Contaminant Cocktails of High Concern in Honey

Subjects: Chemistry, Analytical

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Environmental pollution is a crucial problem in our society, having a better understanding of its consequences, which include the increase of contaminant cocktails present in the environment. The contamination of honeybees can occur through their interaction with the nearby environment. Therefore, if honeybees are previously contaminated, there is a possibility of contamination of their products, such as honey as natural, or minimally processed, product, resulting from the honeybees' activity.

contaminants honey microplastics

1. Introduction

1.1. Honey Contaminants: Overview and Legislation

Over the past decades, the European Union (EU) has been witnessing a decrease in wild pollinator occurrence as well as their diversity, which could be caused by land over exploration, poor management of pesticides application, invasive non-native species, environmental pollution, and, consequently, climate change [1]. Roughly 10,000 honeybees, per beehive, maintain interaction with elements in the surrounding area (over 7 km^2) [2]. This activity results in a contact with a vast environment that, if contaminated with different types of pollutants, such as pesticides, persistent organic pollutants (POPs), veterinary drugs, pharmaceuticals, and other emergent pollutants (such as microplastics and plastic-related chemicals), may affect their well-being [3]. Therefore, honeybees, due to their specific body composition, can keep and transport the contaminants to the beehive, potentially leading to the contamination of bee products, such as honey. On the other hand, the inappropriate use of acaricides in the treatment of beehives during honey collection may lead to cross contamination [4].

Over the last few years, honey has been noticed as a possible environmental bioindicator. In August 2022, a literature search on the *Web of Science* featuring the terms "honey" and "bioindicator" in their title, abstract, and/or keywords were made in order to initiate the following study, resulting in 30 reports of interest. Most of these articles target honey as an environmental bioindicator, regarding the presence of pesticides, heavy metals, radionuclides, and polycyclic aromatic hydrocarbons (PAHs). **Figure 1** summarizes the articles distribution according to the continent of origin—where no portuguese samples were used—while **Figure 2** represents the diverse compounds studied in the different articles. As for the extraction techniques used, five articles mentioned the use of QuEChERS when studying pesticides, but also the liquid–liquid extraction (LLE) and the solid–liquid extraction

(SPE) techniques; when accessing the presence of PAHs, the most common ones were dispersive liquid–liquid micro-extraction (DLLME) and LLE.

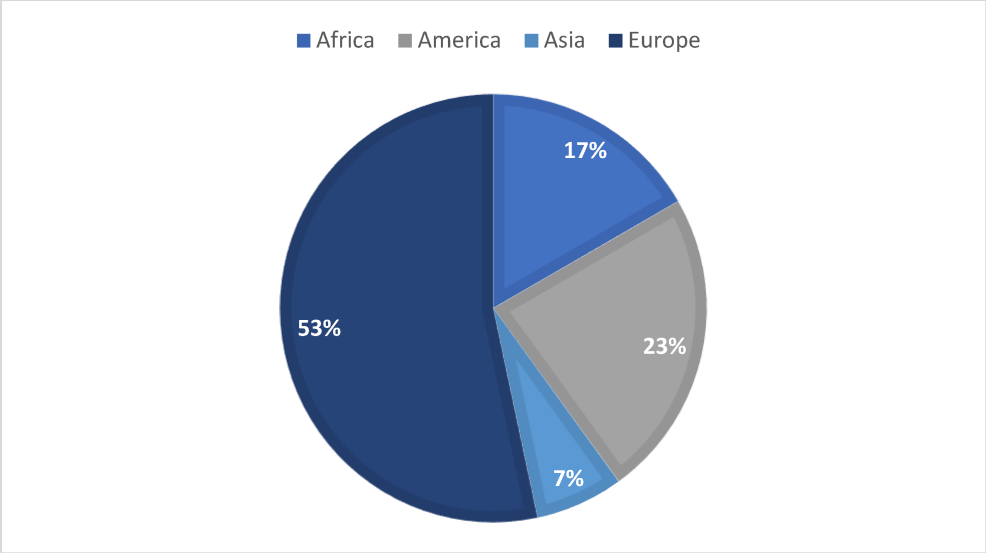

Figure 1. Graphic distribution of the articles according to the continent of origin.

