

Synergistic effects of hypoxia and increasing CO₂ on benthic invertebrates of the central Chilean coast

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Ocean acidification (OA) and hypoxic events are an increasing worldwide problem, but the synergetic effects of these factors are seldom explored. However, this synergetic occurrence of stressors is prevalent. The coastline of Chile not only suffers from coastal hypoxia but the cold, oxygen-poor waters in upwelling events are also supersaturated in CO₂, a study site to explore the combined effect of OA and hypoxia. We experimentally evaluated the metabolic response of different invertebrate species (2 anthozoans, 9 molluscs, 4 crustaceans, 2 echinoderms) of the coastline of central Chile (33°30'S, 71°37'W) to hypoxia and OA within predicted levels and in a full factorial design. Organisms were exposed to 4 different treatments (ambient, low oxygen, high CO₂, and the combination of low oxygen and high CO₂) and metabolism was measured after 3 and 6 days. We show that the combination of hypoxia and increased pCO₂ reduces the respiration significantly, compared to a single stressor. The evaluation of synergistic pressures, a more realistic scenario than single stressors, is crucial to evaluate the effect of future changes for coastal species and our results provide the first insight on what might happen in the next 100 years.

Keywords: hypoxia, ocean acidification, Chile, invertebrates, respiration rate

Introduction

The term Ocean Acidification (OA) is used to describe the decline in seawater pH due to the invasion of ocean waters by anthropogenic CO₂ (Caldeira and Wickett, 2003; Caldeira, 2005; Orr et al., 2005; Raven, 2005). About 1/3 of the CO₂ released by human activity since the industrial revolution has entered the ocean, leading to a decline in surface pH values by ~0.12 units, with a further decrease of 0.3–0.4 units predicted for a doubling of atmospheric CO₂ by the end of the century (Orr et al., 2005; Doney et al., 2009). These decreases in pH are expected to have negative but variable effects specifically on calcifying organisms as altered carbonate chemistry directly affects the deposition and dissolution rates of the CaCO₃ used for structures (Gattuso and Buddemeier, 2000; Orr et al., 2005; Raven, 2005; Kleypas et al., 2006; Gazeau et al., 2007;

Fabry et al., 2008; Range et al., 2011; Andersson and Gledhill, 2013; Kroeker et al., 2013). Calcifying organisms, such as corals (Marubini and Davies, 1996; Gattuso et al., 1998; Marubini and Atkinson, 1999; Langdon et al., 2000), coral reef communities (Langdon et al., 2000, 2003; Leclercq et al., 2000), and planktonic organisms (Bijma, 1991; Riebesell et al., 2000), are known to be among the most vulnerable. Nevertheless, it has been shown in several studies, that some corals and molluscs were able to calcify and grow even faster when transplanted along carbonate saturation gradients (Rodolfo-Metalpa et al., 2011). Even under low pH some species are able to maintain, or even increase, their net calcification, indicating that the use of carbonate saturation state is inconsistent to predict marine calcification (Wood et al., 2008; Cohen et al., 2009; Ries et al., 2009; Rodolfo-Metalpa et al., 2010). As reviewed by Hendriks et al. (2015), these organisms have different mechanism to cope with OA. Close to the organism's surface the pH can be higher through metabolic activity since rate limiting transport in the Diffusive Boundary Layer (DBL) prevents a direct equilibration with the water column (Hendriks et al., 2015). In foraminifera and diatoms the pH in the DBL ranges from 8.0 to 9.1 (Köhler-Rink and Kühl, 2005; Kühn and Raven, 2008), which allows them to create a microenvironment with increased pH (by 0.5 units) compared to ambient seawater. Moreover, calcifying organisms are able to control the pH in extracellular fluids, or control the deposition in a regulated, intracellular environment. Tissues and external organic layers play a major role in protecting shells and skeletons from corrosive sea water, limiting dissolution, and allowing organisms to calcify (Ries, 2011; Trotter et al., 2011). Some organisms can benefit from symbiotic relationships, e.g., coral symbionts remove CO₂ and increase pH due to photosynthesis, enhance conditions for calcification and growth (Gattuso and Jaubert, 1990; Muscatine, 1990).

Whereas increasing atmospheric CO₂ clearly drives OA in the open ocean, drivers of changes in pH and the carbonate system in coastal systems are far more complex (Duarte et al., 2013; Waldbusser and Salisbury, 2014). Coastal ecosystems, unlike the surface waters of the open ocean, may display a diversity of pH trajectories, affected by emissions from volcanic vents, watershed processes, eutrophication, upwelling, and changes in ecosystem structure and metabolism (Duarte et al., 2013). Therefore, the carbon system of the coastal ocean is more dynamic and complex than that of the open ocean (Borges and Gypens, 2010; Cai, 2011), and thereby, a general prediction of the trajectories of pH for coastal systems is difficult to make, as regional differences will be important (Duarte et al., 2013). In these shallow environments benthic engineering species, such as corals, seagrass, macroalgae, salt marshes, mangroves, sponges, and oyster reefs, have the capacity to affect the chemical and physical conditions of the ecosystem (Gutierrez et al., 2011), and exert metabolic control on coastal seawater pH values and variability (Duarte et al., 2013).

Coastal ecosystems are also progressively affected by hypoxia, with a current rate of increase of $5.5 \pm 0.2\%$ year⁻¹ in coastal areas (Vaquer-Sunyer and Duarte, 2008), and predicted of faster increase in the future (Conley et al., 2009). Hypoxia, is a condition characterized by oxygen levels below a threshold where marine organisms show atypical behavior (Riedel et al., 2013) and

eventually leads to mass mortality (Diaz and Rosenberg, 1995; Vaquer-Sunyer and Duarte, 2008). It is typically triggered by respiratory consumption of oxygen to remineralize the excess of organic matter produced in eutrophic coastal systems (Gray et al., 2002). Accordingly, hypoxic coastal waters are characterized by low O₂ concentrations and elevated CO₂, and, therefore, low pH (Pörtner et al., 2005). This is also the case of coastal areas affecting by upwelling of oxygen-poor, corrosive waters, such as the Oregon and Washington coasts (Feely et al., 2008; Gruber et al., 2012) and much of the Chilean coast (Mayol et al., 2012). Yet, the bulk of the literature on the impacts of hypoxia on marine invertebrates focuses on the role of low oxygen, and the impact of concurrent reduced pH has been generally ignored.

