Heavy metal concentrations in the general population of Andalusia, South of Spain
A comparison with the population within the area of influence of Aznalcóllar mine spill (SW Spain)

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Abstract

Levels of metalloids (As – urine) and heavy metals (Hg – urine, Cd – whole blood and Zn – serum) were determined by atomic absorption spectrometry in 601 subjects living in the area affected by the Aznalcóllar mine spill (SW, Spain) and compared with those of a representative sample (960 subjects) selected from the Andalusian community (non-affected area), southern Spain. The characteristic parameters of the analytical method including uncertainty were determined for each metal. Potential associations of metal concentration with age, sex and body mass index as well as life-style habits (smoking, alcohol consumption and food habits) were explored. Concentrations of all the metals studied were statistically higher in the population of the affected area with respect to that of the non-affected area in Andalusia, although levels were always lower or similar to the values reported for the general population and below occupational reference limits. In conclusion, there is a lack of evidence that the spill had any incidence on human health in the population living in the affected area. There are few references in scientific literature reporting values from large series of samples, and hence our data could be useful for further studies.

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1. Introduction

On 25 April 1998, part of a massive holding lagoon containing pyrite ore processing waste failed and released an estimated 5–6 million m³ of acidic and metal-rich sludge and water into the Agrio and Guadiamar Rivers, located in the Aznalcóllar region (Andalusia,
south west Spain), affecting 55 km² (Grimalt et al., 1999; Simon et al., 1999; Vidal et al., 1999; Galán et al., 2002; Taggart et al., 2005). The Guadiamar river flows through part of the Doñana National Park, one of the most important wildlife reserves in Europe, that is located north of the Guadalquivir River Estuary, in the province of Huelva. The main pollutants in the soils after spillage were Zn, Pb, As, Cu, Cd and Hg. Most of the Cu, Zn and Cd entered the soil as part of the solution phase (Aguilar et al., 2004). The concentration of watersoluble metals (Zn, Cd and Cu) increased notably and quickly after the spillage. Arsenic was a major component of the polluting sludge (Pain et al., 1998; Alastuey et al., 1999, Querol et al., 1999). The main source of this As may well have been arsenopyrite, as the pyrite ore deposit mined at Aznalcóllar contained 0.9% arsenopyrite (Almodóvar et al., 1998). Once the heavy metals enter the ecosystems, the biological community including the human population can be affected. The study of the content of metals in different human body fluids (usually whole blood, urine or serum) can be a method to evaluate their effect on health (Gil and Pla, 2001). Since the disaster a great number of studies have been performed on the levels of heavy metals in animals (oysters, clams, molluscs, crustaceans, fish, crayfish, birds, etc.), soils and sediments, etc. (Sánchez López et al., 2003; Aguilar et al., 2004; Solà et al., 2004; Riba et al., 2005a,b), although no information about human exposure has been reported. These studies also report that past mining activity contributed to acid and metal contamination of nearby rivers and therefore the mine influence areas appear to be more contaminated than other areas in Andalusia.

Specific metal analyses in body fluids have been assigned to a number of research groups within Spain. Thus, the determination of urinary As and Hg, blood Cd and serum Zn was assigned to the University of Granada (Department of Toxicology and Legal Medicine and Department of Analytical Chemistry).

The aim of the present study was to compare the levels of selected heavy metals (As, Hg, Cd and Zn) in body fluids from the population affected by the mine spill in Aznalcóllar to those from a representative population of Andalusia used as reference (non-affected area). In addition, it should be pointed out that there are few references in scientific literature reporting values from large groups of individuals, and hence the second objective of the study was to obtain reference values for the general population that may be useful for comparisons in future papers or when facing public health problems in which heavy metal contamination play a role.

The influence of other variables namely sex, age and body mass index (BMI) as well as life-style habits including smoking, alcohol consumption and food habits on the metal concentrations was also studied. It is worth emphasizing that this paper is the first of its kind performed in Spain, as regards the Aznalcóllar mine spill.

