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USE OF *BRACHIONUS PLICATILIS* (ROTIFERA) TO ASSESS THE QUALITY OF MARINE WATER IN CALLAO BAY, PERU

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ABSTRACT: The present investigation used *Brachionus plicatilis* (Rotifera) to evaluate the water quality in Callao Bay, Peru. The water samples were taken in four areas: P_1 in La Punta, P_2 in Chucuito, P_3 in front of the Callao Port Terminal and P_4 in the San Lorenzo and El Frontón Islands, and in four seasonal periods: autumn-2015, winter- 2015, spring-2015, and summer-2016. Physical-chemical parameters, chlorophyll, phycocyanin, heavy metals, and bioassays with seawater at 24 h and 48 h of exposure to *Brachionus plicatilis* obtained from a standardized culture were evaluated in each area and seasonal period. High concentrations of Ag, Pb, Hg, Cu, Cr, Ni and Zn were found at all the sampling sites. The no observed effect concentration (NOEC) values (concentration at which a significant effect of mortality is not observed with respect to the control) and lowest observed effect concentration (LOEC) (minimum concentration at which a significant effect of mortality is observed with respect to the control) at 48 h of exposure to the *B. plicatilis* bioassay were lower for P_1 (winter-2015) and P_2 (winter-2015). Principal components analysis (PCA) showed that principal component 1 (PC_1) contributed 38.70% and PC_2 17.70%. PC_1 was formed by LOEC of Cd, Hg, Ni, Ag, and Pb while PC_2 was related to Cu, Cr and Zn. According to these results *Brachionus plicatilis* can be used as a bioindicator organism to assess water quality in the marine environment.

Keywords: acute bioassay, *Brachionus*, heavy metals, mortality, rotifer.

RESUMO: *A presente investigação utilizou Brachionus plicatilis (Rotifera) para avaliar a qualidade da água na Bahía del Callao, Peru. As amostras de água foram coletadas em quatro locais: P_1 em La Punta, P_2 em Chucuito, P_3 em frente ao Terminal Portuário de Callao e P_4 nas Ilhas San Lorenzo e Frontón, e em quatro períodos sazonais: outono-2015, inverno-2015, primavera-2015 e verão-2016. Os parâmetros físico-químicos, clorofila, ficocianina, metais pesados e bioensaios com água do mar às 24 he 48h de exposição com Brachionus plicatilis obtidos em cultura padronizada foram avaliados em cada área e período sazonal. Os metais Ag, Pb, Hg, Cu, Cr, Ni e Zn foram encontrados em maior concentração em todos os locais de amostragem. Os valores de NOEC (concentração onde um efeito significativo de mortalidade não é observado em relação ao controle) e LOEC (concentração mínima onde um efeito significativo de mortalidade é observado em relação ao controle) em 48 h de exposição com o bioensaio de B. plicatilis foram menores para P_1 (inverno-2015) e P_2 (inverno-2015). A técnica de Análise de Componentes Principais (ACP) mostrou que o componente principal 1 (CP_1) contribuiu com 38,70% e o CP_2 com 17,70%. CP_1 foi formado por LOEC, Cd, Hg, Ni, Ag e Pb. CP_2 relacionou as variáveis Cu, Cr e Zn. Propõe-se a utilização de Brachionus plicatilis como organismo bioindicador para avaliação da qualidade da água no meio marinho.*

Palavras-chave: *bioensaio agudo, Brachionus, metais pesados, mortalidade, rotífero.*

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1. INTRODUCTION

Accelerated industrial development brings with it lifestyle modifications and an increase in the generation of waste and emission of pollutants towards marine and freshwater aquatic systems (Jha, 2008; Escobar-Chávez *et al*., 2019; Pascual *et al*., 2019; Sotelo-Vásquez and Iannacone, 2019), causing negative effects on aquatic life and human health (Jha, 2008; Rojas-Jaimes *et al*., 2019; Panduro *et al*., 2020).

The pollution and accumulation of heavy metals in the oceans must be investigated and analyzed. The term heavy metal (HM) refers to any metallic chemical element that has a high density and is toxic at very low concentrations (Cousseau and Perrotta, 2013). HMs are dangerous because some bioaccumulate and biotransfer and because they are persistent and not destructible (Panduro *et al*., 2020). This leads to an increase in the concentrations of these chemical elements in living organisms over a period of time compared to the concentrations in an environmental matrix (Djinovic-Stojanovic, 2015).

To evaluate the quality of seawater in marine ecosystems, physicochemical parameters are usually used individually and are contrasted with an environmental quality standard, which has limitations because toxic effects to the biota are disregarded as are additive, antagonistic and synergistic interactions between contaminants (Cousseau and Perrotta, 2013; Casanova-Rosero *et al*., 2015; Planes and Fuchs, 2015).

Callao Bay, in Peru, has historically shown that the concentration of pollutants, including HM, has exceeded the environmental quality standards of the Peruvian Water Law (MINAM, 2017). Therefore, adequate effluent treatment and disposal systems must be implemented (*i.e.*, treatment plants and submarine emitters) (Larios-Meoño *et al*., 2015).

Protocols have been established to assess the environmental quality of marine ecosystems using bioindicator species (Cousseau and Perrotta, 2013; Ferrari, 2015). Toxicity bioassays are simple, practical, sensitive, and repeatable and, whenever possible use native species (Cousseau and Perrotta, 2013; Herkovits *et al*., 2015).

Among the indicator species of the aquatic environment, there are rotifers that are components of zooplankton, which are key in trophic webs and present a wide geographical distribution (Toscano and Severino, 2013; Jeong *et al*., 2019ab; Gharaei *et al*., 2020). *Brachionus* is one of the genera with more species in the Phylum and is called "the white mouse" of rotifers (Toscano and Severino, 2013; Jeong *et al*., 2019ab; Alvarado-Flores *et al*., 2019). *Brachionus plicatilis* is a species widely used in aquaculture to feed more than 60 species of bony fish larvae and 18 species of crustaceans (Sharma *et al*., 2018; Yona, 2018), in which they have been used to evaluate nutritional aspects (Ortega-Salas and Reyes-Bustamante, 2013; Rahman *et al*., 2018; Contreras-Sillero *et al*., 2019; Ferrando *et al*., 2019), reproductive (Sun *et al*., 2017; Yona, 2018), as well as developmental biology (Clark *et al*., 2012) and geographical distribution (Toscano and Severino, 2013). *B. plicatilis* is considered a complex of cryptic species with various morphotypes (Guerrero-Jiménez *et al*., 2019; López *et al*., 2019).

