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Biochemical *bio*markers of pollution in Algerian mouse (*Mus spretus*) to assess the effects of the Aznalcóllar disaster on Doñana Park (Spain)

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On April 25, 1998, a tailings dam of the Aznalcóllar pyrite mine partially collapsed and released to the Guadiamar river acidic water (pH < 3) and mud containing toxic metals (Fe, Zn, Pb, As, Cu, Sb, Co, Tl, Bi, Cd, Ag, Hg, Sr), threatening the Do ana National Park, a Spanish wildlife reserve. To assess possible biological effects in terrestrial ecosystems, biochemical biomarkers have been assayed for the first time in Algerian mice (*Mus spretus*), a non-protected and free-living species, from several areas of Do ana and Guadiamar. Biomarkers assayed responded to different types of contaminants: I—metals and oxidant compounds (Se-glutathione peroxidase (SeGSHPx) and antioxidant activities, malondialdehyde (MDA), and glutathione redox status); II—Aromatic chemicals (ethoxyresorufin-O-deethylase (EROD) activity); III—Compounds of both types (glutathione-*S*-transferase (GST) activities). Before the Aznalcóllar spill (October 1997), mice from the 'Brazo de la Torre' had SeGSHPx and EROD activities close to animals from the Huelva Industrial Park, suggesting similar levels of oxidant and aromatic contaminants at both sites. Six months after the spill (October 1998), mice from the lower Guadiamar areas ('Cangrejo Grande' and 'Brazo de la Torre') also showed significant increase of soluble and microsomal GST activities, and altered levels of several antioxidant enzymes. Thus, the spilled chemicals could have induced further biological effects in mice from the exposed areas. Although no significant responses to contamination were found after the spill at Do ana core, further investigations should be carried out to monitor the situation.

Keywords: EROD activity, Se-dependent glutathione peroxidase, glutathione-*S*-transferases, glutathione content and redox status, bioindicators.

Abbreviations: AZN, Aznalcázar; BDT, Brazo de la Torre; CG, Cangrejo Grande; CYP1A, cytochrome P4501A; DBR, Do ana Biological Reserve; EROD, ethoxyresorufin-O-deethylase; G6PDH, glucose-6P dehydrogenase; GSH/GSSG, glutathione in reduced or oxidized form; GSSGrase, glutathione reductase; GSTm/GSTs, microsomal or soluble glutathione-*S*-transferase; HUV, Huelva Industrial Park; IPB, Iberian Pyrite Belt; KAT catalase; NOCs, nitrogen organic compounds; PAHs, polycyclic aromatic hydrocarbons; PCBs, polychlorinated biphenyls; ROS, reactive oxygen species; SeGSHPx, selenium-dependent glutathione-peroxidase; SOD, superoxide dismutase; SOL, Santa Olalla pond.

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Introduction

Do ana National Park is a wildlife reserve (50 720 ha) located at the Huelva province north of Gualdalquivir river estuary (SW Spain), declared a Reserve of the Biosphere by UNESCO due to its variety of ecosystems—marshes, ponds, moving dunes and forest over stabilized dunes—and of plant and animal life. The Park is also a wildlife sanctuary for sedentary and migratory birds, which nest or live there temporarily, particularly at its core, Do ana Biological Reserve (Grimalt *et al*. 1999), with up to six million visiting birds each year. Water comes from rain and streams from the north (Rocina and Partido) or the east, where the Guadiamar river, the last Guadalquivir tributary, supply half of its volume of water (figure 1). To prevent flooding, the low Guadiamar course (so-called 'Brazo de la Torre') was abandoned in the 1970s, and a straight low flow channel was built which runs between two walls (so-called 'Entremuros', 1×17 km) and finally conveys its water to the Guadalquivir river through the 'Brazo de la Torre' (Grimalt *et al*. 1999), 'Entremuros' and 'Brazo de la Torre' are under the tidal influence of the Guadalquivir estuary. A marshland lies between the Guadiamar and Guadalquivir rivers (NE of Do ana), yielding most of the rice grown in Spain, helped by an extensive use of agrochemicals.

The Aznalcóllar mine (130×10^6 metric tons reserves) is at the Iberian Pyrite Belt (IPB), the largest sulphide reserve of western Europe (López-Pamo *et al*. 1999). Ore is milled, washed, treated with reagents, and the metal sulphides separated by flotation. During exploitation, 113×10^6 metric tons of sulphides have been treated, generating 70×10^6 metric tons of acidic wastes, water-rich muds and tailings, which are stocked in a dam, 25 m high and 360 ha area (López-Pamo *et al*. 1999); metals occasionally leak to the Guadiamar river, a process that increases after floods (Cabrera *et al*. 1987). On April 25, 1998, a 50 m wide breach opened at the dam releasing to Guadiamar 4 hm^3 of acidic water ($pH < 3$) and a 2 hm^3 of mud containing 35% Fe, 0.8% Zn, 0.8%Pb, 0.5% As, 0.2% Cu, 0.05% Sb, 0.006% Co, 0.005% Tl, 0.005% Bi, 0.0025% Cd, 0.0025% Ag, 0.0015% Hg and, 0.001% Se (Grimalt
et al. 1999). Mud spread 400 m on both sides of Guadiamar river and 40 km downstream (4500 ha) and water continued 20 km further, being retained by a wall urgently built at 'Entremuros' after the so-called 'Cangrejo Grande'. Oxidation of sulphide to sulphates also lowered the pH and leached and mobilized metals (Grimalt
et al. 1999). 'Entremuros' water was very acidic ($pH = 6.17$), containing 190 ppm Zn, 1.2 ppm Co and 0.36 ppm Mn close to the retaining wall; the 'Brazo de la Torre' was also contaminated, showing that the wall was not fully effective (Garralón *et al*. 1999). In May mud removal was started which was nearly completed by October 1998, before the rainy season; 'Entremuros' water was treated in a plant built on site and poured into 'Brazo de la Torre' before autumn (Grimalt *et al*. 1999).