The Respiration Index (*RI*) was proposed by Brewer and Peltzer (2009) to capture the combined effects of hypoxia and high CO₂ on the efficiency of aerobic respiration, by using the basic oxix respiration equation and the free-energy relation. The *RI* is a simple numeric constraint linearly related to the available energy to support respiration:

$$RI = \log_{10}(pO_2/pCO_2) \quad (1)$$

where $RI \leq 0$ corresponds to the thermodynamic aerobic limit, a formal dead zone; at $RI = 0$ to 0.4, aerobic respiration does not occur; the range $RI = 0.4-0.7$ represents the practical limit for aerobic respiration, and the range $RI = 0.7-1.0$ delimits the aerobic stress zone (Brewer and Peltzer, 2009). The *RI* links hypoxia and CO₂, implying that the thermodynamic constraints for aerobic organisms do not depend on O₂ alone, but also on CO₂. The implication is that high CO₂, by lowering *RI*, affects the vulnerability of marine organisms to hypoxia.

Considering the impact of CO₂ on respiration suggests that the distribution and spatial extent of ocean dead zones will rise, even if the oxygen levels as such do not decline, as a result of rising CO₂ concentrations (Brewer and Peltzer, 2009), which will increase the stress to aerobic organisms and raise the O₂ thresholds for hypoxia. Rising CO₂ concentrations will induce metabolic depression in invertebrate species, reduce the rate of gas exchange across respiratory epithelia, deplete the internal oxygen stores, and accumulate respiratory CO₂ (Pörtner et al., 2005) and, thereby, decrease the buffering capacity in hypoxic bottom water (Hagens et al., 2015).

However, the *RI* index has not been experimentally tested and the underlying expectations have been criticized. Seibel and Childress (2013) argue that CO₂ could never reach concentrations that would limit the thermodynamics of this reaction, because of the large standard free energy change for organic carbon oxidation ($\Delta G^\circ = -686 \text{ kcal mol}^{-1}$), and that a PCO₂:PO₂ ratio of 10503 would be required to reach equilibrium (equilibrium constant, $K_{eq} = 10503$; where $\Delta G = 0$). Thus, they argued that a *RI* of -503 would be the real thermodynamic limit to aerobic life. Although it has been shown that in crabs and catfish the *p*CO₂ in plasma dropped to 45 and 56 mm Hg, respectively, when exposed to elevated CO₂, Pörtner et al. (2005), Seibel and Childress (2013), and Cameron and Iwama (1989) argue that cellular respiration and oxygen provision are kinetically controlled and environmental oxygen and CO₂

concentrations exert little control on intracellular concentrations. Yet, evidence for synergistic effects of low O₂ and high CO₂ includes increased bacterial infections in the pacific white shrimp *Litopenaeus vannamei* (Burgents et al., 2005), inhibition of growth and metamorphosis in the early life stage of bivalves (bay scallops, *Argopecten irradians*, and hard clams, *Mercenaria mercenaria*, Gobler et al., 2014), depressed growth rates for juvenile red abalone (*Haliotis rufescens*, Kim et al., 2013) and synergistic metabolic depression via the effect of adenosine on central nervous functions of the marine invertebrate *Sipunculus nudus* (Reipschläger et al., 1997; Pörtner et al., 2005). In field studies hypoxia and OA seasonally may occur simultaneously in shallow water tidal creeks and lead to sub-lethal effects on organismal and populational levels and reduce oxygen uptake in blue crabs *Callinectes sapidus* (Hypes, 1999). Regardless of the accuracy of the thresholds of *RI* proposed by Brewer and Peltzer (2009), it is clear that the efficiency of aerobic respiratory processes is dependent on the ratio of the partial pressures of both O₂ and CO₂, suggesting that threats from hypoxia will also be aggravated by increasing CO₂ (Brewer and Peltzer, 2009). This is particularly important, as hypoxic and high CO₂ stresses are likely to co-occur (Mayol et al., 2012), with both stresses forecasted to increase in the future (Orr et al., 2005; Vaquer-Sunyer and Duarte, 2008).

Here we evaluate the combined effects of hypoxia and OA on the survival and metabolic rates of benthic invertebrate populations in Central Chile. The invertebrates of the coastline along Chile may be regularly exposed to both stressors, as the Humboldt Current System (HCS) is one of the largest naturally hypoxic areas of the world's oceans (Levin et al., 2002; Thiel et al., 2007; Ulloa and Pantoja, 2009). The HCS is a quite complex dynamic region, characterized by the presence of a system of along-slope currents that brings waters of both tropical and subpolar origin, and by upwelling of cold, oxygen-poor waters supersaturated in CO₂ (Torres et al., 2002; Mayol et al., 2012). Hence, invertebrates in the HCS coastal region may regularly experience high CO₂ and low O₂ and are expected to be adapted to these stressors. All except anthozoans are calcifying species,

believed to be particularly vulnerable to OA (Kroeker et al., 2013). We experimentally tested the effect of these stressors on invertebrate species by exposing them to 4 different treatments (high O₂ and low CO₂, low O₂ and low CO₂, high O₂ and high CO₂, and both low O₂ and high CO₂) and measuring survival and respiration rate after 3 and 6 days.

Materials and Methods

The experiments were conducted between October 17 and December 13, 2012 at the ECIM marine station in Las Cruces, Chile. Organisms were collected during low tide from two sites, the surrounding coastal area of the ECIM marine reserve at Las Cruces and El Tabo, both located on the coastline of central Chile (33°30'S, 71°37'W).

A total of 17 species out of 4 taxonomic groups were tested at control and 3 treatment conditions (Table 1). The selected invertebrate species included 2 anthozoa, 9 molluscs, 4 crustaceans, and 2 echinoderms (Table 2) collected along the coastline of Las Cruces and El Tabo during low tide. These species were selected because of their abundance and significance along the coast, often including a commercial use (e.g., *Tegula atra*, *Prisogaster niger*, and *Concholepas concholepas*). Individuals were acclimated in 25L-tanks with aeration and running seawater, allowing conditions to follow the natural fluctuations occurring in the sea (average ± SD; pH ~ 7.596 ± 0.040, oxygen ~ 8.60 ± 1.10 mg L⁻¹, temperature ~ 15.44 ± 0.07 °C, salinity = 34.26 ± 0.089, see Ramajo et al., 2013; Lardies et al., 2014) for at least 2 days, before being placed into experimental aquaria. Previous to experiments, predators were fed every 1–2 days with bivalves and gastropods, which were collected at the same sites.

Four experimental conditions were used, involving two different levels of pH and oxygen: (1) H₀₂L_{CO2}—involving pH corresponding to atmospheric equilibrium (380 ppm) and saturated oxygen (20% oxygen in the gas mixture); (2) L₀₂L_{CO2}—pCO₂ corresponding to atmospheric equilibrium (380 ppm) and low oxygen (4% oxygen in the gas mixture); (3) H₀₂H_{CO2}—a treatment with elevated CO₂ (low pH), corresponding to

TABLE 1 | Mean (±SE) of the seawater parameters in the aquaria per treatment.

