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Arsenic rich iron plaque on macrophyte roots – an ecotoxicological risk?

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Accumulation of metals with iron plaque on macrophyte roots in wetlands poses an ecotoxicological risk to certain herbivores.

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ABSTRACT

Arsenic is known to accumulate with iron plaque on macrophyte roots. Three to four years after the Aznalcóllar mine spill (Spain), residual arsenic contamination left in seasonal wetland habitats has been identified in this form by scanning electron microscopy. Total digestion has determined arsenic concentrations in thoroughly washed 'root + plaque' material in excess of 1000 mg kg^{-1}, and further analysis using X-ray absorption spectroscopy suggests arsenic exists as both arsenate and arsenite. Certain herbivorous species feed on rhizomes and bulbs of macrophytes in a wide range of global environments, and the ecotoxicological impact of consuming arsenic rich iron plaque associated with such food items remains to be quantified. Here, greylag geese which feed on *Scirpus maritimus* rhizome and bulb material in areas affected by the Aznalcóllar spill are shown to have elevated levels of arsenic in their feces, which may originate from arsenic rich iron plaque.

1. Introduction

In 1998 a tailings dam at the Aznalcóllar mine (Spain) failed and released 5–6 million m^3 (Vidal et al., 1999; Galán et al., 2002) of acidic metal rich sludge and water. The spill contaminated thousands of hectares (ha), travelled tens of kilometres south of the mining complex, and polluted 2754 ha of the Doñana Natural and National Park. Doñana is one of the most important areas for wildlife and conservation in Europe, parts of which are protected as a World Heritage Site, Biosphere Reserve and Ramsar Site (García-Novo and Cabrera, 2006). Some 2656 ha of Natural Park were polluted (Grimalt et al., 1999) including a 900 ha seasonal wetland called the 'Entremuros' (between dykes) which was used as a temporary holding area for sludge and contaminated water. In doing so, the spill was almost completely prevented from entering the more pristine National Park; however, the pH of the water in the Entremuros fell from a normal 8.5 to 4.5, and elevated metal levels were reported in solution (Pain et al., 1998; Garralón et al., 1999). A certain amount of suspended sludge also contaminated this area, which contained 0.4–0.6% As (Pain et al., 1998; Alastuey et al., 1999; Galán et al., 2002). Despite subsequent efforts to remediate, residual contamination remains (Galán et al., 2002; Taggart et al., 2004, 2005; Simón et al., 2008) and the long-term impact of the spill is still to be fully quantified.

Amid fears regarding impacts on local agriculture and fishing, were significant concerns about the affect on wildlife. Affected Natural Park areas were predominantly seasonal and permanent wetlands, which support, amongst other flora and fauna, a range of important avian species, including some which are locally and internationally rare (such as Purple Gallinule (*Porphyrio porphyrio*) and Spanish Imperial Eagle (*Aquila adalberti*) respectively). Research on the immediate impacts on avian species has shown elevated Pb and Cd in blood (Benito et al., 1999), Zn and Cu in liver and eggs (Hernández et al., 1999) and As and Cu in livers (Taggart et al., 2006) of a wide range of birds from within or near impacted areas. Likewise, between 2001 and 2004, Greylag geese (*Anser anser*) feeding in the Entremuros were still being exposed to elevated metal levels when compared to birds feeding outside spill affected areas (Mateo et al., 2006). Avian species can act as biomonitors of pollutants as they move through food chains and are important fauna to study in scenarios such as this one. In this case, herbivorous waterfowl may be especially susceptible, as they consume significant amounts of soil/sediment in association with food (Beyer et al., 1994, 1999). Beyer et al. (1999) reported that soil ingestion by herbivorous grazing ducks was 3%, and higher than in granivorous filtering species, who ingested <1%; also, 8.2% of feed ingested by Canada geese (*Branta canadensis*) comprised of soil

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particles (Beyer et al., 1994). Commonly, the consumption of relatively insoluble soil particles may be of limited toxicological consequence; however, where that soil/sediment is contaminated or where it contains, for example, residual lead shot from hunting, ingestion may have significant ecotoxicological impacts (Pain, 1990; Mateo et al., 2001; Guitart et al., 2002).