Sentinel organisms, or *bioindicators*, are used to assess the presence and biological effects of environmental pollutants (Peakall 1994, Timbrell *et al*. 1994). Molecular or cellular *biomarkers* measured in bioindicators respond rapidly to stress and are used as early-warning signals before irreversible damage to the ecosystems occurs (Peakall 1994, Timbrell *et al*. 1994, López-Barea 1995). Cytochromes P4501A (CYP1A) transform aromatic compounds, being usually assayed by the ethoxyresorufin-O-deethylase (EROD) activity (González and

Gelboin 1994). Glutathione-*S*-transferases (GST), with soluble and microsomal forms, conjugate GSH with electrophilic compounds (Pickett and Lu 1989). Certain pollutants, convert O_2 into reactive oxygen species (ROS), which are highly toxic and mutagenic. Antioxidant enzymes, such as superoxide dismutase, catalase, glutathione-peroxidase and reductase, or glucose-6P dehydrogenase, protect living beings from ROS (Sies 1986). Se-dependent GSH-peroxidases (SeGSHPx) act on H_2O_2 and organic hydroperoxides, while Se-independent forms act only on organic hydroperoxides (Flohé 1989). Low molecular weight antioxidants, such as GSH, cooperate with enzymatic defences (Sies 1986). CYP1A expression is induced by aromatic compounds binding to the Ah receptor, which attaches to xenobiotic responsive elements, present at promoters of their genes (González and Gelboin 1994). Some GST forms are induced by aromatic and oxidative contaminants (Rushmore *et al.* 1994), while metals and oxidative compounds alter the levels of antioxidant enzymes, including SeGSHPx (Mather-Mihaich and DiGiulio 1991). Thus, CYP1A, GST, and antioxidant enzymes signal the biological effects of these types of contaminants (Mather-Mihaich and DiGiulio 1991, Rodríguez-Ariza *et al.* 1993, López-Barea 1995). Finally, the glutathione content and redox status, and the malondialdehyde content respond to oxidative contaminants, either in natural ecosystems or in controlled exposure experiments (Rodríguez-Ariza *et al.* 1993, 1994).

In addition to ponds and marshes, terrestrial ecosystems are very important in the Do ana National Park. Although contamination would enter Do ana via water, biological effects in terrestrial and aerial animals should be studied to assess possible contamination at Do ana core. After the Aznalcóllar spill, bivalves and crustaceans of the Guadalquivir estuary had Zn, Cu and Cd levels beyond the legal maximum (Blasco *et al.* 1999), in agreement with the high concentrations of such metals found at the estuary and Cádiz Gulf (Achterberg *et al.* 1999). Bird-specialists showed that animals feeding in areas affected by the spill have higher blood Zn, Cu, Pb and Cd levels than controls (Benito *et al.* 1999), and the 'comet' assay shows significant DNA damage to lymphocytes of storks born in affected Do ana areas (Pastor *et al.* submitted). To assess possible ecotoxicological effects of the Aznalcóllar spill in mammalian species, the Algerian mouse (*Mus spretus,* F. Muridae), a common and non-protected rodent that typically inhabits marshlands, follows an *r*-type reproductive strategy and attains high population densities (Camacho and Moreno 1989), has been selected as pollution bioindicator. This small animal feeds on plants, seeds and insects (Moreno 1998) and is a key species of the Do ana food web, since it is highly selected by carnivorous predators, including birds of prey (Kufner 1986), that could transfer spill-related contaminants to endangered superpredator species, such as the lynx (*Lynx pardina*). As other micromammals, this rodent responds to metals, radionuclides and plaguicides (McBee and Bickham 1988, Cristaldi *et al.* 1990, 1991, Tull-Singleton *et al.* 1994, Ieradi *et al.* 1991). In fact, before the Aznalcóllar accident, micronuclei frequencies were higher in mice from the Huelva Industrial Park, chronically polluted, than in animals from the Do ana Biological Reserve (Ieradi *et al.* 1998, Degrassi *et al.* 1999).

Since no biochemical pollution biomarkers have been studied before in *Mus spretus,* a wide number of parameters putatively responsive to different types of contaminants have been assessed for the first time in Algerian mouse liver, an organ very active toxicologically that cannot be studied in any protected species. To this end, mice sampled at different areas of the Do ana National Park and the

