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Original Article

Occurrence Of Caffeine In Water And Sediment From Santos Bay (Sao Paulo, Brazil) And Hazard Quotient Estimation

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Abstract

Several studies have demonstrated the environmental impact caused by the disposal of drugs in water bodies after the release of treated and untreated effluents. Caffeine is classified as an emerging contaminant, being present in numerous pharmaceutical products, beverages, foods and cosmetics. After consumption, it is thrown into the sea through submarine sewage outfalls. The objectives of this study were: (i) to detect and quantify caffeine concentrations in marine surface waters in Santos Bay, SP; (ii) verify whether the concentrations found in Santos Bay are capable of triggering toxic effects to marine organisms as per previous studies; (iii) analyze the environmental hazard (HQ) using local concentrations of caffeine; (iv) propose caffeine as a chemical marker of effluent contamination in Santos Bay, SP. It was observed that the concentrations of caffeine found in the Santos Bay ranged from 206 ng L^{-1} to 1,322 ng L^{-1} in water and between 4.10 ng g^{-1} to 13.10 ng g^{-1}in sediment, being considered high compared to others marine ecosystems. It was observed that there is a potential hazard related to chronic effects, since the HQ ranged from 26 to 132 in water and from 27.33 to 87.33 to sediment samples.

Key words: Caffeine; Emerging contaminants; Marine pollution; Domestic effluent.

INTRODUCTION

According to Ortega *et al* (2018), emerging contaminants are substances that may affect non-target organisms and are not explicitly contemplated in any environmental legislation or monitoring. These contaminants are being identified in several countries (Alygizakis*et al*.,2016) and can be recognized as pharmaceutical and personal care products (PPCP), illicit drugs, pesticides and herbicides, flame retardants and industrial additives (Daneshvar*et al*., 2012, Árias 2013, Montagner*et al*., 2017).

The presence of pharmaceutical products in the environment is mainly caused by the fact that wastewater treatment plants (WWTP) are not projected to remove these compounds, resulting in the water bodies contamination, including lakes, rivers, and coastal waters (Petrie *et al*., 2015).The deficiencies of sanitation through domestic sewage in Brazil may cause contamination of aquatic biomes also by untreated effluents.

Caffeine is considered a biologically active substance, contaminating marine ecosystems (Del Rey *et al*., 2012). This substance is classified as an emerging

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contaminant and its presence in water bodies brings important information about environmental health. When present in aquatic ecosystems causes adverse effects in microorganisms, fishes, and amphibian (Canela*et al*. 2014).

Caffeine can be found in food containing cocoa, guarana, or chocolate; drinks as coffee and teas; condiments; tobaccos; and pharmaceuticals products as analgesic and appetite suppressants (Oliveira, 2018). Due to elevated consumption habits, caffeine can reach the aquatic ecosystems in high concentrations if compared with other PPCP molecules (Sauvé *et al*., 2012). The presence of these bioactive substances in the marine environment emphasizes the necessity to evaluate the risks and health impacts (You *et al*., 2015).

In a recent study, Pereira *et al*., 2016 detected the presence of caffeine in Santos Bay in concentrations between 84.4 and 648.9 ng.L^{-1}, while Aguirre *et al*. (2015a,b,c) observed negative effects in marine organisms exposed to concentrations of caffeine between 10 and 30,000 ng.L^{-1}.

In another study, carried out in the municipality of Guarujá, state of São Paulo (Brazil), it was detected near the submarine outfall diffusers, among several other substances of emerging concern, concentrations of caffeine ranging from 42.3 to 141.0 ng / L^{-1} (Roveri et al, 2021).

In this context, the aims of this study were: (i) to detect and quantify the concentration of caffeine in marine surface water of the Bay of Santos, SP; (ii) to verify if the concentrations found in the Santos Bay are able of triggered toxic effects to marine organisms according to previous studies; (iii) to analyze the environmental hazard (HQ) employing local caffeine concentrations; (iv) to propose caffeine as a chemical marker for wastewater contamination in Santos Bay, SP.

MATERIAL AND METHODS

Study Area and sampling procedures

The Santos Bay (São Paulo, Brazil) includes Santos and São Vicente cities characterized by a dense urban area. According to IBGE (2019), the estimated population in 2018 was 432,957 habitants. This region is formed by a petrochemical industrial complex and the major port of Latin America. Besides, the area is densely populated and has summer tourist activity. As reported by Abessa*et al*., (2005) the Santos Bay receives more than 7.367 m^3 of pre-conditioned sanitary sewage. The local wastewater treatment plant (WWTP) receives effluents from Santos and São Vicente cities. After pre-conditioning, the sewage is dumped in Santos Bay through a submarine outfall 10 meter deep in an area located 4.5 km away from the beach (Pereira *et al*., 2016). Santos Bay also receives water from urban drainage channels, in addition to the potential polluting load from the Santos and São Vicente estuarine channels (Harari *et al*., 2013).