The rotifer *B. plicatilis* is a useful model for evaluating aquatic ecotoxicity due to its ecological relevance, biological and practical characteristics, rapid reproduction and short life cycle (Rico-Martínez *et al*., 2013). The toxic effects of particulate matter, other chemical substances and algae toxins have been evaluated using *B. plicatilis* (Rico-Martínez *et al*., 2013; Li *et al*., 2018).

The objective of this study was to perform toxicity bioassays using *B. plicatilis* to evaluate the quality of seawater in Callao Bay, Peru.

2. MATERIAL AND METHODS

2.1 Study area

The city of Callao is located in a strategic site along a long coastline receiving the cold waters of the Humboldt Current, making it the first seaport in Peru and one of the most important ports in South America. The main economic activity is manufacturing industries. Other important economic activities involve the export of frozen fish from Callao, representing 18.2% of the fish consumed in Peru and about 50% in the city of Lima, Peru and manufacturing plants for preparing frozen fish, flour, and canned and cured meats. Fishing in Callao also contributes directly to the generation of added value and employment. The Callao Port Terminal concentrates 90% of the country's maritime transport, both merchant and military, and has an urban environment made up of adjoining private land for residential and industrial use, a Naval Base and the Rímac River, as well as sports facilities on the south side of the port. The quality of the seawater in the province is affected by the reception of drainage, the discharge of industrial effluents and waste transported by the Rímac and Chillón rivers, which cause the values of HMs, thermotolerant coliforms, suspension of oils, fats, and solids to exceed national environmental quality standards according to current regulations. The sea of Callao receives approximately three times more domestic wastewater than the province produces.

The National Institute of Culture of Peru has declared San Lorenzo Island as Cultural Patrimony of the Nation with 20 archaeological monuments present on the island. San Lorenzo Island and El Frontón Island present marine biodiversity. These islands are close to Isla Palomino, which has populations of sea lions, and is part of the National Reserve of the System of “Islas Guaneras, Islotes y Puntas”.

To perform the toxicity bioassays, four seasonal evaluations were carried out in Callao Bay during the fall (May 30)-2015, winter (August 22-23)-2015, spring (November 22-23) in 2015 and the summer (January 5-6) of 2016. The sampling areas were: P_1 at La Punta (Naval School) (12 ° 3’57.2-12 ° 4’47.8 “S, 77 ° 10’8.60” -77 ° 10’35.4 “W), P_2 in Chucuito (in front of IMARPE “Instituto del Mar del Perú”) (12 ° 3 ‘34.91’ -12 ° 3’57.45 “S, 77 ° 9’23.76-77 ° 9’35, 8 “W), P_3 in front of Callao Port Terminal (12 ° 1’59.52” - 12 ° 2’51.67 “S, 77 ° 9’6.1” - 77 ° 9’38.71 “W) and P_4 in the San Lorenzo Islands and the Fronton (12 ° 4 ‘23.6’ ‘- 12 ° 6’34.73 “S, 77 ° 10’38.5” - 77 ° 13’7.5 “W). The sampling areas were georeferenced with a GPS and an echo sounder (GARMIN model map 4215) and spatially located on a map with the ArcGIS 10.8.1 for Desktop and ArcMAP 10.8 programs (Price, 2019).

According to the classification of coastal-marine water bodies in Peru, P_1 and P_3 were located at a site of the marine-port, industrial or sanitation activities, P_2 was in the area allocated for primary contact recreation, and P_4 was located at the site devoted to the extraction and cultivation of mollusks (MINAGRI, 2016).

Only one sample of surface water was collected from each sampling areas without replicates, and physicochemical parameters were determined *in situ* including sea surface temperature (SST) (°C), pH, electrical conductivity (mS·cm^{-1}), salinity (g·L^{-1}), total suspended solids (TSS) (mg·L^{-1}), dissolved oxygen (DO) (mg · L^{-1}), transparency (m), turbidity, phycocyanins (µg·mL^{-1}), chlorophyll (ug · mL^{-1}), oxide-reduction potential (ORP) (mV), ammonia (mg·L^{-1}), and nitrates (mg·L^{-1}). These parameters were evaluated with an EXO 2 multiparameter (YSI ® brand, United States). Chlorophyll and phycocyanins were measured by the Algae Torch R Fluorometric method (EIJKELKAMP® brand, Netherlands).