Further, Taggart et al. (2005) suggested that exposure to potentially toxic levels of As may occur if a herbivore grazed on below ground macrophyte material (roots, rhizomes and bulbs) naturally coated with As rich iron plaque. Studies have shown that iron plaque can accumulate high levels of As, Cd, Cu, Zn, and Pb (Macfie and Crowder, 1987; Crowder and St-Cyr, 1991; Otte et al., 1995; Caçador et al., 1996; Doyle and Otte, 1997; Ye et al., 1998; Caetano and Vale, 2002; Hansel et al., 2002; Panich-Pat et al., 2004), and this important plant–sediment interaction is often used to clean contaminated wetlands and/or treat metal rich discharges (acid mine drainage, landfill leachate, etc.) (Groudeva et al., 2001; Batty and Younger, 2002; Stoltz and Greger, 2002; Manios et al., 2003). However, its potential ecotoxicological significance has not been specifically considered for avian or other herbivores.

In the Entremuros, Taggart et al. (2005) have shown that the concentration of As associated with macrophyte roots can far exceed that found within the sediments in which they are growing. Certain waterfowl in Doñana, such as greylag geese, feed extensively on macrophyte (*Scirpus* sp.) bulbs and rhizomes (Amat, 1995; Esselink et al., 1997). Greylag geese are, in turn, important food items for highly endangered Spanish Imperial Eagles in Doñana, and for humans in the area who commonly hunt and consume various ducks, geese and rallids. Purple gallinules, a species of 'European Conservation Concern' (Birdlife International, 2004), also feed on macrophyte (*Typha* sp.) rhizomes (Viellard, 1974) in the Entremuros. Such herbivores, for whom below ground macrophyte material form an important dietary component, may consume significant quantities of macrophyte roots, and also the sediment particles associated with those roots, rhizomes and bulbs. Whilst iron plaques can accumulate large amounts of potentially toxic metals, the more oxic rhizosphere, or 'near root-rhizome-bulb' sediment, is also likely to become enriched (Otte et al., 1995; Caçador et al., 1996; Doyle and Otte, 1997), and it is this, not the bulk sediment, to which herbivorous species are most exposed (via diet). Although waterfowl commonly 'wash' their food before consumption, Taggart et al. (2005) have shown that despite thorough cleaning, high levels of As can remain associated with macrophyte roots. Beyer and Day (2004) suggested a similar pathway in relation to Pb associated with Mn/Fe oxides deposited on submerged macrophyte tissues (above ground parts), and noted that this may be an unrecognised but important exposure route for mute swans (*Cygnus olor*).

Here, we present As levels in sediment and associated root, rhizome and stem material from macrophytes (*Typha dominguensis* (cattail) and *Scirpus maritimus* (alkali bulrush)) dominant in areas affected by the Aznalcóllar mine spill. Sediment and macrophyte roots are also subjected to analysis using SEM–EDAX (scanning electron microscopy with energy dispersive X-ray spectroscopy) to obtain direct evidence of an association between As and root iron plaque in Doñana, and XAS (X-ray absorption spectroscopy) is utilised to obtain non-destructive information regarding As speciation. The implications of the accumulation of As on root iron plaque are then discussed from an ecotoxicological viewpoint, whilst re-analysing data on As, Fe and Al in greylag geese feces from the Entremuros, previously presented in Mateo et al. (2006).

2. Methodology

2.1. Field sampling

Sampling took place in October 2001 and October 2002. Locations used are shown in Fig. 1, and locations used in each year are given in Fig. 2. Sediment samples