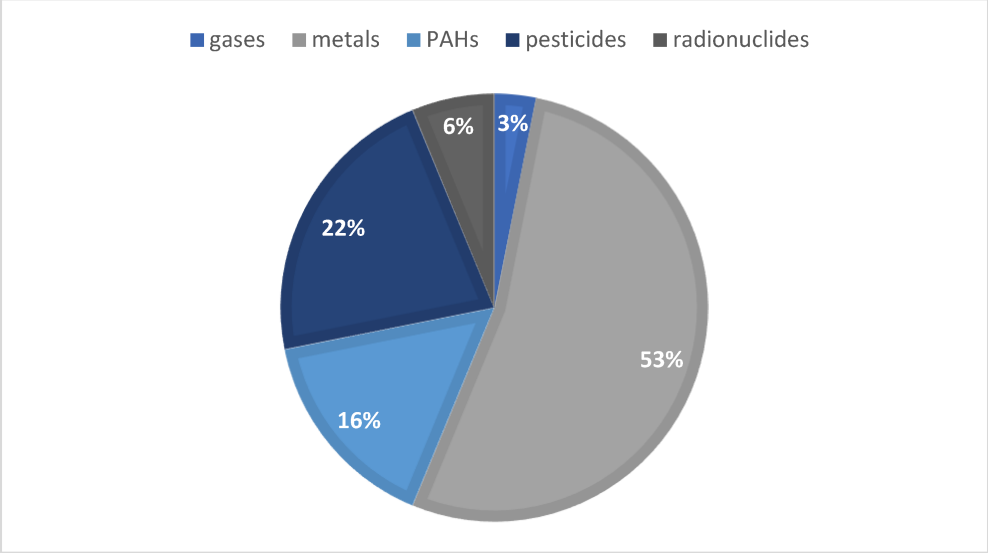

Figure 2. Graphic distribution of the articles according to the compounds studied in honey samples.

As seen in **Figure 2**, more than 50% of the articles mentioned the study of metals, followed by pesticides and PAHs. Neonicotinoids, pyrethroids, organochloride, organonitrogen and organophosphorus were the groups of pesticides analyzed.

Within this search, few articles mentioned the study of POPs, such as polychlorinated biphenyls (PCBs) or brominated flame retardants (BFRs), and no articles mentioned pharmaceutical drugs or microplastics for instance, which are emerging pollutants of growing concern. However, as mentioned below, studies have been conducted with honey and these contaminants, which could prove, even if it was not the main focus, that honey can also be a

bioindicator for these. Nevertheless, more studies should be performed with different pollutants regarding pollinators and their derivatives.

In 2005, two honeybee colonies 100 km apart, in Slovenia, were included in a study [5]. One colony was in Zavodnje, an area known to be polluted by the Šoštanj coal-fired power plant, while the other was in Poljanska dolina, an area without local SO_2 pollution. Researchers recovered data from honey originating in the Zavodnje colony and demonstrated that the sulphate quantified in the honey was correlated to the total yearly emissions of SO_2 detected by the Environmental Information System (EIS), a system including seven stationary emission-measuring stations. Values of sulphate detected in honey from Poljanska were significantly lower compared to the first colony [5]. Throughout the following years, new studies were developed considering honey as a bioindicator for the pollution of PAHs and heavy metals, among others. In 2008 [6], honey originating from six agricultural areas of Greece (north, center, and south) was evaluated regarding the presence of pesticide residues. The analysis performed by Balayiannis et al. [6] detected residues of phorate, chlorpyrifos, chlorfenvinphos, and coumaphos, an acaricide. A more recent project, published in 2021 [7], analysed the presence of organochlorine pesticides (OCPs), a group of compounds considered POPs. The honey samples were recovered from Masindi district, Uganda, an area that includes a forest reserve. Dichlorodiphenyltrichloroethane (DDT), dieldrin, endosulfan isomers, and lindane were quantified in the honey samples studied, concluding that the monitoring of OCPs should continue [7]. Nowadays, the use of OCPs is banned in several countries, but recently these have been reported in honey samples. Therefore, it is possible to conclude that the use of honey as a sample for the study of pesticides is pertinent, even in banned compounds such as POPs.