	H ₀₂ L _{CO2}		L ₀₂ L _{CO2}		H ₀₂ H _{CO2}		L ₀₂ H _{CO2}	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Temperature (°C)	16.2	0.1	16.4	0.1	16.3	0.1	16.3	0.1
Oxygen (mg L ⁻¹)	9.71	0.18	3.11	0.13	9.84	0.17	2.61	0.06
pH (16°C)	8.06	0.01	8.01	0.02	7.72	0.01	7.80	0.01
Alkalinity (μmol kg ⁻¹)	2189.8	46.1	2230.8	27.0	2238.7	43.9	2216.7	32.7
CO ₂ (ppm)	519.2	17.9	604.5	26.8	1239.0	32.2	1011.4	24.3
HCO ₃₋ (μmol kg ⁻¹)	1879.3	7.1	1941.9	8.6	2080.9	3.5	2032.7	4.6
CO ₃₂₋ (μmol kg ⁻¹)	124.7	2.9	116.5	3.4	63.7	1.4	74.1	1.9
Ω Aragonite	1.92	0.04	1.80	0.05	0.98	0.02	1.14	0.03
Ω Calcite	2.99	0.07	2.80	0.08	1.53	0.03	1.78	0.04
<i>RI</i>	1.69	0.02	1.12	0.03	1.31	0.01	0.81	0.01

The treatments are: H₀₂L_{CO2} (ambient O₂ and ambient pH), L₀₂L_{CO2} (low O₂ and ambient pH), H₀₂H_{CO2} (ambient O₂ and low pH), and L₀₂H_{CO2} (low O₂ and low pH).

TABLE 2 | Respiration rate (± SE) and results of the General Linear Model off all tested species after 6 days.

Species	Taxa	Day	Prob. > F		Average respiration rate (± SE)				General linear model (GLM)
					H ₂ LCO ₂	L ₂ LCO ₂	H ₂ HCO ₂	L ₂ HCO ₂	pH*O ₂
<i>Anemonia alicemartinae</i> n = 12	Anthozoa	6	0.0009	Average	0.00091	0.00047	0.00132	0.00008	0.0001
				(± SE)	0.00004	0.00003	0.00016	0.00005	
				Students' T*	B	C	A	D	
				Tukey HSD*	AB	BC	A	C	
				Mortality	0	0	0	0	
<i>Phymactis papillosa</i> n = 24	Anthozoa	6	0.0001	Average	1.38461	0.15462	1.64402	0.53504	-0.1210
				(± SE)	0.10966	0.01999	0.09036	0.13040	
				Students' T*	A	C	A	B	
				Tukey HSD*	A	B	A	B	
				Mortality	0	0	0	0	
<i>Concholepas concholepas</i> n = 12	Gastropoda	6	0.0107	Average	0.08198	0.03974	0.07502	0.03050	0.0023
				(±SE)	0.00521	0.00700	0.01151	0.01178	
				Students' T*	A	B	A	B	
				Tukey HSD*	A	B	AB	B	
				Mortality	0	0	0	0	
<i>Tetrapigus niger</i> (big) n = 12	Echinoidea	6	0.0066	Average	0.00079	0.00056	0.00060	0.00030	0.0001
				(±SE)	0.00008	0.00007	0.00006	0.00005	
				Students' T*	A	B	AB	C	
				Tukey HSD*	A	AB	AB	B	
				Mortality	0	0	0	0	
<i>Tetrapigus niger</i> (small) n = 12	Echinoidea	6	0.0375	Average	0.00094	0.00076	0.00101	0.00045	0.0004
				(±SE)	0.00010	0.00014	0.00015	0.00005	
				Students' T*	A	AB	A	B	
				Tukey HSD*	AB	AB	A	B	
				Mortality	0	0	0	0	
<i>Petrolisthes violaceus</i> (medium) n = 12	Crustacea	6	0.0154	Average	0.04138	0.02853	0.03472	0.02345	-0.0016
				(±SE)	0.00346	0.00261	0.00387	0.00180	
				Students' T*	A	BC	AB	C	
				Tukey HSD*	A	AB	AB	B	
				Mortality	0	0	0	0	
<i>Petrolisthes tuberculatus</i> n = 12	Crustacea	6	0.0446	Average	2.45355	1.81667	1.42996	1.21440	-0.4213
				(±SE)	0.15931	0.02476	0.44057	0.07371	
				Students' T*	A	AB	B	B	
				Tukey HSD*	A	AB	AB	B	
				Mortality	0	-0.07	0	0	
<i>Allopetrolisthes angulosus</i> n = 24	Crustacea	6	0.0015	Average	2.23465	1.12140	2.00113	0.55751	0.2974
				(±SE)	0.40062	0.10822	0.23791	0.08258	
				Students' T*	A	B	A	B	
				Tukey HSD*	A	BC	AB	C	
				Mortality	0	0	-0.07	0	
<i>Pagurus edwardsi</i> n = 24	Crustacea	6	0.1109	Average	2.66199	2.71286	2.63324	1.64802	1.0361
				(±SE)	0.25803	0.73458	0.15050	0.35126	
				Students' T*	A	A	AB	B	
				Tukey HSD*	A	A	A	A	
				Mortality	0	0	0	0	

Levels not connected by the same letter are significantly different (after Student's T and Tukey HSD tests). Mortality Rate was calculated as $\ln(Nt/NO)/\text{days}$. Numbers marked red show significant difference. *Letters mark the significance groups.

atmospheric levels expected by the end of the century (1000 ppm, Orr et al., 2005) and saturated oxygen; and (4) L_{O₂}HCO₂—treatment with low O₂ (4% oxygen in the gas mixture) and high CO₂ (1000 ppm) and low pH. These four experimental conditions conform to an *RI* gradient, ranging from 0.81 ± 0.01 *RI*, indicative of aerobic stress (L_{O₂}HCO₂treatment) to an *RI* of 1.69 ± 0.02 , without limits for aerobic respiration (H_{O₂}LCO₂ conditions). The respiration index was calculated after Equation (1) following Brewer and Peltzer (2009) from the average of the daily *p*O₂ and *p*CO₂ measurements of the four treatments.

To reach the treatment conditions the aquaria were bubbled with a mixture of nitrogen and air to lower the oxygen content, and with pre-determined *p*CO₂ levels. To set the CO₂ content of the air, ambient air was collected via pumps and passed through soda-lime columns to strip the air of CO₂. Precise volumes of CO₂-stripped air and pure CO₂ gas from a commercial 50 L-bottle were administrated using mass-flow controllers (MFCs; Aalborg GFC-17) and mixed in a container filled with marbles to increase mixing efficiency by increasing surface area to achieve *p*CO₂ concentrations of 380 ppm (H_{O₂}LCO₂, L_{O₂}LCO₂) and 1000 ppm (H_{O₂}HCO₂ and L_{O₂}HCO₂). To reach hypoxic conditions, nitrogen was added to the air-CO₂ mixture to reduce the oxygen in the water, maintaining the DO between 2.0 and 3.5 mg L⁻¹, corresponding to sublethal hypoxic levels as defined by (Vaquer-Sunyer and Duarte, 2008; Steckbauer et al., 2011).