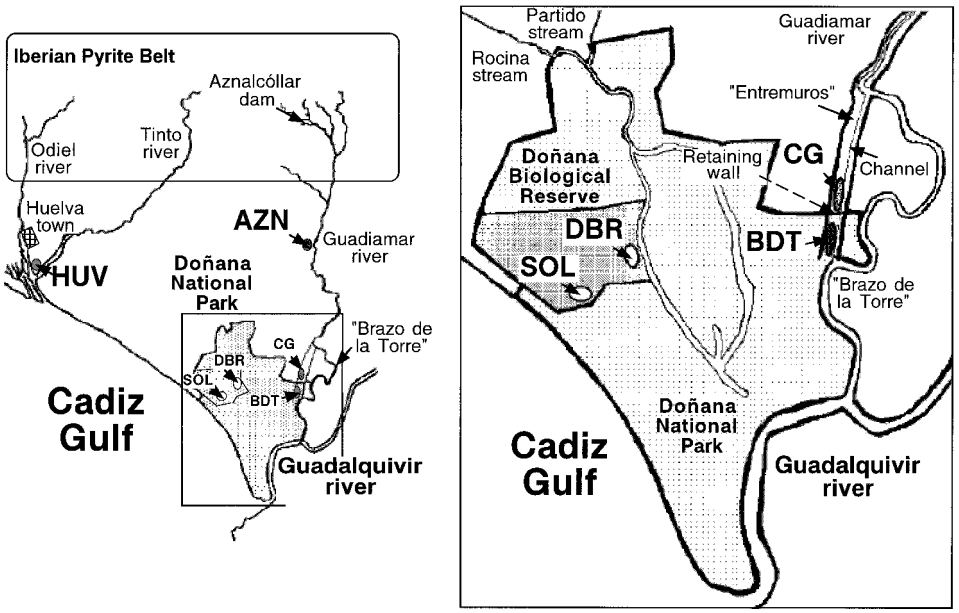

Figure 1. Areas studied. Figure on the left shows the Guadalquivir Estuary, the Tinto and Odiel Estuaries, the Guadiamar river, Do ana National Park and the tailings dam of Aznalcóllar pyrite mine; the area within the rectangle is shown enlarged at the right. Areas sampled are as follow: 1997 investigation, Do ana Palace (DBR), Santa Olalla pond (SOL), 'Brazo de la Torre' (BDT) and Huelva Industrial Park (HUV); 1998 investigation, Aznalcázar (AZN), 'Cangrejo Grande' (CG), 'Brazo de la Torre' (BDT) and Do ana Biological Reserve (DBR).

Guadiamar river 6 months before and 6 months after the Aznalcóllar disaster, were studied to assess previous pollution levels and to monitor possible biological effects of the toxic spill.

Materials and methods

Organisms analysed and sampling areas

Algerian mice (*Mus spretus*) were captured with live traps, and taken alive to the Do ana Biological Reserve, where they were sacrificed, weighed, and dissected. The livers were weighed and frozen in liquid nitrogen. Individual livers were taken frozen to Córdoba, ground in a mortar with liquid nitrogen and stored at −80 °C until analysed.

In October 1997, 106 mice were collected at four areas (figure 1). Next to Do ana Palace at 'la Vera' (DRB), 22 mice were captured and 24 animals were captured at 'Santa Olalla', pond (SOL), both areas are within the Do ana Biological Reserve and considered to be non-polluted. Another 40 mice were from the Right Bank of 'Brazo de la Torre' (BDT), just before the Guadiamar river joins the Guadalquivir river, an area occasionally receiving metals (Fe, Zn and Mn) from the IPB, especially after floods (Cabrera *et al*. 1987, Arambarri *et al*. 1996). As positive control, 20 animals were sampled from an Industrial Park close to Huelva (HUV), 40 km north-west of Do ana and chronically polluted by its chemical industries and the metals leached and carried by the Odiel and Tinto rivers from the IPB (Esturión 1995).

In October 1998, 84 mice were sampled at four areas, two areas being the same as in 1997. Nineteen mice were from the upper Guadiamar course at Aznalcázar (AZN), 25 km south of the broken tailings dam. Another 23 were from 'Cangrejo Grande' (CG), 35 km further south located inside 'Entremuros' just before where the wall was built to retain polluted water (figure 1). Another 23 animals were from the same BDT area sampled in 1997, 4 km further south than CG and immediately after the retaining wall. As negative controls, 19 mice were sampled near the Palace at the Do ana Biological Reserve (DBR).

Since pollution biomarkers other than micronuclei had never been used before in *Mus spretus*, 12

biochemical parameters putatively responsive to several contaminant types were selected. Individual assay of every biomarker in each sample was ruled out, due to the small liver size and the number of parameters to be assayed in five different fractions using two separate extraction protocols. Thus, the livers of six to eight mice from each area studied were randomly grouped, and two separate pooled samples were prepared from each group by mixing 60 mg of every ground liver (a total of 120 mg per mouse). The first pooled sample was used to assay nine enzymatic activities and the MDA content, and the second for HPLC analysis of GSH and GSSG. Animals of 1997 were grouped as follows: DBR, three groups from a total of 22 mice; SOL, three groups from 24 animals; BDT, five groups from 40 mice, HUV, three groups from 20 animals. Animals of 1998 were grouped as follows: DBR, three groups from total of 19 mice; AZN, three groups from 19 mice; CG, three groups from 23 mice; BDT, three groups from 23 mice.

Biochemical biomarkers

The first pooled sample from each geographic group was disrupted in an Ultra-Turrax homogenizer (T25, Janke & Kunkel, Staufen, Germany) with 4 ml of 50 mM Tris/HCl buffer, pH 7.5, containing 0.1 mM EDTA and 0.1 mM PMSF per gram of pooled liver. Malondialdehyde (MDA), a lipid peroxidation product, was determined fluorimetrically as described by Lepage *et al.* (1991) in a separated part of each homogenate; the remainder of it was centrifuged for 15 min at 25 500 × *g* (Beckman J2-21, San Ramón, CA, USA) and the supernatant divided in three fractions. In the first, the activities of five cytosolic enzymes, glucose-6P dehydrogenase (G6PDH), glutathione reductase (GSSGRase), catalase (KAT), Se-dependent glutathione peroxidase (SeGSHPx) and glutathione-*S*-transferase (GSTc), were assayed as described previously (Rodríguez-Ariza *et al.* 1992, 1993). The second fraction was spun for 10 min at 14 000 *g* in Microcon-10 units (Amicon Inc., Beverly, MA, USA) and, after dialysis, used to assay superoxide dismutase (SOD) activity and the possible appearance of more negative Cu,Zn–SOD forms by *in situ* activity staining after separation by fast isoelectrofocusing, pH 3–9 (Pedrajas *et al.* 1993). With the third fraction, microsomes were prepared to assay ethoxyresorufin-O-deethylase (EROD) activity linked to cytochrome P4501A1 and microsomal GSH-transferase (GSTm) activity as previously described (Rodríguez-Ariza *et al.* 1993).

