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PRESENCE OF CAFFEINE IN THE KARST LAKE PUNTA LAGUNA OF THE YUCATÁN PENINSULA AND ITS ENVIRONMENTAL RISK

Presencia de cafeína en el lago kárstico Punta Laguna de la península de Yucatán y su riesgo ambiental

Alma Lilibet SÁNCHEZ-HERRERA, Rosa María LEAL-BAUTISTA and Jesús ALVARADO-FLORES*

Unidad de Ciencias del Agua, Centro de Investigación Científica de Yucatán, Centro de Cancún, Calle 8 núm. 39, Manzana 29, Sm 64, 77524 Cancún, Quintana Roo, México.

*Author for correspondence; email: jesus.alvarado@cicy.mx

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ABSTRACT

Caffeine is known to be a chemical indicator of anthropogenic contamination in water bodies because it is highly soluble and persistent. As such, caffeine is an excellent water quality marker and tool in environmental impact assessments. Our goal was to quantify the caffeine in water from the Yucatán karstic groundwater and determine its environmental impact on indigenous aquatic biota. High-performance liquid chromatography (HPLC) with diode-array detection was used to quantify the caffeine content in water. Acute toxicity tests were performed in six zooplankton rotifer- and ostracod-strain groups native to the Yucatán peninsula that were cultured in our laboratory. In addition, the risk posed by caffeine in water on aquatic biota was determined, using risk assessment of the environmental concentration of caffeine in water (MEC) and the predictive values of toxicity (PNEC) obtained from our acute toxicity study. High concentrations of caffeine were found in the water. The most sensitive species to caffeine exposure was *Diaphanocypris meridiana* (ostracod), with an LC_{50} value of 288.383 mg/L, and the most tolerant organism was *Lecane cornuta* (rotifer) with an LC_{50} value of 3694.611 mg/L. Caffeine contamination of groundwater into aquatic ecosystems suggested, could have others active pharmaceuticals, hazardous substances, and personal care products. The presence of caffeine in Punta Laguna ranges between 1.79 and 3.93 mg/L, which represents a low to moderate risk for rotifers and a high risk for ostracods.

Palabras clave: ecotoxicología, contaminantes emergentes, calidad de agua.

RESUMEN

Se sabe que la cafeína es un indicador químico de contaminación antrópica en cuerpos de agua porque es altamente soluble y persistente. Como tal, la cafeína es un excelente marcador de la calidad del agua y una herramienta útil en las evaluaciones de impacto ambiental. El objetivo fue cuantificar la cafeína en el agua subterránea kárstica de Yucatán y determinar su impacto ambiental en la biota acuática autóctona. Se utilizó cromatografía líquida de alta resolución (HPLC, por su sigla en inglés) con detección de matriz de diodos para cuantificar la cafeína en agua. También se realizaron pruebas de toxicidad aguda en seis grupos de cepas de zooplancton (rotíferos y ostrácodos) nativos de la península de Yucatán que fueron cultivados en nuestro laboratorio.

Se determinó el riesgo que representa la cafeína en el agua para la biota acuática, utilizando la concentración ambiental de cafeína en el agua (MEC) y los valores predictivos de toxicidad (PNEC) obtenidos de nuestro estudio de toxicidad aguda. Se encontraron altas concentraciones de cafeína en el agua. La especie más sensible a la exposición a cafeína fue *Diaphanocypris meridiana* (ostrácodo), con un valor de CL_{50} de 288.383 mg/L, y el organismo más tolerante fue *Lecane cornuta* (rotífero) con un valor de CL_{50} de 3694.611 mg/L. La contaminación por cafeína sugiere que las aguas subterráneas en los ecosistemas acuáticos pueden tener otros activos farmacéuticos, sustancias peligrosas y productos para el cuidado personal. La presencia de cafeína en Punta Laguna fluctúa entre 1.79 y 3.93 mg/L, lo que representa un riesgo de bajo a moderado para rotíferos y alto riesgo para ostrácodos.

INTRODUCTION

Caffeine is a chemical marker of anthropogenic contamination and is considered an emerging contaminant that has high solubility and persistence in water. These attributes make caffeine an excellent water quality marker that can be utilized in risk assessments (Escuadra 2017). Caffeine is the most consumed psychoactive substance in the world; it is found naturally in tropical- or subtropical-origin plants and artificially in foods, food supplements, drinks (e.g., coffee, tea, carbonated sugary drinks), and human medicines. It is estimated that between 0.5 and 20% of caffeine intake is excreted in the urine (Dafouz and Valcárcel 2017). Hence, the widespread use of caffeine by humans results in the presence of caffeine in wastewater. This anthropogenic waste is not eliminated in water treatment plants; rather it is released to aquatic ecosystems. Additionally, there are indirect discharges of caffeine from cesspits leaks (Zarrelli et al. 2014, Dafouz and Valcárcel 2017). It does not appear to be degraded in ocean water (Buerge et al. 2003). Current waste treatment methods do not effectively remove a significant amount of caffeine, and a substantial portion of it enters the ocean (Ogunseitan 1996).

