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Integrative Assessment of Sediments Affected by CO₂ Enrichment: A Case Study in the Bay of Santos—SP, Brazil

Marina Cunha Passarelli ^{1,2}, Estefanía Bonnail ³ , Augusto Cesar ⁴ , T. Ángel DelValls ^{5,*}
and Inmaculada Riba ²

¹ Department of Environment, Petrobras, Santos 11010-310, Brazil; marinapassarelli@gmail.com

² Department of Physico-Chemistry, Aquatic Systems Research Group, UNESCO/UNITWIN WiCop, Faculty of Environmental and Marine Sciences, 11510 Cádiz, Spain; inmaculada.riba@gmail.com

³ Centro de Investigaciones Costeras—Universidad de Atacama (CIC-UDA), University of Atacama, Copiapó 1530000, Chile; estefania.bonnail@uda.cl

⁴ Department of Ocean Sciences, Federal University of São Paulo (UNIFESP), Santos 11030-100, Brazil; acesar@unifesp.br

⁵ Department of Ecotoxicology, Santa Cecília University (UNISANTA), Boqueirão 11045-907, Brazil

* Correspondence: delvalls@unisanta.br

Abstract: CO₂ enrichment in the marine environment caused by leakages from carbon capture and storage technologies may occur over operational procedures. An integrated approach using weight-of-evidence was applied to assess the environmental risk associated with the acidification caused by CO₂ enrichment in coastal sediments from Santos (Brazil). Chemical analyses (metal(loid)s and organic contaminant (e.g., hydrocarbons), toxicity tests (amphipods mortality, sea-urchin embryo-larval development) and macro-benthic community structure alteration assessment were performed with different acidified scenarios (pH 8.0–6.0) for two stations with different contamination degrees. These lines of evidence were statistically analyzed and integrated (multivariate analysis and ANOVA). Results of toxicity showed significant chronic effects starting at pH 7.0 while acute effects were observed starting at pH 6.5. The macro-benthic community integrity showed significant differences for all treatments at the Piaçaguera channel station, considered to be moderately contaminated. Results from the multivariate analysis correlated toxic effects and increase in the mobility of some elements with acidification. Also, the biological indexes were correlated with concentrations of dissolved Zn in seawater. The pH of 6.0 was extremely toxic for marine life due to its high acidification and metal bioavailability. The approach herein identified and discriminated the origin of the degradation caused by the acidification related to the enrichment of CO₂.

Keywords: weight-of-evidence; CO₂ enrichment; coastal zones; metal contamination; sediment toxicity; ecological integrity

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1. Introduction

The increasing of anthropogenic activities since the pre-industrial era produced a series of changes in the ecosystems due to the CO₂ increase in the atmosphere and high anthropogenic emissions such as the use of fossil fuel, electric power plants, and automobiles, among others [1]. Previous studies estimated that CO₂ concentrations in the atmosphere have been raised from 280 ppm, dated in the pre-industrial era, to current concentrations higher than 415 ppm (Mauna Loa–Hawaii–observatory 2021 [2]). In this way, mitigation strategies to combat the impact caused from the increase of greenhouse gases (such as carbon dioxide) and the climate change phenomena have been considered in the last few years [3]. Among the strategies to reduce the CO₂ atmosphere levels, some consist of large-scale carbon capture and storage (CCS) in geological formations, which might contribute to a 19% reduction of CO₂ emissions by 2050 [4]. However, despite of this current practice, there are some gaps in the environmental risk assessment of the technique;

it still remains largely unexplored hampered by complex ecological relationships and different ecosystems [5,6].

Many studies have been performed in the last few years in order to analyze the potential effects from CO₂ enrichment in the ocean caused by both increases in CO₂ atmosphere concentrations and natural uptake on seawater surface, and by CO₂ leakages during the CCS process (e.g., [7–10]), as well as in combination with harmful substances [11,12].

On the other hand, the weight-of-evidence (WoE) approach has been indicated as a good method to achieve precise results of environmental characterization and quality status [6,13–16]. According to [17], several questions commonly asked in an environmental assessment of chemicals include the following: Are contaminants present in the ecosystem and at what levels? Are contaminants bioavailable? Do they cause biological adverse effects? These questions were adapted in this study in relation to the influence of acidification caused by CO₂ enrichment in the seawater. In this sense, these questions were addressed in the present study in the Bay of Santos with particular chemical characterization and its characteristic infauna.