Besides these studies, the EU has established regulations regarding the presence of contaminants and their respective maximum residue levels (MRL), but also releases annual reports that assess the pesticide residues. Regulation (EC) No 396/2005 [8] is the legislation submitted by the EU regarding the MRL of pesticides in food and feed of plant and animal origin. A total of 315 products, including honey, and the respective MRLs for more than 1000 pesticides currently or formerly used in agriculture, can be found in this regulation [8]. By the EU legislation (Article 32, Regulation (EC) No 396/2005 [8]), the European Food Safety Authority (EFSA) requires an annual report evaluating the pesticide residue levels in foods originating from European markets. The 2019 report includes data provided by the national control activities carried out by the EU Member States, Iceland, and Norway [9]. In this EFSA report, 1302 samples of honey were studied, with 277 samples reporting the presence of contaminants, while 265 honey samples presented residues below or at the MRLs and 12 above. The dominant contaminants in honey samples were neonicotinoids and veterinary medicinal residue products, which include acetamiprid, amitraz, azoxystrobin, benzalkonium chloride (BAC), bromide ion, chlorates, chlorpyrifos, coumaphos, dimoxystrobin, flonicamid, fosetyl, glyphosate, and thiacloprid [9]. Even if in the literature it is possible to find different cases where the presence of contaminants, besides pesticides, are noted, no legislation can be found regardless of their potential harm to animal and human health. Therefore, it is imperative to stablish new studies approaching these contaminations in order to implement legislation. There are other official documents addressing additional contaminants, such as brominated flame retardants in food. For instance, there is the Commission recommendation (2014/118/EU) of 3 March 2014 on the monitoring of traces of brominated flame retardants in food [10], but it is only a recommendation and does not even mention honey. There is also a Commission regulation

(EC) No 1881/2006 of 19 December 2006 setting maximum levels for certain contaminants in foodstuffs [11], namely nitrate, mycotoxins, metals, 3-monochloropropane-1,2-diol, dioxins, PCBs, and PAHs, but it is general and does not specify honey.

1.2. Honey Contaminant Analysis—Extraction Methods and Challenges

Honey composition is dependent on its botanical source, geographical conditions, as well as its processing and storage conditions [12]. Either way, honey has a complex composition [13], mainly composed of sugars (with a total of about 80%) [14] and water. Smaller amounts of lipids, nitrogen compounds (which includes proteins and free amino acids), organic acids, minerals, vitamins, and phenolic compounds, among others, can also be found [12]. Therefore, this complex composition is problematic when talking about methods of sample preparation and extraction of contaminants in trace amounts. Therefore, the scientific community is working on the urgent need to validate and establish a method with better efficiency regarding recovery and matrix effects for the desired analyte, in this case, contaminants [15].

Efficient separation through chromatographic columns, especially in food analysis, is widely affected by preliminary sample preparation. Nevertheless, some characteristics of the sample must be primarily taken into consideration, such as particle size and homogeneity, as well as the target analyte that will be analyzed, since this information is crucial for the choice of the solvent, extraction, and clean-up technique [16]. The execution of this preliminary preparation enables (i) a clean-up of the sample; (ii) a transfer of the analytes to the medium of injection; and (iii) an enrichment of the target to a concentration that can be measured [17].

Nowadays, a broad spectrum of different sample preparation techniques can be found in the literature, following a common pathway, despite their differences. The extraction process to obtain the analyte from the sample matrix as well as the clean-up procedures [18] could interfere with the detection of the target analyte [17]. These can be used to detect a specific contaminant, a class, or a multiclass, where the last one, when linked to an appropriate detection and analytic method, can provide a technique capable of detecting and quantifying contaminants with the least steps of extraction and purification, increasing the method efficiency [19].

Souza et al. [19] published a review paper that revised the different techniques for sample preparation and pesticides study in honey. The SPE method allows the combination of the extraction and clean-up steps, employing low amounts of solvent and being capable of efficient analysis of samples directly collected from the apiary. A study using the Purge and Trap technique showed that, for specific conditions and coupled with gas chromatography, it is possible to obtain lower limits of detection (LOD) when compared to SPE. LLE, a conventional technique and one of the most used, is associated with some disadvantages, such as extraction of just one chemical class, the use of larger volumes of organic solvents and the extraction of several interferents from the matrix, being, therefore, a very unselective procedure. Even so, adjustments and progresses have been made in the method-development field to increase the efficiency, enabling the study of more than one class of pesticides and other contaminants and allowing its application in different matrices, increasing its versatility. Furthermore, the review also presents a different number of miniaturized techniques used on honey, namely (i) DLLME, a technique that can present

different variations in order to achieve higher recoveries; and (ii) Microextraction by packed sorbent (MEPS), which consists in a miniaturized version of the SPE method and, when coupled with GC-MS, allow to detect a multiclass residue, with an extraction time close to 4 min, reusing the sorbent and using lower amounts of sample and organic solvent [19].

