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Baseline

Anthropogenic contamination of Portuguese coastal waters during the bathing season: Assessment using caffeine as a chemical marker

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ABSTRACT

Bathing water quality standards are based on international standards and legislations. However, in Europe, only the microbiological parameters are to be accomplished. Recent research has focused on chemical indicators that can determine human fecal contaminants in water. Therefore, the suitability of caffeine as a chemical marker of seawater pollution in the north Portuguese coastal area in the Atlantic Ocean during the bathing season was assessed in this study. The quality of water from the coasts of five cities was monitored. 101 seawater samples were collected from 14 beaches, and their water quality was classified as sufficient, good, and excellent. Caffeine was detected in all samples in concentration range of 18 to 525 ng/L. The highest average concentration of caffeine was found in seawater samples collected in July from beaches classified as having sufficient water quality, which were located in cities with high population density and high tourist affluence.

Oceans and seas hold about 96.5% of the Earth's water. Although oceans cover two-thirds of the Earth's surface, they are easily vulnerable to human influences, e.g., overfishing, pollution from run-off, and dumping of waste from human activities (Boopathy, 2000). Sewage effluent from waste water treatment plants (WWTPs) is recognized as a major source of human pollution (Gaw et al., 2013) and is discharged into marine environments through coastal areas and through rivers receiving WWTP effluents (Benotti and Brownawell, 2007). Moreover, because of their location, coastal areas represent a major advantage as many economic activities may take place, including tourism, commercial ports, and harbors, thus making them more vulnerable to higher levels of pollution than other seawater samples (Loos et al., 2013).

Since the 1970s, the European Union (EU) has laid down rules to safeguard public health and bathing waters. The EU must inform the public about bathing water quality and beach management through bathing water profiles. These profiles contain information on the kind of pollution and sources that affect the quality of the bathing water and that pose a risk to bathers' health (EC, European Commission, 2015, European bathing water quality). The revised Bathing Water Directive of 2006 updated and simplified these rules. It requires the Member States to monitor and assess the bathing water quality. The requirements for award beaches and marine areas with a flag ["Sufficient (yellow flag)," "Good (green flag)," and "Excellent" (blue flag)] are: the accessibility, infrastructure, safety of beaches, environmental information and education, and water quality. The last parameter is a health factor and an important indicator of environmental quality (EC,

European Commission, 2006, Directive 2006/7/EC).

Concerning the management of bathing water quality, *Escherichia coli* (fecal coli bacteria) and *Enterococcus intestinalis* (streptococci) are indicator organisms used for predicting microbiological health risk and to obtain a high level of protection for beaches and marine areas. When "pollution" occurs, it means that there is presence of microbiological contamination or other organisms or waste affecting the quality of the bathing water, presenting a risk to bathers' health (EC, European Commission, 2006, Directive 2006/7/EC). McLella and Eren (2014) stated that the presence of *E. coli* and *E. intestinalis* in water is a strong indicator of sewage contamination. However, these analyses do not indicate the origin of the contaminants, i.e., whether it is animal or human origin (Glassmeyer et al., 2005). Suitable markers are therefore necessary to detect and locate the sources of water pollution (Ferreira and da Cunha, 2005). From all the markers investigated such as fecal sterol coprostanol [a fecal sterol produced in the digestive tract of humans by the microbial biohydrogenation of cholesterol (Leeming and Nichols, 1996)] and pharmaceuticals (Daneshvar et al., 2012; Andreu et al., 2016), the one that received the most attention in recent times is caffeine (Buerge et al., 2003; Ferreira and da Cunha, 2005; Peeler et al., 2006; Kurissery et al., 2012).

Caffeine is an ingredient in a variety of beverages (coffee, tea, and caffeinated soft drinks) and numerous food products (chocolate, pastries, and dairy desserts) (Ferreira, 2005). In the world, 90% of the people consume daily at least one meal or beverage with caffeine in it (Buerge et al., 2003). The global average consumption of caffeine is

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estimated to be between 80 and 400 mg per person per day (Gokulakrishnan et al., 2005). Buerge et al. (2003) mentioned that there are large differences among countries regarding the contribution of different food sources to total caffeine intake. Caffeine is also of further importance in pharmaceuticals. It enhances the effect of certain analgesics used for cough, cold, and headache. Caffeine is used as a cardiac, cerebral, and respiratory stimulant and as a diuretic (Buerge et al., 2003).