To carry out a comprehensive assessment of caffeine contamination in Santos Bay, including the beach zone, water samples were collected at 14 points around the Santos submarine outfall diffuser, as shown in figure 01. if in the center of the bay at an average depth of 10 m, as well as in the estuarine channels and bathing area.

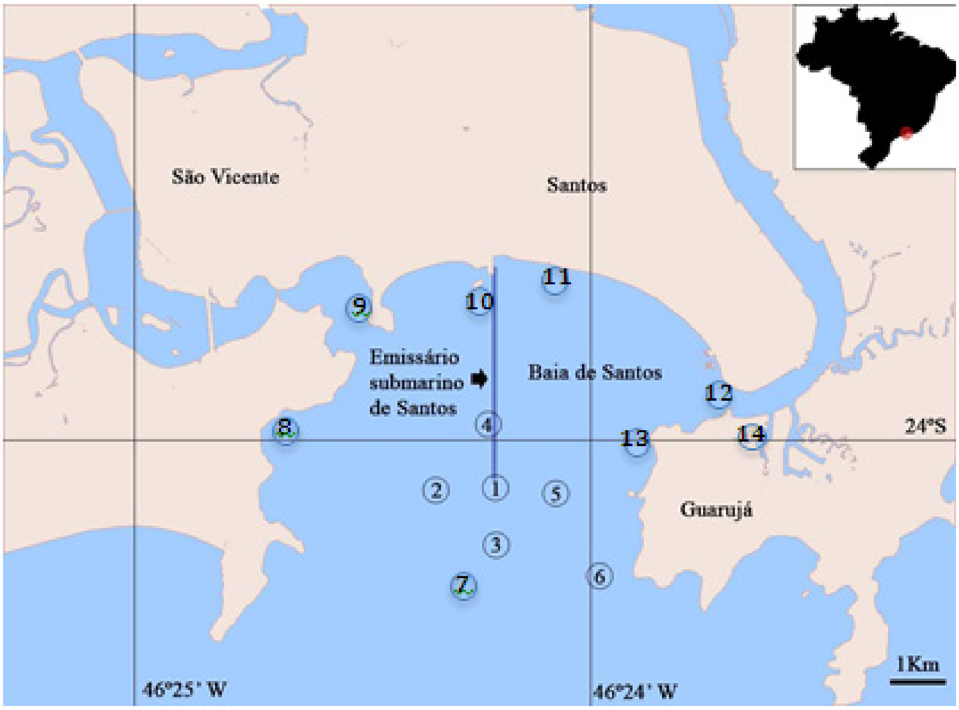

Figure 1. Water sampling stations in Santos Bay, SP.

For each sampling station was collected 1L of water using a Van Dorn bottle. The samples were conditioning into dark bottles, transported to the laboratory, and stocked in a freezer (-20 ºC) until the analysis of caffeine. Physical and chemical parameters (TºC, salinity, pH, dissolved oxygen and redox potential)were measured at the field with a multiparametric probe, model YSI Yellow Spring.

Solid phase extraction (SPE)

The water samples were submitted to solid phase extraction according to the procedure described by Wille *et al.* (2010) and Ghoshdastidar*et al.* (2015). Initially, the samples were filtered with Whatman (diameter GF / C 47 mm, particle retention 1.2 μm, Merck, Darmstadt, Germany). These filters were washed with 2 mL of methanol, added to the filtered sample. Posteriorly, the pH at each sample was adjusted for 7.0±0.5 before the extraction, using the HCl (1M) solution. The water samples were submitted to solid phase extraction (SPE). It was utilized Chromabond HR-X cartridges (3 mL, 200 mg, Macherey-Nagel, Düren, Germany) to extract and obtain the eluates. The cartridges were pre-conditioned with 5 mL of methanol and 5 mL of Milli-Q water. After they had been loaded with 1 L of the filtered sample pooled with the methanol from the filter washing, the cartridges were rinsed twice with 5 mL of Milli-Q water. The cartridges were then dried under a vacuum for 30 min. Elution was performed using 5 mL of acetone and 2 × 5 mL of methanol. After the SPE procedure, the samples were dried under nitrogen flow (at 50 °C) and then resuspended in 1 mL with a solution of water/acetonitrile (95:5, v/v) prior to LC–MS/MS analysis. Before the LC–MS/MS analysis, the samples were filtered in a 0.45 μM filter (Millipore).

