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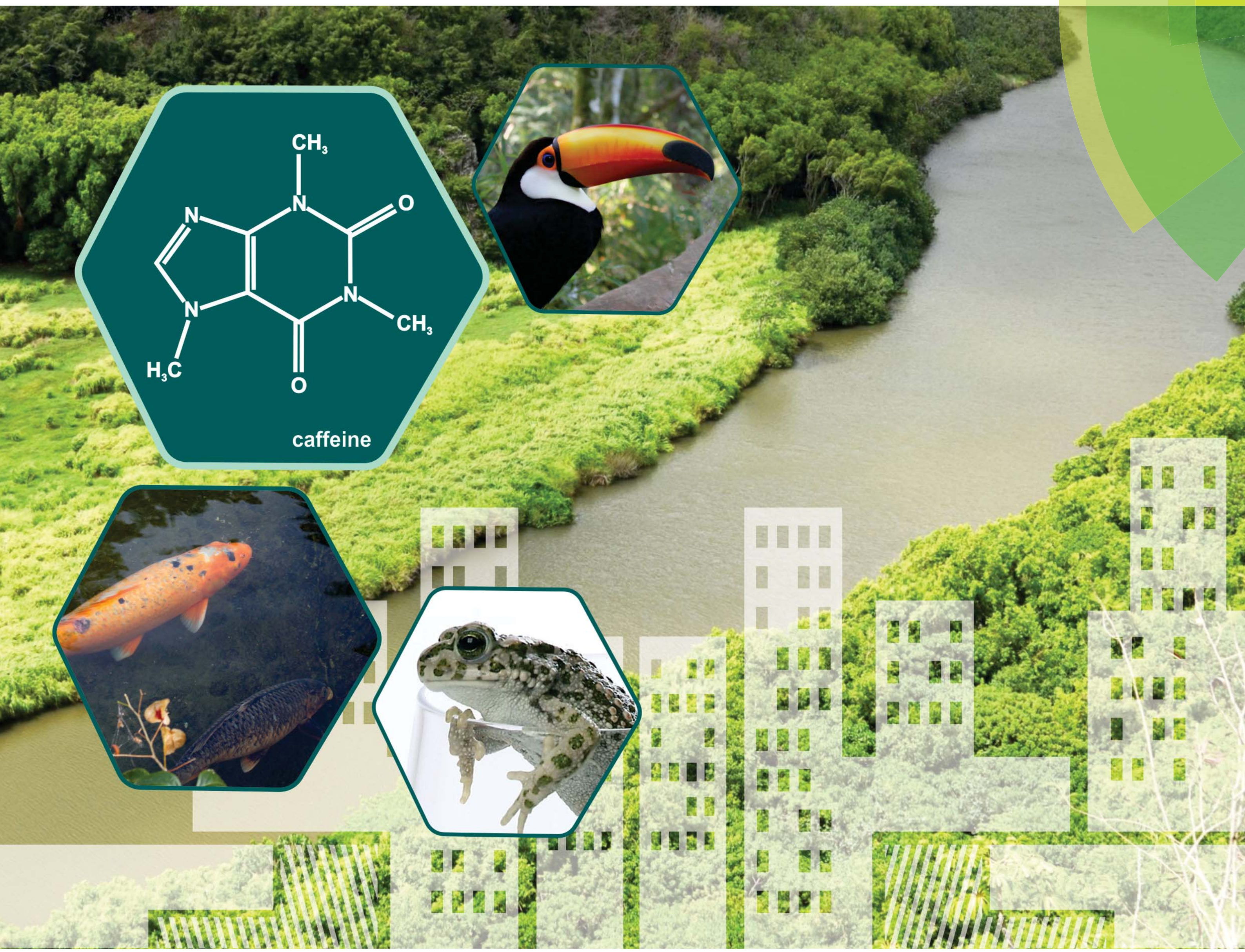

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Caffeine as an indicator of estrogenic activity in source water

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Caffeine has already been used as an indicator of anthropogenic impacts, especially the ones related to the disposal of sewage in water bodies. In this work, the presence of caffeine has been correlated with the estrogenic activity of water samples measured using the BLYES assay. After testing 96 surface water samples, it was concluded that caffeine can be used to prioritize samples to be tested for estrogenic activity in water quality programs evaluating emerging contaminants with endocrine disruptor activity.