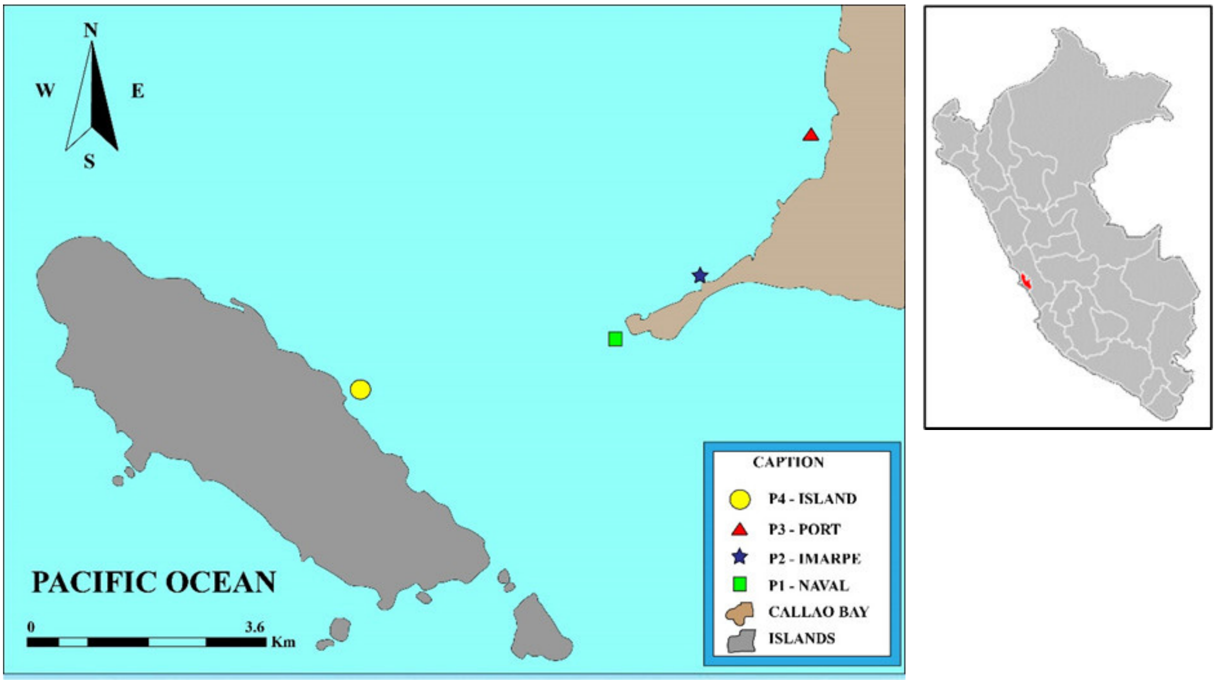

Figure 1. Sample areas in Callao Bay, Peru (ArcGIS 10.8.1 and ArcMAP 10.8). P_1 = Naval School, P_2 = in front of IMARPE, P_3 = Callao Port Terminal and P_4 = San Lorenzo and Fronton Island.

Marine water samples were taken according to standardized protocols for each of the environmental parameters evaluated (Rice *et al*., 2017). For HM analysis, 500 mL of water were collected in polyethylene containers washed and treated with nitric acid. The pH was adjusted to 2.0 with the same acid and the sample was kept at 4°C until transfer in a cooler and subsequent analysis in the laboratory, which followed quality assurance (QA) and quality control (QC) procedures to guarantee the results of the analysis of the water samples based on the Quality Management System designated by the USEPA and by the quality policy of the laboratory. QA/QC practices involve a broad range of activities including, but not limited to: a) manufacturer guidance and user manual, b) calibrations, c) acceptance, use, and maintenance of instruments and equipment, water sample chain of custody, d) pre- and post-deployment procedures, and record-keeping strategies (Rice *et al*., 2007; USEPA, 2002). The total HM content was determined by inductively coupled plasma atomic emission spectroscopy (Rice *et al*., 2017) following standard protocols (EPA 200.7, Rev 4.4, 1994) (INVEMAR, 2003; USEPA, 1994) and for Hg was determined using cold vapor atomic absorption spectrometry (Rice *et al*., 2017).

The results of the physicochemical parameters and HMs were contrasted with the National Environmental Quality Standards for Water according to Supreme Decree No-004-2017 MINAM (MINAM, 2017) and the classification of marine-coastal water bodies of Peru (MINAGRI, 2016). The HMs were also compared

Table 1. Environmental quality standards for heavy metals (mg·L^{-1}) in marine water according to Peruvian and international regulations.

Metals	Peruvian standard		Canadian standard		Australian and New Zealand standard	EC Standard	USEPA		Ecuador standard
	II-C3	IV-E3	Short Term	Long Term			Acute MCC	Chronic CCC	
Sb	0.64	NA							
As	0.05	0.036		0.0125	ID	0.025*	0.069	0.036	0.05
Ba		NA							1
B	NA		GNR	GNR					5
Cd	NA	0.0088	GNR	0	0.0007	0.0002	0.0011	0.05	0.005
Cu	0.05	0.05	ND	ND	0.0003	0.025*	0.0011	0.05	0.05
Cr VI	0.05	0.05	ND	0.0015	0.00014		0.0048	0.05	0.05**
P			ND	ND					
Fe			ND	ND	ID				0.3
Mn			ND	ND					0.1
Hg	0.0018	0.0001	ND	0.000016	0.0001	0.00005	0.0018	0.00094	0.0001
Ni	0.074	0.0082	ND	ND	0.007	0.02	0.074	0.0082	0.1
Ag			0.0075	NRG	0.0008		0.0019		0.005
Pb	0.03	0.0081	ND	ND	0.0022	0.0072	0.140	0.0056	0.01
Se	NA	0.071	ND	ND			0.29	0.071	0.01
Si	NA	NA							
Tl	NA	NA							
Zn	0.12	0.081	Not evaluated	ND	0.007	0.06*	0.09	0.081	

Sb: Antimony, As: Arsenic, Ba: Barium, B: Boron, Cd: Cadmium, Cu: Copper, Cr: Chromium, P: Phosphorus, Fe: Iron, Mn: Manganese, Hg: Mercury, Ni: Nickel, Ag: Silver, Pb: Lead, Se: Selenium, Si: Silica, Tl: Thallium, Zn: Zinc. Peruvian Standard: IIC3 = Category 2: Extraction, crops and other coastal and continental marine activities. C3. Marine port, industrial or sanitation activities in coastal marine waters. IV-E3 = Category 4: Conservation of the aquatic environment. E3. Marine ecosystems. Canadian Standard Short Term and Long Term. Australian and New Zealand standard at 95% species protection. EC: European Community. * = Indicates values suggested by Tueros *et al.* (2009). USEPA: Environmental Protection Agency. MCC: Maximum Concentration Criterion, is an estimate of the highest concentration of a material in surface water that an aquatic community can be briefly exposed to without resulting in an unacceptable effect. CCC: Continuous Concentration Criterion, is an estimate of the highest concentration of a material in surface water to which an aquatic community can be exposed indefinitely without producing an unacceptable effect. Ecuador Standard: Quality Criteria of the Environmental Quality Standard and discharge of effluents to the water resource. ** = Indicates values in total Chromium. ND: No data, GNR: Guideline not recommended. ID: Insufficient data.

with five international standards: (1) the Canadian standard that indicates short-term and long-term Environmental Quality Guidelines (CCMC, 2007), (2) the Australia and New Zealand standard (ANZECC and ARMCANZ, 2000), (3) that of the European Community (EC) as indicated by Crane and Babut (2006) and Tueros (2009), (4) the standard of the United States Environmental Protection Agency (USEPA, 2009) and finally (5) that of Ecuador (AM, 2015) (Table 1).