Preparation and characterization of sediment samples (Sedimentology).

Granulometry

A fraction of 100 g each sample with sediment was separated and dried on the stove for 60ºC for two days. Afterwards, the samples were weighed and submit to a humid sifting of 0.063 mm to remove the muddy fraction and replaced in stuff for two more days at the same temperature at 60ºC. After dried, the sediment was weighed, and the quantity of silt and clay was determined. In sequence, the material was sifted for 15 minutes in beater ROTAP, utilizing a sieve set and following the intervals at the middle Ø in Went-Worth scale. With the fraction retired in each sieve and record with the results obtained was possible calculated the granulometric classification in according to Shepard (1954), and this is possible to obtain the sand contents and mud in according to Folk & Ward (1957).

Calcium carbonate content (C_aCO_3)

The content of C_aCO_3 was measured according to the method of Hirota&Szyper (1975) it was separated fractions of 5 g of sediment for each sample and added 10 mL of hydrochloric acid (HCl 5mol.L^{-1}) for 24h to eliminate C_aCO_3. Thereafter, the sample was washed with distilled water and then it was dry on the stove at 60ºC for 24h. The difference between the initial and final weight was measured to obtain the quantity of C_aCO_3.

Organic Matter (OM)

The determination of OM in each sample was realized according to the method of Luczak*et al.*, (1997). For each sample was separated 5 g of dry sediment (less C_aCO_3) stowing in a ceramic cart, weight previously and incinerated in a muffle at 500 ºC for 3h. The value of OM was obtained by measuring the difference between initial and final weight and calculating the percentage.

Caffeine extraction in sediment

The sediment samples were previously lyophilized two days before the beginning of the extraction and were separated into an aliquot of 10 g each sample.Afterwards, it was utilized a fraction of 2g of the sediment from the lyophilized sample for the extraction process. The samples were macerated to reduce the particle size and were sent to the extraction treatment.

Initially it was prepared a potassium phosphate buffer (0.14 mol L^{-1}) weighing 19.3 g of Na_2HPO_4, diluted on 990 mL water Mili-Q. In this solution was added 10 mL of H_3PO_4 85 % and pH was adjusted for 2. The pellet and tissue were packed in small beakers (separately). Each beaker has received 20 mL of acetonitrile and was placed in an ultrasonic cleaner for 30 minutes. After this time, the solution formed was collected and stowed in falcon tubes, centrifuged for 5 minutes at 2500 rpm and the supernatant was preserved. Then, the beakers with the samples received 20 mL of the phosphate buffer and were placed in the ultrasound for another 30 minutes. After this time, the solution formed was collected and stowed in falcon tubes as described above conditions.The supernatant was removed and placed in the same recipient where the previous supernatant was stowed.

Subsequently, the sediment received an additional 20 mL of acetonitrile and was placed in an ultrasonic cleaner for 30 minutes again. At the end, the supernatant was accumulated and centrifuged at 2500 rpm for 5 minutes. After, an aliquot of 50 mL of this solution was removed and added in a bottle containing 500 mL Mili-Q water. In sequence, was realized the SPE at the same method according described above.

LC-MS/MS analysis

The protocol proposed by as described by Shihomatsu et al. (2017) was employed to determine caffeine, and 10 µLfrom each sample was analyzed by an HPLC Agilent 1,260 (Agilent Technologies, CA, USA) combined with hybrid triple quadruple mass spectrometer ion trap/LC-QqLITAB-Sciex, Ontario (Canada). The seawater/sediment samples were analyzed by a column Agilent Eclipse XDB-C18 4.6 × 50 mm, 1.8 µm at 25°C, and the mobile phase at 0.1% for formic acid (Sigma-Aldrich LC-MS Grade) on water (solvent A) and acetonitrile (JT Baker LC-MS Grade) (solvent B). A linear gradient of 0.7 mL• min−1was used, starting with a mixture of 95% solvent A and 5% solvent B. The concentration of solvent "A" decreased linearly from 95% to 5% over 5 min and this condition was maintained for 1 minute. The mixture was returned to the beginning conditions for 2 minutes and the caffeine was detected and quantified utilizing the ionization source by "electrospray" (ESI - positive) in Multiple Reaction Monitoring Mode (MRM), with the selection of a precursor ion and two ion products to quantify and qualify the compound (Table 1) (Pereira *et al*., 2016). The ion source parameters adopted were (i) interface gas - 20 a.u; (ii) collision gas - 8 a.u; (iii) ESI voltage - 5500; (iv) source gas temperature - 650ºC; (v) nebulizer gas - 45 a.u; (vi) heating gas - 65 a.u.

