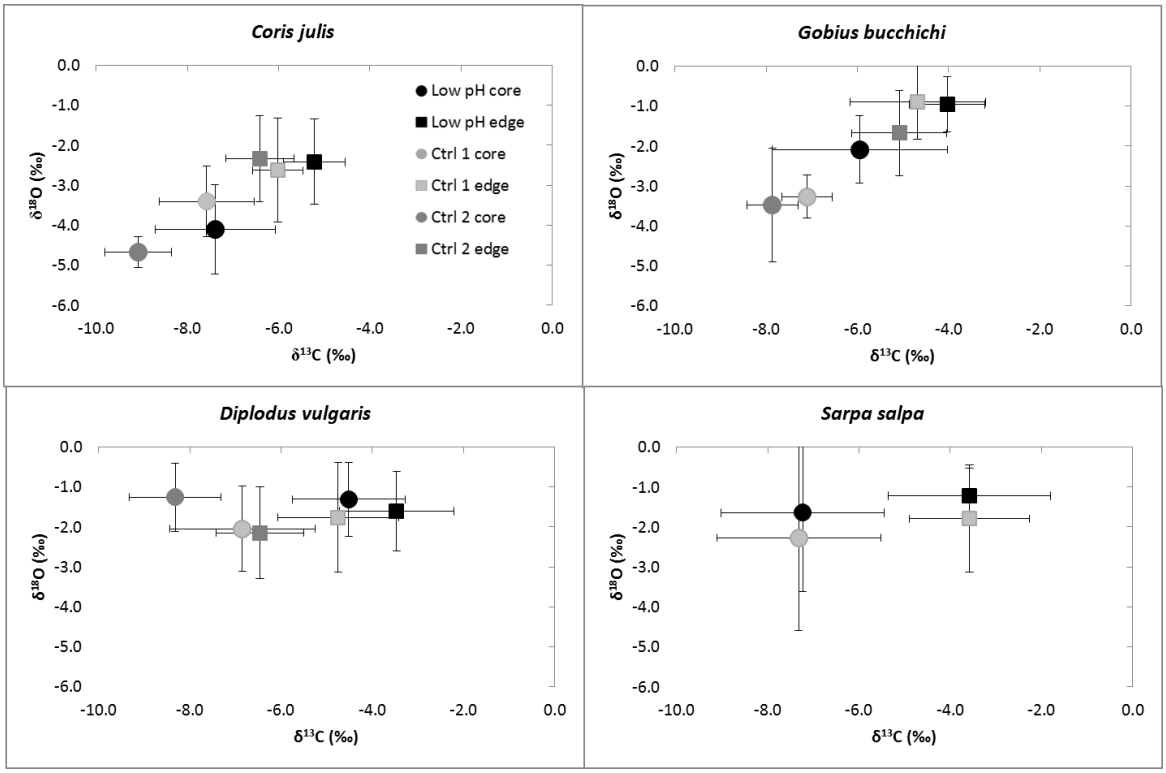

Fig. 5.7 *- $\delta^{13}C$ and $\delta^{18}O$ (mean + SD) in otolith edge and core of* Coris julis, Diplodus vulgaris, Gobius bucchichi, *and* Sarpa salpa *in the three sampling sites (Ctrl 1, Ctrl 2 and Low pH).*

5.4. Discussion

Ocean acidification is known to morphologically alter carbonate structures of most marine invertebrates (like foraminifera, coccolithophorids, echinoderms and molluscs - Doney et al. 2009). However, much less information is available on structural changes that may occur in fish calcified structures, such as otoliths, and it is based exclusively on laboratory experiments (Bignami et al. 2013, Checkley et al. 2009, Maneja et al. 2013, Munday et al. 2011a, 2011b, Réveillac et al. 2015). In this study, we investigated the effects of ocean acidification on otolith morphology and chemistry in fish exposed long-term to high CO_2 / low pH conditions, in a naturally acidified ecosystem. We found an effect of acidification on otolith shape on two out of six species analysed, as found in previous studies, while no clear consistent effects were observed in chemical and isotopic signatures.