Environmental impact

Considering the growing number of emerging contaminants and assuming that around 800 compounds are known or suspected of acting as endocrine disruptors, there is global concern about possible damage caused by these compounds not only to aquatic organisms, but also to human health. Assessing estrogenic activity in both source and drinking water is moving towards becoming routine in the near future, but still poses a tremendous analytical challenge. The use of caffeine as an indicator of estrogenic activity opens a novel approach to optimize not only water screening surveys, but also well-established monitoring programs, thus enabling prioritization of samples to be tested further.

Caffeine is widely consumed due to its presence in several species of plants, coffee, tea, cocoa, herbs and analgesic drugs, acting as a stimulator of the central nervous system.[1] Caffeine is partially metabolized in the human body and is excreted through urine. Because wastewater treatment plants do not completely remove this compound[2] and also because of its high water solubility, caffeine can reach natural water systems such as surface and underground water. Thus, the presence of this compound in natural waters is strongly related to anthropogenic activity, and therefore it has been used as a good indicator of water quality regarding the presence of sewage.[1–9]

Caffeine levels detected in water bodies are related to the sanitation conditions of a region. A study carried out in the Boston Harbor seawater (USA) showed that caffeine was the main contaminant found in this aquatic system, where the presence of raw and treated sewage was consistent with data on the consumption pattern in the region.[3] In surface waters around Madrid (Spain), different concentration ranges measured (675–13 167 ng L^{-1}) correlated with the distribution of the population in the investigated metropolitan area.[10]

Even in countries with high levels of sanitation, caffeine can still be detected in surface waters, as reported in France (13–107 ng L^{-1}),[11] Istanbul (Turkey) (21–20 427 ng L^{-1}),[12] Italy (0.6–1056 ng L^{-1})[13] and the USA (2–225 ng L^{-1}).[14–17]

In Brazil and other countries with poor sanitation conditions, the scenario of contamination of water bodies by sewage is severe, especially due to the high input of raw sewage directly into the surface water systems, where caffeine levels span over up to two orders of magnitude higher than those determined in European countries[11–13,18] and the USA.[14–17] Caffeine was found in the range of 174 and 127 092 ng L^{-1} in surface waters from São Paulo[19] and between 1410 and 753 500 ng L^{-1} in the city of Curitiba, Paraná.[9] The mean concentration of 219 100 ng L^{-1} was measured in surface waters in the State of Rio de Janeiro.[8]

Underground water systems may also be contaminated with caffeine. In the USA, 1231 groundwater samples were collected from May 2004 to March 2010 by the California Groundwater Ambient Monitoring and Assessment (GAMA) Program Priority Basin Project and the median concentration of caffeine reported in these samples was 170 ng L^{-1}, with a maximum concentration of 290 ng L^{-1}.[20]

Caffeine is only partially removed in drinking water treatment plants, being the extent of removal related to the type of treatment and the sewage burden in the raw water. Concentrations ranging from 2 to 260 ng L^{-1} and 5 to 82 ng L^{-1} were determined in source and drinking water, respectively, in France.[21] Concentrations between 10 and 53 ng L^{-1} were measured in drinking water in Italy,[13] from 50 to 396 ng L^{-1} in Spain,[10,22] from 1 to 181 ng L^{-1} in the USA[16,17] and in the range of 7 to 108 ng L^{-1} in Canada.[23] Mean concentrations of 24 and 23

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ng L^{-1} were reported for drinking water in France[11] and China,[24] respectively. In Brazil, caffeine in drinking water was reported to occur at concentrations ranging from 47 to 5845 ng L^{-1}.[25]

The occurrence of caffeine has been compared to other traditional microbiological and physico-chemical water quality parameters and it has been a reliable indicator of pollution in urban aquatic environments.[8] Caffeine has also shown a positive correlation with biochemical oxygen demand (BOD) and nitrate in natural waters.[9] In addition, caffeine could be used as a tracer for the quantification of untreated wastewater input into underground water systems.[26] The presence of this compound has also been considered to be a more effective indicator of the presence of sewage than *E. coli* due to the fact that caffeine is related exclusively to the human body excretion.[2] The correlation between caffeine and faecal coliforms (R^2 = 0.45) showed that caffeine is a promising indicator of recent input of sewage in natural waters.[6]