Subsequently, a calibration curve was used as described by Shihomatsu et al. (2017). Linearity, limit of detection (MDL), limit of quantification (MQL), precision, accuracy, and recovery were the parameters to evaluate the performance of the SPE-LC-MS/MS methodology. The linearity was determined by 3 injections of 5 standard mixture solutions in the matrix, with concentrations in the range from 0.2 to 500 ng mL−1, which corresponds to 0.2 to 500 ng L−1 in water samples, taking into account the 1000-fold pre-concentration factor applied along the procedure. Calibration curve showed satisfactory determination coefficients (0.99 ≤ r2 ≤ 1).The accuracy was 93.8%, the extraction recoveries SPE were obtained in the 89% and 109% range. The uncertainties for the analyzes were ± 3%. The deviation of 7 injections of standard mixture solutions in the matrix at the lowest calibration curve concentration multiplied by 2.447 (Student-t, 6 degrees of freedom and 90% of confidence) result in the detection limit (LOD). The average value result for 7 injections of the standard mixture at lowest concentration plus 5 times of standard deviation represents the quantification limit method (LOQ).

MRM parameters for caffeine positive mode, detection limit (LOD) and limit of quantification (LOQ) are shown in Table 1.

Table 1. MRM parameters for positive ion mode, detection limit, quantification limit and retention time.

Substance	Q1	Q3	DP (V)	CE (V)	CXP (V)	LOD (ng/L)	LOQ (ng/L)	RT (min.)
Caffeine	195.2	138.3	26	19	4	0.1	8.5	3.42
		110.1	26	29	4			

Q1 (First Quadrupole); Q3 (Last Quadrupole); DP (Declustering Potential); CE (Collision Energy); CXP (Collision Exit Potential); LOD (Limits of Detection); LOQ (Limits of Quantification); RT (Retention Time). In Q3, in the upper cell is the quantifier ion and in the lower cell is the qualifier ion.

Environment hazard classification method

To assess the environmental hazard caused by the presence of caffeine in Santos Bay was utilized a deterministic approach evaluation and applied to the samples in this study, according to Dafouz (2018). Following this method, it was calculated the Hazard Quotient – HQ for each sampling site to determine the potential effect caused by the caffeine on the marine biota.

The HQ is defined by Environmental Protection Agency (US EPA) as the ratio between the potential exposure for a substance and its concentration below which no effect is expected (US EPA, 1997a; US EPA, 1997b).

The potential exposure was considered by the Measured Environmental Concentration (MEC) in each local sampled. Due to the continuous input of caffeine in the studied environments, chronic exposure was considered the most appropriate exposure scenario for the evaluation. To measure the risks of chronic effects for the presence of caffeine, some bioindicators were selected in the literature, employing the No Observed Effect Concentration (NOEC) for each organism. The Previous Predicted No-Effect Concentration (PNEC) was estimated by using the lower NOEC found. HQ was then calculated as HQ = MEC / PNEC. Since, if the HQ value is less than 0.1, no adverse effects are expected; if the value is between 0.1 and 1, some adverse effects can be taken into account; if the value obtained is between 1 and 10, adverse effects are likely to occur. Finally, if HQ is greater than 10, adverse effects are more likely to occur (US EPA, 1997a).

RESULTS AND DISCUSSION

Caffeine concentration insurface water

After liquid chromatography mass spectrometry analysis, was obtain the following results for each 14 sampling stations (Table 2).

Table 2: Results of analysis by liquid chromatography mass spectrometry (LC-MS/MS) of the 14 water samplingstations.

Station	Caffeine Concentration (ng L^{-1})
P1	495.0
P2	611.0
P3	624.0
P4	1,322.0
P5	299.0
P6	321.0
P7	263.0
P8	280.0
P9	291.0
P10	305.0
P11	288.0
P12	404.0
P13	333.0
P14	386.0

Analysis of sediment

Sediment Composition

After the granulometry, the sediments were shown on collection points: 3,4,5,7,13,14 and predominantly sandy on points 1,2,6,8,9,10,11,12 with a high concentration of very fine sand, followed by fine sand, medium sand and coarse sand. Regarding the composition of the sediments, the biggest quantities of organic material (OM) were found on sediments points 4 (14.99%), 5 (12.22%), 7 (17.34%) and 14 (17.42%). The high fractions of sand were found on sediments points 8 (81.13%), 11 (75.03%), 10 (74.82%) and (72.98%) (Table 3).

