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Could Acidified Environments Intensify Illicit Drug Effects on the Reproduction of Marine Mussels?

Lorena da Silva Souza [1], Estefanía Bonnail [2,*], Julia Alves Luzzi [3], Augusto Cesar [4], Tomás A. DelValls [3] and Camilo Dias Seabra Pereira [3,4]

[1] Department of Physico-Chemistry, Aquatic Systems Research Group, UNESCO/UNITWIN WiCop, Faculty of Marine and Environmental Sciences, University of Cádiz, 11500 Cádiz, Spain
[2] Centro de Investigaciones Costeras-Universidad de Atacama (CIC-UDA), University of Atacama, Copiapó 1530000, Chile
[3] Department of Ecotoxicology, Santa Cecília University (UNISANTA), Santos 11045-907, SP, Brazil
[4] Department of Marine Sciences, Federal University of São Paulo (UNIFESP), Santos 11030-100, SP, Brazil
* Correspondence: estefania.bonnail@uda.cl

Abstract: The increasing oceanic uptake is a direct response to the increasing atmospheric burden of CO_2. Oceans are experiencing both physical and biogeochemical changes. This increase in CO_2 hosts in oceans promotes changes in pH and seawater chemistry that can modify the speciation of compounds, largely due to dependent element speciation on physicochemical parameters (salinity, pH, and redox potential). So, ocean acidification can trigger enhanced toxicity of illicit drugs to non-target marine organisms due to the combined effects of crack cocaine and low pH (from 8.3 to 7.0 pH values) on the reproduction of the marine mussel *Perna perna*. Fertilization rate and embryo–larval development were used as endpoints to assess the effects of crack-cocaine concentrations (6.25, 12.5, 25, 50, and 100 mg L^{-1}) and its association with pH values variation (8.3, 8.0, 7.5, and 7.0). The IC_{50} was calculated from the results of an embryo–larval assay in different methods of acidification (CO_2 and HCl), which evidenced that HCl treatment was more toxic than CO_2 treatment for the same drug concentrations. Results showed that the gametes of *P. perna* react to acidification when exposed to crack-cocaine concentration and pH reductions.

Keywords: CO_2 enrichment; crack cocaine; early life stages; climate change effects; *Perna perna*

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1. Introduction

Mauna Loa observatory [1] observed a peak in atmospheric CO_2 in May 2021 (monthly average 419 ppm). This and other greenhouse gases are causing global environmental change [2]. However, the exchange of CO_2 between the atmosphere and the hydrosphere promotes ocean acidification (OA) by decreasing the pH of average surface seawater [3]. We estimate a decrease of 0.67 units of pH in seawater by 2300 with respect to preindustrial pH levels [4].

The increase in CO_2 atmospheric burden promotes the increasing oceanic uptake, so that oceans experience both physical and biogeochemical changes: surface and deep water warming, reduced subsurface oxygen, and a reduction in $CaCO_3$ saturation levels and pH [5]. The oceanic biogeochemical dynamics are increasingly relevant in the assessment of the ecosystem health, climate impacts, mitigation strategies, and planetary sustainability [4]. Some previous studies have indicated that OA over-calcifies marine organisms [6–9], marine bacteria [10–12], amphipods [13], macro-algae [14,15], or macro-fauna [16,17].

OA interacts with local stressors, such as xenobiotics and other emerging compounds. Nevertheless, nowadays, these interactions between OA, environmental changes, and contaminants require greater scientific knowledge [18]. The seawater pH decrease caused by increasing CO_2 can modify the speciation of compounds, due to largely dependent elements' speciation on physicochemical parameters (salinity, pH, potential redox) [19,20],

and therefore their bioavailability for organisms [21]. Previous studies have shown the effect of OA on the speciation of metals [7,20,22]; however, this is still poorly investigated for contaminants acting as emerging compounds, and as pharmaceuticals.