The purpose of this work was to evaluate the feasibility of using caffeine and other emerging contaminants to predict the estrogenic activity in natural waters sampled in Brazil. A group of 16 compounds was selected to be monitored: caffeine (I), estrone (II), 17β-estradiol (III), estriol (IV), progesterone (V), testosterone (VI), mestranol (VII), levonorgestrel (VIII), 17α-ethynylestradiol (IX), diethylstilbestrol (X), triclosan (XI), bisphenol A (XII), 4-*n*-octylphenol (XIII), 4-*n*-nonylphenol (XIV), phenolphthalein (XV) and atrazine (XVI). Ninety-six samples were collected from seven different surface water sampling sites in the State of São Paulo: Atibaia River, Capivari River, Piracicaba River, Corumbataí River, Cotia River, Sorocaba River and Tanque Grande Reservoir from March 2010 to April 2011.

Using 1 L of sample, the selected compounds were extracted using Solid Phase Extraction (SPE) with OASIS HLB (Waters) and eluted with methanol and acetonitrile. Details of sampling procedures and sample pre-treatment are described elsewhere.[25,28] The quantification was carried by Liquid Chromatography tandem Mass Spectrometry (LC-MS/MS) using an Agilent 1200 Series LC system coupled to an Agilent 6410 Triple Quadrupole mass spectrometer with an electrospray ionization source.[25] *Saccharomyces cerevisiae* bioluminescent bioreporter assay (bioluminescent yeast estrogen receptor assay – BLYES) was used to evaluate the estrogenic activity (EA) of the same water extracts. This specific bioluminescent strain was previously described[27] and the procedures used in this work were already published.[28,29] Sample extracts were split into two, one part being used for the chemical analysis and the other for the BLYES assay.

The results obtained for the different compounds were evaluated by univariate and multivariate (Principal Component Analysis, PCA) analyses using the Unscrambler 10.3 chemometric software (CAMO, Norway). Fig. 1 shows the direct univariate correlation between the caffeine concentration and EA. A reasonable correlation (correlation coefficient, R^2 = 0.4664) was found.

Fig. 2 depicts the distribution of the loadings for the first and second principal components (which explains together 45% of the data variability) for the 8 (of the 16) contaminants that were quantified at least once and the measured EA. Again, it is possible to conclude that there is a significant correlation between the caffeine concentration and estrogenic activity, considering the closeness of the loading values of these parameters observed for the first and second PCs, and the significant variability of the dataset they describe. In addition, it is also possible to observe that some compounds that are considered to be low inducers of EA may be present in water without presenting a significant correlation with the measured estrogenic compounds present in the samples, such as XV, XII, II and XIV. On the other hand, estriol (IV) appears to show a significant correlation with EA, as expected.

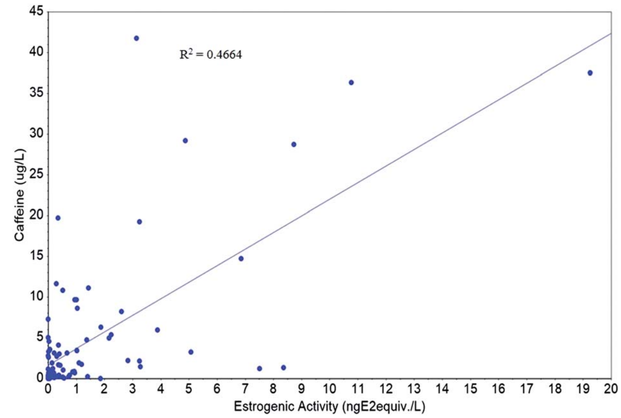

Fig. 1 Scatter plot of the concentration of caffeine and estrogenic activity for the 96 tested samples.

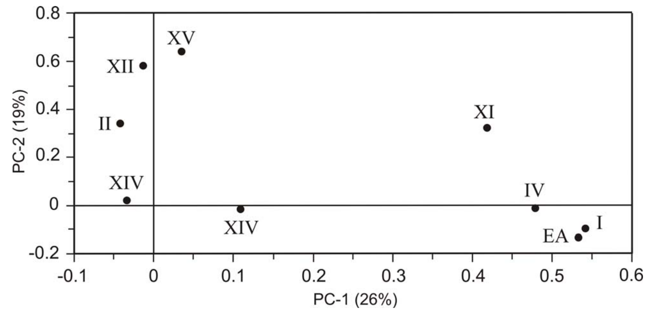

Fig. 2 Plot of the loadings resulting from the PCA analysis performed on 9 variables of the dataset (8 contaminant concentration and EA).