Recent studies have evaluated the relationship between decrease in seawater pH, adverse effects on marine organisms, and changes in the metal concentrations [7,8,18–23]. However, none of these studies have been designed or applied under an integrative point of view using multiples lines of evidence as recommended by [24].

Therefore, the use of an integrated approach using the maximum amount of information could be an effective way to contribute with the studies of environmental risk related to CO₂ enrichment in the ocean, which would support stakeholders and policymakers in finding appropriate solutions for the conservation and protection of marine ecosystems. In the present study, the relationship between CO₂ enrichment in the ocean and increases of the contamination levels was assessed based on the WoE and using sediment and water physicochemical analysis, ecotoxicological analysis, and macro-benthic community alteration as the mains lines of evidence linked in the WoE.

2. Materials and Methods

2.1. Study Area and Sampling Stations

The Santos Estuary System (SES), located on the central coast of the state of São Paulo (SE Brazil), has been widely characterized in terms of contamination in water and sediment [14,16,25–27]. Sediment samples were collected from two sampling sites according the contamination levels (Figure 1): low contaminated (Palm Island, PAI) and intermediate contamination (Piaçaguera channel, CPI). Chemical characterization of the sampling sites can be found in [22]. Briefly, seawater elements were below detection limits, 24 °C (± 1) of temperature, pH 8.0 ± 1 , and a DO below 60% when sampling. Sediment from PAI registered 1.11% of OM, and 26.8% of fines, and sediments from CPI were characterized by 13.1% and 34.6% for OM and fines, respectively. Based on the ecotoxicological assessment of sediments from the Santos and São Vicente estuarine system study [23] maximum element concentration was 167.2 mg kg^{−1} Cu, 6.02 for Ni, 154.2 for Zn, 0.950 for PAHs, 4.00 for PCBs; meanwhile organic carbon content was ranged between 0.85 and 3.75 (close to PAI), and the percentage of fines varied from 1.42 to 11.56. These high levels are attributed to the mangroves and sewage disposal [25].

2.2. CO₂ Injection System

Aiming to mimic scenarios of acidification associated with CO₂ enrichment in the marine environment, a CO₂ injection system was employed for ecotoxicological assessment and for mesocosms using macro-benthic community structure (Figure 2). The system was previously described by [19] and updated by [21]. Briefly, the AT Control System from Aqua Medic (Bissendorf, Germany) was used to independently manipulate and control the pH in each chamber test. The electrodes (NBS scale) are placed inside the chamber test and connected to the computer system to control the pH in each test vessel. The CO₂ gas is added in the chambers through a solenoid valve that opens when the pH increases

0.01 units above the predetermined pH values and closes when target pH is reached. This system was used for all the treatments and toxicity tests (Table 1).

Figure 1. Location of sampling sites selected in the Bay of Santos (SP, Brazil): Piaçaguera channel (CPI) station (intermediate contaminated area) and Palma Island (PAI) station (no contaminated area).

Figure 2. Schematic design of the CO₂ injection system for the different biological adverse effect lines of evidence selected: (i) to assess the effects of CO₂ enrichment on the benthic integrity; (ii) design used with elutriate assays. At the first step was acidified the samples before mixed and then the supernatant fraction was used to analyze the larval development of sea urchin; and (iii) design used for acute toxicity tests with amphipods.

Table 1. Summarized description of the toxicity test conditions and endpoints determined.

Organisms	pH Range	T (°C)	DO (%)	Duration	Endpoint
<i>H. youngi</i>	8.0–6.0	25 ± 1	>80	10 days	Mortality
<i>L. variegatus</i>	8.0–6.0	25 ± 1	>80	24 h	Pluteus stage
Macrobenthos	8.0–6.0	25 ± 1	>80	21 days	Biological indexes

T, temperature; DO, dissolved oxygen saturation.