Here, otolith shape responded significantly to acidification in *G. bucchichi* and *D. vulgaris*, with other two species revealing moderate effects (*C. julis* and *S. salpa*). These species are

benthic or nekton-benthic, and in particular, *G. bucchichi* is a highly site-attached species, showing a territorial behaviour. and the juveniles specimens of *D. vulgaris* are highly territorial (elevated site fidelity for *Diplodus* spp. as shown by Di Lorenzo et al. 2014). In contrast, *C. chromis*, the only species without clear otolith shape variations between acidified and control sites is a pelagic and mobile species in comparison to the others investigated in this study. Results follow previous laboratory studies (*i.e.* Bignami et al. 2013, Checkley et al. 2009, Maneja et al. 2013, Réveillac et al. 2015) showing that otolith morphology can be affected by different CO_2 levels, with observed differences in shape likely determined by the duration of exposure to the elevated CO_2 conditions, with more mobile species (*i.e. C. chromis*) showing less structural differences as they experience elevated CO_2 for shorter time periods. Comparisons of otolith relative length highlighted a difference between sites only for *D. vulgaris*. However, other laboratory studies have not find differences in otolith shape of species reared in low pH conditions (*i.e.* Atlantic cod *Gadus morhua* and the clownfish *Amphiprion percula*; Maneja et al. 2013 and Munday et al. 2011b, respectively), suggesting species-specific responses, probably depending on time of exposure, levels of pCO_2 or species sensibility (Munday et al. 2011b).

Otolith size and relative densities change the ability of fish to detect sounds (Bignami et al. 2013), and otolith asymmetry can have deleterious effects on fish survival (Gagliano et al. 2008), with possible consequences on fish population recruitment (Munday et al. 2010). Although adults can survive at high CO_2 levels, acidification can affect their sensory systems, with consequences on olfactory (Dixson et al. 2010) and auditory (Simpson et al. 2011) processes. This sensory alteration can have important implications on fish behaviour (Munday et al. 2014). Nowadays, there is a need to integrate laboratory, mesocosm and field experiments to improve our understanding of the effects of ocean acidification (Riebesell & Gattuso, 2015). CO_2 vents are naturally acidified environments that provide a great opportunity to investigate organisms normally exposed long-term to future ocean conditions.

Although we found differences between the vent and the low pH compared to the other sites in water chemistry, mostly in winter and fall, and there was a clear influence of proximity to the primary vent in terms of water characteristics, our analysis revealed that otolith elemental composition was not sufficiently different to successfully differentiate fish captured in sites with different pH conditions. This is in accordance with a previous laboratory experiment where no significant differences were found in otolith chemistry (Li, Mg, Mn, Sr and Ba) of fish reared at different pH levels (pH = 8.15, 7.7 and 7.6 - Munday at al. 2011). Overall, the

small spatial scale of the study, together with variations in water chemistry over time, may have contributed to the homogenizing of otolith elemental fingerprints (*i.e.* Hamer et al. 2003, Reis-Santos et al. 2012, Swearer et al. 2003). In addition, seawater chemistry is relatively constant compared to estuarine environments, and at short distances variations among sites can be difficult to detect, as found in previous studies in marine environments (Gillanders et al. 2001).

Significant differences between sites were confirmed by edge otolith chemistry only for juvenile *D. vulgaris*, while for other species (*C. chromis*, *C. julis* and *S. salpa*) multivariate ordination plots suggested a separation between low pH and control sites, although significant differences were not found. Surprisingly, the most sedentary species studied (*G. bucchichi*) showed significant differences in shape analysis, but not in elemental signature. In contrast, core elemental compositions did not reveal spatial differences. This lack of difference is probably indicative of fish having a common natal origin (*i.e.* same spawning area) or chemical variations not being sufficient to distinguish between natal origin sites (Papetti et al. 2013). All the species investigated in this study have a pelagic larval stage that ranges between 10 and 30 days (Raventos & Macpherson, 2001), which could mean that the natal origin is common coastal spawning areas away from Aeolian Archipelago.