The occurrence of pharmaceuticals and personal care products (PPCPs) in the environment is increasingly recognized as an important issue [23–26]. This includes illicit drugs; emerging pollutants are also considered [27,28]. Pharmaceuticals and illicit drugs, once ingested, are partially metabolized due to their particular solubility, and enter the sewage stream [29], but the inefficient or incomplete treatment causes marine pollution. Once in the environment, illicit drugs promote adverse effects in non-target organisms, as demonstrated by some studies with cocaine byproducts [30–34]. The marine mussels *Perna perna* exposed to 0.5 and 5 ng L^{-1} exhibited cytotoxicity linked to the instability of the lysosomal membrane [31], biomarker responses (EROD, DBF, GST, GPx) [23], and how detoxification, oxidative stress, and cytogenotoxicity are caused [32]. However, CC metabolites [33] also promoted sublethal effects in *D. polymorpha*. Additionally, crustaceans exposed to CC showed an increase in motility [23]. CC effects in mammals, such as mice, display genotoxicity and mutagenicity induced by acute crack cocaine exposure [34]. However, the effects in mollusks under acidified environments with illicit drug presence required further research.

Understanding factors affecting the survivorship and growth of juvenile mussels through vulnerable early life stages is critical both for aquaculture efforts and ecology equilibrium. This work aims to assess how acidification can trigger enhanced toxicity of illicit drugs to non-target marine organisms due to the combined effects of crack cocaine (CC) and low pH (from 8.3 to 7.0 pH values) on the reproduction of the marine mussel *Perna perna*. To achieve this, two acidification methodologies were applied (HCl and CO_2 injection). Additionally, the toxicity test endpoints for mussels were survival, fertility, and embryo–larval development.

2. Material and Methods

2.1. Experimental Set Up

The seawater used in the experiment was artificially prepared in the laboratory by dissolving natural salt (Red Sea salt®) in deionized water up to 35 ppm, which is the optimum value for the target species as described by [35]. Filtered reconstituted seawater was used as control (N), but was also used to dilute the different CC treatments. For the experiments, the pH was modified as acidified scenarios (8.3-Control-, 8.0, 7.5, and 7.0) with two different methods (CO_2 injection and HCl addition) and different CC concentrations (Figure 1).

The CO_2 injection system used was an adaptation of the experimental setup described by [14], pH was continuously monitored. Experiments conducted with the strong acid HCl to modify the total alkalinity used the methodology adapted from [36]. Thus, the different pH values were achieved by adding 2M HCl (37%), and the pH measurements were recorded every 5 h.

2.2. Chemical Determination

The CC concentration selection criteria for the experiments with marine mussels were based on previous experiments of [31,37,38]. Concentrations of CC were detected in Santos Bay between 12.60–537 ng L^{-1}, and up to 5896 ng L^{-1} in Igarapé (Manaus, Brazil) [23]. Five different concentrations (6.25, 12.5, 25, 50, and 100 mg L^{-1}) of the illicit spiked CC were used to determine the toxic effects in different acidification scenarios. The drug was donned for research purposes as a courtesy by the Criminal Department of Limeira city (Sao Paulo State-Brazil). Measured concentrations and solvent extraction are further detailed in the Supplementary Materials (Table S1). Briefly, crack-cocaine concentrations were analyzed by LC-MS/MS and HPLC Agilent 1260 (Agilent Technologies, Santa Clara, CA, USA) combined with a 3200 QTRAP hybrid triple quadrupole/LIT mass spectrometer ABSciex (Vaughan, ON, Canada), according to the procedure described by [38] and employed by [39]. The cocaine primary standard was purchased from Cerilliant–Sigma Aldrich (Lot

FE07271503, St. Louis, MO, USA). Further detailed information about CC content can be found in the Supplementary Materials.