According to Ferreira (2005) and Kurissery et al. (2012), the caffeine contamination of groundwater suggests that many other active pharmaceuticals, hazardous substances, and personal care products are certainly present in water and aquatic ecosystems. In relation to this, ecotoxicological studies on the effects of caffeine and other personal care products on freshwater species reported non-adverse reactions in short-term exposures; however, the increase in the discharge of chemical substances into aquatic systems causes deleterious effects due to persistent exposure (Zarrelli et al. 2014).

Toxicity nominal values for the median lethal concentration (LC_{50}) of caffeine in zooplankton are high; in consequence, the toxicity or hazard is considered low. For example, LC_{50} values of 4451 mg/L (24 h) for caffeine were calculated for the rotifer *Brachionus calyciflorus* (Chakraborty et al. 2011) and of 60 mg/L (48 h) of the same substance were determinedfor the cladoceran *Ceriodaphnia dubia* (Russom et al. 1997). On the contrary, the metabolites derived from caffeine by chlorination (a frequently used process in primary wastewater treatment) exhibit high toxicity for *B. calyciflorus* (LC_{50} = 3.5 mg/L) compared with previous toxicity reports (Chakraborty et al. 2011). That is, caffeine concentrations in water are not high. Similarly, caffeine toxicity in zooplankton is low, as indicated by reported high LC_{50} values. However, caffeine toxicity using native species from the Yucatán Peninsula has never been studied. The reported LC_{50} values are significantly higher than the reported concentrations of caffeine in water. For example, in Madrid, Spain, maximum caffeine values of 1.3167E-05 mg/L in surface water and 7.5E-05 mg/L in drinking water have been reported (Valcárcel et al., 2011). By comparison, caffeine concentrations in influent water has been reported as 3.84E-06 mg/L in Seville (Santos et al. 2007), 0.0368 mg/L in Korea (Choi et al. 2008), 0.017 mg/L in Canada (Crouse et al. 2012), 0.0049 mg/L in the USA (Spongberg and Witter 2008), and 0.024 mg/L in Colombia (Blarasin 2012).

Caffeine is an emerging contaminant associated with anthropogenic pollution that poses significant risks to aquatic organisms, including vertebrates such as fish. Studies have shown that, in fish larvae, exposure to caffeine at concentrations of 20.0, 67.0, and 100.0 mg/L can cause hypoactivity, erratic movements, and possible signs of anxiety or neuronal alterations (Santos et al. 2023). At lower concentrations, similar to those found in aquatic environments (0.0005-0.3 mg/L), caffeine has been observed to reduce exploratory conduct in zebrafish and alter feeding behavior, which is associated with weight loss, increased aggression, and changes in sociability

(Santos et al. 2023). In addition, adverse effects on the behavior of organisms, such as the water flea *Daphnia magna*, have been reported when exposed to relevant environmental concentrations of caffeine at levels lower than 0.05 mg/L (Nunes et al., 2022). The presence of caffeine in water is a clear indicator of human contamination, and its concentration can reflect the magnitude and frequency of such a condition. For example, studies indicate that the average caffeine concentration in water varies by region: in Europe, it is 0.01032 mg/L, in America, 0.00349 mg/L, in Asia, 0.007 mg/L, and in Oceania, 0.01113 mg/L. Extreme values exceeding 1.0 mg/L have been detected in certain areas of America, Asia, and Africa (Diogo et al., 2023).

Research also shows that caffeine can accumulate in sediments, indicating its potential for long-term persistence in aquatic ecosystems. However, experimental studies using microcosms have reported a relatively short half-life for caffeine in water, averaging around 1.5 days (Korekar et al. 2020). Additionally, Kurissery et al. (2012) found that caffeine degrades more rapidly with exposure to sunlight, suggesting that it may persist longer in areas where water is shielded from direct sunlight. In this context, caffeine, as previously noted, serves as an excellent marker of anthropogenic contamination and poses an environmental concern for aquatic life.

Caffeine is removed from aquatic systems through various processes, including sedimentation, volatilization, biological degradation, and chemical and photochemical breakdown. Given these findings, it is essential to prioritize research on caffeine's adverse effects on indicator organisms to better assess the potential risks to aquatic ecosystems in the short and medium term.