The second pooled sample from each geographic group was extracted with 5 ml of 1 M $HClO_3$ containing 2 mM EDTA per gram of pooled liver and centrifuged for 15 min at 25 500 × *g*. Reduced and oxidized glutathione levels were analysed in the supernatant by HPLC with electrochemical detection, and the ratio of oxidized to total glutathione was calculated, both as GSH equivalents (Rodríguez-Ariza *et al.* 1994). A Supelcosil LC-18 reversed-phase column (Supelco, Bellefonte, PA, USA) and isocratic elution with 0.15 M sodium phosphate buffer, pH 2, and 1% methanol (GSH) or 2% (GSSG) was used at 1 ml min^{-1}. Potential settings used for Coulochem II detector (ESA, Bedford, MA, USA) were: GSH, guard cell 0.9 V, detector 0.8 V; GSSG, guard cell 1.1 V, detector 1.025 V.

All steps were carried out at 4 °C. Each biochemical analysis was performed in triplicate and average values were calculated; results are expressed as mean ± SD of the values of the number of pooled samples prepared from each area. Protein was determined by the Bradford (1976) procedure, and the results are expressed as specific activities, U or mU per mg of protein as indicated. All reagents used were of the highest purity available.

Results and discussion

Comparison of Doñana and Huelva areas in 1997

Several biochemical pollution biomarkers were assayed in the different pooled samples prepared from livers of mice collected in 1997 at three areas from the Do ana National Park (DBR, SOL, BDT), plus another located at the Huelva Industrial Park (HUV) (figure 1).

Table 1 shows the results obtained with seven biomarkers, G6PDH, GSSGRase, KAT, GSTc, GSTm, SOD and MDA. The lowest enzymatic activities were usually found at the DBR and SOL areas, both within the Do ana Biological Reserve and considered free of contaminants; no significant differences were found between DBR and SOL values. In contrast, 'Brazo de la Torre' mice showed significantly higher GSSGRase, KAT and GSTm activities than animals of the DBR area. Curiously, most biomarkers were lower in HUV mice, with significantly lower KAT, GSTc and GSTm activities but higher SOD activity, than BDT animals. Nevertheless, most values in HUV mice were not statistically

Table 1. Biomarkers analysed in 1997 mice.

Biomarker assayed	Areas sampled			
	DBR (*3*)	SOL (*3*)	BDT (*5*)	HUV (*3*)
G6PDH (mU mg^{-1})	5.6±0.7	6.5±0.8	6.5±1.7	4.3±0.8[b]
GSSGRase (mU mg^{-1})	38.8±3.5	44.8±4.5	52.2±7.8[a]	46.4±3.1[a]
KAT (U mg^{-1})	285±73	295±73	417±74[a]	197±40[c]
GSTc (U mg^{-1})	1.6±0.1	1.9±0.4	2.1±0.4	1.4±0.2[c]
GSTm (mU mg^{-}1)	363±34	466±154	524±89[a]	329±55[c]
SOD (U mg^{-1})	34.9±13.2	30.5±4.7	26.0±4.7	37.9±2.9[c]
MDA (arbitrary fluorescence units)	19.0±5.1	7.4±0.6	16.8±2.8[b]	13.1±2.2[b]

Data show the means±SD of several biomarkers analysed in animals from Do ana Palace (DBR), Santa Olalla pond (SOL), 'Brazo de la Torre' (BDT), and Huelva Industrial Park (HUV). The number of pooled samples from each area is shown in parenthesis and italics. Statistical significances were determined by Student's *t*-test ($P<0.05$) are expressed as [a](compared with DBR), [b](compared with SOL), and [c](compared with BDT).

different from those of DBR, except for the increased GSSGRase activity found in mice from the Huelva Industrial Park. In addition, animals from BDT and HUV areas had significantly higher MDA levels than SOL mice. Acidic Cu,Zn–SOD isoenzymes, that signal protein oxidative damage (Pedrajas *et al*. 1993), were not found after isoelectrofocusing separation and *in situ* activity staining at any area studied. While the results shown in table 1 are not fully conclusive, as in many field studies with free-living animals, they suggest that areas sampled in 1997 were either not contaminated, such as DBR and SOL, or more polluted, such as HUV (Esturión 1995) and BDT (Arambarri *et al*. 1996). The areas studied in 1997 were clearly separated by two biomarkers, SeGSHPx and EROD activities, as shown in figure 2. Low SeGSHPx activity was found in DBR and SOL mice, but several-fold higher activities were detected in BDT and HUV animals: 4.9– ($P=0.0008$) and 6.4-fold ($P=0.0006$) higher in BDT mice than in DBR or SOL, respectively, and 3.8-fold ($P=0.0002$) and 4.9-fold ($P=0.0015$) higher in HUV animals than in DBR or SOL, respectively. Figure 2 shows also that EROD activity was very low in DBR mice, moderately higher in SOL animals ($P=0.035$), and drastically higher 6.5-fold ($P=0.019$) and 7.3-fold ($P=0.0001$) in mice from 'Brazo de la Torre' and Huelva Industrial Park, respectively, with respect to DBR.