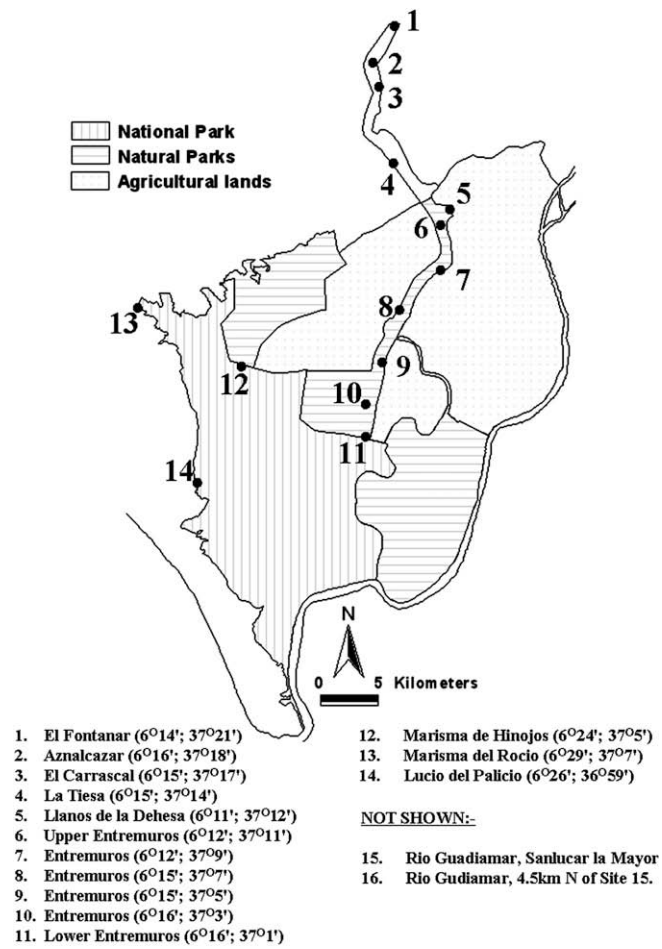

Fig. 1. Sediment and macrophyte sampling locations used in 2001 and 2002. Locations 12–14 are considered to be control locations, unaffected by the Aznalcóllar mine spill. Locations 1–11 and 15–16 were affected by the mine spill. Co-ordinates (locations 1–14) and names given are taken from the Junta de Andalucía 1:75,000 Doñana, Parque Naturales de Andalucía map (1998 edition). Locations 15 and 16 are on the Rio Guadiamar, due north of site 1 at the locations described.

(0–5 cm) were collected at each location using a core sampler; and, where present, whole plant samples of *T. dominguensis* and *S. maritimus* were taken. Sampling locations were characteristically at the edges of slow flowing shallow water courses, where conditions were adequate for growth of these macrophytes. At each location, triplicate sample points were taken at 10 m spacing along a 20 m transect. Hence, at each location, there were three sites, at which three sediment and three macrophyte samples were taken within a 1 m area. Macrophytes were cleaned of adhering sediment in the field, and then cleaned again, thoroughly, in running deionised water in the field laboratory, before being frozen. Sediment samples were also frozen. Greylag goose feces were sampled in the Entremuros during the same sampling period, details of which are given in Mateo et al. (2006).

2.2. Laboratory procedures

Sediments were air dried, sieved to <2 mm, and a subsample oven dried at 85 °C. Macrophyte samples were defrosted, underwent further cleaning in deionised water, then subsamples were dried at 85 °C. Approximately 0.2 g of sediment or macrophyte material was weighed to ±0.001 g into an acid cleaned digest tube, 2.5 ml of analytical grade 70% nitric acid were added, the tubes covered, and left overnight to digest. A further 2.5 ml of analytical grade 30% hydrogen peroxide were then added and the digest tubes heated to 160 °C. This temperature was maintained for 4 h. Digest solutions were decanted into 15 ml centrifuge tubes, made up to 10 ml with deionised water, and stored at 4 °C until analysis. Arsenic was determined using a Perkin-Elmer Hydride Generation AAS 300 system, after pre-reduction with a solution of 10% potassium iodide, 10% hydrochloric acid, and 5% ascorbic acid. All data are in mg kg^{-1} dry weight (DW). Values below the limit of detection (LOD) were considered as one-half of the LOD in statistical analyses. Arsenic recoveries from samples made up using certified 1000 mg l^{-1} stock solutions were 92.1 ± 8.9% ($n = 8$), whilst recovery from certified soil standards (GBW07406-GSS-6) was 84.3 ± 10.2% ($n = 8$); (geometric mean ± SD).

2.3. SEM–EDAX and XAS analysis

To provide information regarding elemental As associations and speciation, samples of sediment and root from 2001 found to contain high As (>250 mg kg^{-1}) using the digest procedures, were further analysed using SEM–EDAX and XAS. The SEM–EDAX was conducted on a ISI-ABT55 with a Link Analytical (AN10/55S) energy dispersive X-ray analyser. Samples were attached to aluminium stubs and carbon coated. In backscattered electron mode, particles high in As stood out brightly, compared to particles containing elements with a lower atomic number. These As rich particles were analysed for elemental composition using the X-ray analyser and a 3–4 μm electron beam (Fig. 3).

XAS was conducted at the Synchrotron Radiation Source, Daresbury Laboratory, UK. Frozen samples were mounted in aluminium holders using Sellotape® and plunged into liquid nitrogen. Samples were maintained in this state until analysis, and all experiments were performed in a liquid nitrogen cooled cryostat. X-ray absorption spectra at the As K-edge were collected on Station 16.5, operating at 2 GeV with an average current of 150 mA, using a vertically focusing mirror and a sagitally bent focussing Si (220) double crystal monochromator detuned to 80% transmission to minimise harmonic contamination. Data were collected in fluorescence mode using an Ortec 30 element solid state Ge detector. Several scans were summed for each sample.