The rotifer *B. plicatilis* was acquired from the *Universidad Científica del Sur* and from the germplasm bank of the Marine Institute of Peru (IMARPE), Lima, Peru. The culture was kept at 25°C ± 2°C in a bioclimatic chamber in continuous darkness and in 1 L Erlenmeyer flasks according to the protocol of Pérez-Legaspi and Rico-Martínez (1998). The preparation of artificial seawater for cultivation and for ecotoxicological bioassays was carried out using distilled water with 35 $g \cdot L^{-1}$ of artificial sea salt (Fluval Sea® Sal Marina, Rolf C. Hagen (USA) Corp., Mansfield, MA, USA) sterilized with ultraviolet radiation.

The experimental design for the ecotoxicological bioassays consisted of the exposure of *B. plicatilis* to seawater from each of the sampling areas in dilutions with the artificial seawater in completely randomized blocks: six concentrations or treatments with four repetitions: 100%; 50%; 25%; 12.5% and 6.25%, and a negative control; and two exposure times of 24 h and 48 h (Ferrari, 2015; Escobar-Chávez *et al*., 2019). The preparation of each concentration was carried out in 6.5 mL well plates (flat bottom 12 multi-well plates, Cole Parmer®, IL, United States). A battery of tubes was used, in which 6 mL of seawater was placed in the first tube (100%), repeated four times, then half was extracted and placed in the next tube and this was completed up to 6 mL again, and so on until the last concentration. Then, for each concentration, 10 juveniles of *B. plicatilis* from parthenogenetic females were introduced at between 0 and 24 h after birth. The temperature of the bioassays was 23 ± 1° C. Rotifers were not fed during ecotoxicological tests under dark conditions, to avoid increased swimming activity and the effect of photolysis (Snell *et al*., 1991).

The endpoint of the toxicity tests was mortality (immobility), considering individuals dead when only the presence of loric or absence of coordinated movement was evidenced when punctured with an entomological pin for 15 s under microscope observation. Based on the mortality of the rotifers, the toxicity of seawater was determined by quantifying the LC_{50} (mean lethal concentration) (Planes and Fuchs, 2015). The LC_{50} values were expressed as a percentage of dilution (volume / volume).

The general data obtained was processed in a database in EXCEL (Office, 2010). The statistical package SPSS version 20.0 for Windows 7® was used to determine the acute toxicity parameters (LC_{50} in % of the water sample). The statistical Probit model was used for each concentration of seawater and to determine the percentage of mortality (Rice *et al*., 2017) and the lower and upper 95% confidence limits at 24 h and 48 h of exposure ($p < 0.05$). In addition, the NOEC and LOEC values were obtained by means of analysis of variance (ANOVA) of a factor and a subsequent Tukey's honestly significant difference test. Before calculating the ANOVA, the Levene homogeneity of variances test and the Shapiro-Wilks normality test were performed to properly use the parametric tests (Zar,1996). Principal components analysis (PCA) was used as a data ordering and reduction technique of the number of variables employing toxicity values of LOEC of seawater with *B. plicatilis* at 48 h of exposure with eight HM from surface water in Callao Bay, Peru. HM (Cd, Cu, Cr. Hg, Ni, Ag, Pb and Zn) were selected from among those presenting Environmental Quality Standards of Canadian or European Community guidelines. The first two components were selected by PCA for the nine variables analyzed (Jolliffe and Cadima, 2016).

2.2 Ethical aspects

This study followed all the national and international ethical aspects of ecotoxicology. The use of invertebrates such as rotifers in the laboratory is allowed in ecotoxicological studies without ethical restrictions (Franco *et al*., 2014). The number of organisms used in bioassays was in accordance with the principle of the three "r's" (Oliveira and Goldim, 2014). For proper management of rotifer culture, reagents and seawater samples, as well as their disposal, the "Safety Plan for Laboratories and Workshops (SSST-PLC-01)" was followed the Rectoral Resolution No. 10026-2009 -UNFV and the "Security Protocol for Engineering, Architecture and Natural Sciences Laboratories and Workshops (SSST-PS-02)". The respective national permits were obtained from the competent national authority for collecting the water samples in Callao Bay.

3. RESULTS

Table 2 shows the variations of the physicochemical and phycocyanin and chlorophyll parameters in the four sampling areas and for the four seasonal evaluations in Callao Bay, Peru. The average SST ranged from 19.5 to 21.5°C for the areas sampled. The sequence from lowest to highest pH in the sampling areas was: $P_2 > P_3 > P_1 > P_4$. The highest values for the mean OD and for the mean transparency were for the P_4 sampling area. Phycocyanin and chlorophyll values were low in all evaluations. The highest phycocyanin and chlorophyll values were found in P_3, while the highest NH_3 levels were obtained in P_4 and for P_1.

The HMs and Ag, Pb, Hg, Cu, Cr, Ni and Zn concentrations that did not meet the water quality standards of Callao Bay, for at least some of the comparison standards and mainly for the Canadian and Australia-New Zealand regulations, were found in higher concentrations in the P_1, P_2 and P_3 sampling sites (Table 3).

Table 2. Variations of the physicochemical parameters of marine water in the sampling areas and in the station evaluations in Callao Bay, Peru.