SeGSHPx detoxifies H_2O_2, and DNA- and lipid-hydroperoxides generated by compounds inducing oxidative stress (Flohé 1989). Mice lacking SeGSHPx activity are highly sensitive to several oxidants, and paraquat induces its synthesis *in vivo* and in cell culture (de Haan *et al*. 1998). This enzyme increases also in fish from polluted littoral areas (Rodríguez-Ariza *et al*. 1993) or industrially-polluted rivers (Almar *et al*. 1998), and is a useful biomarker for oxidative compounds, such as metals (Mather-Mihaich and DiGiulio 1991, López-Barea 1995). Thus, the increased SeGSHPx activity suggests the presence of oxidative contaminants in the BDT and HUV areas. Cytochromes P4501A transform organic aromatic xenobiotics (PAHs, arylamines, nitrosamines), that enhance transcription of the corresponding genes via binding to an aromatic hydrocarbon (Ah) receptor that binds to xenobiotic responsive elements present at their promoter regions (González and Gelboin 1994). Thus, increased CYP1A or its linked EROD activity is a standard biomarker of responses to organic contaminants (Peakall 1994, Timbrell *et al*. 1994, López-Barea 1995), although inhibition does also occur. The

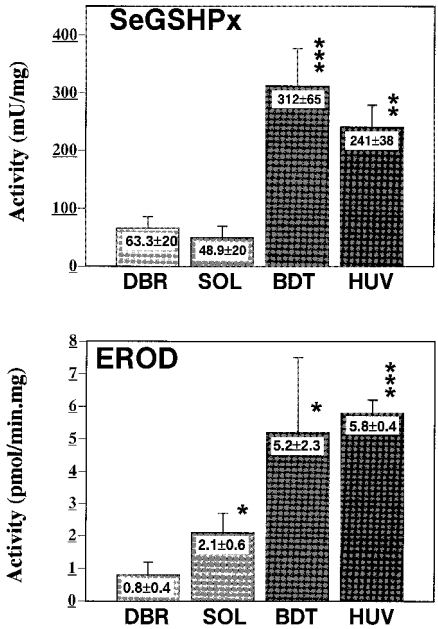

Figure 2. Biomarkers showing variations between the groups sampled in October 1997. The SeGSHPx and EROD activities were assayed in mice from Do ana Palace, Santa Olalla pond, 'Brazo de la Torre' and Huelva Industrial Park. Statistical significances with respect to DBR values were determined by the Student's *t*-test and are expressed as: *(P<0.05), **(P<0.01) and ***(P<0.005).

results with EROD activity suggest that the DBR area, at Do ana core, was less contaminated than the area SOL and, possibly, BDT and HUV areas were highly polluted by aromatic compounds, as suggested by the very high inductions detected. In fact, EROD increases (2.9 to 5.4-fold) below those found in our study occur in rodents exposed under controlled conditions to soils highly polluted with PCBs (Fouchécourt *et al*. 1998).

The results obtained in 1997 samples with SeGSHPx and EROD activities showed similar patterns, low in DBR or SOL areas in the Do ana Biological Reserve but several-fold higher in polluted areas. Both biomarkers showed very high activities in mice from 'Brazo de la Torre', an area known to occasionally receive metals carried by the Guadimar river from IPB and the Aznalcóllar mine (Cabrera *et al*. 1987, Arambarri *et al*. 1996), and in animals from the Huelva Industrial Park, an area known to be chronically polluted by the concerted action of the chemical industries located there and the metals leached and carried from the IPB by the Tinto and Oldiel rivers that join to form the Huelva Estuary (Esturión 1995). Actually, higher frequencies of total and kinetochore-positive micronuclei have been found in HUV mice compared with DBR animals (Ieradi *et al*. 1998, Degrassi *et al*. 1999), suggesting that genetic damage in the HUV area may be due to pollution. The SeGSHPx and EROD activities indicate that in 1997 the BDT and HUV areas were significantly affected by both oxidative and organic aromatic contaminants, although with slightly higher levels of oxidative compounds in the BDT area (higher SeGSHPx response), and of aromatic contaminants in the HUV area (higher EROD response).

Table 2. Biomarkers analysed in 1998 mice.

Biomarker analysed	Areas sampled			
	DBR (*3*)	AZN (*3*)	CG (*3*)	BDT (*3*)
G6PDH (mU mg^{-1})	3.5±0.9	8.0±2.4[a]	5.5±0.2[a]	4.0±0.1[b,c]
GSSGRase (mU mg^{-1})	55.7±3.7	55.1±1.9	56.2±3.8	58.2±1.8
KAT (U mg^{-1})	185±24	154±22	135±24	126±3.3[a]
SOD (U mg^{-1})	38.9±7.1	35.1±17	36.3±6.5	42.7±9.0
MDA (arbitrary fluorescence units)	7.5±0.8	7.1±2.7	7.6±1.5	11.2±1.9[a,c]

Data shows the means ± SD of several biomarkers analysed in animals from Do ana Palace (DBR), Aznalcázar (AZN), 'Cangrejo Grande' (CG) and 'Brazo de la Torre' (BDT). The number of pooled samples analysed in each area is shown in parenthesis and italics. Statistical significances ($P < 0.05$) are expressed as [a](compared with DBR), [b](compared with AZN), and [c](compared with CG).