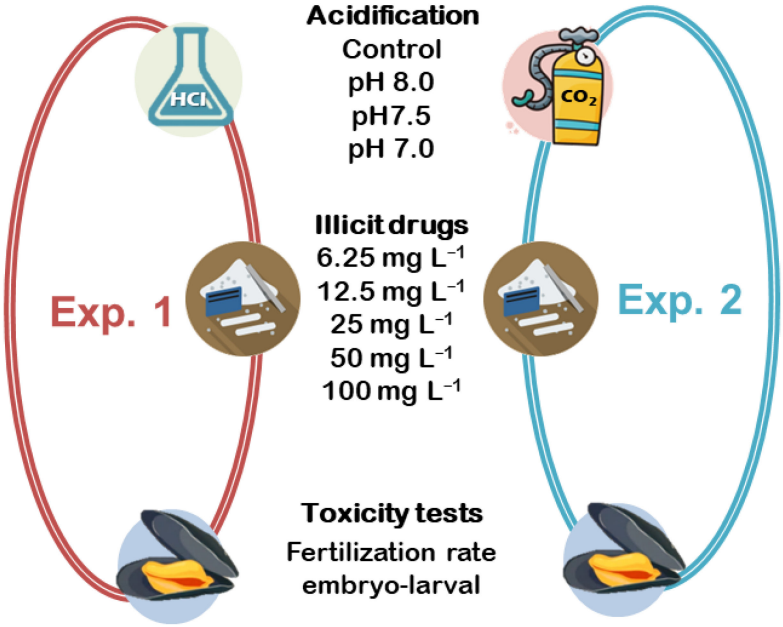

Figure 1. Schematic design of toxicity tests carried out in the study: HCl (**left**) and CO_2 (**right**) injection acidification. Simplified parameters selected for experiment 1 and 2 with acidification sources (CO_2 and HCl), crack concentrations (6.25, 12.5, 25, 50 and 100 mg L^{-1}) and the targeted endpoints for mussels (fertilization rate and embryo larval development).

Total alkalinity (TA) was determined (in triplicate) by the potentiometric titration system (Metrohm 794 Basic Titrino, Herisau, Switzerland) with a glass electrode (Metrohm, ref. 6.0210.100) calibrated in NBS scale. Carbonate chemistry parameters were calculated based on the pH and the TA with the CO_2SYS software [40] using the dissociation constants from [41] refit by [42], and KSO_4 according to [43].

2.3. Toxicity Tests

Specimens of marine mussel (*Perna perna*) were purchased from an aquaculture facility (Cocanha beach at Caraguatatuba, SP/Brazil) and held in a 500 L tank filled with clean aerated seawater for 24 h before toxicity tests. The gametes were obtained according to [44], with minor adaptations proposed by [35]. Four replicates were used for each CC concentration with different pH values (Figure 1). The pH value of 8.3 was used as a control, where no CO_2 or HCl was added (natural pH of the reconstitute water) (Table 1).

2.4. Fertilization Bioassay

The fertilization rate was conducted according to [35] in 10 mL test tubes with CC solution at different pH values, besides a control with reconstituted seawater and without CC to ensure the quality of the experiment. Then, 50 µL sperm solution was added to each test tube; after 1 h of sperm solution exposure to different treatments, oocytes were added. After 40 min, 40% formaldehyde at pH 7.0 was added to finalize the assay. By using a light microscope with 100× magnification, the presence of the polar body of the fertilization membrane or the first cell divisions in 100 eggs was evaluated. The test was considered valid, with ≥80% of the eggs successfully fertilized in the control [45].

2.5. Embryo–Larval Development Bioassay

The following assay was performed as described by [44,46]. Briefly, adults were induced to spawn by thermal stimulation, and fertilization was achieved by adding 1.5 mL of sperm solution into 250 mL of the eggs suspension for 60 min (RT). Then, approximately

25,000 embryos were introduced into 1.3 L glass chambers containing 500 mL of filtered (0.22 µm) reconstituted seawater (25 °C, S of 35 ± 1, 7.5 mg L^{-1} DO, 12 h photoperiod), with the desired pH and CC concentration for each treatment (Figure 1). After exposure time (42 h), 40% formaldehyde was added and larvae counted. The test was considered valid for ≥80% larvae successfully developed in the control.