Table 3. Sediment Composition

Sediment Composition (%)				
Points	OM	$CaCO_3$	Sand	Mud
P1	4.02	7.39	61.78	26.81
P2	4.03	5.08	62.12	28.77
P3	5.22	7.30	22.05	65.43
P4	14.99	1.80	19.07	55.14
P5	12.22	4.90	27.90	54.99
P6	4.70	48.09	35.85	11.36
P7	17.34	4.74	14.59	63.33
P8	3.10	4.96	81.13	10.81
P9	6.41	59.69	27.07	6.83
P10	1.64	7.23	74.82	16.30
P11	2.27	5.12	75.03	17.58
P12	2.17	6.43	72.98	18.42
P13	2.99	32.18	38.62	26.21
P14	17.42	13.57	13.87	55.14

OM (organic material), $CaCO_3$ (Calcium carbonate).

Sediment contamination

The concentration of caffeine in the sediments is shown in Table 4.

Table 4. Caffeine concentration in sediment samples on each sampled points.

Station	Caffeine Concentration (ng g^{-1})
P1	10.80
P2	13.10
P3	8.48
P4	5.97
P5	7.67
P6	4.03
P7	4.36
P8	4.10
P9	6.44
P10	4.44
P11	4.66
P12	4.26
P13	11.90
P14	8.48

The highest caffeine concentration in the water was observed close to the submarine emissary diffusers, being the point 4 the most prominent, which may be related to the tidal current moving towards the coast in the moment of the sampling (Tábua de Marés, 2019). There is a downward trend in concentrations as the points move away from the submarine outfall. The difference between the found concentrations can be related to the abiotic factors like solar radiation, temperature, salinity and hydrodynamic, which influence the dispersion plumes, mainly with the effluent jets pressure throw on the submarine outfall (Ferreira, 2015). The influence of pressure jets on emissaries can be explained by diffuser proximity, where the pressure is strong and hence the concentration is higher on this point (Bleninger, 2006).

In a previousstudy, the maximum concentration identified was 649 ng L^{-1} (Pereira *et al*., 2016). This result was lower than the highest concentration found in the present study of 1,322 ng L^{-1}, the difference between both studies was 204%.

In a study carried out in Boston Harbor – USA, Cantwell *et al*. (2016) identified caffeine in concentrations of 15 ng L^{-1} on the external port and 185 ng L^{-1} on the internal port. In accordance with this study, the sewage overflow, and the irregular discharge of sanitary effluents into the city's rainfall runoff influenced on the results. However, public actions were made to improvement of the residual waters and the opening of 14 km tunnel systems for effluent release into the treatment systems. After this improvement, Boston Harbor had a reduction of 86% in the contamination on 27 years.

In Portugal, caffeine concentration in water samples rangedfrom 18 to 525 ng L^{-1}, and the highest concentration was detected in beaches with excellent quality, located in densely populated and touristic cities (Paiga*et al*., 2017). These results were due to the effluent discharges in submarine outfalls at sea. It was observed that the highest concentration found in coastal zones in Portugal (525 ng L^{-1}) was lower than the highest concentration found in Santos.

In Matosinhos, Portugal, the primary treatment of effluents is in operation only in port regions (European Commission, 2007; Coelho, 2008). Whereas in Santos Bay, the sewage is preconditioned and chlorinated before bedischarged through the submarine sewage outfall (SABESP, 2019).

Studies performed in the Coastal zone of the Lesbos Islands - Greece, indicated the presence of caffeine between 6.1 ng L^{-1} and 522 ng L^{-1} close to emissaries' pipelines (Nödler*et al*., 2016). Ghoshdastidar*et al*. (2015) performed a study in New Scotland (Canada) and found caffeine concentrations up to 115,141 ng L^{-1} at some points. In the port region of Metropolitan Halifax was detected sewage dumping incidents without treatment. The study collected samples at different points in receiving waters of municipal sewage treatment plants, detecting pharmaceutics, metabolites and a strong presence of caffeine in 100% of samples.

In 2012 was performed a study in Barbados Island, Caribe, where was detected the presence of caffeine in wastewater samples between 100 ng L^{-1} and 6,900 ng L^{-1}. According to this study the population growth is the main cause of contamination of the marine ecosystem (Edwards *et al*., 2015).

Alygizakis*et al*., (2016) quantified caffeine concentrations ranging from 5.2 to 78.2 ng L^{-1} at Saronikos Gulf and ElefsisBayonEgeu Sea, Greece. It was demonstrated in this study that the anthropic contamination over wastewater treatment plant (WTP) inputs were the several pollution sources in Saronikos Gulf as well as contamination by shipping activities, industrial effluents, dredging and / or inputs.