Sampling area	Seasonal assessments	T° (°C)	pH	Cond. ($ms \cdot cm^{-1}$)	Sal ($g \cdot L^{-1}$)	TSS ($mg \cdot L^{-1}$)	DO ($mg \cdot L^{-1}$)	Transp. (m)	Turb (FTU)	Phyco ($ug \cdot L^{-1}$)	Chlorof ($ug \cdot L^{-1}$)	ORP (mV)	NH_4 ($mg \cdot L^{-1}$)	NH_3 ($mg \cdot L^{-1}$)	NO_3 ($mg \cdot L^{-1}$)
P_1	1	21.5	8.1	53.4	35.1	38142	2.49	0.86	2.4	0,1	0.30	ND	ND	ND	ND
	2	18.1	7.9	53.7	35.5	34203	2.28	0.75	4.0	0	1.40	241.3	52.38	0.32	77.88
	3	20.8	8.5	53.8	35.3	33737	1.72	5.00	0.7	0	0	184.4	60.04	2.50	2105.56
	4	21.5	8.7	53.1	34.7	34000	2.15	1.25	1.0	0	0.09	178	52.65	1.81	417.42
	Mean	20.5	8.3	53.5	35.1	35020.5	2.16	1.97	2.0	0,025	0.45	201.2	55.02	1.54	866.95
	SD	1.6	0.3	0.32	0.34	2089.73	0.33	2.03	1.5	0,05	0.65	34.85	4.35	1.11	1086.02
P_2	1	20.4	7.9	53.7	35.2	38357	1.35	3.12	2.3	0	0.20	ND	ND	ND	ND
	2	16.9	8.0	53.9	35.1	34773	2.21	2.75	2.7	0	1.60	225.3	55.43	0.30	73.98
	3	20.1	8.1	53.0	34.8	33808	0.90	2.25	7.0	0	0.68	182.7	54.89	4.25	957.67
	4	20.8	8.1	53.2	34.8	34010	1.33	3.75	2.0	0	0	176.0	51.39	1.35	365.58
	Mean	19.6	8.0	53.4	34.9	35237	1.45	2.97	3.5	0	0.62	194.6	53.90	1.97	465.74
	SD	1.7	0.1	0.42	0.21	2121.11	0.55	0.63	2.3	0	0.71	26.7	2.19	2.05	450.28
P_3	1	20.1	8.0	53.8	35.2	38428	1.74	2.87	2.9	0	0.70	ND	ND	ND	ND
	2	17.2	8.0	53.7	34.9	34180	1.44	3.00	7.6	0	1.1	211.2	64.52	0.45	156.22
	3	19.6	8.2	53.5	34.9	33893	0.89	3.00	2.5	0	0.88	176.2	54.61	3.77	672.19
	4	21.2	8.2	53.4	35.3	34076	0.65	2.25	0.7	0	0	170.9	50.22	1.26	321.85
	Mean	19.5	8.1	53.6	35.0	35144.25	1.18	2.78	3.4	0	0.67	186.1	56.45	1.83	383.42
	SD	1.6	0.1	0.18	0.21	2192.38	0.50	0,36	2.9	0	0.48	21.9	7.33	1.73	263.44
P_4	1	24.6	8.2	53.9	35.0	38500	3.61	7,50	ND	ND	ND	ND	ND	ND	ND
	2	18.4	8.1	53.7	35.1	34702	3.11	4,75	2.4	0	2	234.4	33.57	0.29	114.75
	3	19.9	8.6	53.3	34.9	34011	2.40	5,00	ND	0	0.48	170.8	53.71	4.42	562.84
	4	23.0	9.1	53.6	35.3	34104	4.32	2,75	ND	0	0	164.0	48.37	2.20	304.01
	Mean	21.5	8.5	53.6	35.0	35329.25	3.36	5,00	2.4	0	0.83	189.7	45.22	2.30	327.20
	SD	2.8	0.4	0.25	0.17	2135.89	0.81	1,95	0	0	1.04	3.8	10.43	2.07	224.94
Peruvian Standard	II-C3	Δ3	6.8-8.5			70	≥2.5								NA
	IV-E3	Δ3	6.8-8.5	NA		≤30	≥4.0								200

T° = Sea surface temperature. pH = acidity of the medium. Cond = Electrical conductivity. Salt = Salinity. TSS = Total Dissolved Solids. DO = Dissolved oxygen. Transp = Transparency. Turbi = Turbidity. Phyco = Phycocyanins. Chlorof = Chlorophyll. ORP = Oxide reduction potential. NH_4 = Ammonium. NH_3 = Ammonia. NO_3 = Nitrates. SD = Standard deviation. ND = Not determined. P_1 = Naval School. P_2 = IMARPE (Institute of the Sea of Peru). P_3 = Callao Port Terminal and P_4 = San Lorenzo Island. 1 = Fall-2015, 2 = Winter-2015, 3 = Spring-2015, 4 = Summer-2016. Peruvian Environmental Standard SQA (MINAM, 2017): Category 2: Extraction, cultivation and other coastal and continental marine activities. C3. Marine port, industrial or sanitation activities in coastal marine waters. Category 4: Conservation of the aquatic environment. E3. Marine ecosystems.

Table 3. Heavy metals (mg·L^{-1}) in seawater in the sampling areas in Callao Bay, Peru.