A different method was used by Chiesa et al. [20] to analyze the presence of multiresidue pesticides in organic honey from German and Italian beekeepers. In this study, the technique used was Accelerated Solvent Extraction (ASE), where extraction times and solvent consumption are reduced, being characterized by high temperatures that increase the diffusion rates and the solubility of the analytes into the solvent, as well as high pressure, keeping the viscosity and surface tension of the solvent reduced due to the elevated temperatures employed. Moreover, the method performed was adjusted to combine the extraction and cleanup steps, resulting in an “in-line” method. This allowed to remove the interferences from honey samples, whose recoveries did not depend on the analyte concentration, being overall a cost-effective and minimized-waste method.

Another technique that became popular due to the reduced extraction time and solvent consumption was the Quick, Easy, Cheap, Effective, Rugged and Safe (QuEChERS) method, described in 2003 for the extraction of pesticides from food matrix [21]. Despite its original purpose, nowadays it is also used to recover other analytes from food matrices, such as environmental pollutants (e.g., pesticides, PAHs, etc.) and antidepressants, among others. Similar to other sample preparation methods, QuEChERS can be divided into extraction and clean-up [22]. In the extraction step, a salting-out effect occurs (partitioning of salts to the extract), where the solvent and the inorganic salts (employed to induce the separation between phases and to transfer hydrophobic analytes to the organic layer) are added to the sample. For the clean-up step, a dispersive solid-phase extraction (d-SPE) occurs, adding sorbents (for example, C18 and Z-Sep–remove hydrophobic interferences such as fat; PSA–removes polar interferences such as sugar and organic acids) that remove the matrix interferences to further clean-up and obtain the desired analyte in the extract solution [23]. Since it was first mentioned, various QuEChERS were designed, with different compositions, and the selection of the best one takes into consideration the analyte properties, the matrix, and the analytic technique conditions [16]. Comparing this technique with the traditional ones, QuEChERS are quicker [16], less expensive, employ lower volumes of solvents, and are less toxic [19], but also can provide greater recovery rates and increase the analytical performance [22].

2. QuEChERS Approach for the Analysis of Several Contaminants in Honey Samples

2.1. Pesticides

To extend the production area, the volume of production, shelf time and, simultaneously, improve the appearance of the product, farmers often reach for pesticides–chemicals designed to attack pathogens that could be a threat to their plantations, such as bacteria, weeds, fungi, insects, etc. [16]. Bioaccumulation, high lipophilicity, the long half-life, and the potential for long-range transport are characteristics presented by some pesticides on the market, which increase the possibility to contaminate the environment, being possibly a risk to human health [24].

Pesticides are designed to interfere with important mechanisms of several pathogens and, as a side effect, they may also be able to interfere with non-target organisms and plants. Another important point is that these can move freely in the environment, through wind currents and water leaching or runoff, making their transportation over the globe possible [25]. Therefore, and due to their persistence, we can understand that residue levels have been detected in different areas, such as in the air, soil, water, and non-target organisms [26]. Organisms' contamination can occur through main mechanisms: biomagnification–the higher in the food chain, the higher levels can be found in tissues and organs; and bioconcentration–the accumulation into the organism happens from the neighboring medium [27].

Pesticide contamination in humans can occur directly–such as inhalation, ingestion or dermal absorption–or indirectly–through contaminated food and water[26]. Different negative, both acute and chronic health, effects have been reported in the literature, including nausea, nervous system depression, endocrine disruption, and cancer [28]. For example, OCPs are highly associated with the stimulation of the central nervous system; breast, prostate, stomach, and lung cancer, and diabetes type 2, liver malfunctions, and endometriosis, among others [24]. Considering these effects on health and their persistence, OCPs have been banned since 1997 [28]. Many pesticides have also the capacity to interfere with the human reproductive process since they are designed to intervene with the pathogens' reproductive system [26]. Another interesting effect is the association with psychiatric problems such as depression and depression–anxiety [29].