Table 1. Physicochemical water parameters (nominal cocaine–crack CC, pH in seawater, salinity—S, dissolved oxygen—DO, temperature—T) monitored in the different assay treatments (control as reconstituted seawater, CO_2 injection and HCl treatment). pH average ± SD values were calculated from 30 min period measurements taken during a 48 h period.

pH	CC (µg L^{-1})	pH Seawater	S (PSU)	DO (mg L^{-1})	T (°C)
Reconstituted seawater		8.30 ± 0.03	34	5.14	25
	6.25	8.30 ± 0.02	34	5.78	25
	12.5	8.30 ± 0.01	34	5.78	25
	25	8.30 ± 0.04	34	5.35	25
	50	8.30 ± 0.03	34	5.14	25
CO_2 treatment					
8.0	Control	8.00 ± 0.05	34	5.80	25
	6.25	8.00 ± 0.03	34	5.82	25
	12.5	8.00 ± 0.02	34	6.60	25
	25	8.00 ± 0.03	34	5.91	25
	50	8.00 ± 0.05	34	5.80	25
7.5	Control	7.50 ± 0.01	34	5.99	25
	6.25	7.50 ± 0.06	34	5.74	25
	12.5	7.50 ± 0.03	34	5.32	25
	25	7.50 ± 0.08	34	5.66	25
	50	7.50 ± 0.01	34	5.99	25
7.0	Control	7.00 ± 0.03	33	5.67	25
	6.25	7.00 ± 0.06	34	6.03	25
	12.5	7.00 ± 0.03	34	6.00	25
	25	7.00 ± 0.01	33	5.91	25
	50	7.00 ± 0.03	33	5.67	25
HCl treatment					
8.0	Control	8.00 ± 0.04	35	5.49	24
	6.25	8.00 ± 0.03	35	5.19	24
	12.5	8.00 ± 0.03	35	6.34	24
	25	8.00 ± 0.02	35	5.09	24
	50	8.00 ± 0.04	35	5.49	24
7.5	Control	7.50 ± 0.02	35	5.64	25
	6.25	7.50 ± 0.04	35	5.59	25
	12.5	7.50 ± 0.03	35	5.79	25
	25	7.50 ± 0.04	35	5.45	25
	50	7.50 ± 0.02	35	5.64	25
7.0	Control	7.00 ± 0.05	35	5.23	26
	6.25	7.00 ± 0.02	35	6.09	26
	12.5	7.00 ± 0.03	35	5.99	26
	25	7.00 ± 0.06	35	6.01	26
	50	7.00 ± 0.05	35	5.23	26

2.6. Statistical Analysis

Regarding the effects caused by the variation in the pH values, the term EpH_{50} was used. The IC_{50} and EpH_{50} were calculated through the polynomial interpolation method. Normality and homogeneity of variances were tested using Shapiro–Wilke's and Levene's tests, respectively. Two-way ANOVA with Tukey's post hoc test was performed to compare

results of the different acidification levels and CC concentrations with the control using the statistical software SPSS 15.0 for Windows.

3. Results and Discussion

3.1. Chemical Analysis

Optimal water-quality conditions, which are essential to the success of a toxicity test, were predetermined and maintained through a CO_2 injection system. Water chemistry was continuously monitored (Table 1).

The values for the carbonate species, calculated by CO_2SYS software, are shown in Table 2. A decrease in pH seawater resulted in a reduction in the concentration of OH^- and CO_3^{2-}, as presented by our results, since we observed an increase in CO_2 concentrations and a decrease in CO_3^{2-} rates. The higher the concentration of CO_2, the lower the CO_3^{2-} stability, which decreased its capacity to connect with other chemical elements such as calcium, since the carbonate (CO_3^{2-}) has a higher chemical affinity for H^+ ions than for calcium (Ca^{2+}).