Model compound spectra were collected in transmission mode. XANES (X-ray absorption near edge structure) data were fitted using a linear combination of XANES spectra collected from model systems (Table 1 shows models). Background subtracted EXAFS (extended X-ray absorption fine structure) spectra were analysed in EXCURV98 using full curved wave theory (Gurman et al., 1984; Binsted, 1998). Phaseshifts were derived from 'ab initio' calculations using Hedin-Lundqvist potentials and von Barth ground states (Hedin and Lundqvist, 1969). Fourier transforms of the EXAFS spectra were used to obtain an approximate radial distribution function around the central As atom (the absorber atom); the peaks of the Fourier transform were related to "shells" of surrounding back scattering atoms characterised by atom type, number of atoms in the shell, the absorber–scatterer distance, and the Debye–Waller factor, $2\sigma^2$. The data were fitted for each sample by defining a theoretical model and comparing the calculated EXAFS spectrum with the experimental data. Shells of backscatterers were added around As, and by refining an energy correction E_f (the Fermi energy), the absorber–scatterer distance, and the Debye–Waller factor for each shell, a least squares residual (the R factor (Binsted et al., 1992)) was minimised. The coordination numbers were chosen assuming that any arsenate would be 4-coordinate, any sulphur-bound species would be 3-coordinate, etc., as in the model systems. The proportion of As in each site was refined as a free variable, keeping the total central As occupation number as 1.

3. Results

3.1. Sediment and macrophyte As levels

In 2001 (Fig. 2) sediment As ranged from 1.6 to 532.2 mg kg^{-1} and locations 2 and 4 had points >50 mg kg^{-1}, (considered here to be contaminated to a level where remedial action should be considered). A range of historical guidance limits for soil As exist between 10 and 55 mg kg^{-1}, depending on use of the soil, soil type, origin of limit, etc. (Adriano, 1986; ICRCL-59/83, 1987). The USEPA (2005) also presented ecological soil screening levels for As in relation to the protection of avian ground insectivores and avian herbivores at 43 and 67 mg kg^{-1}, respectively. In 2002, concentrations ranged from 0.1 to 956.4 mg kg^{-1}; and locations 1, 3, 15 and 16 were found to have points >50 mg kg^{-1}. Only location 4 in 2001 and locations 3, 15 and 16 in 2002 contained mean As levels >50 mg kg^{-1}, and these sites were outside Doñana Natural/National Park. At background locations (12–14), As ranged from 1.6 to 9.3 mg kg^{-1}.

In 2001, *S. maritimus* and *T. dominguensis* stem As ranged from 0.01 to 8.1 and 0.29 to 28.5 mg kg^{-1}, respectively; rhizomes were 0.08–123.2 and 0.02–139.6 mg kg^{-1}. In 2002, *S. maritimus* and *T. dominguensis* stem concentrations ranged from 0.06 to 21.0 and 1.37 to 9.5 mg kg^{-1}; rhizomes were 0.07–141.2 and 1.48–18.5 mg kg^{-1}. Root levels varied from 1.39 to 572.0 and 6.61 to 1088.6 mg kg^{-1} for *S. maritimus* in 2001 and 2002, and between 1.74–355.03 and 6.21–422.87 mg kg^{-1} for *T. dominguensis*. Root As concentrations at discrete sampling points were up to >11 times higher than they were in the sediment from which they originated, and ≈50% of roots from both macrophyte species had As concentrations >50 mg kg^{-1}. Chaney (1989) suggests the maximum dry diet level tolerated by livestock (cattle, sheep, swine and chicken) is ≈50 mg kg^{-1}.

3.2. SEM–EDAX and XAS analysis

Despite analysing approximately 200 sediment particles from four sediments with the highest As levels, a distinct As mineral phase could not be detected. However, SEM–EDAX of macrophyte roots showed that As was locally associated with iron plaque (Fig. 3), and that certain plaque particles had an estimated As concentration of >1% (>10,000 mg kg^{-1}).