Effects of Aznalcóllar spill in the Guadiamar river and Doñana

After the Aznalcóllar spill released mud and acidic water with pyrite-related metals (Garralón *et al*. 1999, Grimalt *et al*. 1999), a new investigation was designed to assess possible impacts on terrestrial fauna at different points of the Guadiamar river and Do ana. Thus, mice were sampled at three river areas which were probably affected, Aznalcázar, 'Cangrejo Grande' and 'Brazo de la Torre', using as controls mice from the Do ana Biological Reserve (figure 1).

Table 2 shows the results obtained in each area with five biomarkers, G6PDH, GSSGRase, KAT, SOD and MDA. Although no significant differences of GSSGRase or SOD activities were found among the four areas studied, G6PDH activity increased significantly in AZN (2.2-fold, $P = 0.038$) and CG (1.6-fold, $P = 0.019$) with respect to DBR animals, returning to control levels in BDT mice. In addition, MDA content increased in BDT mice, being significantly higher in this area (1.5-fold, $P = 0.036$) than in DBR or CG animals. Compared with DBR mice, catalase activity decreased steadily at AZN (0.83-fold), CG (0.73-fold) and BDT (0.68-fold) animals, but only BDT was significantly lower than DBR ($P = 0.014$). No acidic Cu, Zn–SOD forms were found by fast IEF and *in situ* staining for SOD (results not shown). Figure 3 shows that SeGSHPx, EROD, GSTc and GSTm activities, yielded clear geographic discrimination in the 1998 investigation. SeGSHPx activity was higher in animals from AZN (3.2-fold, $P = 0.006$), CG (3.6-fold, $P = 0.001$), and BDT (3.2-fold, $P = 0.002$) than in mice sampled as controls at the Do ana Biological Reserve. These results suggested that these three Guadiamar areas were closely affected by oxidative contaminants from the Aznalcóllar spill. Similarly, EROD activity was higher in CG and BDT mice than in those from Do ana or Aznalcázar, being 2.8-fold ($P = 0.019$) and 4-fold higher ($P = 0.007$) than in DBR or AZN animals, respectively. These results suggested that the lower Guadiamar areas were more polluted by organic aromatic compounds than the Do ana core or areas closer to the collapsed dam.

While SeGSHPx and EROD activities had been also highly responsive in 1997, after the Aznalcóllar spill two new biomarkers signalled the differences. Activities of soluble and microsomal glutathione-*S*-transferases increased linearly with the distance to the collapsed dam. Thus, compared with AZN, GSTc increased 1.9-fold at CG ($P = 0.004$) and 2.5-fold at BDT ($P = 0.002$); similarly, GSTm increased 1.8-fold at CG ($P = 0.006$) and 2.4-fold at BDT ($P = 0.013$), with respect to AZN. It is well established that some soluble glutathione-*S*-transferases respond to both

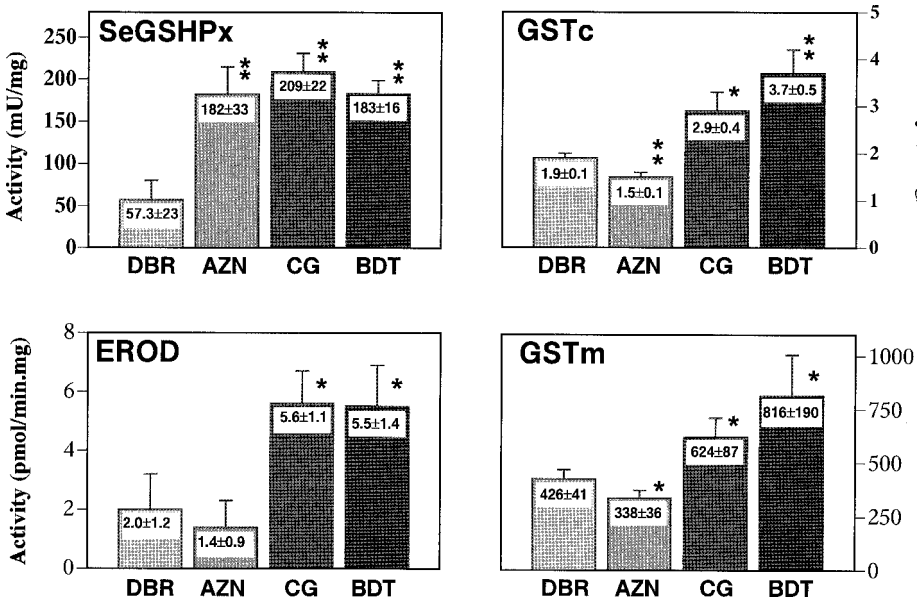

Figure 3. Biomarkers showing variations between the groups sampled after Aznalcóllar spill (October 1998). The SeGSHPx, EROD, GSTs and GSTm activities were assayed in mice from Do ana Palace, Aznalcázar, 'Cangrejo Grande', and 'Brazo de la Torre'. Statistical significances with respect to the DBR values are expressed as follows: *(P<0.05), **(P<0.01) and ***(P<0.005).

aromatic compounds and oxidative contaminants, due to the presence at their 5´-controlling elements of xenobiotic responsive elements, where the activated Ah receptor binds, and of antioxidant responsive elements, which sense ROS-generating compounds (Rushmore *et al.* 1994). Thus, the parallel increases in GSTc and GStm activities could reflect the combined presence of organic contaminants and of metals released by the collapsed dam in the Aznalcóllar mine (see further discussion later).