Contrary to our expectation, otolith isotopic signatures ($\delta^{13}C$ and $\delta^{18}O$) did not show a clear pattern among sites with different pH. Although dissolved inorganic carbon changes along the Levante Bay, with a clear gradient from the primary vent towards the control area, otoliths did not record this information, probably due to the high variability of these natural sites in terms of both pH and DIC. Fish analysed in this study seem to exploit the low pH site (confirmed both by underwater visual census and stable isotope analysis on muscle tissue carried out in the same study area for the same fish - see Chapters 2 and 3), however otolith isotopic composition seems to have a limited ability to characterize collection areas or movements in this volcanic area. This result is in contrast with a previous study conducted by Fraile et al. (2016) where a depletion in $\delta^{13}C$ found in otoliths was correlated to higher CO_2 anthropogenic emissions.

As found in previous studies, otolith elemental composition can vary among species, depending on physiological mechanisms (Chang & Geffen, 2013, Reis Rantos et al. 2008, Sturrock et al. 2015). Here, the six fish species showed a different response to the low pH environment, which may be related to species-specific capacity for pH regulation. The natural

variability in exposure to CO_2 in the vent area, together with the different response of each species to similar conditions, habitat range or use patterns could explain the diversity of our results. Some species, like the most site-attached *G. bucchichi*, showed significant differences in otolith shape between sites, but did not seem affected by high CO_2 / low pH conditions. Indeed, Nagelkerken et al. (2015) found that, although the escape speed is lower compared to control sites, the density of this species is higher at the vent site. In addition, juveniles of *D. vulgaris*, another abundant species at the Vulcano low pH site (Chapter 2), does not seem disturbed by low pH. This was the only species that differed both in terms of otolith composition (elements and isotopes) and shape, and is also renowned for its site fidelity as juveniles (Di Lorenzo et al. 2014). On the other hand, the herbivore *S. salpa* and other more mobile species (*C. chromis* and *S. ocellatus*) did not show differences in terms of shape or otolith signature. These species likely do not use this particular site permanently but may roam there for foraging as suggested by the stable carbon and nitrogen isotope analysis on muscle (Chapter 3).

Otolith chemistry does not seem a good proxy to follow a pH / DIC gradient in this naturally acidified area, but the different shape in otoliths indicates that these could be valid natural tags to identify habitat use and discriminate among populations. Other species, likely less tolerant, do not permanently occupy the acidified environment, but likely use it to their benefit (*i.e.* foraging, protection) and take advantage from their mobility to escape long-term exposure to high CO_2 / low pH conditions. The lack of difference in chemistry has important implications on the applicability of using otoliths as natural tags for connectivity assessments, habitat use and reconstruction of fish environmental life histories, in a context of global change. It is also important to understand what are the consequences of differences in otolith shape and morphology on fish physiology and behavior, or indirect consequences on species interactions and fish survival. Moreover, there is a need to understand the implications these differences could have on fishery resources and socioeconomic issues in future acidified oceans.