2.3. Toxicity Tests

Acute and chronic toxicity tests were carried out to analyze the effect of the acidification caused by CO₂ enrichment using different marine organisms. The pH values of the treatments ranged between 8.0 and 6.0 (8.0 ± 1, 7.5 ± 1, 7.0 ± 1, 6.5 ± 1 and 6.0 ± 1) using three replicates. Table 1 shows a summarized description of the different biological responses and the toxicity test conditions.

2.3.1. Acute Test

Amphipods *Hyale youngi* were collected from stalks of the macro-algae *Ulva* sp. from the rocky reefs in Palm Island from Bay of Santos (Figure 1). The organisms were acclimated to laboratory conditions for a week before testing. Tests were performed using 2-L glass beakers in a ratio of 1:4 v/v sediment samples/seawater following [19]. Briefly, the chambers tests were left for 24 h under constant aeration before starting the CO₂ injection, and then, amphipods were introduced. Organisms ($n = 10$) were exposed for 10 days to different acidification scenarios described (in triplicate). Amphipod survival rate was determined after exposure.

2.3.2. Chronic Test

Specimens of *Lytechinus variegatus* were collected from Palm Island from the Bay of Santos, transported to the laboratory, and placed in tanks with aerated seawater before testing. Sediment-seawater (ratio 1:4 v/v) was first acidified by the injection of CO₂ gas until the target pH was reached. Then, sediment elutriate was obtained by mixing sediment and filtered seawater. The mixture was kept under agitation (50 rpm) for 30 min, followed by a settling period of 12 h [20]. After this procedure, the supernatant fraction was extracted and placed in glass tubes (20 mL of sample per replicate). Elutriates were acidified with CO₂ bubbles to maintain the target pH treatments during the test. The gametes were obtained by KCl (0.5 M) injection following [28].

2.4. Macro-Benthic Community

Sediments infauna (top layer from 0 to 5 cm depth) were collected using a 0.025 m² van Veen grab and exposed to different pH values in order to analyze the impact of the CO₂-induced acidification in the benthic community in the marine environment. Briefly, 25-L plastic aquarium replicates were filled with sediment grabs. Two of these aquaria were randomly selected as day 0 (D0) and the others were exposed to the different pH treatments and control. Sediments from D0 were sieved immediately and the retained fauna were fixed. The experiments were performed following [29]. After the acclimation time (1 day), mesocosms were performed in duplicate and maintained at 21 °C with natural photoperiod, and constant aeration for 21 days. Water was replaced every three days. Abundance and richness using Margalef's method (d) and the Shannon's diversity (H0) were calculated for each replicate.

2.5. Chemical Analyses

Overlying seawater (50 mL) was collected and preserved in darkness, avoiding any headspace for posterior analysis of total alkalinity (TA). TA was determined by automatic titration (Mettler Toledo, T50, Schwerzenbach, Switzerland) using a combined glass electrode (Mettler Toledo, DGi115-SC) calibrated on the NBS scale.

The metal(loid) concentrations (As, Cr, Cu, Ni, Zn) in the total fraction sediments, water, and elutriates were determined due to presence and potential adverse effects over biota found in previous studies [23]. These elements are also associated with concentrations of PAHs and PCBs.

The sediment samples (0.1 g of 40 °C dried sediment sample) were digested with a mixture of acids (nitric, hydrochloric, and hydrofluoric acid) under controlled temperature (first at 180 °C, and posterior heating at 110 °C), and complexation with boric acid (10 min). After digestion by microwaves (Speed wave of Berghof), samples were analyzed using inductively coupled plasma–mass spectrometer (ICP- MS) (Thermo Elemental Series-X, Santa Clara, CA, USA). These elements were also analyzed in overlying and elutriate seawater at the end of the toxicity tests in filtered (0.45 mm) subsamples and acidified (pH < 2). Analyses were performed using inductively coupled plasma optical emission spectrometry (ICP-OES). These analyses were performed in the Scientific Instrumentation Center of the University of Granada.

2.6. WoE Approach

Three main LoEs were determined in the present study to characterize and distinguish the impacts related to the contamination degree and the acidification caused by the CO₂ enrichment (Figure 3).