Table 2. Carbonate system speciation in assays exposed to the different scenarios by CO_2 enrichment treatments for the mussel toxicity tests.

pH Treatment	CC (µg L^{-1})	TA (µmol L^{-1})	TIC (µmol kg^{-1})	HCO_3^- (µmol kg^{-1})	CO_3^{2-} (µmol kg^{-1})	CO_2 (µmol kg^{-1})	pCO_2 (µatm)	Ω_{cal}	Ω_{arag}
8.0	Control	1641	1516	1413	85.6	18.1	655	2.06	1.36
	6.25	1654	1525	1419	88.3	17.7	646	2.13	1.41
	12.5	1312	1475	1291	7.5	176.2	6541	0.18	0.12
	25	1754	1650	1547	79.2	23.8	880	1.91	1.27
	50	1799	1794	1697	39.3	57.8	2152	0.95	0.63
	100	1862	1883	1776	33.6	74.2	2767	0.81	0.54
7.5	Control	1685	1604	1511	65.9	27.5	1030	1.59	1.06
	6.25	1672	1632	1545	48.1	39.1	1451	1.16	0.77
	12.5	1744	1713	1622	47.1	44.1	1641	1.14	0.75
	25	1717	1691	1600	44.6	45.6	1715	1.08	0.72
	50	1855	1888	1776	30.7	81.9	3083	0.74	0.49
	100	1874	1924	1803	27.5	93.3	3481	0.67	0.44
7.0	Control	1713	1707	1615	37.7	54.0	2007	0.92	0.61
	6.25	934	911	863	22.9	25.3	952	0.56	0.37
	12.5	1751	1800	1686	25.1	88.9	3333	0.61	0.41
	25	1793	1736	1641	58.9	36.0	1355	1.44	0.95
	50	1796	1846	1728	26.1	91.5	3473	0.63	0.42
	100	1925	1910	1808	46.0	56.1	2117	1.12	0.74

On the other hand, after adding HCl, concentrations of H^+ and H_2CO_3 in seawater increased, and HCO_3^- and CO_3^{2-} decreased. However, few studies [14,47–49] focus on and explain the main difference in the carbonate speciation between the HCl and CO_2 methodologies.

The decrease in $CaCO_3$ saturation levels, confirmed in this study by an under-saturation in the index for calcite (Ω_{cal}) and aragonite (Ω_{arag}), poses a major threat to marine organisms, particularly shell-forming and calcifying organisms [50]. As seen in the study from [6], there was an alteration in the calcification process in *Millepora alcicornis* (calcareous hydrozoan) triggered by CO_2-driven acidification.

The aliquot of CC analyzed by LC-MS/MS contained 37.99% of cocaine. Determination of the real concentrations of cocaine in the exposure aliquot was unmeasurable; however, a low decrease in CC concentrations in the bioassays is expected, as [51] reported for wastewater, as did [31] when referring to marine water.

3.2. Toxicity Assays

3.2.1. Fertilization Rate Assay

Figure 2 shows results from fertilization assay under pH 8.3 with the different CC concentrations. The treatments did not present any significant difference ($p < 0.05$) compared to N treatment considered negative control for this assay.

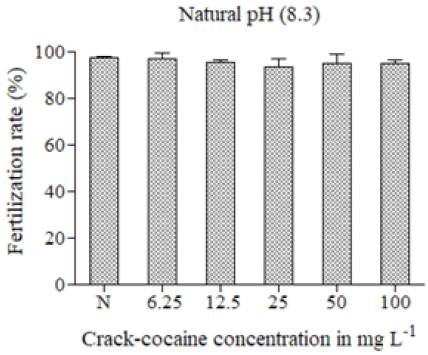

Figure 2. *Perna perna* fertilization rate (in %) results obtained after exposure to different concentrations of CC (6.25, 12.5, 25, 50, and 100 mg L^{-1}) without acidification at pH 8.3 considered the negative control. (N represents natural pH 8.3, with no acidification method applied).

Figure 3 shows the fertilization rate results for both acidification methodologies. The CO_2 acidification methodology at pH 7.5 with the highest CC concentration (50 mg L^{-1} and 100 mg L^{-1}) presented a significant decrease in the fertilization success of the mussel when compared with the control (N). In the case of the HCl acidification methodology, there was a significant decrease in the fertilization rate for all pH treatment and CC concentrations when compared to the control.