Del Rey *et al*., (2012)performed a study in 14 places on Oregon Coast (USA). It was detected caffeine concentrations at 44.7ng L^{-1}. In this study was also identified 152.2 ng $L^{-1}$of caffeine in rivers, which revealed that in open sea locations,were observed lower levels of caffeine than in semi-closed bays.

In Singapore it was identified caffeine in 100% of samples on natural water reservoir with a maximum concentration of 2,980 ng L^{-1}, possibly due to effluent leaks along the river, decreasing the concentration up to 644.5 ng L^{-1}along the river. This decrease in concentration is due to the effects of photolysis, as well as dilution and biodegradation, which may be eliminating the caffeine (You *et al*., 2015).Cesen*et al*., (2019) observed the caffeine presence on 83% of samples, where the maximum concentration found was 49,600 ng L^{-1} in Slovenia and Croatian sewage treatment plants draining into rivers.

Gonçalves *et al*., (2017) performed a study in Terezópolis (Rio de Janeiro, Brazil), where detected caffeine concentrations in an interval between 160 and 47,500 ng L^{-1}in the Paquequer River, results higher than those found in Santos. Another study realized in Piraí and Jundiaí rivers, located in São Paulo state, Brazil, detected caffeine presence in all samples collected. In this study the highest concentration was 662 ng L^{-1} in Piraí river and 14,050 ng L^{-1} in Jundiaí river (de Sousa *et al*., 2018). In this same study, it was identified caffeine in sediment samples in concentrations between 2.57 and 6.59 ng g^{-1}. In other study analyzing sediment samples from Cádiz Bay (Spain), it was found caffeine concentration between 1.9 and 12.20 ng g^{-1}(Maranho*et al*., 2015b).

Comparing the highest concentration found in Santos samples with the highest concentrations found in other coastal zones, it was observed that Santos had a higher concentration than Portugal (525 ng L^{-1}), Greece (522 ng L^{-1}), Boston Harbor (185 ng L^{-1}), Mediterranean Sea (78 ng L^{-1}), and Oregon (45 ng L^{-1}). However, in some places the maximum concentrations were higher than the Santos Bay, such as New Scotland (115,141 ng L^{-1}) and Caribbean (6,900 ng L^{-1}). . In parallel of the concentrations found in Santos marine ecosystems with the waters of lentic and lotic ecosystems from other studies, it appears that, except for Oregon and Piraí River, the other places showed highest concentrations. These difference of caffeine concentrations can be explain by some factors, such as climatology and hydrography, the hydrodynamics, geological characteristics, population concentration, wave energies, level of wastewater treatment, among other factors, which influence the input and dispersion or persistence of these substance in the aquatic ecosystems (Neves, 2008).Therefore, according to the results found in Santos Bay, there is a need for greater attention and investments in sanitation for the degradation of these contaminants.

Since caffeine has been reported in concentrations ranging from ng to ug in water and sediment, ecotoxicological studies are necessary to assess the risk and effects on aquatic organisms. According to the study carried out by Aguirre *et al*., (2015a), in the laboratory, the activities of detoxification enzymes GST – Glutathione S-Transferase (GST) and lysosomal membrane stability (LMS) were evaluated, with exposure of 21 days to a concentration of 100 ng L^{-1} of caffeine in *Corbicula fluminea*, with effects of cytotoxicity and physiological stress being identified, with significant damage to the LMS at all concentrations tested. Comparing with the present study, all points collected were above 100 ng L^{-1}, which may cause sub lethal effects in organisms present in Santos Bay, considering that caffeine has a half-life of 30 days.

In another laboratory study, Aguirre *et al*., (2015b) evaluated the effects of caffeine in the bioluminescence of *V. fischeri bacteria*, as well as a growth inhibition test with *I. galbana* and *P. subcapitata* and fertilization tests with sea urchin *Paracentrotuslividus sp*. In this study it was observed that caffeine caused differences in bioluminescence in *V. fischeri* exposed to concentrations ranging from 200-18,000 mg L^{-1}. The growth inhibition of *I. galbana* and *P. subcapitata* was observed after exposure to concentrations of 100-500 mg L^{-1}. It was also notice that in order to have an acute effect on the fertilization of the sea urchin, exposures to 10,000 and 50,000 mg L^{-1} were necessary. These concentrations are higher than the values found in Santos (μgL^{-1}). A chronic effect was observed during the embryo-larval development of sea urchin, where there was a significant decline in the percentage of normal pluteal larvae exposed to caffeine between 0.00001 mg L^{-1} (10 ng L^{-1}) to 0.015 mg L^{-1} (15,000 ng L^{-1}), showing a decrease in embryo-larval development from 63% to 29% respectively. Therefore, caffeine is not supposed totrigger acute effects on the organisms tested at environmental concentrations, however, chronic effects on early life stages were observed, thus being able to compromise reproductive processes.