Figure 3. Schematic representation of the lines of evidence (LoEs) applied in the study to assess the environmental risks of CO₂ enrichment in marine ecosystem: Contamination addresses the chemical concentration in sediments to answer two main questions, which contaminants and level of contamination? Sediment toxicity addresses the adverse biological effects under laboratory conditions and alteration on the macro-benthic community effects under in-situ conditions.

The hypothesis of this work predicted that marine organisms and environment might be affected by exposure to the acidification caused by CO₂ enrichment that was statistically tested using an integrated approach (WoE) based on significant differences and a multivariate analysis. The obtained results from each LoEs were linked for each derived component assessed using three different methods as outlined by [6,17,30]: (a) tabular matrix based on statistical significance ($p < 0.05$) compared to the reference site; (b) pie charts, showing statistical differences ($p < 0.05$ significant different and positive effect; $0.1 \geq p > 0.05$ significant different but moderate effect; and $p > 0.1$ not significant different among the samples sites and pH values; and (c) linking the results using a multivariate analysis approach [6].

The significant differences found between the pH treatments and the reference site (PAI station, pH 8.0), and to develop the pie charts and tabular matrix ANOVA and Dunnet's tests were used through TOXSTAT software, version 3.5.+

The values used for these analyses were determined through index of contamination, toxicity, and ecological integrity. The indexes were calculated considering the values

of ratio to reference (*RTR*) following [17]. For it, the results from each LoE in each pH treatment were divided by the values presented in the reference case (PAI, pH 8.0) and then deriving the minimum value to calculate the index. Then, these values were averaged for metal contamination in sediment and mobility, acute toxicity, chronic toxicity, and ecological integrity.

The contamination index was calculated considering two conditions: the concentration of As, Cr, Cu, Ni, and Zn in the sediment (RTR_{sediment}) and in seawater and elutriates (RTR_{mobility}). The index of acute toxicity was derived from the results of amphipod survival rate and to chronic toxicity was estimated from the larval development inhibition results of sea-urchins. In relation to the index of benthic integrity, the inverted average values of *RTR* were calculated for total abundance, diversity, and richness. The formulas used to determine the indexes were:

$$RTR_i = \frac{v_i}{(v_i)_0} \text{ and } I = \frac{\sum_{i=1}^n RTR_i}{n} \forall i$$

where i = parameter and V_i = value of the parameter in the reference case, and I [Index] is equal the sum of number calculated for each *RTR* per the total variable number considered in the respective case.

Multivariate analysis was conducted following methods previously described by [17,26]. The results were analyzed using factor analysis method with extraction of the variables by principal component analysis (PCA). Factors were rotated using the varimax normalized procedure. The component loading cutoff of 0.40 was used in the selection of variables for inclusion into the factors. Also, the factor scores for each sampling site were calculated. Statistical analyses were carried out by means of the STATISTICA[®] software package version 13.2.

3. Results

Summarized results for sediment and seawater chemistry, acute and chronic toxicity, and benthic community structure related to CO₂ enrichment in the marine environment are described in Table 2. Results of the concentration of metal(loid)s in elutriate treatment and seawater were shown for the different pH tested. In general, greater concentration of metal(loid)s in overlying seawater were found than elutriate treatments, except in the case of Zn and As, that were higher in the elutriates. Moreover, higher concentrations of metals were found in the sediment samples from CPI than PAI, with the exception of Ni and As. Regarding to biological responses, significant ($p < 0.05$) mortalities were found at pH 6.0 when using the PAI sediment, and at pH 6.5 using the CPI sediment. However, significant ($p < 0.05$) effects were found for the larval development of sea-urchin at pH 7.0 and 6.5 for PAI and CPI sediments, respectively. No significant ($p < 0.05$) decreases were found in the community indexes of PAI sediment fauna when compared with the control treatment (i.e., no CO₂ added), except in the total abundance which decreased considerably at lowest pH level. However, the results found for the benthic community from CPI showed significant decrease in the diversity at pH 6.0. On the other hand, an increase in the total abundance of species was showed at pH 6.0 from CPI.