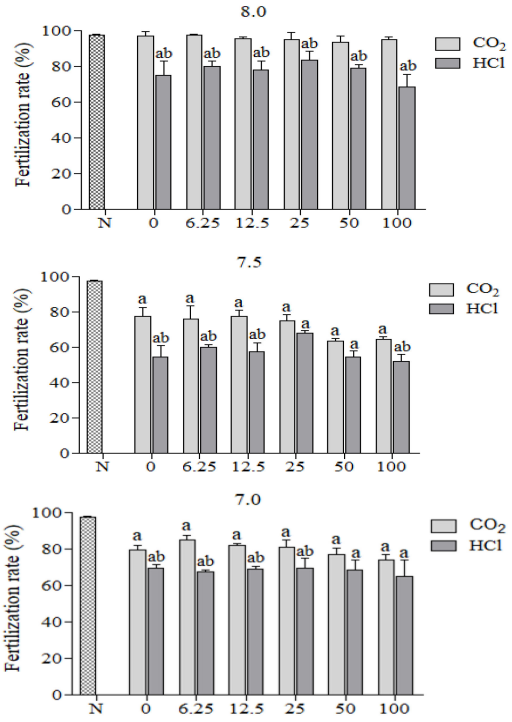

Figure 3. *Perna perna* fertilization rate results obtained after exposure to different concentrations of CC and acidification at different pH values 8.0, 7.5, 7.0 promoted by CO_2 and HCl supplies. Concentration 0 means acidified but with no CC. A statistically significant difference ($p < 0.05$) compared with N (reconstituted seawater considered as negative control) is shown with "a"; and between CC concentrations in different acidified methods is shown with "b".

Agreeing with our results, ref [52] showed a reduction in the percentage of fertilized eggs of *C. gigas* when the pH value was decreased by 0.7 from the original value compared to the other treatments. This might support the hypothesis proposed by [53], who affirmed that seawater acidification would affect the intracellular pH of sperm and alter sperm motility, fertilization, and embryo development. Similar species, such as the green-lipped mussel, have demonstrably increased respiration rates and reduction in growth and reproductive output at elevated pCO_2 [54].

Regarding CC concentrations that the sperm was exposed to during a one-hour period, our results showed that even the highest concentration of CC (100 mg L^{-1}) was not able to affect the sperm's ability to fertilize the oocyte. On the other hand, [31] presented the effects on the fertilization rate of *P. perna* mussel already in the CC concentration of 1.25 mg L^{-1}.

3.2.2. Embryo–Larval Toxicity Test

The results of this toxicity assay using *Perna perna* in reconstituted seawater (N) with different CC concentrations are presented in Figure 4. Significant differences were observed in CC concentrations of 12.5, 25 and 50 mg L^{-1} when compared with N (no CC concentration added). The authors of [31] found significant differences in *P. perna* embryo–larval development when exposed to 1.25 mg L^{-1} CC.

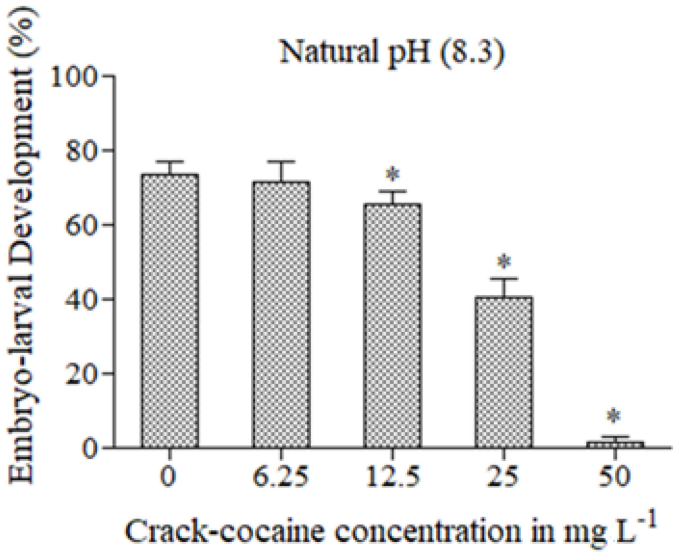

Figure 4. *Perna perna* embryo–larval development results after exposure to the different CC concentrations in reconstituted seawater (pH 8.3) without acidification and considered as negative control. Asterisks indicate significant differences ($p < 0.05$).