In contrast to the reports for the aforementioned countries, caffeine has been detected in wastewater effluents and underground waters at much higher concentrations in Quintana Roo, Mexico, ranging from 0.52 to 3.55 mg/L (Leal-Bautista et al., 2011, 2013; Dafouz and Valcárcel, 2017). This is due to two important conditions: (*a*) the use of sinkholes as recreational waters where people are used to swimming (Leal-Bautista et al. 2011), and (*b*) the highly permeable karstic environment, where direct infiltration and fast transport affect water conditions by dumpsites at the well-field in the Tulum area (Leal-Bautista et al. 2013, Dafouz and Valcárcel 2017).

Considering previous reports of caffeine occurrence in groundwater (Leal-Bautista et al., 2011, 2013) and the scarce information regarding the sensitivity of native zooplankton to caffeine toxicity, it becomes essential to assess and integrate the potential environmental impacts of this contaminant in Punta Laguna, a karstic lake recognized as a protected area and located in Valladolid, Yucatán, Mexico.

Punta Laguna Lake is surrounded by a rural community that offers a number of multidisciplinary ecotourism activities. In the area immediately adjacent to the reserve, approximately 300 inhabitants are spread across seven communities, with about 100 residing specifically in Punta Laguna, according to 2007 data. However, there is no available census data to provide updated statistics. Over the past decade, tourism in the nearby city of Valladolid has increased significantly, partly driven by its designation as a "magical village" by tourism authorities in 2012, and additional tourism growth is expected with the development of infrastructure for the Tren Maya (García-Frapolli et al. 2007, Cervantes-Cocom and Chan-Ceh 2020). The impact of tourism is considerable, with waste generation potentially reaching up to 800 g per person per day, totaling 34 920 kg per month, along with the production of approximately 200 L of wastewater (Chacón 2021). For example, visitors' data for the period 2007-2011 show a total of 9512 tourists (Pavón et al. 2013). However, it is crucial to update these figures to reflect more recent trends and provide a current overview of tourism activity. In this sense, risk assessments have been a critical challenge for environmental managers whose labor is devoted to protecting the subtle balance between biological systems at risk and public health (Ferreira 2005, Kurissery et al. 2012). Additionally, the geological characteristics of the area enable the constant and rapid infiltration of caffeine, along with the absence of water treatment. To the best of the authors' knowledge, this research has the potential to serve as a baseline for other aquatic karst systems on the Yucatán Peninsula, both with and without water treatment.

MATERIALS AND METHODS

Study area

Punta Laguna is a karstic lake designated as a Ramsar site (No. 1763), encompassing a protected area of 5367 ha characterized by seasonal wetlands and low to mid-mature rainforests interspersed with secondary vegetation (García-Frapolli 2007). The lake itself covers a surface area of approximately 90 ha (0.9 km^2), with sodic-chlorinated waters (Curtis et al. 1996) and sediments enriched in calcium carbonate and organic matter (Hodell et al. 2007). The geographic coordinates of the site are 20.648226º N, –87.642963º W.

Water sample gathering for caffeine quantification

Water samples were collected by submerging the open mouth of the sampling flask (amber glass) into the surface of the lake's water. The samples were then placed on ice and returned to the laboratory. Then, the samples were processed to extract caffeine and analyzed (see section 2.5). All samples were filtered through a 0.45 µm filter (Whatman) before the extraction. In total, samples were collected at 15 sites once. The following physical and chemical parameters were measured directly (in situ) on the water surface, using a Hanna Temp/pH/EC/TDS (HI98129) tester at each collection site: temperature (ºC), pH, and electrical conductivity (µS/cm).

Zooplankton gathering

Zooplankton samples were collected at 15 sites scattered across the Punta Laguna karstic lake during November 2017. Fifty liters of water samples were collected with a plastic bucket once from the littoral and limnetic zones, and subsequently filtered using a Wisconsin sampler with a 55-µm mesh aperture to obtain a final volume of 250 mL per sample. Organisms were isolated by adding an individual organism to each well of 24-well polystyrene Costar plates containing a final volume of 1 mL of fresh medium (USEPA 2002) and filtered medium from the collection site (filtered with a 55-µm mesh). The zooplankton species were identified using pictorial and dichotomous keys (Koste 1978, Stemberger 1979, Segers 1995, Elías-Gutiérrez et al. 2008).