Aquaria were filled water filtered over 20 μm filters, equilibrated to the treatment conditions, and placed in temperature-controlled tanks set to ambient temperature. Three replicas were used per treatment, resulting in a total of 12 experimental aquaria per species. We used an optic fiber oxygen-meter (Microx TX3, PreSens, Germany), with diameter tips of 20–50 μm. Zero calibration was performed using a sodium sulfite (Na₂SO₃) solution (0% saturation) and 100% was calibrated using vigorously air-bubbled seawater. Experimental pH was measured at 5 min intervals with pH_{NBS} sensors (Metrohm and Hanna Instruments), connected to a Consort D130 datalogger. At least once per week, pH in total scale was measured using a pH-meter (pH mobile 826, Metrohm), connected to a combined electrode (double junction), calibrated using buffers Tris (pH = 8.089) y 2-Aminopiridine (pH = 6.786) at 25°C in a temperature controlled water bath (Torres et al., 2011). Water samples for alkalinity analyses were taken at least once per week, fixed with 20 μL HgCl₂ and analyzed within 3 months, using a Metrohm Titrando 808 after Dickson et al. (2007). pH_{NBS}, temperature, alkalinity and salinity values were used to calculate *p*CO₂, the saturation state of aragonite (Ω_{Ar}) and calcite (Ω_{Ca}) in each treatment using CO₂SYST (Pierrot et al., 2006), with K₁ and K₂ constants from Mehrbach et al. (1973), as revised by Dickson and Millero (1987), and the K_HSO₄ constant from Dickson (1990).

After 3 and 6 days, individuals were transferred to 300 or 1000 mL air-tight vessels and incubated in treatment water for 1–5 h, depending on the size of the animal and vessel, to measure oxygen consumption at 14°C. Temperature was stabilized using a temperature-controlled water bath (JioTech, Co). Oxygen was measured using calibrated PreSens micro-optodes at the beginning and the end of the incubation and the difference was

used to calculate the consumption rate using dry weight (DW) and size (in mm) as mg O₂ g⁻¹ DW min⁻¹ and mg O₂ mm⁻¹ min⁻¹. After the experiment, the body size (maximum length, mm) and wet weight (g) of the animals were measured, and the organisms were kept frozen until further processing. To evaluate the dry weight, organisms were dried for at least 24 h at 60°C and weighted. For gastropods, shell and soft parts were treated separately.

Statistical Analysis

To compare the results of the 3 treatments to the H_{O₂}LCO₂ data across species ranging broadly in size and other traits, we calculated the log “effect size” after Hedges et al. (1999) and Gurevitch and Hedges (1999). Response ratios quantify the proportional change resulting from experimental manipulations and ln-transformed response ratios are commonly used because of their robust statistical properties and ease of biological interpretation (Hedges et al., 1999; Kroeker et al., 2010). The effect of the different water conditions on the oxygen consumption was measured for each treatment as the ln-transformed response ratio,

$$\ln RR = \ln(X_E) - \ln(X_C), \quad (2)$$

where X_E and X_C are the mean values of the response variable in the experimental and H_{O₂}LCO₂ treatments, respectively. As our goal is to test the effects of low O₂ and high CO₂ as stressors we designated high O₂ and low CO₂ as the control treatment, even though ambient values in the ecosystem where the organisms grow are closer to the high O₂ low CO₂ treatment (see below).

Three-Way ANOVAs were conducted to test the effect of species, treatment and time (i.e., difference in the responses measured between day 3 and 6) on the respiration rate. A One-Way ANOVA was used to test for differences in respiration rate between treatments for each species. Where the respiration showed significant differences, a Student's *t*-test and post-hoc Tukey HSD test were conducted to resolve which treatments resulted in different respiration rates. Moreover, a General Linear Model (GLM) was used to quantify response to changes in pH, oxygen and their interaction. If the interaction term was significant and positive, then there were synergistic effects between the stressors, and if the interaction term was significant and negative the effects were antagonistic. All analyses were done using RStudio (version 0.97.336) and JMP (version 10.0) with the level for significance set at 0.05.

Results

Seawater temperature averaged (±SE) $16.31 \pm 0.06^\circ\text{C}$ during the experimental period and did not differ among treatments (Table 1). Mean dissolved oxygen concentration varied from 9.77 ± 0.12 mg O₂ L⁻¹ in the normoxic treatment to 2.86 ± 0.08 mg O₂ L⁻¹ in the hypoxic treatments, respectively (Table 1), and were significant different from each other (*p* < 0.001, ANOVA). Mean pH was 8.03 ± 0.01 in the ambient and 7.75 ± 0.01 in the high CO₂ treatments (*p* < 0.001, ANOVA), respectively. The average alkalinity was 2219.0 ± 18.7 μmol

kg⁻¹ throughout the treatments and experimental duration. The mean *p*CO₂ in the water was 562 ± 17 μatm in the normal and 1142 ± 25 μatm in the high CO₂ treatments, respectively. Ω_{Ar} and Ω_{Ca} averaged 1.86 ± 0.04 and 2.89 ± 0.05 in the normal and 1.05 ± 0.02 and 1.63 ± 0.03 in the high CO₂ treatments, respectively (Table 1). The *RI* averaged 1.69 ± 0.02 for the H₂O₂LCO₂, 1.12 ± 0.03 for the L₂O₂LCO₂, 1.31 ± 0.01 for the high CO₂ and 0.81 ± 0.01 for the L₂O₂HCO₂ treatment (Table 1). The *RI* values for the hypoxic and high CO₂ treatment were similar as the differences in *p*O₂ and *p*CO₂ had a similar effect on *RI*. All treatments matched the target values and were held to an acceptable level and variability within each treatment (Table 1).

The animals held at H₂O₂LCO₂ conditions of high oxygen and normal pH did not experience mortality, indicating that mortality observed in the L₂O₂LCO₂, H₂O₂HCO₂, and L₂O₂HCO₂ treatments was due to the low pH and/or low DO concentration and not to other potential. Yet, survival rates were very high, with only 10 individuals dying out of a total of 320 specimens tested in the experiment after 3 or 6 days. As most of the individuals survived 3 days even in the L₂O₂HCO₂ treatment, they were kept in the aquaria up to 6 days. The species mortality was observed were limpets *Fisurella* sp. (1x H₂O₂HCO₂ and 1x L₂O₂HCO₂ on day 4), the polyplacophora *Chiton granosus* (1x L₂O₂HCO₂ on day 3) and *Tonica* sp. (2x L₂O₂LCO₂ and 1x L₂O₂HCO₂ on day 3); and the anomura crustaceans *Petrolisthes violaceus* (1x L₂O₂HCO₂ on day 3), *Petrolisthes tuberculatus* (1x L₂O₂LCO₂ on day 3), and *Allopetrolisthes angulosus* (2x H₂O₂HCO₂ on day 3), respectively. However, survival rates were higher than 97% across treatments and species (Table 2), indicating that the experimental conditions represented sublethal stresses.