As already discussed by Bergamasco *et al.*,[29] the higher sensitivity of the BLYES assay in comparison to the expected activity of each individual compound that was measured could be explained by synergistic effects or by the presence of strong estrogenic substances, such as 17α-ethynylestradiol, which could produce some response even at concentrations lower than the limit of detection of the LC-MS/MS method employed in this study. It is important to emphasize the fact that the BLYES assay is around 200 times more sensitive than LC-MS/MS in terms of 17β-estradiol (0.01 ng $E2_{equiv.}$ L^{-1} and 1.8 ng L^{-1}, respectively).[25]

From a total of 12 samples with caffeine concentrations between <LOD (0.005 μg L^{-1}) and 0.1 μg L^{-1}, 67% presented positive results for BLYES; next, for a total of 37 samples showing caffeine concentrations between 0.1 and 1.0 μg L^{-1},

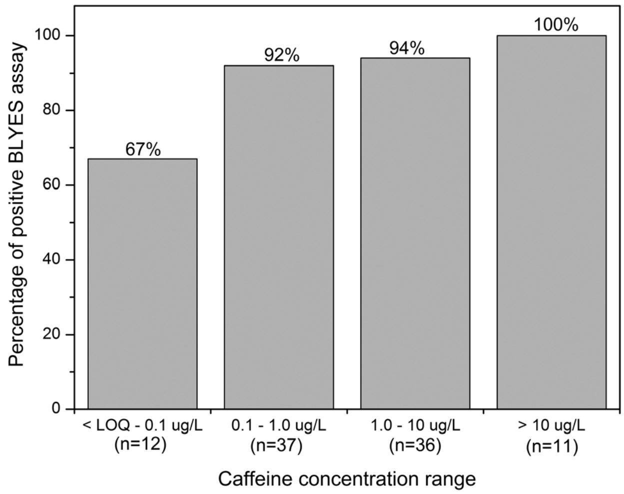

Fig. 3 Percentage of source water samples (n) tested positive for the BLYES assay as a function of the caffeine concentration range. A total of 96 samples.

92% were positive for BLYES; in water samples ($n = 36$) presenting caffeine concentrations between 1.0 and 10 μg L^{-1}, 94% were tested positive for BLYES; for the 11 samples showing caffeine concentrations above 10 μg L^{-1}, 100% presented positive results for BLYES (Fig. 3). Considering all samples that showed caffeine concentrations above 1 μg L^{-1} ($n = 47$), 96% showed positive results for estrogenic activity using the BLYES assay. This trend is important from both environmental and monitoring perspectives, as the caffeine concentration can be used to prioritize sites to be studied. Also, using only caffeine as an indicator of estrogenicity, it is possible to verify seasonal periods that deserve more attention concerning endocrine disrupting activity. In summary, high concentrations of caffeine in source water samples indicate a high input of sewage, and consequently higher concentrations of compounds such as hormones and other pharmaceuticals that positively respond to the BLYES bioassay.

Conclusions

The correlation observed between caffeine levels and estrogenic activity (EA) strongly suggests that in natural waters, this compound is a powerful candidate to be used as a tracer to screen samples with high probability of showing estrogenic activity in water monitoring programs. However, it is important to mention that at the concentration levels found in the tested waters, caffeine poses no risk to human health neither is it an endocrine disruptor candidate. One advantage to be highlighted is the fact that caffeine can be determined using simple and low cost analytical methods such as SPE HPLC/DAD.[19] The prioritization based upon caffeine levels has a direct effect in lowering the required number of samples to be assessed by a more sophisticated and expensive method.

Although a correlation between caffeine and estrogenic activity was established using the BLYES assay, one should bear in mind that this correlation depends on the type of estrogenic assay used, and a database should be constructed for each different EA assay chosen.

Finally, assuming the possibility of having water quality criteria for EA, it will be possible to use this correlation to define a threshold value for the caffeine concentration to predict the estrogenic activity with high levels of confidence.

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