3.1. Tabular Matrix

Significant differences ($p < 0.05$) between the reference station (PAI, pH 8.0) and the treatments with both sediments were represented as showing a positive response either in contamination, toxicity, and integrity (Table 3). The results obtained for the metal(loid) concentrations in the sediment samples showed no significant difference between the treatments used in this study. However, significant differences were found in the results of metal(loid) mobility between the reference (PAI, pH 8.0) and the lower acidification treatments (pH 6.5 and 6.0) for both stations. Regarding the results of toxicity, significant chronic effects were found from pH 7.0 for the PAI sediment and from pH 6.5 for CPI sediment. Meanwhile acute effects were observed at pH 6.0 in PAI sediment and at

pH 6.5 for CPI sediment. Moreover, the results of macro-benthic community integrity showed significant differences for all treatments for CPI sediment, pointing out that the contamination presented in CPI sediments is caused by mobility and bioavailability.

Table 2. Summarized results for chemical concentrations in sediment (mg kg^{-1} dry weight), overlying water and elutriate ($\mu\text{g L}^{-1}$), acute and chronic toxicity tests (mortality of amphipods- Mort_A, and sea urchins -Inh_SU) and, ecological integrity exposed to the pH treatments (pH 8.0, 7.5, 7.0, 6.5, and 6.0) using the PAI and CPI sample sites. (-) Reflects concentrations below of detection limit of the equipment.

	pH Values									
	PAI					CPI				
	8.0	7.5	7.0	6.5	6.0	8.0	7.5	7.0	6.5	6.0
Contamination										
Cu _{elu}	3.0	2.8	4.8	3.0	5.7	2.5	5	1.4	1.1	2.5
Cu _{sw}	6.7	2.3	2.5	2.3	4.6	5.7	5	6.86	6.84	7
Cu _{sed}	7.2	7.2	7.1	7.2	7.3	11	11.1	10.8	10.9	11
Cr _{elu}	0.8	0.8	0.8	3.12	2.81	-	1.66	1.47	2.5	1.53
Cr _{sw}	1.85	4.3	6.92	5.88	2.3	2.1	1.6	2.23	2.6	2.28
Cr _{sed}	27.6	27.4	27.7	27.9	27.4	30.9	30.9	31.1	30.8	30.9
Zn _{elu}	-	-	34	25	32	40	91	54	60.4	38.8
Zn _{sw}	24	23	26	40	35	39	26	48	54	96
Zn _{sed}	123	122	126	127	125	133	134	133	137	138
Ni _{elu}	-	-	1.12	-	0.16	1.22	1.18	1.23	1.12	1.14
Ni _{sw}	-	-	1.8	2.12	4.9	1.7	1.82	2.13	3.75	6.2
Ni _{sed}	13.7	13.7	13.6	13.7	13.8	5.92	5.96	5.96	5.94	5.95
As _{elu}	40	75	45	100	142	65	65	60	60	95
As _{sw}	57	-	-	-	150	58	48	59	64	130
As _{sed}	3.16	3.14	3.15	3.17	3.16	2.83	2.83	2.85	2.89	2.85
Toxicity										
Mort_A (%)	18	27	24	38	100	20	26	22	47	100
Inh_SU (%)	17.25	16	70	72	100	6	10	17	100	100
Macrobenthic										
Diversity	0.59	0.61	0.63	0.57	0.60	0.63	0.67	0.79	0.22	0.31
T. abundance	54	57	65.5	54	21	6	8	8	8	20
Richness	0.64	0.65	0.69	0.51	0.67	0.91	1.01	1.07	0.27	0.51

Table 3. Matrix results for the different pH treatment used and sediment samples, representing significant differences ($p < 0.05$) were found when compared to the reference case (PAI, pH 8.0). Responses are shown as either positive (+), negative (-) and (+/-) when the result was close to be positive but not showed significant difference.

Site, pH Treatment	Metals		Toxicity		Benthic
	Sediment	Mobility	Acute	Chronic	
PAI, pH 8.0	—	—	—	—	—
PAI, pH 7.5	—	—	—	—	—
PAI, pH 7.0	—	±	—	+	—
PAI, pH 6.5	—	+	—	+	—
PAI, pH 6.0	—	+	+	+	—
CPI, pH 8.0	—	—	—	—	+
CPI, pH 7.5	—	±	—	—	+
CPI, pH 7.0	—	±	—	—	+
CPI, pH 6.5	—	+	+	+	+
CPI, pH 6.0	—	+	+	+	+

3.2. Pie Charts

Figure 4 represents significant differences based on the weights for the different lines of evidence (element contamination, acute and chronic toxicity, and macro-benthic integrity). The PAI sampling site was selected as reference site at control pH (no CO₂ added; pH 8.0 ± 0.1). Each pH treatment for both sampling sites were compared with the control treatment. Significant differences were represented as black sections when $p < 0.05$, gray sections when significant differences were $0.01 \geq p > 0.05$, and white section for no significant difference ($p > 0.01$).