Metals	Detection limits	Callao bay, Peru															
		1				2				3				4			
		$1P_1$	$1P_2$	$1P_3$	$1P_4$	$2P_1$	$2P_2$	$2P_3$	$2P_4$	$3P_1$	$3P_2$	$3P_3$	$3P_4$	$4P_1$	$4P_2$	$4P_3$	$4P_4$
Sb	0.006	0.01	<0.006	<0.006	<0.006	0.017	<0.006	0.020	0.032	0.01	0.022	0.025	0.016	0.023	<0.006	0.010	0.0007
As	0.0092	<0.0092	<0.0092	<0.0092	<0.0092	<0.0092	<0.0092	<0.0092	0.0283	<0.0092	<0.0092	<0.0092	<0.0092	<0.0092	<0.0092	<0.0092	<0.0092
Ba	0.0013	0.0090	0.0067	0.0059	0.0071	0.0097	0.0052	0.0066	0.0053	0.0062	0.0074	0.0069	0.0056	0.0069	0.0067	0.0079	0.0047
B	0.0016	4.25	4.22	4.34	4.31	4.34	4.33	4.30	4.33	5.24	5.82	5.11	5.70	5.11	5.09	5.32	5.91
Cd	0.0015	<0.0015	<0.0015	<0.0015	<0.0015	<0.0015	<0.0015	<0.0015	<0.0015	***0.0024***	***0.0026***	***0.0015***	***0.0036***	***0.0031***	***0.0028***	<0.0015	0.0028
Cu	0.0014	0.0067	0.0150	0.0040	0.0100	0.0058	0.0038	0.0042	0.0031	0.0105	0.0087	0.0072	0.0112	0.0067	0.0037	0.0089	0.0069
Cr	0.0016	0.0020	0.0033	0.0022	0.0220	0.0027	0.0036	0.0016	0.0016	0.0032	0.0082	0.0018	0.0053	0.0062	0.0066	0.0071	0.0049
P	0.0243	0.0890	0.1264	0.1031	0.2700	0.1308	0.1165	0.0947	0.0893	0.1684	0.1554	0.1493	0.0596	0.2716	0.1956	0.2412	0.1041
Fe	0.0083	0.2224	0.1175	0.0679	0.2000	0.1483	0.0352	0.0793	0.0108	0.06	0.1429	0.1645	0.021	0.2012	0.1422	0.1595	0.065
Mn	0.001	0.011	0.002	0.002	0.010	0.002	<0.001	0.001	<0.001	0.006	0.005	0.008	0.002	0.007	0.003	0.006	0.009
Hg	0.0001	<0.0001	***0.0004***	***0.0006***	<0.0001	***0.0007***	***0.0009***	***0.0008***	***0.0002***	<0.0001	***0.0001***	<0.0001	<0.0001	***0.0001***	***0.0001***	***0.0002***	<0.0001
Ni	0.0046	0.0115	0.0095	***0.0224***	0.0200	0.0057	0.0171	0.0091	0.0098	***0.0237***	***0.0301***	***0.0331***	***0.0304***	***0.0204***	***0.0219***	0.0185	0.0198
Ag	0.001	0.0010	<0.001	0.0020	<0.001	0.003	<0.001	0.002	0.003	0.005	0.006	0.003	0.004	0.002	0.004	0.003	0.005
Pb	0.004	***0.026***	***0.042***	***0.029***	***0.030***	***0.03***	***0.012***	***0.059***	***0.034***	***0.042***	***0.081***	***0.024***	***0.043***	***0.029***	***0.071***	***0.076***	***0.055***
Se	0.01	<0.010	<0.010	<0.010	<0.010	<0.01	<0.01	<0.01	<0.01	0.05	0.06	0.02	0.08	0.03	0.02	0.04	0.05
Si	0.0202	1.5580	1.2040	0.9301	1.1500	0.9607	1195	1335	0.6665	1.239	2.126	1.54	1.01	1.146	1.066	1.393	0.9925
Tl	0.0009	0.015	<0.015	<0.015	<0.015	0.020	<0.015	<0.0009	<0.0009	<0.0009	<0.0009	0.011	<0.0009	0.0037	0.0015	0.0016	<0.0009
Zn	0.0075	0.0164	0.0192	0.0090	0,01	0,0127	0.0113	0.0118	0.0082	0.0107	0,0122	0,0223	0.0104	0.0113	***0.0823***	0.0191	***0.1741***

Sb: Antimony, As: Arsenic, Ba: Barium, B: Boron, Cd: Cadmium, Cu: Copper, Cr: Chromium, P: Phosphorus, Fe: Iron, Mn: Manganese, Hg: Mercury, Ni: Nickel, Ag: Silver, Pb: Lead, Se: Selenium, Si: Silica, Tl: Thallium, Zn: Zinc. 1 = autumn-2015 season, 2 = winter-2015 season, 3 = spring-2015 season and 4 = summer-2016 season. P_1 = Naval School, P_2 = IMARPE (Peruvian Sea Institute), P_3 = Callao Port Terminal, P_4 = San Lorenzo Island. Numbers in bold identify values higher than the Canadian guidelines and numbers in italics indicate values above European Community guidelines.

As shown in Table 4, in the 16 toxicity bioassays with *B. plicatilis* performed after 24 h of exposure, it was observed that 93.75% (n=15) of the LC_{50} values were higher than 100%, while after 48 h of exposure, 62.5% (n = 10) of the LC_{50} values were higher than 100%.

Table 4. Marine water toxicity in Callao Bay, Peru based on LC_{50}, NOEC and LOEC on the mortality of *Brachionus plicatilis* (Rotifera) at 24 and 48 h of exposure.

Sampling	Exposure time (h)	LC_{50}	LC_{50}-lower	LC_{50}-higher	NOEC	LOEC	F	Sig
$1P_1$	24	>100	89.95	>100	12.5	25	3.11	0.04
	48	>100	99.66	>100	12.5	25	2.93	0.04
$1P_2$	24	>100	>100	>100	12.5	25	3.42	0.03
	48	70.17	32.05	>100	12.5	25	3.85	0.02
$1P_3$	24	>100	>100	>100	100	>100	2.33	0.09
	48	>100	>100	>100	50	100	2.07	0.12
$1P_4$	24	>100	>100	>100	50	100	1.87	1.53
	48	61.36	30.23	>100	12.5	25	7.80	0.00
$2P_1$	24	88.38	34.36	>100	6.25	12.5	14.28	0.00
	48	47.67	23.79	95,53	0	6.25	20.00	0.00
$2P_2$	24	>100	97.64	>100	25	50	4.09	0.02
	48	>100	18.39	>100	0	6.25	17.06	0.00
$2P_3$	24	>100	74.39	>100	6.25	12.5	16.41	0.00
	48	93.59	43.13	>100	25	50	7.96	0.00
$2P_4$	24	>100	>100	>100	50	100	1.93	0.14
	48	>100	61.19	>100	6.25	12.5	14.34	0.00
$3P_1$	24	>100	79.57	>100	6.25	12.5	6.05	0.00
	48	>100	74.88	>100	6.25	12.5	4.97	0.01
$3P_2$	24	>100	>100	>100	100	>100	1,55	0.23
	48	84.87	33.91	>100	25	50	4.14	0.02
$3P_3$	24	>100	>100	>100	6.25	12.5	7.09	0.00
	48	>100	>100	>100	100	>100	1.42	0.27
$3P_4$	24	>100	>100	>100	25	50	3.54	0.24
	48	>100	99.60	>100	50	100	3.00	0.04
$4P_1$	24	>100	>100	>100	100	>100	0.79	0.56
	48	>100	>100	>100	50	100	2.10	0.14
$4P_2$	24	>100	>100	>100	100	>100	0.91	0.50
	48	>100	>100	>100	100	>100	1.00	0.44
$4P_3$	24	>100	47.35	>100	6.25	12.5	33.39	0.00
	48	87.13	46.30	>100	6.25	12.5	24.76	0.00
$4P_4$	24	>100	99.19	>100	50	100	3.03	0.04
	48	>100	>100	>100	50	100	1.60	0.22

LC_{50} = Medium lethal concentration. LC_{50}-lower = Lower mean lethal concentration. LC_{50}-upper = Upper mean lethal concentration. NOEC = Concentration at which no significant effect is observed based on mortality with respect to the control. LOEC = Minimum concentration at which a significant effect is observed based on mortality with respect to the control. F = value of the Fisher statistic of ANOVA to calculate the NOEC and LOEC. sig = significance. Values in bold were less than or equal to 100%.