After the exposition to experimental conditions, the metabolic rate differed between species and taxa. Generally, echinoderms displayed lower respiration rates and the gastropod species *Tegula atra* and *Diloma nigerrima* the highest (Table S1). There were significant differences in metabolic rates between treatments (*p* < 0.001) and species (*p* < 0.001) but not with the duration of the experiment (*p* = 0.69; Table 3). The majority of species (65%) showed metabolic depression, which was reflected in reduced respiration rates, when exposed to hypoxia, high CO₂ or both stressors (Figure 1). Their negative responses increased over time, although not significant. The fraction of species showing a significant difference in respiration rate with high CO₂ increased from 41% after 3 days to 60% after 6 days and those

showing significant responses to hypoxia increased from 65% after 3 days to 90% after 6 days, with 100% of the species showing significant responses to both stressors acting together already

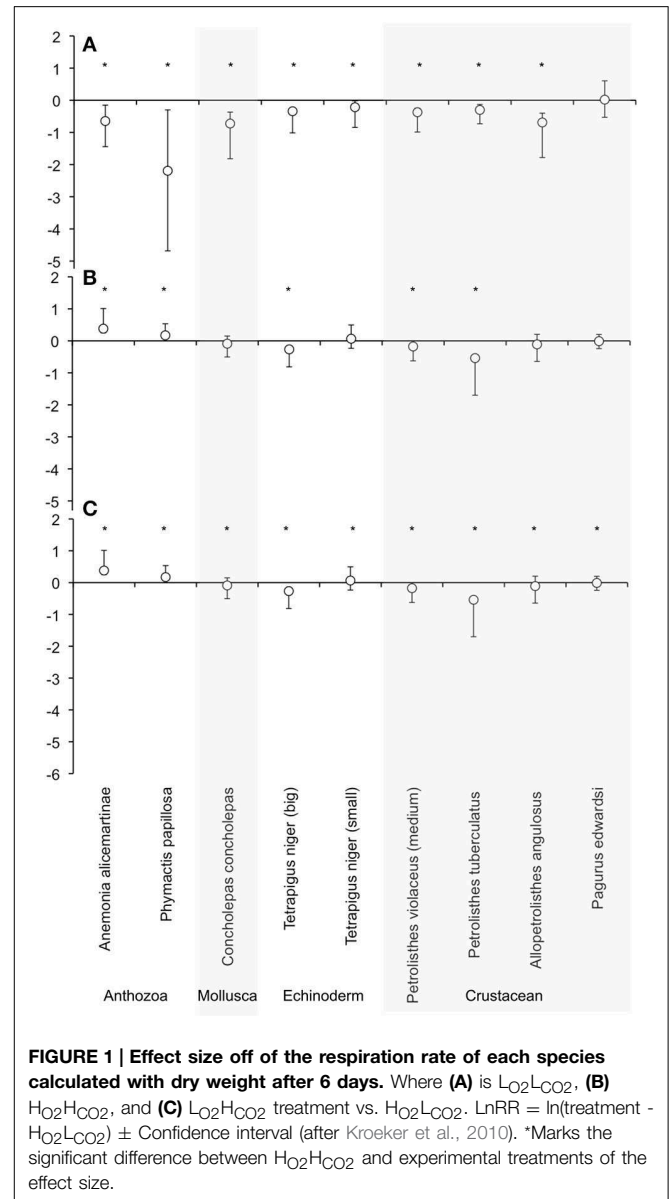


FIGURE 1 | Effect size off of the respiration rate of each species calculated with dry weight after 6 days. Where (A) is L₂O₂LCO₂, (B) H₂O₂HCO₂, and (C) L₂O₂HCO₂ treatment vs. H₂O₂LCO₂. LnRR = ln(treatment - H₂O₂LCO₂) ± Confidence interval (after Kroeker et al., 2010). *Marks the significant difference between H₂O₂HCO₂ and experimental treatments of the effect size.

TABLE 3 | Results of the Three-Way ANOVA describing the effects of species, treatment and day on the respiration rate.

	Df	Sum Sq	Mean Sq	F-value	Pr(>F)
Species	19	1670.8	87.94	84	< 0.00001***
Treatment	1	11.3	11.35	458	0.00108**
Day	1	0.2	0.17	10.898	0.68748
Treatment:Species	19	41.6	2.19	0.162	0.00504**
Treatment:Day	1	0.0	0.00	2.103	0.98996
Species:Day	8	1.8	0.22	0.000	0.98801
Treatment:Species:Day	8	0.2	0.03	0.216	1.00000
Residuals	299	311.3	1.04	0.025	

Signif. codes: 0 '****', 0.001 '***', 0.01 '**', 0.05.