In a study performed to assess the effect of caffeine on *Ruditapesphilippinarum*, Aguirre, *et al*, (2013) observed that after 35 days of exposure to15,000 ng L^{-1} of caffeine decreased in 50% the stability of the membranelysosomal in hemolymph. In a study carried out in Spain, in continental waters, the effects of caffeine were tested for two species: *Daphnia magna* and *Diacyclopscrassicaudiscrassicaudis*. Acute effects were identified in *Diacyclopscrassicaudiscrassicaudis* at a concentration higher than 395,000 μg L^{-1} of caffeine. An acute effect was observed in *Daphnia magna*, at a caffeine concentration higher than 5,280 mg L^{-1}(Di Lorenzo et al., 2019). In a study carried out to evaluate the effects of caffeine on *Prochiloduslineatus* exposed to caffeine in concentrations of 300, 3000 and 30,000 ng L^{-1} for a period between 24 to 168 hours, it was found increased activity of EROD (Ethoxyresorufin-O-deethylase)in the liver. With the results of this study, the interference of caffeine in biotransformation processes in *Prochiloduslineatus* was verified. After 168 hours of exposure, however, caffeine was not able to cause oxidative stress (Santos-Silva *et al*., 2018).

In tests carried out by Maranho*et al*. (2014) with marine sediments contaminated with caffeine, lethal and sublethal responses related to different phases of metabolism (enzymatic activities of phases I and II), neurotoxicity (acetylcholinesterase activity), oxidative stress (lipid peroxidation and activity of antioxidant enzymes) and genetic damage (DNA strand breaks) were analyzed to assess the possible adverse effects of caffeine. In these tests, the amphipod *Ampeliscabrevicornis* was chosen as a bioindicator species. This study demonstrated the bioavailability of caffeine in marine biota as follows: phases I and II, where oxidative stress was observed with adverse effects at a concentration of 1 ng g-1 and mortality at a concentration of 624 ng g^{-1}, in addition to changes in DNA strand breaks with adverse effects at a concentration of 1 ng g $^{-1}$ and mortality at a concentration of 307 ng g^{-1}. Thus, it is evident that the concentrations found in marine sediments that causedsublethal are similar to the concentrations found in Santos Bay.According to the previous studies, caffeine may cause effects on detoxification enzyme (GST - glutathione S-transferase) and on the stability of the lysosomal membrane, causing cytotoxicity and physiological stress in *Corbicula fluminea* in 100 ng L^{-1}, thus being able to cause the same effects to organisms in Santos, with a caffeine concentration was up to 1,322 ng L^{-1}.

A chronic effect of abnormal development was observed during the embryo-larval assay of sea urchin, exposed to a caffeine concentration from 0.001 mg L^{-1} (1,000 ng L^{-1}) to 0.015 mg L^{-1} (15,000 ng L $^{-1}$) (Aguirre *et al*. 2015b). With the concentration found in Santos Bay (Station 4), the contaminant studied may cause chronic effects to organisms during embryo-larval phase, affecting their development.

In a study carried out to assess the environmental risk of pharmaceutical products, including caffeinein marine sediments, after 30 minutes of exposure, to caffeine concentrations between 1,500; 150; 15; 1.5; e 0.15 ng g^{-1}, absence of fertilization membrane in eggs was observed, as well as the abnormal development of *P. lividus* larval when exposed to all tested caffeine concentrations (Maranho*et al*., 2015a).With regard to bioaccumulation, Santos-Silva *et al.,* (2018) carried out a study in the Paraná and Acaraguá rivers, which cross protected areas in Argentina, to identify the influences of human activities on fishes in this biome. Presence of several substances was evidenced, as drugs, antibiotics, drugs, and caffeine in the fishes of the region. In this study, caffeine represented 91% of the total measured of pharmaceutical concentrations, ranging from 1.2 to 13 μgKg^{-1} of body weight. The highest levels of caffeine were found in the liver (mean: 8.1 μgKg^{-1}) followed by gills and 5.7 and 2.2 μgKg^{-1}. However, caffeine was found mainly in the muscle of all Prochiloduslineatus from the Paraná River (0.8 to 6 μgKg^{-}

^{1}), except in the female liver (0.6 μgKg^{-1}). These caffeine concentrations were lower than the fish from the Acaraguá River. The presence of caffeine in all sampled fish indicates the existence of a source of effluent discharges in the region, demonstrating the absorption and bioaccumulation in fish.