Figure 4. Pie charts showing statistical differences among the pH levels using the different LOEs. (A) Representing PAI sediment samples and (B) representing CPI sediment samples. The segments shown in black were associated with significant difference ($p < 0.05$) compared to the control, gray segments were related based on differences using different p values ($0.01 \geq p > 0.05$), white segments indicated results not significantly different to the reference case.

These results confirm those reported by tabular matrix and provide more information related to the cases with moderate effects ($0.01 \geq p > 0.05$). The results showed significant effects ($p < 0.05$) for chronic toxicity and moderate ($0.01 \geq p > 0.05$) effects for mobility of metals at a pH of 7.0 using PAI sediment. In the case of CPI sediment, significant effects ($p < 0.05$) for the most of LoEs tested were found at pH 6.5, showing a strong environmental degradation at the treatment within this pH level. Furthermore, moderate increases in the metal mobility were found in the CPI control and at higher values of pH when compared with the reference case.

3.3. Multivariate Analysis

A multivariate analysis approach was performed in order to identify the correlation among sediment and water contamination, biological effects in the laboratory and the pH reduction in seawater caused by CO₂. The original variables are represented by three new principal components (Table 4).

Table 4. Sorted rotated factor of the original variables on the three principal factors. The loading >0.4 are highlighted in the table.

Variables	Components		
	#1	#2	#3
Variance	43.38	28.56	10.80
Mobility of metals			
As _{sw}		0.82	
Cr _{sw}	−0.65		
Cu _{sw}	0.76		
Ni _{sw}		0.85	
Zn _{sw}	0.54	0.48	0.54
As _{elu}		0.88	
Cr _{elu}		0.62	
Cu _{elu}	−0.45	0.37	−0.57
Ni _{elu}	0.84		
Zn _{elu}	0.74		
Sediment metal concentration			
As _{sed}	−0.99		
Cr _{sed}	0.99		
Cu _{sed}	0.99		
Ni _{sed}	−0.99		
Zn _{sed}	0.92		
Toxicity			
Larval Inhibition		0.72	0.60
Amphipod Mortality		0.96	
Acidification (H ⁺)		0.96	
Ecological Integrity			
Diversity			−0.84
T. Abundance	−0.88		
Richness			−0.87

The first three components explained 82.74% of the variance in the original data set. The first component #1 represents a 43.38% of variance and combined the results of Cu and Zn in overlying seawater and in sediment, Zn and Ni in elutriates, and Cr, Cu, and Zn in sediment. The second component #2 explains a 28.56% of variance and linked As, Zn and Ni in seawater, Cr, Cu, and As in elutriate, the increase in the concentration of H⁺ protons (pH reduction) and the biological effects (mortalities of amphipods and sea-urchin larval development inhibition), and the third component #3 explains a 10.80% of variance linking Zn in overlying water and the larval development inhibition of sea-urchins.

Moreover, negative values were found for the diversity, total abundance, and richness at #1 and #3, which indicates correlations among these results. These variables were

linked as negative due to the form in which the results were represented (as the values of ecological integrity decrease, more effects were found to be correlated with these factors).

These results suggest that the toxic effects were correlated with the increase in the concentrations of protons associated with the enrichment of CO₂. The results also correlate the toxic effect with the increase in the mobility of some metals (As, Zn, and Ni in seawater and As, Cr, and Cu in elutriate). Furthermore, the larval development of sea-urchin and the diversity and richness of the macro-benthic communities was shown to have also been affected by the concentration of Zn in seawater that was not correlated with the pH reduction.

Factor scores estimated for each case are represented in Figure 5. This is a clear visualization of the relationship between the components (factors) and the cases (treatments) tested in the study.