Glutathione content and redox status

Glutathione is the most important low molecular weight soluble antioxidant. Since its content and redox status is altered by prooxidant forces, both parameters, measured by HPLC with electrochemical detection, are sensitive biomarkers of natural pollution or exposure to oxidative contaminants, such as metals or oxidative organic compounds (Rodríguez-Ariza *et al.* 1993, 1994). Table 3 shows the GSH and GSSG contents and the glutathione redox status in mice from both investigations.

In 1997, DBR and SOL mice had similar GSH and total glutathione contents, which were significantly higher in HUV animals. In contrast, BDT mice had significantly lower GSH and total glutathione but higher GSSG contents, i.e. a more oxidized redox status, confirming that they suffered oxidative stress, as indicated by other biomarkers, such as the increased GSSGRase, KAT and SeGSHPx activities and MDA level (table 1, figure 2). In fact, freshwater bivalves transplanted to polluted sites have lower GSH and higher GSSG contents (Cossu *et al.* 1997), fish from metal-polluted littoral areas or exposed to Cu have an

Table 3. Hepatic glutathione content and redox status of mice from different areas in 1997 and 1998.

Investigation and area studied	GSH	2×GSSG	Total glutathione	2×GSSG / Total glutathione
1997				
DBR (*3*)	1776±227	635±25	2411±224	0.265±0.027
SOL (*3*)	2179±115	373±44[a]	2552±103	0.146±0.018[a]
BDT (*5*)	1014±479[a,b]	952±242[b]	1966±274[b]	0.503±0.182[b]
HUV (*3*)	2507±235[a,c]	310±6.3[a,c]	2817±298[c]	0.109±0.011[a,b,c]
1998				
DBR (*3*)	2079±558	1027±410	3106±955	0.325±0.032
AZN (*3*)	2140±514	1015±272	3155±755	0.322±0.037
CG (*3*)	604±326[a,b]	783±106	1387±299[a,b]	0.581±0.135[a,b]
BDT (*3*)	2091±638[c,*]	444±51[b,c,*]	2535±632[c]	0.184±0.060[a,b,c],*

Data show the content±SD of GSH, GSSG (as GSH equivalents) and total glutathione, in nanomole per gram of fresh weight, and the ratio of oxidized to total glutathione; the number of pooled samples analysed in each area is shown in parenthesis and italics. Statistical significances ($P < 0.05$) are expressed in 1997 as [a](compared with DBR), [b](compared with SOL), [c](compared with BDT), and in 1998 as [a](compared with DBR), [b](compared with AZN), [c](compared with CG). Statistical significances ($P < 0.05$) of each 1998 parameter in comparison to 1997 are shown by an asterisk.

oxidized redox status (Rodríguez-Ariza *et al.* 1993, 1994), and fish from polluted rivers show lower glutathione content (Almar *et al.* 1998). The higher GSH content and lower oxidized redox status of Huelva mice suggested that, in agreement with their higher SeGSHPx activity (figure 2), such animals could have adapted to metals and oxidative contaminants present at the Industrial Park by producing more GSH, as shown in Cu-exposed fish (Rodríguez-Ariza *et al.* 1994), to compensate for their decreased G6PDH, KAT, GSTc and GSTm activities (table 1). In 1998, glutathione contents and redox status were almost identical in DBR and AZN animals. Nevertheless, mice from 'Cangrejo Grande', where water from the collapsed dam had been retained, showed significantly lower contents of GSH and total glutathione and 58% of their glutathione was oxidized. Such results suggested that CG animals were subjected to oxidative stress promoted by the toxic spill, according to the higher G6PDH, SeGSHPx, GSTc and GSTm activities (table 2 and figure 2). In contrast to 1997, BDT animals showed GSH and total glutathione contents similar to DBR or AZN mice and a lower oxidized redox status. Such a recovery could be due to the spilled As, since higher GSH level has been found in As-exposed murine macrophages (Bannai *et al.* 1991).

Comparison between the 1997 and 1998 investigations

In both investigations (October 1997 and 1998, 6 months before and 6 months after the spill) mice were from two areas, BDT, at the Guadiamar river and probably affected by the spill, and DBR, probably unaffected. Thus, the effect of the toxic spill could be distinguished from previous contamination (figure 4), since nutritional or reproductive status would be similar because the animals were sampled at the same period of the biological cycle. In 1998, the activity of several antioxidant enzymes, G6PDH, KAT and SeGSHPx, decreased at BDT with respect to 1997 values, all changes being significant, particularly those of catalase activity. The biotransforming enzymes responded in different ways: EROD activity was low in DBR and remained very high in BDT in both investigations (5.2 ± 2.3 in 1997 vs 5.6 ± 1.1 in 1998), while GSTc and GSTm activities remained

Figure 4. Evolution of antioxidant and biotransforming enzymes with Aznalcóllar spill. The activities of several antioxidant and biotransforming enzymes were assayed in mice from Do ana Palace (DBR, clear histograms) and 'Brazo de la Torre' (BDT, dark histograms) during the investigations of 1997 (no pattern) and 1998 (hatched). Statistical significances with respect to the 1997 values at each area are expressed as follows: *($P < 0.05$), **($P < 0.01$) and ***($P < 0.0005$).

low at DBR but increased significantly at BDT. Differences between 1997 and 1998 were more clearly observed at BDT than at DBR, where changes were usually not significant, suggesting that they could be due to the toxic compounds released by the collapsed reservoir.