2. Material and methods

2.1. Apparatus

Total As and Cd were determined by means of a Perkin-Elmer AAAnalyst 800 Atomic Absorption Spectrometry (Perkin Elmer, Norwalk, USA) equipped with Zeeman background correction and an AS-800 autosampler. Arsenic was measured with direct flow injection through hydride generation system (Perkin-Elmer FIAS-100) and cadmium by graphite furnace and graphite tubes with integrated L’vov platform.

Zinc and mercury were determined in a Perkin Elmer LB1100 Atomic Absorption Spectrometry (Perkin Elmer, Norwalk, USA) equipped with Power Supply Lamp System and MHS-10 Mercury Hydride System.

Urine creatinine was determined by the method of Jaffé using a Hitachi 917 autoanalyzer.

2.2. Reagents

Atomic absorption spectrometry standard solutions for As, Hg, Cd and Zn (Titrisol grades from Merck) were used to build up the calibration curve. They were prepared from a stock solution of 1000 mg/L for each metal by successive dilutions with water. All aqueous solutions of reagents and standards were prepared using reverse-osmosis type quality water produced by a Milli-RO 12 plus Milli-Q purification system (Millipore, Bedford, MA).

The chemicals used were all of analytical reagent grade. High-quality concentrated (65% w/v) nitric acid (Merck, Darmstadt, Germany), (96% w/v) sulphuric acid (Merck) and (37% w/v) hydrochloric acid (Merck), sodium borohydride (Merck), sodium hydroxide (Panreac, Barcelona, Spain), ascorbic acid (Panreac), potassium iodide (Panreac), magnesium nitrate (Merck), palladium nitrate (Merck), Triton X-100 (Merck), potassium permanganate (Merck) and silicone antifoaming agent (Merck) were used.

Volumetric polyethylene material (including autosampler cups) and micropipettes with plastic tips were used. The glass material was cleaned by soaking in 20% v/v HNO₃ for 24 h. It was finally rinsed with Milli-Q® water and dried in a polypropylene container.
2.3. Validation of analytical methods

The characteristic parameters of the analytical methods were determined for each metal (As, Hg, Cd and Zn) by means of analysis of blanks and standard solutions at different concentrations. These parameters included the limit of detection (LOD) and quantification (LOQ) as determined following the recommendations of the IUPAC (Sánchez López et al., 2003), linear range, precision (minimal, intermediate and reproducibility), accuracy, recovery and characteristic mass. Uncertainty of methods was calculated for each metal. The overall uncertainty must be estimated considering every source of uncertainty and treating it separately to obtain the contribution from that source. Each of the separate contributions to uncertainty may be referred to as an uncertainty component. It is often possible to evaluate the combined effect of several components as a global standard uncertainty (Eurachem/ Citac Guide, 2000). Once the parameters and their associated uncertainties that contribute to the uncertainty for the method as a whole are listed, the individual uncertainties are combined in the uncertainty budget in which we include the relative reference standard uncertainty and the relative balance calibration standard uncertainty (gravimetric steps) and the volumetric sample relative uncertainty (volumetric steps). Statistical evaluation of the relative uncertainty associated with recovery involves the relative uncertainty associated with the calibration curve (including addition method in the particular case of As), the relative uncertainty associated with the reproducibility of the method and statistical evaluation of the relative uncertainty associated with the intermediate precision of the method (quantification step).

2.4. Population study and biological samples

A total number of 1561 individuals were included in the present study. 601 of them were from the area affected by the toxic spill (Aznalclázar, Aznalcóllar, Benacazón, Carrión de los Céspedes, Huévar, Pilas, Sanlúcar la Mayor, Villamarrine de la Condesa and Hinojos) and 960 from representative Andalusian areas not affected by the spillage. A minimum sample size of 500 per group was calculated in order to achieve enough statistical power to detect differences below 10% with an α error of 0.05 and a β error of 0.1. A random double sampling was carried out. In the first stage 50 Andalusian towns were selected and in the second one a representative sample of individuals was randomly selected from the 1996 census (according to gender and three age categories: 12–24, 25–59, and 60–75 year olds).

Different types of biological samples were analyzed in this study, urine, whole blood and serum where As and Hg, Cd and Zn were measured, respectively.