Figure 5. Estimated factor scores for each sediment sampling sites used in this study (PAI and CPI). The factor scores quantify the prevalence of every component for each sediment site in relation to the pH levels and are used to confirm the factor description (Table 4).

The factors scores showed that the results indicated in the component #2 that were the toxicity effects and the mobility of certain metals were associated with the pH 6.0 in both stations studied. Moreover, a higher influence of the factors #1 and #3 in the CPI station that were related to the contamination of metals in both cases, sediment and mobility, was observed. These results confirmed Table 3 and Figure 4 since significant effects ($p < 0.05$) were found at pH 6.0 for both stations, and greater effects associated with the contamination were shown for CPI station.

4. Discussion

The CCS technologies may cause punctual potential acidification events due to CO₂ enrichment in the marine environment caused by leakages. These CO₂ leakages may occur over time during some steps of operations technology [31]. In this context, studies of environmental risk characterization in the areas nearby CCS operations are highlighted [2].

In Brazil there are projects with operating status of CCS system in Rio de Janeiro and Bahia [32]. The sediment stations selected for this study are located in the Santos estuary and Bay; this zone is considered relatively close to Rio de Janeiro and with tropical characteristics as well. Moreover, both sediment stations selected for this study are areas usually known to receive large quantities of organic matter from the surrounding mangrove and urban activities.

According to the report on coastal waters quality of the State of São Paulo [33], the pH value found in the areas selected in this study varies between a pH of 6.5 and 8.5. Furthermore, a previous study has shown a value of 2.2% and 7.5% of total organic carbon (TOC) in the PAI and CPI stations, respectively [16]. Therefore, studies of the impact

caused by enrichment of CO₂ in areas that present tropical characteristics as Brazil and with organic carbon concentrations in sediments are also highlighted.

The results found in the study were expressed using the WoE approach, based on different lines of evidence, to characterize the environmental degradation associated with the enrichment of CO₂ in the coastal zones. The use of this integrated approach has been widely indicated as an efficient method to identify and distinguish the origin of contamination and provide a better monitoring and protection of the marine environment [6,15,25,27,34]. Previous studies have characterized the sediment contamination by means of metals, PAHs and PCB concentrations in the Santos Estuary [16,27,35]. These authors and their results agree with our study showing that the lowest metal concentrations were found for the control sediment station (PAI). The concentrations obtained in the other station (CPI) were also in agreement with previous studies showing the sediments at this station as an intermediate contamination. Besides, sediment quality guidelines based on *Tiburonella viscana* [23] determined no pollution for concentrations below 63 mg kg⁻¹ Cu, 73.3 for Zn, below 0.5 for ΣPAHs and 4 µg.kg⁻¹ ΣPCBs. However, no significant differences were showed in the concentration of metals in the sediment between the reference case, and all pH treatments tested for both stations when using the tabular matrix and pie charts. Additionally, the concentration of metals found in both stations were below limit values defined by [36] (used in Brazil to classify the levels of metal concentrations in dredged sediment).

The results from the multivariate analysis showed no correlation between the biological indexes of macro-benthic communities and pH reduction (#2, Table 3). The diversity and richness of the macro-benthic communities were associated with concentrations of Zn in seawater (#3), while the total abundance of the organisms was associated with the sediment contamination and the mobility of Cu, Zn, and Ni (#1). These results can explain those demonstrated in the tabular matrix and pie charts that were found a significant difference ($p < 0.05$) in the ecological integrity in all pH treatments when using the CPI sediment station. Moreover, these findings may indicate the contamination and bioavailability of elements present in this station, which was confirmed with the estimated factor scores (Figure 5).

Benthic organisms may be exposed to contaminants on different routes, such as by their contact with pore water, sediment-water interface through walls of the body, and respiratory surface or by the ingestion of contaminated sediment particles [37]. Although no significant difference was found in the metal concentration in the sediment stations, the higher difference in the macro-benthic communities could be related to the effects of other environmental factors and contaminants that were not measured in this study. The Santos estuary is influenced by a number of factors, such as port and dredging activities, human sewage effluents, contribution of nutrients from the mangroves, and others [16,25,34]. It was reported by [16,23] a greater bioavailability of PAHs at CPI station, such as benzo(a)anthracene, benzo(a)pyrene, and dibenzo(a,h)anthracene, which are considered very toxic for both biota and humans [38,39]. Furthermore, these authors reported higher concentrations of Hg bioaccumulated in the oysters. Thus, all of these factors reported and the high carbon concentration (7.5%) found in the CPI station could contribute to the low biological indexes of macro-benthic community present there.