As for water impact, it has been reported that different insecticides and herbicides can be harmful to different aquatic species, but an alarming point is the report that lower concentrations of malathion can impact plankton populations, an important point of the food chain [26]. Another relevant point is the fact that, besides alteration on aquatic fauna and flora [28], water contamination can not only alter the quality of drinking water but can also transfer the contaminants to the soil and other living organisms [25]. Soil biodiversity is widely affected by pesticide contamination, impacting different microorganisms present in the soil biota (with interference on microbial metabolism, molecular interactions, and symbiotic association) [26].

Risk assessments are needed when approving active substances, including pesticides. Their approval is also dependent on criteria relating to honeybees and, for future use, they cannot result in a nefarious exposure for the honeybees and present acute or chronic effects on the colony [30].

Honeybees present an important role in the environment, biodiversity, and food production [31] and, in 2012, neonicotinoids and fipronil were considered high risks for their health [30]. Neonicotinoids, a group of pesticides such as nicotine, are considered more toxic to invertebrates than mammals [32]. These compounds target the central nervous system, resulting in paralysis and death [32]. In 2018, the EU officially banned the use of three neonicotinoids (clothianidin, imidacloprid, and thiamethoxam) on all crops grown outdoors, due to their effect on bees' health [31]. Honeybees' exposure to these compounds happens through pollination and, therefore, they can be quantified in honeybee products, such as honey and beeswax. Considering this, it is important to explore methods of extraction, quantification, and monitoring pesticides, to better understand the risks associated with these products.

2.2. Persistent Organic Pollutants and Polycyclic Aromatic Hydrocarbons

Persistent organic pollutants are organic chemicals with the ability to remain in the environment for long periods, being widely distributed and toxic to humans and wildlife (with the capability to accumulate in the fatty tissue), due to their specific combination of chemical and physical proprieties [33]. Firstly, the Stockholm Convention targeted nine OCPs, PCBs and polychlorinated dibenzo-*p*-dioxins, and polychlorinated dibenzo-*p*-furans (PCDD/Fs) as the "dirty dozen", but throughout the years, new chemicals have been added, such as BFRs, endosulfan isomers, hexabromocyclododecane, among others [34]. Today, the Stockholm Convention considers 35 POPs that can be divided in three different groups: "pesticides", "industrial chemicals", and "unintentional production" [35]—by-products resulting from combustion processes. Polycyclic aromatic hydrocarbons are not considered in the list of POPs by the Stockholm Convention, oppositely to the Aarhus Protocol [36]. PAHs typically result from natural wildfires or incomplete combustion of fossil fuels and are semi-volatile, persistent, organic pollutants widely distributed in the environment [37].

POPs contamination can cause damage in a molecular level, such as neurotoxic effects and metabolic diseases [38]. Due to their nefarious actions, PCBs production has been banned, but their bioaccumulation and persistence in the environment prove to be a problem. For humans, the main route of exposure is inhalation, but others can be considered, such as dermal absorption and oral ingestion [39]. Within the effects of PCBs exposure, researchers can find epidemiological studies referring to metabolic and neuro system diseases [40], and these have already been classified, by IARC, as carcinogenic to humans [41]. PCBs can be associated with insulin resistance and diabetes mellitus type 2, due to their capacity to interfere with the expression of genes related to these phenomena [42]. Another problem is their neurotoxicity, which could be associated with the fact that PCBs metabolism increases the formation of reactive oxygen species (participating in processes within the nervous system), resulting in an oxidative stress environment leading up to inflammation of the cells [43].

When dispersed in the environment, PAHs can affect human health [44]. Naturally, their effects are dependent, for example, on exposure route and duration, as well as their concentration. Among the acute effects that have been associated with these, researchers can observe skin irritation and inflammation (where naphthalene is considered a direct skin irritant) [45]. Furthermore, naphthalene can induce the disruption of red blood cells, when ingested or inhaled in large quantities. PAHs (such as benzo[a]pyrene) can have a carcinogenic nature when activated, producing epoxides and diols that can bind to DNA. Another chronic effect is the capacity to induce, in humans, reproductive and immune damage [45].