Zooplankton sentinel species

Zooplankton cultures were isolated and established in 2015 under laboratory conditions at the Ecotoxicology Laboratory of the Water Sciences Unit, Yucatán Scientific Research Center (Cancún, Quintana Roo, Mexico). This also included two ostracods (*Diaphanocypris meridiana* and *Cypridopsis vidua*, both from Rancho Viejo). In addition, a strain of the freshwater rotifer *Lecane cornuta* from the cenote zone in Puerto Morelos and three strains of the rotifer *Lecane bulla* from three places, including Chemuyil, Punta Laguna (culture established in 2017), and Canal Cocodrilo, were isolated. The geographic locations of the sites are as follows: Rancho Viejo: 21.202787 N, –86.840858 W; Cenotes of Puerto Morelos: 20.848990 N, –86.879421 W; Chemuyil: 20.373039 N, –87.330818 W; Punta Laguna: 20.648226 N, –87.642963 W, and Canal Cocodrilo: 21.183975 N, –86814241 W. The rotifers were fed only with the algae *Nannochloropsis oculata* (strain from Florida Aqua Farms, Texas), which was cultivated by adding MicroGrow (modified F2 medium, AquaFarms, Florida) to a water medium reconstituted according to Nichols' (1973) protocol. All species were placed inside a bioclimatic chamber (Thermo Scientific) at a temperature of 25 ± 2 ºC, with a 12-h photoperiod of light and 12 h of darkness. The typical concentration of the algae to feed rotifers was 5×10^6 cells/mL. Finally, ostracods were fed fresh lettuce, cut into pieces of 1.0 × 1.0 cm (three pieces per 10 mL), according to the method described by López-Gutiérrez et al. (2018).

Samples processing and caffeine analysis

Even though the analysis of caffeine is still limited by the lack of standardized and validated analytical methods that can be readily implemented in environmental laboratories, it was performed using high-performance liquid chromatography with a diode-array detector (Agilent-Technologies, model 1290, Infinity), with a C18 column of 150 mm (Zorbax) and a methanol/water ratio of 35/65%. Sample pretreatment consisted of solid-phase extraction (SPE), which followed the EPA (2010) Method 539. All samples were filtered through a 0.45 µm filter (Whatman) before the extraction. The extraction process began by adding 5 mL of methanol, followed by 5 mL of distilled water. Previously, 1 mL of H_2SO_4 (CAS: 7664-93-9; Meyer, 95.0-98 %) was added to the sample, and then it was homogenized. Finally, the extract was eluted by adding 5 mL of methanol to recover it in an amber vial. Polymeric syringes were used as sorbent (SPE Strata-X Phenomenex) for the SPE. Caffeine standards were prepared for the calibration curve (1, 2, 3, 4, 5, and 6 mg/L). An acceptable linearity was established with a determination coefficient $R^2 = 0.95$ (equation curve: $y = 1\,671\,757.83x - 59\,695.07$; y =area, x = mg/L). Additional information on the signal analysis of caffeine: retention time, 0.373 min; 273 nm/Bw: 4.00 nm, ref 360 nm/Bw: 100 nm. The reading of the standards and samplings was carried out in triplicate. The equipment conditions were: 1 mL min-1 flow rate, and UV detection at 295 mm. Caffeine's standards and 15 samples were done in triplicate; the limit of detection (LOD) was 0.00015 mg/L, and the limit of quantification (LOQ) was 0.0005 mg/L.

Caffeine toxicity testing

The experimental models were designed based on EPA-821-R-02-012 methods (USEPA 2002) and adapted to the organism used in each test. The stock solution was prepared at a concentration of 10 g/L of caffeine for toxicological tests. The acute testing was

carried out as follows: 10 (for rotifers) and five (for ostracods) neonates were placed in a final volume of 1 mL for each concentration in 24-well polystyrene plates (Costar). An initial exploratory range of caffeine concentrations from 1 to 10 000 mg/L was first tested in order to determine the sensitivity thresholds of the organisms. Based on these preliminary results, a final exposure range of 10 to 5000 mg/L was established. Test organisms were then individually allocated by species into 24-well plates and exposed for 24 h under controlled laboratory culture conditions, using caffeine as a standard (SUPELCO, Pharmaceutical Secondary Certified Reference Material, CAS 58-08-2, UNSPSC code: 12352210). This final range was used to estimate median lethal concentration (LC_{50}) values. The authors decided to use the final exposure concentrations according to the mortality data of the exploration range. For *C. vidua* we used the following exposure concentrations: 500, 800, 1200, 2000, and 3200 mg/L. For *D. meridiana*, concentrations were 50, 100, 300, 500, and 800 mg/L. Finally, for rotifers we used 100, 500, 750, 1500, 3000, and 5000 mg/L. Specimens were incubated in the bio-climatic cabinet for 24 h. T Tests were considered effective only if the mortality rate in the control medium was less than 10%, in accordance with the USEPA (2002) standards. In addition, organisms were not used for testing if they showed signs of unhealthiness, discoloration, or stress.