A previously validated food frequency questionnaire (Gómez Aracena, 1990) was given to all participants in order to provide the following information: sex, age, BMI and life-style habits (smoking, alcohol consumption and food habits). The two population samples (affected and non-affected) can be taken as comparable in health, life-style and living conditions. None of the subjects reported occupational exposure to any of the metal elements determined in this study.

2.5. Analytical procedures

Metal concentrations in urine were adjusted for creatinine levels.

2.5.1. Arsenic

A direct flow-injection atomic absorption spectrometric technique (FI-HGAAAS) was used to measure urinary levels of total arsenic. The arsenic contained in standard solutions (calibration curve 0, 0.5, 1.5 and 2.5 μg/L) or urine samples was reduced to As⁺³⁺ prior to analysis with a mixture of potassium iodide and ascorbic acid. To 1 mL of sample or reference solution 1 mL of concentrated HCl and 1 mL of 5% (w/v) KI – ascorbic acid was added. After 45 min at room temperature the mixture was diluted to 10 mL with water. The reducing agent was an aqueous solution of 0.2% (w/v) NaBH₄ in a 0.05% (w/v) NaOH solution freshly prepared and filtered. Standard addition was required. The parameters for As were wavelength 193.7 nm, integration time 15 s, smoothing 19 points or 0.5 s and temperature cell of 900 °C. An electrodeless discharge lamp was used.

2.5.2. Mercury

Mercury was determined using an aqueous solution of 3% (w/v) NaBH₄ in a 1% (w/v) NaOH solution freshly prepared and filtered as reducing agent. One to two drops of silicone antifoaming was dispensed into a reaction flask before introducing any solution. All solutions were stabilized in the reaction flask by adding 250 μL of 5% KMnO₄ solution before starting the determination. The mercury standard calibration plot (0, 20, 50, 100, 200 μg/L) was prepared in 10 mL of acid mixture containing 1.5% HNO₃ and 1.5% H₂SO₄, 9 mL of acid mixture was added to 1 mL of urine sample. The parameters for Hg were wavelength 253.6 nm with time delay of 12 s. No flame was required and an electrodeless discharge lamp was used.
2.5.3. Cadmium

A calibration curve with different cadmium concentrations (0, 1, 3, 5 μg/L) was prepared in 0.2% HNO₃. Aliquots of 20 μL of whole blood were introduced directly into the graphite furnace with an equal volume of matrix modifier (a mixture of 3.3% Pd and 0.03% Mg as nitrates in 0.2% HNO₃). The temperature program was previously optimized. Two pyrolysis steps were required at 600 and 700 °C. Atomization temperature was set at 1600 °C with 0 mL/min argon flow rate. An electrodeless discharge lamp was used and the wavelength set at 228.8 nm.

2.5.4. Zinc

Calibration standards (0, 50, 100, 200, 400 mg/L) were prepared in 5% glycerol solution to match serum viscosity. Serum samples were diluted 1:5 by using the same solution. Operational conditions were a wavelength of 213.9 nm with a time delay of 1 s. A hollow cathode lamp was used.

2.6. Reference materials

Reference samples for whole blood (three levels, ref. 201505, 201605 and 201705), urine (ref. 201205) and serum (ref. 201405) were supplied by Seronorm (Billingstad, Norway). As they were supplied freeze-dried, they were reconstituted by adding 5 mL of water.

2.7. Statistical analysis

Data were analysed by using the software package SPSS 11.0 (SPSS, Chicago, USA). Measurements below the detection limit were replaced by LOD/2. Metal concentrations in urine were adjusted for creatinine concentration and regressions were performed on creatinine-corrected data. Log-transformed metal concentrations were used to normalize their distributions.

The statistical power to detect differences in the heavy metal levels of the affected population as compared to the non-affected population was 79.6% for arsenic, 56.6% for mercury, 89.3% for cadmium and 89.6% for zinc.

The potential influence of classical confounders (tobacco, alcohol and BMI) on heavy metal levels was assessed by Student’s t test in non-affected population which was considered as control. The association between levels of heavy metals and BMI was assessed by Spearman’s rank correlation test.