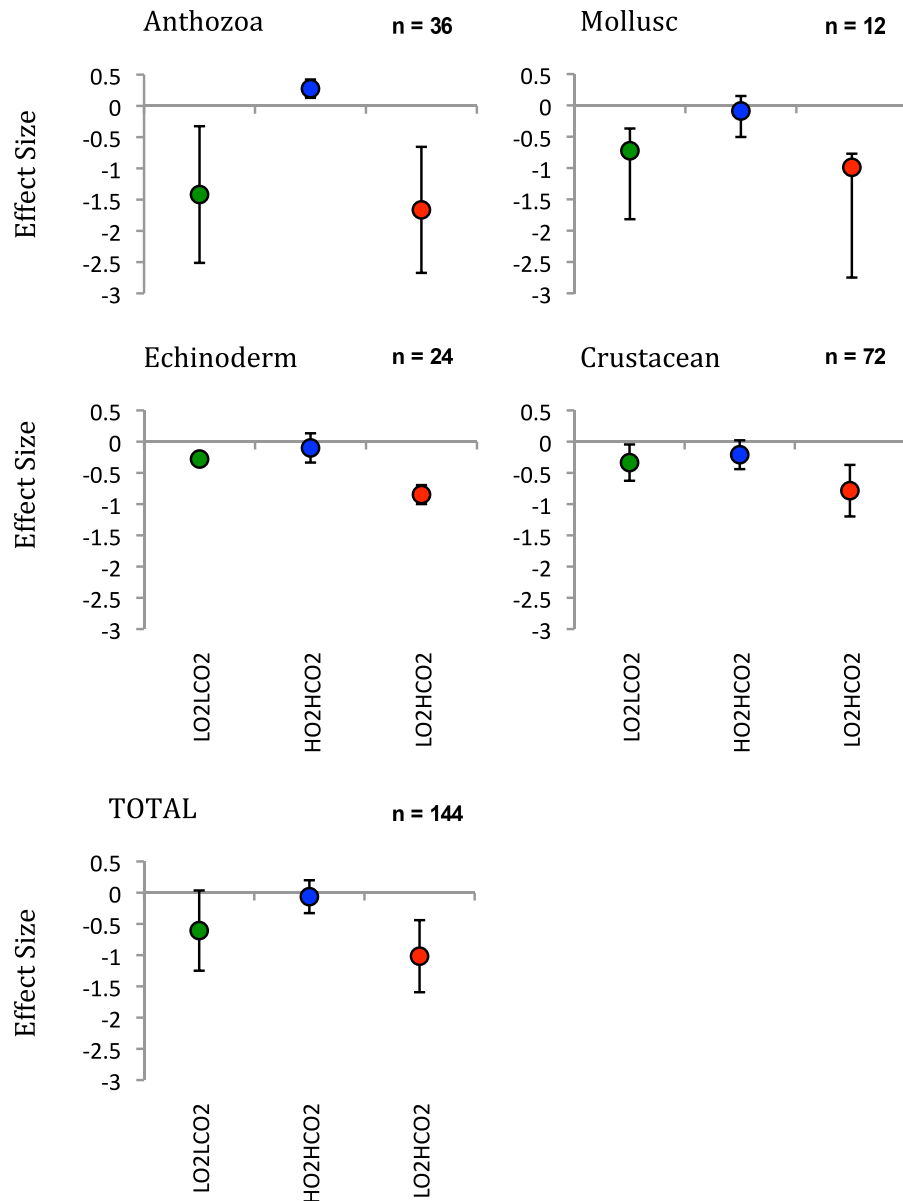
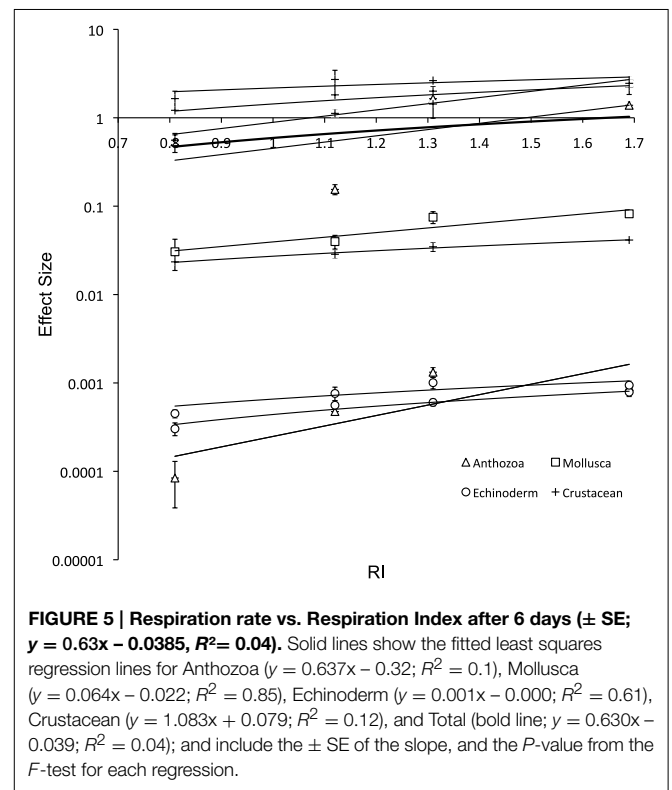
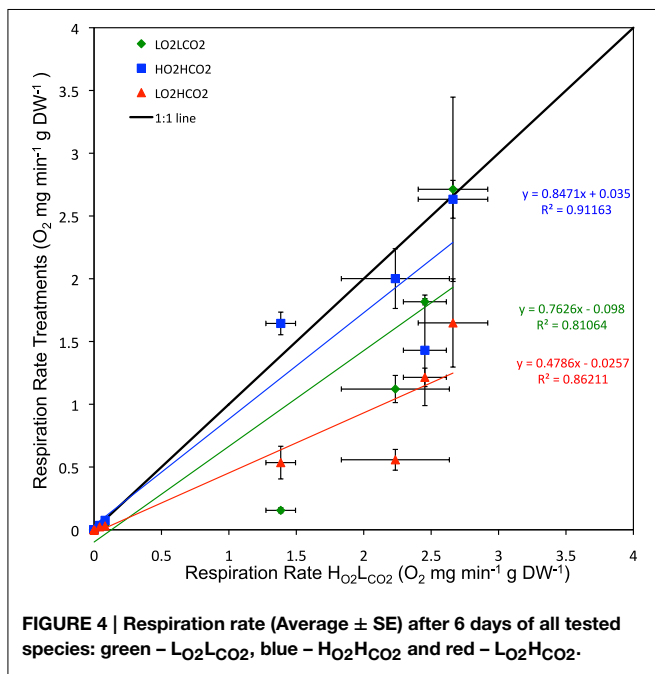
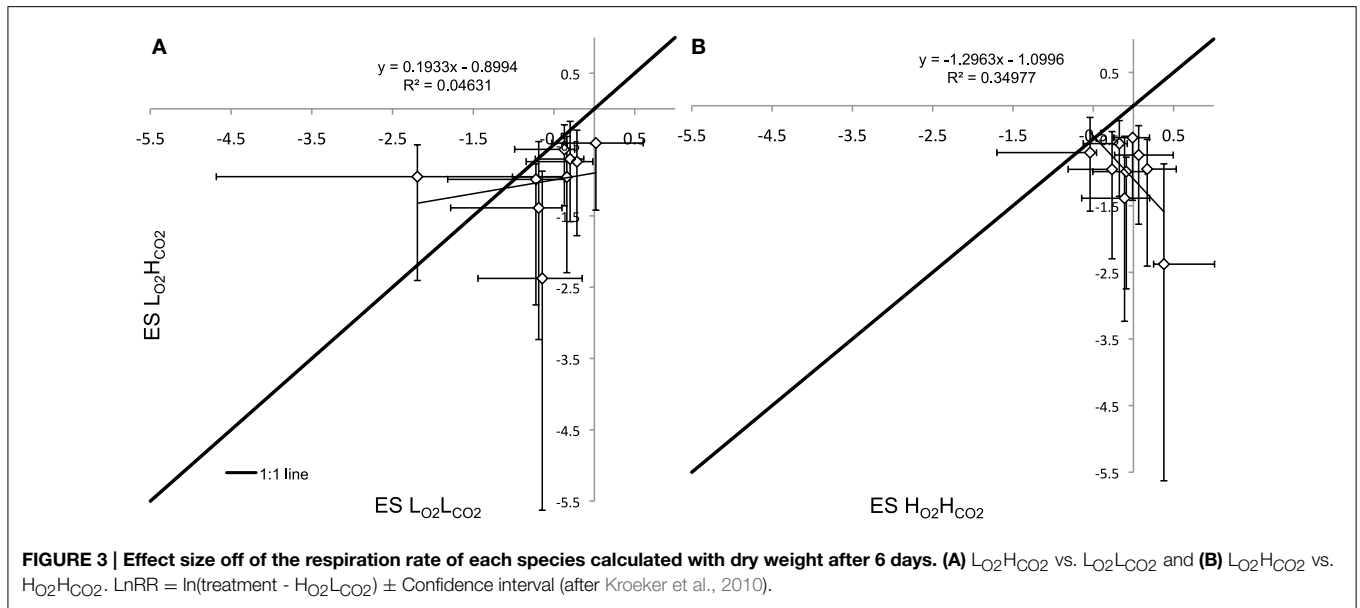