Afterwards, five concentrations were produced, and three replicas with two repetitions were made for the calculation of LC_{50} values, not observed effect concentration (NOECs), and lowest observed effect concentration (LOEC). Values for LC_{50} were calculated using linear regression analysis and probit models, whereas NOEC and LOEC were ascertained by a one-way variance analysis (ANOVA) and a Duncan test, using Statistica v. 6 (StatSoft 2001).

Caffeine spatial representation

An interpolation map was generated using the data on caffeine concentration and the geographic location of each sampling site, with the aid of software QGIS 2.14.0. In brief, raster interpolation and clipper extraction were executed. The color interpolation was performed using linear uniband pseudocolor rendering, and the interpolation weighting was inversely proportional to distance (IDW) via the neighbor-joining algorithm.

Risk quotient assessment

The risk quotient (RQ) was assessed following the methodology proposed by Bouissou-Schurtz et al. (2014). The RQ was calculated as RQ = MEC/PNEC, where MEC represents the measured environmental concentration of the contaminant and PNEC denotes the predicted no-effect concentration. In this study, PNEC values were derived exclusively from LC_{50} data, applying the formula PNEC = LC_{50}/1000. The lowest LC_{50} values were selected and divided by an assessment factor of 1000 to account for intra- and inter-laboratory variability, biological variation, and laboratory-to-field extrapolation, as recommended by Beyer et al. (2014). Risk interpretation was based on the following thresholds: RQ < 0.1 = insignificant risk; 0.1 –1 = low risk; 1 –10 = moderate risk; and RQ > 10 = high risk.

RESULTS

In the Punta Laguna karstic lake, the presence of caffeine was reported at concentrations ranging from 1.79 to 3.93 mg/L (**Table I**). **Figure 1** presents a map of caffeine concentration in the sample water. The results show that the eastern zone of the lake has the heaviest caffeine concentrations, while the northwestern zone has the lowest concentrations, and the remaining sampled areas have undetectable caffeine levels. The mean physical and chemical parameters (n = 15) were as follows: temperature, 30.42 ± 0.43 ºC; electrical conductivity, 1134.33 ± 5.83 µS/cm; and pH, 8.74 ± 0.12.

During the collection in Punta Laguna, 22 species were identified. Taxonomic analyses revealed the presence of 13 rotifer species, four cladoceran

TABLE I. CAFFEINE CONCENTRATIONS AT LAKE (mg/L) AND STANDARD DEVIATION.

Sample	Average	Standard deviation
1	3.9	0.17
2	3.5	0.09
3	3.3	0.06
4	3.9	0.31
5	3.2	0.09
6	3.0	0.14
7	2.6	0.06
8	3.2	0.10
9	2.1	0.05
10	ND	ND
11	ND	ND
12	ND	ND
13	ND	ND
14	ND	ND
15	1.8	0.15

ND: not detected.

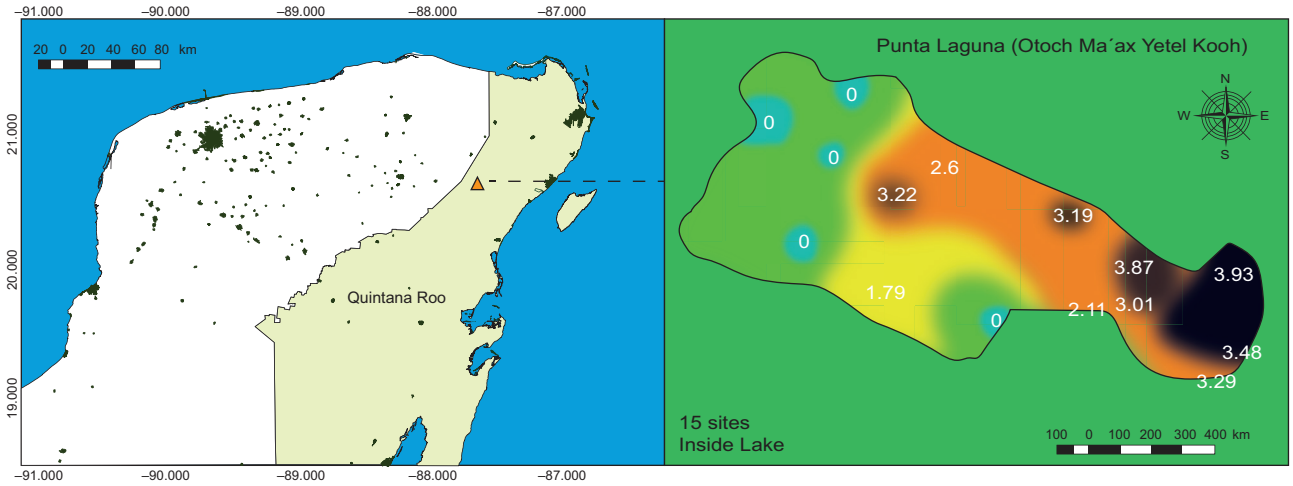

Fig. 1. Map of the spatial distribution of caffeine (mg/L) in the Punta Laguna karstic lake. The eastern zone of the lake has the heaviest caffeine concentrations (shown in black), and the northwestern zone has the lowest concentrations (shown in orange-yellow). Other areas show undetectable levels of caffeine (shown in blue). Geographic location: 20.648226 N, 87.642963 W.