Differences in the mean heavy metal levels between the affected and non-affected populations were assessed by the analysis of covariance (ANCOVA) after adjusting for age, gender and any other confounder which previously showed any association in the bivariate analysis. Since the effect of each covariate may not be the same for the two populations studied, the interaction effect between each covariate and the population was checked.

3. Results and discussion

The characteristic parameters of the analytical method for each metal are presented in Table 1. In all cases the correlation coefficients were higher than 0.99 and the results showed that the instrumental response can be considered linear in the range studied. The standard addition method was applied to calculate the recovery of each metal.

Table 2 shows the influence of smoking, alcohol consumption and sex on geometric mean levels of the

<table>
<thead>
<tr>
<th>LOD/LOQ (µg/L)</th>
<th>As</th>
<th>Hg</th>
<th>Cd</th>
<th>Zn</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.03/0.10</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Linear range (µg/L)</td>
<td>4</td>
<td>20</td>
<td>7</td>
<td>400</td>
</tr>
<tr>
<td>Linear equation</td>
<td>y = 0.0800x + 0.0021</td>
<td>y = 1.5941x + 0.0083</td>
<td>y = 0.0430x + 0.0035</td>
<td>y = 0.0002x + 0.0029</td>
</tr>
<tr>
<td>r</td>
<td>0.9998</td>
<td>0.9992</td>
<td>0.9996</td>
<td>0.9982</td>
</tr>
<tr>
<td>RSD (%) (n = 10)</td>
<td>0.0008</td>
<td>0.0802</td>
<td>0.0004</td>
<td>0.0893</td>
</tr>
<tr>
<td>Precision (%)</td>
<td>Minimal</td>
<td>3.5</td>
<td>1.98</td>
<td>2.62</td>
</tr>
<tr>
<td>Accuracy (%)</td>
<td>1.33</td>
<td>5.80</td>
<td>3.34</td>
<td>3.06</td>
</tr>
<tr>
<td>Characteristic mass (pg)</td>
<td>25.37</td>
<td>12.00</td>
<td>2.96</td>
<td>7271.20</td>
</tr>
<tr>
<td>Uncertainty (%)</td>
<td>5.10</td>
<td>11.49</td>
<td>2.71</td>
<td>12.28</td>
</tr>
</tbody>
</table>

LOD: limit of detection; LOQ: limit of quantification; RSD: standard relative deviation.

* n = 10.

* The analyses were carried out in 1-week periods for 5 weeks.
metals studied (As, Hg, Cd and Zn) in the non-affected population as well as the correlation between age and BMI with those metal levels. Blood Cd levels were significantly higher in current smokers (6-fold with regard to non-smokers) and alcohol consumers (3-fold). Our data are in accordance with previous studies that reported an increased Cd level in whole blood from smokers with respect to non-smokers (Graasmick et al., 1985; Schumacher et al., 1993; Benedetti et al., 1994; Chia et al., 1994; Dell’Omo et al., 1999; Moreno et al., 1999). Urinary Hg levels showed a significant correlation to age, so that older people presented increased Hg levels. Likewise, those that had greater BMI showed higher urinary Hg levels. These two findings support the accumulative potential of this metal. Moreno et al. (1999) found that age strongly influenced serum Zn levels but made only a small contribution to blood Cd concentrations. Regarding sex differences, Zn concentration was significantly higher in Andalusian males than females and the reverse was true for Hg levels.

Table 3 shows geometric mean levels of As, Hg, Cd and Zn adjusted for age, gender, BMI, tobacco and alcohol consumption.
alcohol in our target populations (affected and non-affected by the mine spill). The affected population showed significantly higher levels of all the metals studied with respect to the non-affected population. Nevertheless, levels fall within their respective reference ranges. Reference values from healthy population as well as exposed and non-exposed workers are also shown in Table 3. For comparison purposes, values from general population are more appropriate, nevertheless we have also included occupational reference values because of the scarcity of existing data for the general population. The latter are not often comparable owing to a lack of homogeneity, as there are differences in units, body fluids, sample size, life-style influences (dietary intake, smoking habits) and even age.