FIGURE 2 | Effect size off of the respiration rate of taxonomic groups calculated with dry weight after 6 days. $\text{LnRR} = \ln(\text{treatment} - \text{H}_2\text{O}_2\text{LCO}_2) \pm$ Confidence interval (after Kroeker et al., 2010).

after 3 and 6 days (**Figure 1** and Figure S1). Anthozoans were the most sensitive taxa as there was a significant difference in all 3 treatments compared to the $\text{H}_2\text{O}_2\text{LCO}_2$ (**Figure 2**). For all other taxa, the result was significantly different for the $\text{L}_2\text{O}_2\text{LCO}_2$ and $\text{L}_2\text{O}_2\text{HCO}_2$ treatment but not always for the $\text{H}_2\text{O}_2\text{HCO}_2$, respectively (**Figure 2**). The combination of both stressors ($\text{L}_2\text{O}_2\text{HCO}_2$) led to a greater metabolic depression than either stressor alone (**Figure 3**).

Regression analysis of respiration rate in the presence of stressors vs. that in the $\text{H}_2\text{O}_2\text{LCO}_2$ treatment showed that the effects of hypoxia and high CO₂ were additive (**Figure 4**), as the deviations of the slope of the regression line for the

$\text{L}_2\text{O}_2\text{HCO}_2$ treatment from 1 ($1 - \text{slope } \text{L}_2\text{O}_2\text{HCO}_2 = 0.48 \pm 0.11$) does not differ from that resulting from the sum of those of the individual treatments ($1 - \text{slope } \text{L}_2\text{O}_2\text{LCO}_2 = 0.24 \pm 0.17$; $1 - \text{slope } \text{H}_2\text{O}_2\text{HCO}_2 = 0.15 \pm 0.20$; expected $\text{L}_2\text{O}_2\text{HCO}_2 = 0.39 \pm 0.18$). This finding shows that, overall, the metabolic responses to the stressors tested was additive, and not synergistic or antagonistic, as also confirmed by the general linear model of the metabolic responses of the individual species to the stressors, where after 6 days only in 4 out of the 9 species tested (4 out of 17 species after 3 days, Table S2) showed a significant interaction term between the two stressors (**Table 2**). The most tolerant taxa after 6 days was the Crustacea, as 3 out of 4 species didn't show significant



differences and in *Petrolisthes violaceus* a significant antagonistic effect was observed.

The respiration rates decreased with decreasing RI in all species as expected (Figure 5), although the relationships between metabolic rates and RI was relatively weak within taxa, due to the different intensity of metabolic rate.

Discussion

The tested benthic invertebrates from the central Chilean coast were relatively resistant to hypoxia, high CO₂ and

their combined effects, as the mortality rate was low across species and metabolic depression, while present, was relatively modest (Table 2). Anthozoans and Crustaceans were relatively vulnerable to hypoxia, while Molluscs and Echinoderms were tolerant. This is consistent with results from Vaquer-Sunyer and Duarte (2008), who showed Molluscs and Echinoderms to be particularly tolerant to hypoxic events compared to Crustaceans.

The organisms were comparatively resistant to high CO₂ as they showed no significant mortality or metabolic depression when exposed to high CO₂. Indeed, exposure to high CO₂ showed increased respiration rate in Anthozoans and Echinoderms, as also reported in a recent meta-analysis (Kroeker et al., 2013), rather than a metabolic depression. Although it has been reported that food supply and not pCO₂ appears to be the primary factor driving biomass and biogenetic CaCO₃ production (Melzner et al., 2011; Thomsen et al., 2013), the effect on respiration rate is controversial (Lampert, 1984). Animals were fed previously, but not during the experiments, as feeding previously to the oxygen measurements was shown to increase respiration rate compared to starved animals. In the H₂O₂HCO₂ treatment the concentration of aragonite (Ω_{Ar}) was under-saturated ($\Omega_{Ar} < 1$) and in the L₂O₂HCO₂ treatment close to under-saturation ($\Omega_{Ar} = 1.14 \pm 0.15$), where calcifiers are expected to be under physiological stress (Doney et al., 2009). Molluscs, depositing mostly aragonite, are expected to be more vulnerable to high CO₂ (Porter, 2007) than Echinoderms and Crustaceans, which deposit calcite (Raup, 1959; Raabe et al., 2005).

Most importantly, our results showed that hypoxia and high CO₂ have additive effects and revealed no consistent synergistic or antagonistic effect for these stressors. Moreover, the observation of very low mortality rates and relatively modest metabolic depression (on average 52% reduction compared with the values in H₂O₂LCO₂ treatments) with both stressors reveals that the Chilean invertebrate species tested are relatively resistant to these stressors. The resistance of invertebrates in the central Chilean coast to hypoxia and high CO₂ is nonetheless not surprising as these organisms may experience these conditions in their natural habitat. Whereas pCO₂ of 1200 ppm as tested here are used in OA experiments to characterize values expected beyond year 2100 (Kroeker et al., 2013), these values are reached regularly in the Chilean coast (Torres et al., 2011; Mayol et al., 2012). Indeed, in the year preceding this experiment high pCO₂ values, of the order of those used in the high treatment here, were found twice, associated with upwelling conditions (N. Lagos, unpubl. data). Moreover, oxygen and pCO₂ are closely correlated in the water mass along the Chilean coast (Mayol et al., 2012), so that upwelling events leading to pCO₂ values around 1200 ppm are associated with oxygen values of ~2 mg L⁻¹ (Mayol et al., 2012). Hence, the hypoxia and high CO₂ treatments used here represent stresses already experienced by these organisms. Comparison of the CO₂ and O₂ conditions in the treatments with those experienced by the organisms in their habitat shows that the treatment best representing their environment is involving both high O₂ and low pH (Figure 6). Indeed, the pH environment in their environment is even lower than that imposed in the high CO₂ treatment in our experiment. Shall the organisms be vulnerable to high CO₂ they would have been already sieved from the community and would not occur in this ecosystem. Indeed, the prevalence of high CO₂ in coastal waters (e.g., Borges, 2005) suggest that the use of CO₂ levels close to present atmospheric equilibrium as H₂O₂LCO₂ (cf. Hendriks et al., 2010) may not represent ambient conditions in many coastal ecosystems (Duarte et al., 2013), possibly confounding the interpretation of results. We suggest that the variability in