species, three copepod species, one ostracod, and one oligochaete species. The scientific names of these species are shown in **table II**.

In total, six zooplankton strains from Quintana Roo were used in the caffeine toxicity tests: two species of ostracods, three rotifers of the genus *Lecane*, and four clones of the genus *Lecane*, one of which was established and cultivated from Punta Laguna. Later on, risk assessment was performed. In total, six LC_{50} values were obtained (**Table III**). The most sensitive species to caffeine was the ostracod *D. meridiana*, whereas the most tolerant was *L. cornuta*

TABLE II. LIST OF ZOOPLANKTON TAXA FOUND IN THE PUNTA LAGUNA KARSTIC LAKE, VALLADOLID, YUCATÁN.

Taxonomic group	Scientific name
Monogonont rotifer	*Plationus patulus* (Müller 1786)
Monogonont rotifer	*Brachionus havanaensis* (Rousselet 1911)
Monogonont rotifer	*Euchlanis dilatata* (Ehrenberg 1832)
Monogonont rotifer	*Keratella americana* (Carlin 1943)
Monogonont rotifer	*Lecane quadridentata* (Ehrenberg 1832)
Monogonont rotifer	*Lecane bulla* (Gosse 1886)
Monogonont rotifer	*Lecane leontina* (Turner 1892)
Monogonont rotifer	*Lecane hamata* (Stokes 1896)
Monogonont rotifer	*Lecane paradoxa* (Steinecke 1916)
Monogonont rotifer	*Lecane cornuta* (Müller 1786)
Monogonont rotifer	*Lecane spinulifera* (Edmondson 1935)
Monogonont rotifer	*Lecane crepida* (Harring 1914)
Monogonont rotifer	*Macrochaetus collinsi* (Gosse 1867)
Cladoceran	*Macrothrix triserialis* (Brady 1886)
Cladoceran	*Ceriodaphnia cornuta* (Sars 1885)
Cladoceran	*Alona guttata* (Sars 1862)
Cladoceran	*Bosmina tubicens* (Baird 1845)
Ostracod	*Cypridopsis vidua* (Müller 1776)
Copepods	Calanoida (Order)
Copepods	Cyclopoida (Order)
Copepods	Harpaticoida (Order)
Oligochaeta	*Pristina leidyi* (Smith 1896)

TABLE III. RESULTS OF THE ACUTE TESTS WITH SIX FRESHWATER SPECIES OF THE ZOOPLANKTON INDICATOR FROM PUNTA LAGUNA KARSTIC LAKE.

Taxa	Class	LC_{50} (mg/L)	R^2	*LC_{50} (mg/L)	R^2	NOEC	LOEC	LC 95 %
Cypridopsis vidua C1C	Ostracod	1579.343	0.90	1402.327	0.96	800	1200	1458.75-1699.93
Diaphanocypris meridiana C1C	Ostracod	288.383	0.71	230.063	0.94	100	300	236.06-340.70
Lecane cornuta C1Coz	Rotifer	3694.611	0.79	2813.715	0.92	750	1500	3154.18-4235.03
Lecane bulla C1C	Rotifer	3293.76	0.75	ND	ND	750	1500	2709.81-3877.70
Lecane bulla C2PL	Rotifer	3632.344	0.80	ND	ND	750	1500	3112.827-4151. 862
Lecane bulla C3C	Rotifer	3375	0.74	ND	ND	750	1500	2776.803-3973.197

*LC_{50} values were estimated using probit analysis.
LC_{50}: median lethal concentration; R^2: determination coefficient; NOEC: no observed effect concentration; LOEC: lowest observed effect concentration; (LOEC); LC: limit of confidence.

C1Coz from Cozumel. In contrast, the LC_{50} value for the *L. bulla* clone C2PL species from Punta Laguna was 3632.344 mg/L.