For every metal, the interaction effect between population from the affected or non-affected area and all the covariates listed in Table 2 was checked. However, no significant association was found for any of the interaction terms assessed.

The mean urinary Hg levels were also significantly higher in the affected population, although they are similar to values reported for the general population (Ewers et al., 1999) and 4-fold lower when compared to occupational reference values (<5 μg/g creatinine) (Lauwerys and Hoet, 1993). The population of the affected area presented higher blood Cd levels. Nevertheless, the mean levels were below the reported reference values (Baselt, 1980; Minoïa et al., 1990; Staessen et al., 1990; Ikeda et al., 1997; Ewers et al., 1999). The higher levels of mercury and cadmium in the population affected by the mine spill might be due to the cumulative potential of these metals. Both of them were two of the main soil pollutants after the spillage, so that their long biological half-life and risk of accumulation in the body could account for the differences found in the study. Anyway these differences should not raise any concern for public health as their Hg and Cd levels fall within reference values for the general population.

Serum Zn levels were significantly higher in the affected population but similar to those reported for the general population (Baselt, 1980; De Mateo Silleras et al., 2000; Barany et al., 2002) and 50% lower than the occupational reference level (Lauwerys and Hoet, 1993). These differences cannot be attributed to dietary patterns since Zn levels failed to be associated with any of the dietary items listed in Table 5. An alternative explanation can be found in background contamination, since Zn levels from the general population of Seville province were about 20% higher than those found in the affected population (see later) which also belongs to the province of Seville (the province where the spillage took place).

Urine concentrations of As were statistically higher in the affected area with respect to the non-affected area. Nevertheless, levels were lower than reported values for general population (Baselt, 1980; Concha Quezada, 2001) and 20-fold lower than occupational reference levels (Lauwerys and Hoet, 1993). We do not have a satisfactory explanation for these differences. Although As is strongly related to dietary items (Table 5), no differences exist in the dietary pattern between the affected population and the remaining non-affected population from Seville. Environmental contamination by As can also be discarded since arsenic levels from the non-affected population of the province of Seville are roughly half those found in the affected population. We could also speculate with industrial pollution, but the main province with pyrite mines is Huelva and arsenic levels of their population were also lower than those from the affected area. The only explanation that might support our findings are differences in the arsenic level in drinking water, although there is no evidence to rule out or confirm this possibility. No known water

### Table 4

Geometric mean levels – adjusted for the potential confounders (see footnote) – of urinary As and Hg (μg/g creatinine), blood Cd (μg/L) and serum Zn (μg/L) in Andalusian Population (excluding the affected area)

<table>
<thead>
<tr>
<th>Province</th>
<th>As (μg/g creatinine)</th>
<th>Hg (μg/L)</th>
<th>Cd (μg/L)</th>
<th>Zn (μg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Average (95% CI)</td>
<td>n</td>
<td>Average (95% CI)</td>
<td>n</td>
</tr>
<tr>
<td>Almeria</td>
<td>1.34 (0.84–2.13)</td>
<td>40</td>
<td>2.81 (1.96–4.01)</td>
<td>38</td>
</tr>
<tr>
<td>Cádiz</td>
<td>1.52 (1.12–2.05)</td>
<td>96</td>
<td>0.73 (0.58–0.91)</td>
<td>95</td>
</tr>
<tr>
<td>Córdoba</td>
<td>3.20 (1.94–5.28)</td>
<td>35</td>
<td>0.84 (0.60–1.22)</td>
<td>35</td>
</tr>
<tr>
<td>Granada</td>
<td>2.10 (1.47–3.01)</td>
<td>69</td>
<td>1.23 (0.94–1.62)</td>
<td>65</td>
</tr>
<tr>
<td>Huelva</td>
<td>0.96 (0.65–1.41)</td>
<td>58</td>
<td>0.62 (0.46–0.82)</td>
<td>56</td>
</tr>
<tr>
<td>Jaén</td>
<td>0.41 (0.28–0.60)</td>
<td>57</td>
<td>1.38 (1.03–1.85)</td>
<td>57</td>
</tr>
<tr>
<td>Málaga</td>
<td>1.57 (1.22–2.02)</td>
<td>140</td>
<td>1.08 (0.89–1.30)</td>
<td>136</td>
</tr>
<tr>
<td>Sevilla</td>
<td>0.81 (0.55–1.20)</td>
<td>57</td>
<td>2.86 (2.13–3.83)</td>
<td>56</td>
</tr>
</tbody>
</table>

Adjusted for: (a) age, (b) sex, (c) body mass index, (d) tobacco, and (e) alcohol.
supply with typically higher levels of arsenic exists in Andalusia.