Caffeine is a very good tracer because its detection in the aquatic system indicates human waste source (Peeler et al., 2006). Caffeine concentration previously found in seawater samples and estuarine systems ranged from nanograms to micrograms per liter (Weigel et al., 2002; Benotti and Brownawell, 2007). The first study that reported the presence of caffeine in seawater samples was presented by Weigel et al. (2001). The authors mentioned a group of compounds that were not reported before in marine ecosystems, in which caffeine was included. In literature, caffeine was found in the North Sea of the United Kingdom, the Netherlands, Germany, Denmark, and Norway (Weigel et al., 2001; Weigel et al., 2002); in the Mediterranean Sea of Swiss midland region (Buerge et al., 2003); in the Hanalei Bay of Kauai in Hawaii (Knee et al., 2010); in the Atlantic Ocean of Costa da Caparica in Portugal (Neng and Nogueira, 2012) and of Todos os Santos Bay in Salvador of Brazil (Ferreira, 2005); in west coast of Vancouver Island in British Columbia (Verenitch and Mazumder, 2008); in the coastal watersheds in Canada (Comeau et al., 2008); in the Marina Bay of Singapore (Wu et al., 2008); in Oregon coastal region (Rodriguez del Rey et al., 2012); in the Biscayne Bay of Florida (Gardinali and Zhao, 2002); in South Florida coastal region (Singh et al., 2010); and in the Jamaica Bay of New York in the United States of America (Benotti and Brownawell, 2007).

As mentioned by Ribeiro et al. (2015), estuaries, rivers, and coastal areas are subjected to intensive anthropogenic pressure from a wide range of pollutants discharged into them. An increase in recreational activities during the sea bathing season has been coupled with an increase in the worries about the dangers of bathing in waters contaminated by sewage discharges.

Portugal is located in southwestern Europe and has an extended coastal area in the Atlantic Ocean. Most of the Portuguese population settles in the coastal areas, where most of industrial, agricultural, and port activities are implemented. To the authors' knowledge, this is the first time that caffeine was used as an anthropogenic marker of human pollution in seawater samples collected from the Portuguese coastal area. A total of 101 seawater samples were collected during the bathing season (from the middle of June to the middle of September) in 2013, and quality of water from 14 beaches of four cities in the North (Vila do Conde, Matosinhos, Porto, Vila Nova de Gaia) and one city in the Center (Espinho) of Portugal was monitored. Therefore, our primary objective was to monitor the levels of caffeine in seawater samples, as a human pollution marker, collected from beaches classified as having sufficient, good, and excellent water quality. The secondary objective was to highlight the importance of including others parameters, such as caffeine, in the legislation of the Blue Flag Program.

Sampling frequency was determined by considering the bathing water quality. Thus, for excellent quality bathing water samples were collected only once a month; for good quality bathing water every 2 weeks; and for sufficient quality bathing water every week (EC, European Commission, 2006, Directive 2006/7/EC). Seawater samples were collected by the Portuguese Environment Agency (APA, Agência Portuguesa do Ambiente-Portuguese Environment Agency). The samples were directly collected from the sea, preferably away from the surf caused by the waves, where the depth reaches at least 1 m to avoid the presence of suspended sediments in the samples. The distance to the shore varies according to the beach type, although the main concern was to respect the 1-meter depth requirement (ISO 5667-3, 2012).