With the aforementioned information, risk assessments were conducted using the caffeine concentrations in water (MEC), along with the previous information on caffeine concentrations in water and the toxicity values (LC_{50} values for rotifers and ostracods) for the region's aquatic biota. All values are shown in **table IV**. According to our results, the zooplankton groups exhibited different risk characterizations, based on the toxicity observed in

TABLE IV. RISK LEVEL OF CAFFEINE ON NATIVE ZOOPLANKTON SPECIES FROM THE YUCATÁN PENINSULA.*

Sample	MEC mg/L	Lecane bulla from Punta Laguna (RQ = MEC/PNEC=3.632344 mg/L)	Rotifers (RQ = MEC/PNEC = 3.29376 mg/L)	Ostracods (RQ = MEC/PNEC = 0.288383 mg/L)
1	3.90	1.07	1.18	13.52
2	3.50	0.96	1.06	12.14
3	3.30	0.91	1.00	11.44
4	3.90	1.07	1.18	13.52
5	3.20	0.88	0.97	11.10
6	3.00	0.83	0.91	10.40
7	2.60	0.72	0.79	9.02
8	3.20	0.88	0.97	11.10
9	2.10	0.58	0.64	7.28
10	ND	ND	ND	ND
11	ND	ND	ND	ND
12	ND	ND	ND	ND
13	ND	ND	ND	ND
14	ND	ND	ND	ND
15	1.80	0.50	0.55	6.24

*Risk quotient (RQ) values: 0.1-1.0, low risk; 1.0-10, moderate risk; > 10, high risk.
MEC: environmental concentration of caffeine in water; PNEC: predictive values of toxicity; ND: not detected.

toxicological tests. However, specifically, the LC_{50} value of *L. bulla* clone C2PL from Punta Laguna was used to estimate the hazard of caffeine concentration. Two sites from that lake had a moderate risk, while eight had a low risk. This indicates that only 13% of sites had a moderate risk to species of rotifers of the genus *Lecane* due to the concentration of caffeine in water, and this area corresponds to the northeast of the Lake, as shown in **figure 1**.

Using the minimum LC_{50} values for all freshwater rotifers, risk assessment indicated that five sites exhibited moderate risk, while five sites presented low risk. When ostracods were used with their minimum LC_{50} values for caffeine, seven sites (46.7%) in the northeastern region of the lake were classified as high risk, and three sites (20%) as moderate risk. Sites 10-14, located in the northwestern part of the lake, were considered to have insignificant risk due to the absence of detectable caffeine concentrations.

DISCUSSION

Our study shows the presence of caffeine in the water of Punta Laguna karstic lake. The highest concentration detected was 3.93 mg/L in the northeast of the Lake. Previous studies on caffeine quantification in Quintana Roo by Leal-Bautista (2013) have shown the presence of caffeine in three municipalities: Puerto Morelos, Playa del Carmen, and Tulum, at concentrations ranging from 0.52 to 3.55 mg/L. It is important to highlight that caffeine in the water of the Punta Laguna system is probably indirect, as the community has neither sewer systems nor wastewater treatment plants (the inhabitants have cesspits for their waste), and that the aim of the study was to use caffeine as an anthropogenic impact tracer in aquatic systems (Blarasin 2012). Although caffeine is primarily associated with human effluents, it can also be linked to the use of vitamin complexes in agriculture or livestock rearing, as well as pesticides that contain caffeine. However, the main source of surface water contamination with caffeine is related to the continuous use of caffeine in drinks such as coffee, tea, and carbonated water (sodas), as well as through medical or personal hygiene products (Pardo and Ricardo, 2007). Therefore, the presence of caffeine in Punta Laguna is the result of the anthropogenic activities occurring at the site.

According to our results, the presence of caffeine confirms that there is indeed contamination from anthropogenic influences on the system, possibly originating from leaks at septic tanks and dumpsites. The concentrations found were high (3.93 mg/L), but they coincide with other reported values from the Yucatán Peninsula. Caffeine, as mentioned earlier, has been proven to be an effective marker for tracing surface water pollutants originating from human wastewater (Buerge et al. 2003). Caffeine is perhaps the most widely consumed alkaloid due to its extensive use in beverages, food, and pharmaceuticals, and it is not totally degraded by wastewater treatments (Korekar et al. 2020). Septic tanks do not treat water as efficiently as a wastewater treatment plant, so there is a source of caffeine from semi-treated wastewater and from dumpsites. Additionally, the geological characteristics of the area enable rapid and consistent infiltration of this substance.