Previous studies have reported high metal concentrations in the Guadiamar River in the late 1970s, well before the accident, due very likely to past mining activities when this river became one of the world’s most metal-polluted (Solà et al., 2004). Riba et al. (2005a) have reported that the upper river Guadiamar still has some traces of toxic mud from the mining spill.

Since 1999 the metal contamination caused by the spill is undergoing both fluvial and aerial redistribution and a continued input from the heavily contaminated Guadiamar Valley/River may still be occurring and may continue to do so for many years (Taggart et al., 2005, 2006). As a result, potential transfer of pollutants from metal-accumulating macrophytes to herbivores may occur although it still remains to be studied (Taggart et al., 2005). This may be a significant food chain transfer pathway which could possibly reach the human population close to the affected area. Aerial redistribution may also have contributed to this environmental contamination. Both routes of exposure might explain the higher metal levels in the population affected by the spill.

Table 4 shows mean levels of As, Hg, Cd and Zn in the eight Andalusian provinces. Differences in dietary habits among these provinces (especially as regards to the consumption of fish and seafood, which present higher As content) may have contributed to the differences observed and could account for the variability of As levels among the provinces. Interestingly, the average As levels in the affected area (Table 3) were double those found in the remaining province of Seville (Table 4), which seems to pinpoint a background contamination in the former area, although these levels were of no public health concern as they were lower than either general population or occupational reference values (see Table 3). Urinary Hg also showed a high variability, because their levels were significantly higher in the provinces of Seville and Almeria. Hg concentration from the affected area was roughly half that from the remaining province of Seville. Regarding serum Zn, lower levels were also observed in the affected population when compared to the rest of the Seville population.

Table 5 summarizes the correlation between metal levels (As, Hg, Cd and Zn) and food intake evaluated by a food questionnaire. Data correspond to the population from the non-affected area. The ingestion of sardines and mussels exerted a strong influence on urine As levels. However the intake of meat (pork) was inversely related to As concentration in urine. A weaker association was also observed with the intake of eggs, chicken, hake and anchovies. These findings suggest that the intake of animal products (chicken, pork, and eggs) is usually associated with lower levels of arsenic in urine which may be related to less fish consumption. A significant association was found between whole blood Cd levels and fish, seafood and mixed salad intake, which was extremely notable in the case of pink ling – Genypterus blacodes ($r=0.206; P<0.001$). In turn, an inverse correlation between urinary Hg and eggs and beef intake was observed. Finally, serum Zn levels failed to be associated with food intake.

Juresa and Blanusa (2003) found the highest concentrations of Hg and As in hake (Merluccius merluccius; 0.37 and 23.30 mg/kg fresh weight, respectively) and cadmium levels were about 10 fold higher in shellfish (Mytilus galloprovincialis; 0.14 mg/kg fresh weight) than in fish from the Adriatic Sea. However, the estimated dose of those trace elements from sea food consumption by the general population did not exceed the provisional weekly intake recommended by the Joint FAO/WHO Expert Committee on Food Additives. Llobet et al. (2003) found that the consumption of fish (hake and sardine) and shellfish (mussel) was the main food item responsible for As, Hg and Cd intakes.

In conclusion, the metal concentrations of the populations studied from both affected and non-affected areas by the mine spill were in all cases below reference values. Therefore, there is no evidence that the spill had any impact on the population from the affected area and that remediation measures carried out after the Aznalcóllar pyrite mine spill could have contributed
to the reduction of the environmental contamination capable of affecting the human population.

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