A total of 101 grab seawater samples were collected from the 14 beaches of five cities (Fig. 1). Two beaches in City 1 (Vila Nova do

Conde) (both beaches classified as excellent water quality), four beaches in City 2 (Matosinhos) (one beach classified as excellent, two beaches classified as good, and one beach classified as sufficient water qualities), two beaches in City 3 (Porto) (one beach classified as excellent and one beach classified as sufficient water quality), 3 beaches in City 4 (Vila Nova de Gaia) (two beaches classified as excellent and one beach classified as sufficient water qualities), and 3 beaches in City 5 (Espinho) (one beach classified as excellent, one beach classified as good, and one beach classified as sufficient water quality) were assessed (Table SM1, Supplementary material).

Microbiological determinations and the information on the studied beaches were supplied by the APA (APA, Agência Portuguesa do Ambiente-Portuguese Environment Agency) and are given in Table SM1 (Supplementary material). Information on the studied beaches was presented in the work of Lolić et al. (2015). It is noteworthy that seawater samples were collected at the same time for (a) microbiologic, (b) pharmaceutical (the study of Lolić et al. (2015)), and (c) caffeine (present study) analysis.

Amber glass bottles pre-rinsed with ultrapure water were used for sample collection. After reception in the laboratory, the seawater samples were filtered through 0.22- μm nylon membrane filters (Fioroni Filters, Ingré, France).

Acetonitrile LC-MS grade was supplied by Biosolve (Valkenswaard, Netherland), methanol LC-MS Ultra CHROMASOLV[®] and formic acid 98% PA-ACS were purchased from Sigma-Aldrich (Steinheim, Germany), and hydrochloric acid 37% was obtained from Carlo Erba (Rodano, Italy). Ultrapure water (resistivity of 18.2 M Ω -cm) was produced using a Simplicity 185 system (Millipore, Molsheim, France).

Caffeine and isotopically labeled standard (caffeine ¹³C₃, certified reference material, 1.0 mg/mL in methanol) were purchased from Sigma-Aldrich (Steinheim, Germany). Standard stock solutions of caffeine and caffeine ¹³C₃ at concentrations of 1 g/L in methanol were prepared and stored in the dark at -20 °C. Working standard solutions used to prepare the calibration and fortification standards were prepared in the initial mobile phase conditions.

All chromatographic solvents were filtered through a 0.22- μm nylon membrane filter (Fioroni Filters, Ingré, France) using a vacuum pump (Dinko D-95, Barcelona, Spain) and degassed for 15 min in an ultrasonic bath (Sonorex Digital 10P, Bandelin DK 255P, Germany). Before chromatographic analysis, sample extracts were filtered through 0.22- μm PTFE syringe filters (Specanalitica, Carcavelos, Portugal). Solid-phase extraction (SPE) cartridges (Strata-X; 200 mg, 3 mL) from Phenomenex (California, USA) were used for SPE.

Caffeine concentration in the collected seawater samples was performed following a procedure based on SPE and liquid chromatography (LC) coupled to tandem mass spectrometry (MS/MS).

SPE procedure was adopted as described in the work of Paíga et al. (2015). Seawater samples (pH 2, adjusted with hydrochloric acid) were preconcentrated onto Strata-X SPE cartridges (200 mg, 3 mL), which were previously preconditioned with 5 mL methanol, followed by 5 mL ultrapure water and 5 mL ultrapure water at pH 2, using a vacuum system manifold (Chromabond, Düren, Germany). After loading 500 mL seawater samples, cartridges were rinsed with 5 mL ultrapure water and dried under vacuum for 60 min to remove excess water. After elution with 10 mL methanol, the extracts were evaporated under a gentle stream of nitrogen and further reconstituted to a final volume of 1 mL of the initial mobile phase conditions. The achieved enrichment factor was 500. Finally, 10 μL of caffeine ¹³C₃ standard was added to obtain a final concentration of 200 $\mu\text{g/L}$.

Quantification of caffeine was performed using a Nexera Ultra-High Performance Liquid Chromatography system (Shimadzu Corporation, Kyoto, Japan) equipped with two solvent delivery modules, a degasser, an autosampler, a column oven, and an LC-MS/MS detector (LCMS-8030) with an electrospray ionization source.

Identification and quantification were performed under Multiple Reaction Monitoring (MRM), which recorded the transitions between

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