No guidelines are available regarding analytical control and method validation procedures for target analytes, other than pesticides, which is a fault in the literature, so the following analysis regulations for the pesticides will be considered.

According to the SANTE/11312/2021 [46] regulation, the methods with the most effective recoveries were the ones presented by Petrovic et al. [47], dos Santos et al. [48], and Surma et al. [49]. Regarding the lowest LOD and LOQ registered for PAHs, 0.07 and 0.23 ng/g were reported by Al-Alam et al. [50], respectively, presenting lower

recoveries than those that are satisfactory. This method [50] also registered the lowest LOD and LOQ for PCBs. Al-Alam et al. [51] also developed a multiresidue detection method for PAHs and PCBs, where low LOD and LOQ were achieved, but recoveries were not within the SANTE/11312/2021 [46] regulation.

When looking to the honey samples findings, PAHs were more common than PCBs. The most commons PAHs detected were naphthalene, fluorene, phenanthrene, anthracene, fluoranthene, pyrene, benz[a]anthracene, and chrysene [47][50][51], with a higher concentration of chrysene (140.6 ng/g) [47]. PCB 28, 77, 81, and 101 were detected by dos Santos et al. [48], with maximum levels of 635, 65, 50, and 194 ng/g, respectively. PFOA residues were also detected at a maximum concentration of 0.223 ng/g by Surma et al. [49]. According to the IARC classification, naphthalene, benz[a]anthracene, and chrysene are possibly carcinogenic to humans (group 2B) [52].

Commission Regulation (EU) No 1259/2011 [53] and No 2020/1255 [54] are the regulations regarding the MRLs of PCBs and PAHs, respectively. Nevertheless, none of these documents were about MRLs allowed on honey. This is a problem that should be taken into consideration since, as seen above, residues of these compounds can be found in this matrix.

2.3. Pharmaceuticals

The negative impact of the presence of pharmaceutical products on the natural environment is well stablished. However, this remains largely unregulated, despite the extremely toxic impact on both animals and humans.

The presence of pharmaceuticals on honey can occur since honeybees can be susceptible to several different microorganisms and parasites if the environmental conditions are not the best, and in order to control these plagues, throughout the years, different veterinary drugs have been developed [55]. Within these molecules, it is possible to find macrolides, nitroimidazoles, lincosamides, quinolones, sulphonamides, tetracyclines, among others [55][56][57].

Residuals of these pharmaceuticals on honeybees' products need to be monitored since their presence can result in malicious effects on consumers and bring negative impacts to the bees themselves. For example, macrolides are able to produce allergic reactions [57] but also can induce gastrointestinal disorders as well as residual lincosamides [56]. Sulphonamide contamination can result in an allergic reaction, bacteria resistance to antimicrobial resistance, and possible carcinogenicity [57]. Nefarious effects associated with tetracyclines include drug resistance, and allergic and toxic reactions if the individual is hypersensitive [55]. Quinolones have been associated with hepatoxicity, while nitroimidazoles with cell mutation and carcinogenic radionuclides [58].

All the studies presented good recoveries, within the 70–120% limits pointed in the SANTE/11312/2021 [46] regulation, except for the method described by Lombardo-Agui et al. [59], which recoveries are between 61.2–99.8%. The lower values described of LOD and LOQ were 0.14 and 0.50 ng/g, respectively, in a multiclass method, for the detection of sulfonamides, macrolides, nitroimidazoles, tetracyclines, etc. [55].

The most common drugs detected among the different articles were enrofloxacin (354.5 [57] and 281.4 ng/g [55]) and ciprofloxacin (18.7 [57], 74.2 [55], and 89.43 ng/g [58]). Gawel et al. [60] developed a multiclass method, with good recoveries and low LOQs, which allowed the detection of different classes of pesticides as well as veterinary drugs and growth regulators. With this method, it was possible to quantify multiple contaminants alongside amitraz, an acaricide, at a concentration of 600 ng/g.

According to the Commission Regulation (EU) No 37/2010 [61], the only pharmacologically active substances allowed on honey samples are amitraz and coumaphos–both acaricides–with MRLs of 200 and 100 ng/g, respectively. Both drugs have been identified [60] in concentrations higher than the MRLs presented. Besides these drugs, others were quantified in the samples analysed that were not allowed by the regulation [61]. Once again, these results represent a pollution concern that may affect human health.

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