The results of embryo–larval successes applying the two different methods of acidification (CO_2 and HCl) are shown in Figure 5. It was demonstrated that the CO_2 acidification method promoted a significant decrease (with respect to control treatment) ($p < 0.05$) for CC concentrations up to 12.5 mg L^{-1}. Results obtained for HCl acidification methodology showed significant differences for CC concentrations up to 6.25 mg L^{-1} at pH 7.7 compared to control treatment. In the figure were pH values of 8.3 (N-control), and 8.0 and 7.7, excluding pH 7.5 (and less), since this pH value was highly toxic and presented no normal larval development for both acidification strategies.

The IC_{50} was calculated from the results of embryo–larval assay and presented in Table 3. The different methods of acidification presented different values of IC_{50} associated with CC, evidencing that HCl is more toxic than CO_2 when associated with the same concentrations of CC.

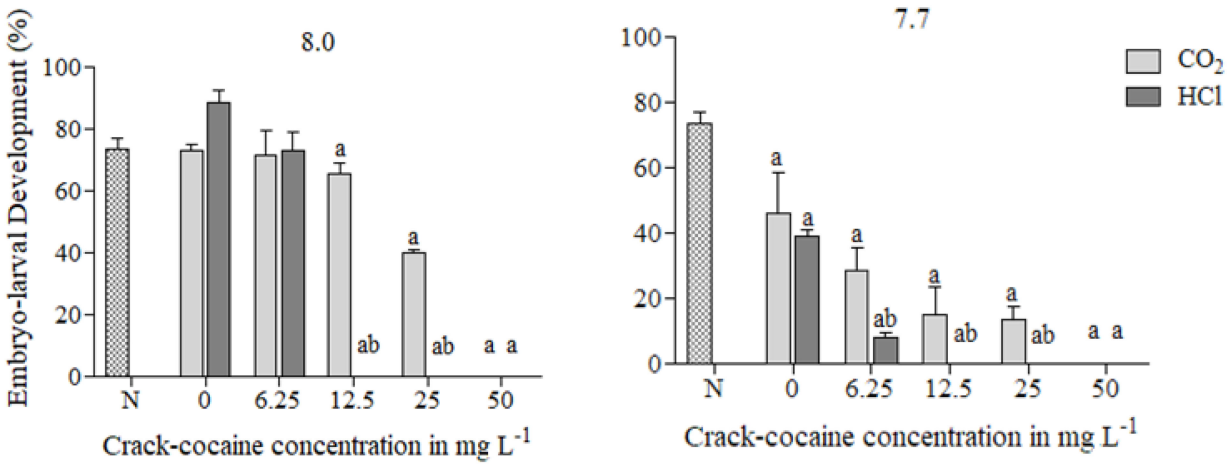

Figure 5. *P. perna* larval development success results after exposure to CC and acidification at two different pH values (8.0 on the (**left**) and 7.7 on the (**right**)). The letter "a" indicates a significant ($p < 0.05$) difference compared to results at the N treatment (reconstituted seawater). Similarly, letter "b" indicates a significant ($p < 0.05$) difference between CC concentrations in different acidified methods.

Table 3. Values of IC_{50} derived from CC concentrations at the different pH values promoted by HCl and CO_2 acidification methodologies.

pH Values	IC_{50} (mg L^{-1})	
	CO_2	HCl
8.3	14.08 (12.66–15.30)	8.85 (8.64–9.01)
8.0	13.85 (12.50–14.60)	8.72 (8.44–8.95)
7.7	9.37 (4.66–16.15)	3.92 (3.73–4.14)
7.5	-	-

Regarding pH effects, Table 4 shows the EpH_{50} and $E[H^+]50$ derived from the different treatments; that is, acidification causes effects in more than 50% of the embryos after 44 h exposure. The HCl acidification method presented a greater effect on the organisms (including control groups) when compared to the CO_2 method. In addition, CC showed more severe toxic effects when associated with acidification by HCl (Tables 3 and 4). This increase in toxicity may be related to the chemical reaction of the HCl acid, which released protons H^+ and ions of Cl^-, against the bicarbonate (HCO_3^-) released from CO_2.