Table 2 shows the XANES fits and EXAFS analyses collected for sludge from the spill (the pollutant source), highly contaminated sediment (956.4 mg kg^{-1} As, 2002, location 15), and three *S. maritimus* and two *T. dominguensis* roots. The *T. dominguensis* roots were (2002, location 16), from the same plant with 422.9 mg kg^{-1} As. This plant exhibited two distinct root morphologies, one with virtually no fine lateral secondary roots branching from the primary roots (the first result in the table), and a second with a high density of secondary lateral roots. The first two *S. maritimus* roots shown were from the same plant, analysed fresh, and then analysed dry (location 6, 2002, upper Entremuros, 651.2 mg kg^{-1} As). The third *S. maritimus* sample was fresh (2001, location 5, adjacent to upper Entremuros) and contained 572.0 mg kg^{-1} As. The XANES fits showed the sludge and sediment samples fitted a mix of arsenate (85%) with a lesser amount of arsenopyrite (15%). The EXAFS analyses was in line with this, but showed a slightly higher proportion of arsenopyrite. Most macrophyte root samples were best fitted to arsenate, but the second *T. dominguensis* root (with a high density of fine lateral roots) also showed evidence of arsenite.

4. Discussion

Our results confirm previous findings showing that the Aznalcóllar spill clean-up has been somewhat ineffective in relation to As, and a significant pollutant load remains in affected areas. Although none of the consistently elevated sediment sample locations were in National/Natural Park areas, the Rio Guadiamar valley to the north, where consistently elevated locations were noted, is undergoing development as a protected "Green Corridor". Such schemes must consider whether spill residues are liable to have an ecotoxicological impact on wildlife encouraged to migrate through or reside in As contaminated areas. Also, whilst mean sediment As levels were <50 mg kg^{-1} at the majority of locations, residual contamination was occasionally highly spatially heterogeneous. At site 4 for example (in 2001), As varied from 36.0 to 532.2 mg kg^{-1} over a distance of 10 m. Future monitoring should assess these variations and take account of the "hot-spot" nature of residual contamination, especially if samples are from protected areas where patchy contamination may still have ecotoxicological significance.

Many locations had consistently high levels of root As (>50 mg kg^{-1}; Fig. 2). Whilst consistently elevated As in sediment has not been identified in protected areas, locations 6, 8 and 9, within the Entremuros (Natural Park), all showed elevated mean *S. maritimus* root levels. In 2002, *S. maritimus* at site 6 had a maximum As root concentration of 651.2 mg kg^{-1}, and only site 2 (Aznalcazar) had levels above this. Levels were also high at location 5, where in 2001, *S. maritimus* roots had up to 572.0 mg kg^{-1}, the highest level that year. This location is within a drainage/irrigation ditch which serves the rice fields adjacent to the Entremuros. These fields are important feeding grounds for species such as greylag geese and white stork (*Ciconia ciconia*). As in Taggart et al. (2005), results show that *S. maritimus* tends to accumulate higher amounts of root As than *T. dominguensis*. This is probably related to increased root surface