Although Punta Laguna is a rural area, recreational activities have contributed to the rapid introduction of caffeine, as visitors consume beverages, foods, and dietary supplements containing this compound. Consequently, caffeine enters the environment directly and can be detected in groundwater from wells and sinkholes (Leal-Bautista, 2013). The northeastern region of the lake, which serves as the main entrance and hosts the majority of the local population, exhibited the highest caffeine concentrations, according to the authors' observations. These findings suggest a flow gradient from areas of higher to lower concentrations, reflecting transport, degradation, and seasonal fluctuations of caffeine within the karstic aquatic system, as well as inputs from anthropogenic sources. This pattern highlights the environmental impact of insufficient sanitation infrastructure, such as the use of septic tanks, in this highly permeable area. Furthermore, the continuous and increasing discharge of caffeine, both spatially and temporally, at concentrations sufficient to persist in the environment, is likely to exert effects on the aquatic microbiota.

According to the acute toxicity data (from short-term exposure) on caffeine in ostracods in the present study, *C. vidua* is the most tolerant species, with an LC_{50} value of 1579.343 mg/L, whereas *D. meridiana* is the most sensitive species, with an LC_{50} value of 288.383 mg/L. It is worth mentioning that there are no reports of the presence of the *D. meridiana* species in Punta Laguna, whereas the presence of *C. vidua* is reported there. In the case of rotifers, *L. bulla* from Punta Laguna karstic lake was the most tolerant strain to the toxicity of caffeine in freshwater rotifers from Quintana Roo, compared to *L. cornuta* from Cancún. However, it is more tolerant to caffeine exposure compared to a different strain of *L. bulla* from another area. These data suggest that *L. bulla* from Punta Laguna had previous acclimation to

caffeine, compared to the other strain. In other words, the differences in LC_{50} values were only 250-300 units of magnitude (mg/L). These caffeine sensitivity and tolerance data from our toxicity testing are the first evidence of zooplankton species native to Quintana Roo and the first data regarding LC_{50} values for the *Lecane* species.

The toxicity values for all zooplankton species tested are substantially higher than the caffeine concentrations detected in aquatic systems of the Yucatán Peninsula (Leal-Bautista et al. 2013) and those measured in Punta Laguna during this study. LC_{50} values in some cases were up to 400 times higher than the caffeine concentrations determined in water. Supplementary toxicological parameters (NOEC and LOEC) are also reported (**Table III**). When compared to environmental caffeine concentrations, LOEC values were approximately 400 times higher; for instance, for the rotifer *L. bulla* from Punta Laguna, the LOEC was 1500 mg/L, 384 times greater than the maximum caffeine concentration measured in water (3.9 mg/L). Similarly, the NOEC for caffeine (750 mg/L) exceeded the maximum environmental concentration by 192-fold, indicating that the observed caffeine levels in Punta Laguna are well below thresholds associated with measurable toxic effects.

With these baseline caffeine toxicity values in zooplankton, it is possible to design periodic experiments to determine the adverse effects of extended exposures to lower doses. Additionally, NOEC and LOEC values are commonly used in environmental risk assessments and ecotoxicology studies, making it easier to compare results with other studies. Moreover, these values are within national and international environmental protection regulations.

Finally, our estimation of environmental risk for the Punta Laguna karstic lake was conducted using data on caffeine quantification and toxicity in zooplankton of a species native to Quintana Roo, following the proposal by Bouissou-Schurtz et al. (2014). According to the present results, the danger of caffeine in water is low to moderate for rotifers and high for ostracods, despite using a risk factor of 1000 in the toxicity parameters to compensate for the intra- and inter-laboratory variations, biological variations, and laboratory-field extrapolation, according to the suggestions of Beyer et al. (2014). These results indicate that the caffeine concentrations in the water of Punta Laguna are sufficiently high to underscore the importance of replicating the study area assessment and including similar sites in Quintana Roo. The limnetic richness recorded in Punta Laguna was similar to that of other karstic lakes in the Yucatán Peninsula (Cervantes-Martínez and Gutiérrez-Aguirre, 2015). Hence, understanding the behavior of point vs. diffuse contaminant sources using a chemical tracer such as caffeine could benefit the protection of aquatic biota and water quality.

CONCLUSION

In summary, caffeine levels in Punta Laguna range from 1.79 to 3.93 mg/L, with a low to moderate risk for rotifers and a high risk for ostracods. The predicted no-effect concentration (PNEC) is 3.63 mg/L. However, assessing the hazard of caffeine is complex, as its impact on zooplankton cannot be summarized by a single metric; further studies on other species in the karstic lake are required. This concern extends beyond Punta Laguna; many communities on the Yucatán Peninsula utilize septic tanks, which can lead to potential contamination of aquatic ecosystems by caffeine and other hazardous substances being discharged. Given the karstic nature of the region, contaminants can infiltrate quickly, emphasizing the need to identify chemical substances and use biological indicators for environmental risk assessments. This integrated approach will elucidate the extent of contamination impacts, regardless of the scale of human activity or population size.

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