1 = autumn-2015 season, 2 = winter-2015 season, 3 = spring-2015 season, 4 = summer-2016 season. P_1 = Naval School, P_2 = IMARPE (Peruvian Marine Institute), P_3 = Callao Port Terminal and P_4 = San Lorenzo Island. h = hours.

NOEC and LOEC values were lower at 48 h of exposure in the P_1 and P_2 sampling sites in winter-2015 (Table 4). At 24 h of exposure, the following LOEC values were observed: 12.5% in four samples (25%), 25% in three samples (18.75%), 50% in two samples (12.5%), 100% and 25% in three samples (18.75%), and higher than 100% of marine waters in four samples. On the other hand, while, at 48 h of exposure, LOEC values were seen in two samples (12.5%) presented values of 6.25, three samples (18.75%) with 12.5%, three samples (18.75%) with 25%, two samples (12.5%) with 50%, five samples (31.25%) with 100% and, 12.5% higher than 100% of marine waters (two samples) (Table 4).

PCA showed that principal component 1 (PC_1) contributed 38.70% and PC_2 17.70%. PC_1 was formed by LOEC, Cd, Hg, Ni, Ag and Pb, while PC_2 was made up of Cu, Cr and Zn (Fig. 2).

4. DISCUSSION

The sampling sites P_1, P_2, P_3 and P_4 showed higher concentrations of HMs, especially Cd, Cu, Cr, Hg, Ni, Ag, Pb, Se and Zn, that did not meet the quality standards in Callao Bay water for at least some of the comparison standards and mainly for Canadian regulations and EC guidelines. High HM concentrations have been recorded in association with industrial and domestic discharges detected in Callao Bay, Peru (OECD, 2016). Three main sources of contamination have been identified in the Callao area: (1) waters of the Rímac and Chillón rivers, mainly due to the intense mining activity in the Andean zone of the valleys of these two rivers which have low salinity, high nutrient content, low DO content and high HM concentrations, (2) waters of the interior of the port, with contamination by port activities, industrial waste and the fishing terminal with high HM concentrations, and (3) area in front of the dock contaminated by the discharge of waste from ships, oil spills and periodic discharges from bars and restaurants (Guillén *et al*., 1978).

At 48 h of exposure, 87.5% of LOEC values were observed to less than or equal to 100%, with HMs such as Cu, Cr, Hg and Zn exceeding some of the international standards for comparison (Table 1). Similarly, the DO evidenced hypoxia levels (2.28 $mg{\cdot}L^{-1}$) in $3P_1$ sampling area, the lowest transparency values (0.75 m) and the highest salinity value (35.5 $g{\cdot}L^{-1}$). The literature shows that salinity and dissolved organic matter can also influence the toxicity of Cu and other HMs, which could be explained by the binding sites available for free Cu complexes in water, forming colloids induced by salts (Kovalenko *et al*., 2020;

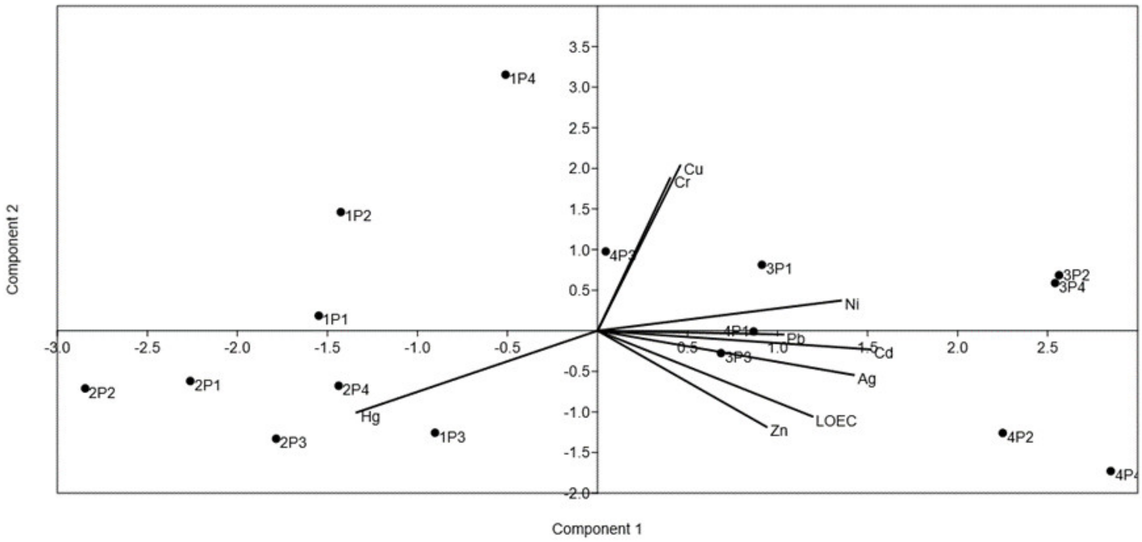

Figure 2. Principal component analysis (PCA) graph of LOEC values at 48 h of exposure with *Brachionus plicatilis* assay and heavy metals in the marine water of Callao Bay, Peru.

LOEC = Minimum concentration at which a significant effect is observed based on mortality with respect to the control. 1 = autumn-2015 season, 2 = winter-2015 season, 3 = spring-2015 season, 4 = summer-2016 season. P_1 = Naval School, P_2 = IMARPE (Peruvian Marine Institute), P_3 = Callao Port Terminal and P_4 = San Lorenzo Island.