The decreased G6PDH, KAT and SeGSHPx activities in BDT could be due to their inhibition or inactivation by the toxic metals released by the spill. Several antioxidant enzymes diminish in freshwater bivalves transplanted to sites polluted by PAHs, PCBs and metals, a process related to lipid peroxidation (Cossu *et al.* 1997). The lack of further increase of EROD activity in 1998 BDT mice could be due to the induced levels already found in 1997, which could have attained their maximum values (Fouchécourt *et al.* 1998), thus preventing further increases after the Aznalcóllar spill. The increased GST activities found after the accident in BDT animals could be due to the combined effect of metals, that generate ROS to activate the antioxidant responsive elements of such genes, and organic contaminants, which cooperate to the induction via the xenobiotic responsive elements also present in the promoter region of such genes (Rushmore *et al.* 1994).

Several nitrogen organic compounds (NOCs, such as alkyldiphenylamines, mono-and dihalogen triphenylamines and 9-phenylcarbazole) have been detected in mud and surface waters affected by the Aznalcóllar spill (Alzaga *et al.* 1999). The acidic pH of water further increased the solubility of NOCs, explaining the high concentrations of such compounds found in 'Entremuros' water (3–7 ppb, June 1998), which increased 10-fold after the summer (Alzaga *et al.* 1999). NOCs were also detected at 'Brazo de la Torre' and Guadalquivir river, with the higher concentrations being found closer to the BDT area. Although the source of such compounds is unknown, they are probably not related to the flotation agents or to

the explosives used in the Aznalcóllar mine. Instead, they are probably linked to technical products used in the mine, such as lubricant additives, hydraulic fluids, or else derived from combustion. It cannot be excluded that they could be derived from industrial wastes dumped in the tailings dam before the accident (Alzaga *et al*. 1999).

Aromatic amines are a broad class of genotoxic contaminants that are bioactivated in rodents by several cytochrome P450 forms and other biotransforming enzymes, whose expression is induced by arylamines (Steele an Ioannides 1986). Glutathione transferases could also be induced by the electrophilic halogenated compounds released, with similar reactivities to some substrates used for GST assay (Pickett and Lu 1989). NOCs could have been flowing through the Guadiamar river before the Aznalcóllar spill, as previously shown for metals (Cabrera *et al*. 1987), thus explaining the high SeGSHPx and EROD activities already found in the BDT area in 1997. Nevertheless, the toxic metals and NOCs released by the spill in 1998 could make the situation more critical, eliciting the responses of new biomarkers and inducing some chronic adaptations. It cannot be excluded that fertilizers (such as Cu^{2+} ions) and plaguicides (such as organoarsenicals) used in the extensive rice fields grown in the left bank of the Guadiamar river upstream the Do ana National Park could also have contributed to contamination of the DBT area before the Aznalcóllar spill.

Final remarks and future prospects

Possible biological effects of Aznalcóllar spill in terrestrial ecosystems of the Do ana National Park have been initially assessed using a common, free-living and non-protected rodent, the Algerian mouse (*Mus spretus*), as a bioindicator. This small animal inhabits marshlands, attains high population densities, feeds on plants, seeds and insects, and could quickly be affected by the spilled metals that could enter Do ana via polluted waters. This species has a key role in Do ana food web, being selected by many predators, including carnivorous mammals (*Felis silvestris*, *Herpestes ichneumon*, *Genetta genetta*) and birds of prey (*Falco tinnunculus*, *Atene noctua*, *Strix aluco*, and *Tyto alba*) (Herrera and Hiraldo 1976, Kufner 1986). Thus, detection in *Mus spretus* of biological effects linked to the Aznalcóllar spill could warn of possible risks to other predators, and to endangered and protected superpredators, such as the lynx (*Linx pardinus*), or the Spanish imperial eagle (*Aquila adalberti*), that could be affected by the spill due to their highest position in the food web.

Several biochemical parameters have been assessed for the first time as pollution biomarkers in *Mus spretus* liver, an organ toxicologically very active which cannot be studied in any protected species. Biomarkers were selected according to their alleged selective responses to metals and oxidant compounds, organic aromatic compounds or both types of contaminants. Three biomarkers showed variations between the sampled groups, and could replace them for future studies. SeGSHPx activity detected oxidative chemicals more efficiently than G6PDH, GSSGRase, KAT and SOD activities, MDA and glutathione content and redox status. EROD activity responded to organic aromatic contaminants, and GSTc and GSTm activities both to oxidant and organic aromatic compounds. Microassay adaptation could allow these biomarkers to be individually assessed in future studies, and to spare enough biological material to analyse some contaminants. Before the Aznalcóllar spill (October 1997), the high EROD and SeGSHPx

activities suggested that the lower Guadiamar course (BDT) had similar levels of aromatic and prooxidant contaminants to the Huelva Industrial Park, chronically polluted. Six months after the spill (October 1998), the activities of several antioxidative enzymes decreased and those of soluble and microsomal GST increased significantly, particularly in the CG and BDT areas, suggesting that compounds released by the collapsed dam induced further biological effects in the exposed areas.

No significant contamination has been detected after the spill at Do ana Biological Reserve, although further investigations should be carried out to monitor possible alterations of such a Reserve of the Biosphere. Additional biomarkers should be included in future such as micronuclei induction or the 'comet' assay, two cellular biomarkers that indicate the genotoxic effect of chemicals. Such an approach will be used to follow the evolution of the possible ecotoxicological effects of the Aznalcóllar spill at the Do ana Biological Reserve and Do ana National Park, and also to follow the recovery of affected Guadiamar areas. Controlled exposure of *Mus spretus* to Aznalcóllar mud in microcosm experiments could also help to clarify the uptake and release processes of spill-related metals. In addition, mice could be exposed to metals or associations of the most dangerous ones to study their toxicological and synergic effects.

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