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CHAPTER 6: General Conclusion

We used a naturally acidified environment to study the structural and functional organization of fish assemblages exposed to different pH levels, comparing a low pH site and two controls. Overall our findings suggest that the fish community seems able to withstand the projected increase in seawater CO_2 and the concomitant lowering of pH. Fish community showed a slight decrease in species richness in the Low pH site compared to controls, and single species abundance and frequency did not show a unique spatial trend, although different among sites, suggesting a species-specific responses (**Chapter 2**). Moreover, fish exploit the vent area for feeding, as confirmed by the carbon and nitrogen isotopic depletion found in almost all the species. However, marked structural changes did not occur although a slight lower trophic level and higher species packing as highlighted by the isotopic niche analysis was found in the Low pH (**Chapter 3**). Accordingly to trophic organization analysis, the greater accumulation of mercury in fish exposed to acidified conditions corroborates the result that fish exploit the vent area. Although we found an increasing in Hg accumulation along the food chain, levels were always below the European Union Maximum Residue Limits and trophic magnification factor was comparable among sites (**Chapter 4**). Also otolith analysis revealed a species-specific response, morphologic alterations was recorded only in two out of six species analysed, showing that sensitivity to high CO_2 emissions / low pH conditions can vary among species (**Chapter 5**). Moreover, elemental chemical signature of the otoliths exposed to different pH levels was similar, suggesting important implications towards the applicability of using otoliths as natural tags for connectivity assessments, habitat use and reconstruction of fish environmental life histories, in a context of global change.

Results on otoliths of some fish species analysed in this study suggest that each species can tolerate in a different way particular conditions of high CO_2 levels (Chapter 5). For instance, the small-sized specimens *D. vulgaris* showed differences in the otolith shape, suggesting that this species is more tolerant than others and spend more time in the low pH conditions, registering the otolith morphological alteration. As found by Nagelkerken et al. (2015) in the same vent of Vulcano Island, the density of *Gobius bucchichi* (a small-sized, cryptic and highly territorial species not censused in this study) is higher in the Low pH site than in controls. Hence, each species can be more or less tolerant and use this area in a different way for different time. On the other hand, large-sized species (*i.e. S. salpa*) with a wider home range, did not show alteration in otolith shape and could exploit this area for feeding. Another

possible explanation, indeed, is that fish are attracted by abundant prey found in the Low pH site (Vizzini et al. submitted), exploiting the site for feeding as confirmed by stable isotopes (Chapter 3). In this way, the higher cost to balance the acid-base regulation may be compensated by a high prey availability. Indeed, a higher abundance of invertebrates (mainly Amphipoda and Polychaeta) associated to macrophytes was found in the low pH site of Vulcano Island, due to a combined effect of high CO_2, which on one hand drives bottom-up forces boosting primary producers (*i.e.* resource-effect), and on the other hand hinders top-down controls through loss of carnivorous species (*i.e.* stressor-effect), generally less tolerant to ocean acidification.

Contrary to expectation, slight differences were found in the structure and species composition at community level, but more remarkable differences were found at species level. Moreover, although expected negative impacts on larvae and juveniles (Baumann et al. 2012, Ishimatsu et al. 2004, Pankhurst & Munday, 2011), the abundance of small-sized individuals in the Low pH site was comparable to that of the two controls. In addition, the behavioural differences found both *in situ* experiments and in field observations (Munday et al. 2014, Nilsson et al. 2012), did not alter directly the fish community structure. Although species richness was slightly lower, similarity of Low pH and Ctrl 1 sites may be due to mobility of large-sized individuals. This may be one of the possible explanations for the lack of differences at community level. Highly mobile organisms such as fish, cephalopods and some crustaceans that are capable of controlling extracellular pH through active ion transport are predicted to be less sensitive to ocean acidification (Melzner et al. 2009, Portner, 2008). On the other hand, population replenishment may occur from outside the vent area, from 'not acidified populations' as suggested by Kroeker et al. (2012) and Munday et al. (2014) in other vents worldwide. Indeed, most fish species analyzed have a long pelagic larval stage ranging between 10 and 30 days (Raventos & Macpherson, 2001).