Table 4. Values of EpH_{50} and $E[H^+]50$ for the experimental treatments.

CC (mg L^{-1})	CO_2		HCl	
	EpH_{50}	$E[H^+]50$ (mol kg^{-1})	EpH_{50}	$E[H^+]50$ (mol kg^{-1})
Control	7.34	4.56×10^{-8}	7.53	2.95×10^{-8}
6.25	7.55	2.81×10^{-8}	7.65	2.21×10^{-8}
12.5	7.61	2.41×10^{-8}	8.18	0.66×10^{-8}
25	7.58	2.61×10^{-8}	-	-
50	8.18	0.66×10^{-8}	-	-

According to [52], two reasons might be promoting the morphological abnormalities of the larvae: (a) damage to the embryonic ectodermic cells rendering them unable to produce sufficient amorphous calcium carbonate, avoiding the proper development of the shell, or (b) shell dilution due to corrosion caused by acidified seawater. However, these factors are the result of an incomplete calcification process, which would not be enough to cover the entire mantle of the larva [55]. This feature would also promote a swimming skill decrease in the larvae, i.e., a fitness decrease [55,56].

Larval physiology components are affected by the carbonate speciation (and probably life stage). Any failure to embryo–larval develop represents a significant bottleneck in the population dynamics; however, other carbonate system parameters may act as stressors. For example, the saturation state (Ω_{arag}) appears to matter most for the rapid shell building of prodissoconch I phase in bivalve larvae [57]. Our results prove that the gametes of *P. perna* react to acidification when exposed to expected realistic pH reductions.

Besides the carbonate system parameters, according to [58], the destabilization of the lysosomal membrane may cause a nutrient imbalance during embryogenesis, leading to disturbances in larval development in bivalves. A growth delay or abnormal shapes from early stages indicate intense morphogenetic activity. The authors of [31,32] demonstrated that the lysosomal membrane stability of mussels is affected by the different CC concentrations. This could mean larval starvation affecting not only embryonal development, but also population growth inhibition.

Regarding the combined cocaine with different acidification scenarios, toxicity was also tested for the reproduction (fertilization and embryo development) of the sea urchin *Echinometra lucunter* [37]. However, further research is required to understand possible ecological scenarios with other species, as the CC have become a common compound in urban sewage. Even more, the role of other illicit drugs (and their metabolites) [58] and new emerging compounds in warming and acidifying environments might have a dramatic combined effect on other species and other life stages, even algae (irgarol and *Ulva lactuca* [59]) and mussels (cocaine, benzoylecgonine and *Mytilus galloprovincialis* [60]).

4. Conclusions

The HCl acidification method was found to be more toxic than CO_2 enrichment to early life stages of *P. perna* mussel. Our results demonstrate the combined effects of a psychoactive substance (crack cocaine) in the first life stages of *P. perna* exposed to ocean acidification scenarios. Considering the taxonomic position of the species, it is quite possible that early development of other bivalve species might be similarly affected by strong acidified seawater (CO_2), although further verification might be necessary for other species, drugs, and life stages.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/app122111204/s1, Table S1: Measured concentrations of crack cocaine.

Author Contributions: Conceptualization, L.d.S.S., C.D.S.P. and T.A.D.; Methodology, L.d.S.S. and C.D.S.P.; Software, L.d.S.S., C.D.S.P., A.C. and E.B.; Validation, all authors; Formal Analysis, L.d.S.S., E.B. and C.D.S.P.; Investigation, L.d.S.S., E.B. and T.A.D.; Resources, L.d.S.S., C.D.S.P. and T.A.D.; Data Curation, E.B. and A.C.; Writing—Original Draft Preparation, L.d.S.S., C.D.S.P., T.A.D.; Writing—Review and Editing, E.B. and T.A.D.; Visualization, E.B. and T.A.D.; Supervision, C.D.S.P., A.C. and T.A.D.; Project Administration, E.B. and T.A.D.; Funding Acquisition, C.D.S.P., A.C. and T.A.D. All authors have read and agreed to the published version of the manuscript.

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