Li *et al*., 2020). Nevertheless, more studies with the rotifer *B. plicatilis* are required to evaluate the absorption, distribution, metabolism, and excretion pathways of xenobiotics, such as many HMs, by this marine species that are determinant and essential for their toxicity (Hwang *et al*., 2016; Jeong *et al*., 2019ab; Varea *et al*., 2020). It has been reported that in other components of marine zooplankton, such as the copepods *Acartia tonsa*, *Acartia hudsonicaque*, *Notodiaptomus conifer* and *Centropages ponticus*, Cr, Hg and Cd cause changes in the egg production rate, hatching success and survival of nauplii under natural conditions and in the presence of these contaminating HMs (Hussain *et al*., 2020).

The PC_1 related Cd, Hg, Ni, Ag, Pb and LOEC. These results show that based on the LOEC, *B. plicatilis* is a suitable species to evaluate the presence of these HMs in seawater samples since a relationship was found between the LOEC of this marine rotifer and the concentrations of these HMs. The ecotoxic mortality observed based on the LOEC values could be due to a mixing effect of several chemical HM substances and not only one HM (Fu *et al*., 2014). Jeong *et al*. (2019a) showed that the marine rotifer *in vivo* exposure of *Brachionus koreanus* to a seawater sample collected from a polluted region in South Korea was not acutely toxic to adult rotifers, but both fecundity and lifespan were reduced in a concentration-dependent manner. This suggests that the main toxicity of the field-collected seawater is likely associated with Zn toxicity as well as contamination with a variety of metals. Jeong *et al*. (2019b) investigated the adverse effects of contaminated natural sea water of Youngil Bay, South Korea. This bay has shown pollution by metals due to industrial discharges from nearby steel industry complexes in seawater samples with the marine rotifer *B. koreanus*, which presented decreased population growth rates. On the other hand, no significant effects were shown in the reproduction rate or life span. Li *et al*. (2020) suggested that the rotifer *B. plicatilis* is an ideal species for use in marine ecotoxicological evaluations contaminated with HM, and reported that toxicity is higher when HMs are combined or mixed. Therefore, this rotifer is an attractive organism for these studies due to its wide distribution, ease of culture, adequate size, short generation time, and complex life cycle (Hwang *et al*., 2016; Li *et al*., 2020). However, there are very few studies on the effect of toxic metals on marine zooplankton (Hussain *et al*., 2020).

5. CONCLUSIONS

The variations in the results of HM in the bioassays with *B. plicatilis* in the different sampling areas and seasonal periods demonstrate the various industrial, recreational, transport and sports activities carried out in the coastal marine area of Callao bay. This area is one of the main localities on the Peruvian coast with marine pollution by HMs such as Cd, Cu, Cr, Hg, Ni, Ag, Pb, Se and Zn, main source of which is the wastewater of domestic, agricultural and industrial origin from the Rímac and Chillon rivers. The flow of these rivers is greater in the summer months as a result of the rains in the mountains of the province of Lima that are discharged into the sea through collectors, rivers and ditches that contribute these HMs. The activities of maritime transport and boat moorings stay of boats of different lengths of time and use also contribute HMs such as Cu, Ni and Zn. The current system in front of Callao Bay influences the movement of the HM between the different evaluation areas, and is mainly influenced by the Peruvian Coastal Current that flows in a northerly direction (Orozco *et al*., 1998; Argüelles *et al*., 2012). Among HMs, Cu is used commercially as an algaecide in antifouling paints, and Cr is used in a variety of industries as a tanning agent for leather, in electrolytic coatings of metals (electroplating), and in the production of glass, pigments, fungicides and batteries (Hussain *et al*., 2020).

The evaluation of the presence of HMs based on the different seasons, it was seen that the winter of 2015 presented the highest toxicity values based on the LOEC at 48 h of exposure compared to the summer of 2016. When evaluated by sampling areas, the P_1, and P_2 sampling areas showed a greater number of toxicity values based on LOEC at 48 h of exposure compared to points P_3 and P_4. High NH_3 values were observed in the case of P_2-spring-2015, P_3-spring-2015 and P_4- spring-2015. One study suggested that high NH_3 values significantly reduce the shelf life and egg production of *B. plicatilis* (Li *et al*., 2020). Other authors have described that the season of the year does not directly influence the toxicity of HMs. Marine waters from different geographic locations, sources of pollution and HM levels have shown lethal and sublethal toxic effects in bioassays with marine invertebrates (Beiras *et al*., 2001; Fathallah *et al*., 2011).

Reproduction and ingestion endpoints for the marine rotifer *Proales similis* were found to be generally more sensitive to HMs such as Cu, Cd, and Hg compared to hatching mortality or egg diapause (Snell *et al*., 2019). Li *et al*. (2020) reported that investigations with *B. plicatilis* could use, in addition to mortality, other biomarkers, such as behavior, enzymatic activity or gene expression, including genetic methods such as transcriptomics, with a high potential application to detect key molecular mechanisms.

The occurrence of the phenomenon of the “Niño” between the summer of 2015-2016 (ENFEN 2015), coincided with the seasons in which the water samples were collected. During this climatic phenomenon the surface temperature of the sea during the study period was 18.1 °C (16.5 to 19.9) with a thermal anomaly of 2.4 °C (1.7 to 3.5). The average annual rainfall was also exceeded by 608 mm with a maximum flow of 134.8 $m^3 \cdot s^{-1}$ in the upper part of the Rímac River. In relation to the upper part of the Chillón River, the average annual rainfall was exceeded by 506 mm with a maximum flow of 84.9 $m^3 \cdot s^{-1}$, intensifying the presence of pollutants due to the dragging of substances (discharges from the city, fertilizers, mining activity, electronic equipment or paints, etc.).

The use of *B. plicatilis* in marine ecotoxicological tests is recommended as a tool for the evaluation of environmental risks, produced by HMs and/or various pollutants, because its use in bioassays is reliable, replicable, sensitive and short in duration. In addition, it has greater ecological precision compared to other bioassays.

AUTHOR CONTRIBUTIONS

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