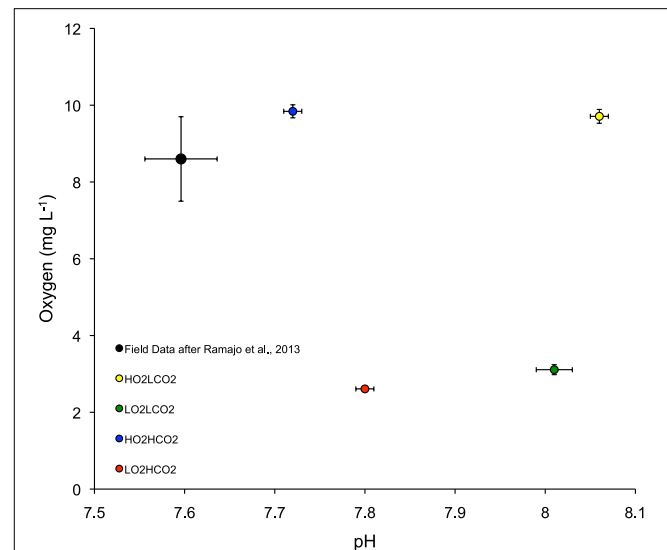


FIGURE 6 | The experimental range of variables from this experiment compared to the ecosystems ambient range of oxygen and pH from the study sites in central and southern Chile during November 2009 and January 2010 (Ramajo et al., 2013).

responses to OA and hypoxia experiments (cf. Vaquer-Sunyer and Duarte, 2008; Kroeker et al., 2013, respectively) should be re-examined in terms of the conditions experienced *in situ* by the population from which the individuals were derived.

The fact that the ecosystem supports healthy populations of these invertebrate species despite regular upwelling events already suggests that they must be relatively resistant to at least short term exposure to these conditions. Indeed, exposure to such extreme conditions during upwelling events is typically in the order of 3–7 days (Narváez et al., 2004), the time scale to evaluate responses used here. That the previous history of exposure to the stressors affects the resistance of the organisms was shown experimentally by Brady and Targett (2013), who showed that previous diel-cycle hypoxia lowers the avoidance threshold from <2.8 mg O₂ L⁻¹ (in saturation-acclimated fish) to ~1.4 mg O₂ L⁻¹ (in diel-cycling hypoxia acclimated fish) in the juvenile weakfish *Cynoscion regalis*, showing that they become more resistant to hypoxia.

Whereas hypoxia and high CO₂ are expected to co-occur in nature (Brewer and Peltzer, 2009; Mayol et al., 2012), the responses of marine organisms to these stressors has been largely studied in isolation where either hypoxia (Vaquer-Sunyer et al., 2012) or high CO₂ (Doney et al., 2009; Hendriks et al., 2010; Kroeker et al., 2013) are tested. High CO₂ and hypoxia in the environment, affect the metabolic rates as they lead to a shift in the steady state acid-base equilibrium (Pörtner and Grieshaber, 1993; Pörtner and Heisler, 1998; Pörtner et al., 2005). The combination of hypoxia and increasing CO₂ reduces the rates of relevant trans-membrane ion exchange (Pörtner et al., 2000) and causes a synergistic metabolic depression via the effect of adenosine on central nervous functions if anoxia occurs (Reipschläger et al., 1997). Nevertheless, the examination of the

responses to combined hypoxia and high CO₂ is based on a limited set of studies thus far. Kim et al. (2013) exposed juvenile abalone (*Haliotis rufescens*) to short term (3–6 h to 24 h) hypoxia and low pH and found that hypoxia had the greater influence on mortality (pH 7.5 vs. 8.0), but growth was lowest when both stressors were combined. Frieder et al. (2014) showed that low O₂ in combination with low pH did not affect the development and size of 2 mytilid mussels from the Scripps Institution of Oceanography pier (*Mytilus californianus*) and San Diego Bay (*M. galloprovincialis*), USA. Gobler et al. (2014) reported that the bay scallop, *Argopecten irradians*, showed additive responses on survivorship, growth and metamorphosis to low O₂ in combination with low pH, consistent with our findings. However, Gobler et al. (2014) reported that the later stages of the hard clam *Mercenaria mercenaria* were resistant to hypoxia or acidification separately but experienced significantly reduced growth rates when exposed to both conditions simultaneously. This indicates that responses to hypoxia, high CO₂ and their combined effects might be species specific.

The additive nature of the effects of hypoxia and high CO₂ lends weight to the use of the Respiration Index, *RI*, to reflect their combined stress on metabolic processes. Whereas the merit of the *RI* has been challenged recently (Seibel and Childress, 2013) no experimental test had been reported to date. Our results show that metabolic rates decline with decreasing *RI*, as expected (Brewer and Peltzer, 2009), confirming that the *RI* holds power as a predictor of effects, separate or combined, of hypoxia and high CO₂ on metabolic rates. However, our results also support the criticisms of Seibel and Childress (2013) to the predictive power of the thresholds proposed by Brewer and Peltzer (2009). The lowest *RI* we reached in our experiment was 0.81 ± 0.06, reached in the L₀₂H_{CO2} treatment. This is within the range of 0.7–1.0 where Brewer and Peltzer (2009) propose that aerobic respiration must be severely compromised. Yet, we observed little or no mortality, suggesting that the *RI* thresholds for marine invertebrates are well below those postulated by Brewer and Peltzer (2009). The test provided here is, to the best of our knowledge, the first experimental test, and more tests are required to confirm the merit of the *RI* index and to establish reliable thresholds for marine organisms. Moreover, in future studies measurement of calcification rates would be a good

addition to assemble more data on the effects of future scenarios on marine invertebrates.

In summary, marine invertebrates inhabiting the upwelling ecosystems of the Chilean coast show additive but negative responses to hypoxia and high CO₂ and are relatively resistant to the combined effects of these stressors. We suggest that responses to the combined effects of hypoxia and high CO₂ are likely to be dependent on the conditions previously experienced by marine invertebrate populations and that organisms in upwelling-affected areas, such as those along the Chilean coast, are likely adapted, at least to brief exposures, to the occurrence of both stressors.

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Supplementary Material

The Supplementary Material for this article can be found online at: <http://journal.frontiersin.org/article/10.3389/fmars.2015.00049>

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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