Caffeine hazard assessment

PredictedNon-Effect Concentration (PNEC)

In accordance with the hazard assessment method, some bioindicator organisms were chosen in the literature in order to observe the chronic effects after exposure to caffeine, enabling the determination of the PNEC value by the lowest NOEC observed (Table 5).

Table 5: Concentration Effect Non Observation (and responses in aquatic organisms after exposure to caffeine NOEC ng L-1(water) and ng g-1(sediment).

Species	Matrix	Group	NOEC	Endpoint	Reference
P. lividus	Water	Echinoderms	10 (ng L^{-1})	Embriolarval development	Aguirre-Martinez *et al*. 2015
Corbicula manilensis	Water	Mollusk	50,000 (ng L^{-1})	Mortality	Aguirre-Martinez *et al*. 2015
I. galbana	Water	Seaweed	$5x10^{7}$ (ng L^{-1})	Growth	Aguirre-Martinez *et al*. 2015
Bioluminescence *V. fischeri*	Water	Bacterium	$1x10^{8}$ (ng L^{-1})	Mortality	Aguirre-Martinez *et al*. 2015
P. subcapitata	Water	Seaweed	$1x10^{8}$ (ng L^{-1})	Growth	Aguirre-Martinez *et al*. 2015
P. lividus	Water	Echinoderms	$1x10^{10}$ (ng L^{-1})	Fertilization	Aguirre-Martinez *et al*. 2015
P. lividus	Sediment	Echinoderms	0.15 (ng g^{-1})	Absence of fertilization membrane in eggs; abnormal larvae development.	Maranho*et al*. 2015a

NOEC (non-observed effect concentration)

For the purpose of determining the PNEC of chronic exposure, the literature that evaluated the toxicity of caffeine in marine aquatic organisms. Six different species were employed, with seven effects observed (endpoints). To determine the PNEC, the lowest NOEC found was considered, that is, the embryo-larval development of *P. lividus*, with the lowest concentration of non-observed effect (NOEC) of 10 ng L^{-1} of caffeine in water (Aguirre *et al*., 2015b) and 0.15 ng g^{-1} in sediment (Maranho*et al*. 2015a).

Table 6 shows the HQ values for each sampling station in Santos Bay.

Table 6: HQ measurement of caffeine at each water and sediment sampling station

Station	MEC Water (ng L^{-1})	MEC Sediment (ng g^{-1})	PNEC Water (ng L^{-1})	PNEC Sediment (ng g^{-1})	HQ Water	HQ Sediment
01	495	10.80	10	0.15	49.5	72.00
02	611	13.10	10	0.15	61.1	87.33
03	624	8.48	10	0.15	62.4	56.53
04	1,322	5.97	10	0.15	132.2	39.80
05	299	7.67	10	0.15	29.9	51.13
06	321	4.03	10	0.15	32.1	26.87
07	263	4.36	10	0.15	26.3	29.07
08	260	4.10	10	0.15	26.0	27.33
09	291	6.44	10	0.15	29.1	42.93
10	305	4.44	10	0.15	30.5	29.60
11	288	4.66	10	0.15	28.8	31.07
12	404	4.26	10	0.15	40.4	28.40
13	333	11.90	10	0.15	33.3	79.33
14	386	8.48	10	0.15	38.6	56.53

As verified in table 03, values above 10 were evidenced in all sampled points, thus denoting the possibility of adverse effects, according to the criteria adopted by the United States Environmental Protection Agency (US EPA), indicating threats to biota inhabiting this coastalarea.Comparing the HQ values calculated in the present study with the work perfomed by Dafouz 2018, the mean HQ verified in Santos was 44, higher than the study performed byDafouzet al. (2018) with HQ average of 15, excluding outliers and considering PNEC 10 for all samples.

Higher quotients were found to Station 4(water) with HQ of 132 and Staion 2 with HQ of 87,33 (sediment), both located in the zone of influence of the submarine sewage outfall.

Considering the HQ values found in this study are higher than the threshold established by the US EPA (HQ> 10), it is considered that there is an environmental hazard in all sampled stations with the possibility of adverse chronic effects as observed by Aguirre *et al*. (2015b).

CONCLUSION

It was observed that the concentrations of caffeine found in the Santos Bay ranged from 206 ng L^{-1} to 1,322 ng L^{-1} in water and between 4.10 ng g^{-1} to 13.10 ng g^{-1}in sediment, being considered high compared to others marine ecosystems. It was observed that there is a potential hazard related to chronic effects, since the HQ estimates were higher than 10. Therefore, there is a need for greater attention to the monitoring of this class of bioactive substances and the alternatives of sewage treatment, in order to eliminate or mitigate adverse effects. Caffeine is also recommended as a chemical marker of contamination by domestic effluent in this coastal zone.

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