It is known that the reduction in the concentration of both hydroxide and carbonate (OH⁻ and CO₃²⁻) could change the speciation of some metals and, consequently, raise up the fraction in free forms at lower pH [40]. One of the lines of evidence selected for this study was to analyze the mobility of metals and their toxicity in relation to acidification by enrichment of CO₂. The results showed a significant increase in the metals concentrations in overlying water and elutriate treatment at pH 6.5 and 6.0 using the tabular matrix and the pie charts for both sediment stations when compared with the reference case (PAI, pH 8.0). Moreover, moderate effects in the mobility of metals were observed in the gray areas (Figure 4) at pH 7.5 and 7.0 using the CPI station.

A recent study showed that there are more sulfides forms in the sediments from SES than other complex forms [16]. Furthermore, these authors reported that when there is

more sulfide complex than divalent metals in the environment it is supposed that these metals are complexed to the sulfides, which are less bioavailable. According to [40], metals form strong complexes with hydroxide and carbonate, which undergo significant changes in speciation as the pH of seawater decreases. Thus, for an environmental risk assessment in case of a CO₂ enrichment, it is important to analyze the speciation of metals to understand interactions with lower pH in seawater and how they affect the biota. The tabular matrix (Table 3) and pie charts (Figure 4) showed chronic effects associated with pH values of 7.0 and pH 6.5 for PAI and CPI sediment, respectively. However, the sediment from PAI is considerate lesser contaminated than CPI sediment, so higher bioavailability of some elements are present as carbonate forms. In addition, the results using the multivariate analysis indicated strongly correlation between certain metals (As, Zn, and Ni in seawater, and Cr, As, and Cu in elutriate) and the biological effects related to the increase in the concentration of H⁺ protons (pH reduction). These results may confirm the statistical results reported (Table 3 and Figure 4) and provide an estimation of the metal(loid)s related biological responses (included in the 'gray and black' areas of the WoE analysis from Figure 4).

Furthermore, the results of factor scores from component 2# also confirm a relationship between metal mobility, toxicity, and acidification as a positive score only at the lowest pH (6.0) in both stations. This means that the CO₂ enrichment in these stations could cause effects under extreme acidified conditions-.

5. Conclusions

The weight-of-evidence approach was successfully applied in this study using different lines-of-evidence that allows an environmental risk characterization related to the enrichment of CO₂ in the studied coastal zones.

The results showed chronic biological adverse effects associated with values of pH 7.0, significantly differenced to the reference case (PAI station, pH 8.0) using a sediment with low contamination levels (PAI station). Furthermore, moderate effects were also observed in the mobility of metals associated with the pH 7.0 using both sediment stations.

The mobility of certain metals (As, Zn and Ni in seawater, and Cr, As, and Cu in elutriate) and the toxic effects were significantly related to the increase in the concentrations of protons. These findings suggest that metals might be responsible for the adverse effects as demonstrated from the WoE analysis, probably promoted an increasing of metal bioavailability and toxicity at pH values of 7 or lower.

Regarding the ecological integrity of the macro-benthic community, there was no correlation between the biological indexes and the pH reduction when using the multivariate analysis. The results correlated the biological indexes with the metals contamination in the sediment and the dissolved Zn in seawater. Although no significant differences were found in the metals concentration in the sediment in both stations studied, other contaminants may have influenced in the results.

The WoE approach identified a source of contamination responsible for the pollution and for the adverse effects on marine organisms studied in this work. The results applying this technique showed that the pH value of 6.0 is significantly toxic to the exposed marine organisms and associated with both the increase of the proton concentration itself and direct toxicity to organisms (and, consequently, the increase of the mobility and bioavailability of metals driving pollution in the coastal zones).

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