Another possible explanation for the lack of difference could be related to plastic or adaptive responses after long-term exposure to high CO_2 environmental conditions. Indeed, contrary to laboratory experiments that investigate effects of short-time exposure to low pH and sharp lowering of pH (Ishimatsu et al. 2004), fish may take advantage from the natural fluctuations in carbonate chemistry at the vents to tolerate better or adapt, generation after generation, to these particular conditions (Calosi et al. 2016, Kroeker et al. 2012). Field observations permit to evaluate the rate of 'evolutionary adaptation' (Crozier & Hutchings, 2014, Sunday et al. 2014). As an example, the ocellated wrasse *S. ocellatus* offspring brooded in different CO_2

conditions had similar responses, but after transplanting portions of nests to the high-CO_2 site, embryos from parents that spawned in ambient conditions had higher metabolic rates (Cattano et al. 2016). Moreover, Allan et al. (2016) suggested transgenerational acclimation for some behavioural traits in juveniles reef fish exposed long-term to high CO_2 levels. Thus, adaptive mechanisms can have a crucial role for fish, improving their resilience to environmental conditions. Emblematic is the case of *G. bucchichi*, which is a highly sedentary fish: it does not seem negatively affected by high CO_2 / low pH conditions in terms of population width, although some altered behavioural aspects were found in field experiments (Nagelkerken et al. 2016). On the other hand, some species, likely less tolerant, can exploit their higher mobility to 'escape' adverse conditions (*i.e. S. salpa* or *C. chromis*). As a consequence, of species tolerance, indirect effects on species interactions are difficult to forecast.

Moreover, species can be indirectly affected by other factors, such as food availability and quality. For instance, copepods, which are basal sources in marine ecosystems and a major food source for fish, appear resilient to ocean acidification, but can be indirectly impacted by the lowered quality of microalgae, which they feed on (Rossoll et al. 2012). In addition, ocean acidification is expected to act synergistically with other stressors such as warming and pollution, and the combined effects of multiple pressures may pose a crucial issue to seafood security and quality with economic repercussions (Branch et al. 2013, Zeng et al. 2015). As an example, Kang (2011) correlated the main consequences of climate change (increase in atmospheric carbon dioxide, UV irradiation, and ocean temperatures) with a decrease in omega-3 fatty acid contents in phytoplankton (basal source in food web), and consequently in higher trophic levels including species consumed by humans. Therefore, a decrease in these essential molecules for optimal human growth and development may have detrimental effects on health, increasing risk for various diseases.

Here we dealt with the effect of a single stressor (acidification) and we found minor changes in terms of direct effects of lowered pH on fish community and trophic structure. Some differences were found at the species level and may be indirectly related to ocean acidification (*i.e.* through habitat modification - Munday et al. 2014, Nagelkerken et al. 2015, Sunday et al. 2016). Many studies highlight that most species will be more sensitive when subjected to multiple stressors (*i.e.* acidification and warming, considered as two of the greatest threats to marine biodiversity - Harvey et al. 2013, Kroeker et al. 2013, Nagelkerken & Connell, 2015). Accordingly, it is important to consider that acidification is not expected to

act alone, but in a synergetic way with other global and local stressors (*i.e.* warming, invasive species, eutrophication and pollution - Kroeker et al. 2013, Nagelkerken et al. 2015, Russell et al. 2009).

This study provided a complete and exhaustive frame of fish assemblages structure and trophic organization at different pH levels. As scant data are available in the literature on this topic, the results of this research provide information about the ecological effects of long-term exposure to high CO_2 levels on fish, a key biological component whose monitoring is relevant not only from the ecological side, but also for the economic one and for the implications on human health. Moreover, this study confirms the importance to use the naturally acidified environments to test ecological hypotheses on the effects of ocean acidification on communities and ecosystems. However much caution must be used to discuss the results as they can be bias by confounding variables characterizing the vent area (such as trace elements). Finally, we recommend an integration of experiments from different research approaches to better understand and forecast the effects of ocean acidification on marine life. At the same time, we need to reverse the trajectory of global CO_2 emissions, starting to maintain under control our own carbon footprint and to follow green choices towards a more sustainable way of life.

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