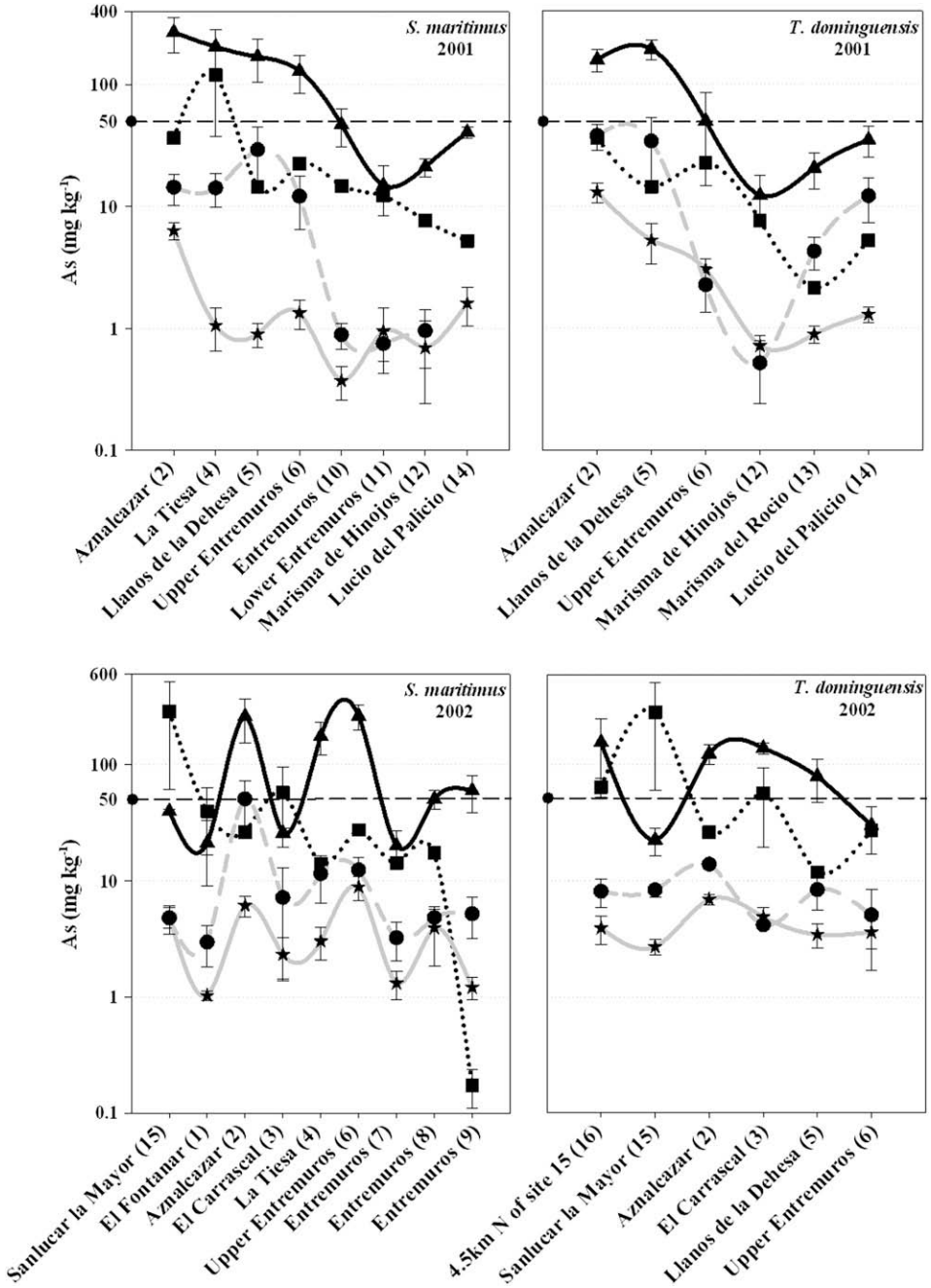

Fig. 2. Mean levels of arsenic (with standard error bars; $n = 9$) in the sediment (squares), roots (triangles), rhizomes (circles) and stems (stars) of two dominant macrophytes in and near Doñana in 2001 (upper) and 2002 (lower). Locations are ordered such that those nearest the source of the Aznalcóllar mine spill are presented on the left side of the graphs, whilst those least affected/control locations are on the right.

area, as *S. maritimus* roots are much finer than those of *T. dominguensis*.

Widely accepted thresholds for As in plants which are consumed by herbivores, do not currently exist. However, Chaney (1989) suggests that a 'normal' inorganic As concentration in dry foliage is 0.01–1 mg kg^{-1}, a phytotoxic level may be 3–10 mg kg^{-1}, and the maximum level tolerated by livestock (cattle, sheep, swine and chicken) is $\approx$50 mg kg^{-1} in dry diet. Taggart et al. (2005) found no evidence that macrophytes in the Entremuros were accumulating As in above ground parts to levels which might be considered ecotoxic, given these limits. Here, data also show that As is <50 mg kg^{-1} in above ground parts; however, concentrations were found to be >1 mg kg^{-1} in 36 and 89% of *S. maritimus*, and 77 and 100% of *T. dominguensis* stems in 2001 and 2002, respectively. In terms of stems >10 mg kg^{-1}, data show 0 and 9% for *S. maritimus* and 16 and 0% for *T. dominguensis*, in 2001 and 2002. For rhizomes, only 2 and 4% of *S. maritimus* and 13 and 0% of *T. dominguensis* in 2001 and 2002 had levels >50 mg kg^{-1}. However, where roots were concerned, 42 and 52% of *S. maritimus* and 46 and 54% of *T. dominguensis* sampled in 2001 and 2002 had As >50 mg kg^{-1}.

The SEM–EDAX analysis of roots with up to 572 mg kg^{-1} As, has shown numerous particles, rich in As, which remain attached to roots despite thorough washing and having undergone several freeze–thaw cycles (Fig. 3). These particles are estimated to contain >1% As (see As peak, Fig. 3a), which suggests they may constitute a distinct mineral phase, rather than simply an iron (hydr-)oxide

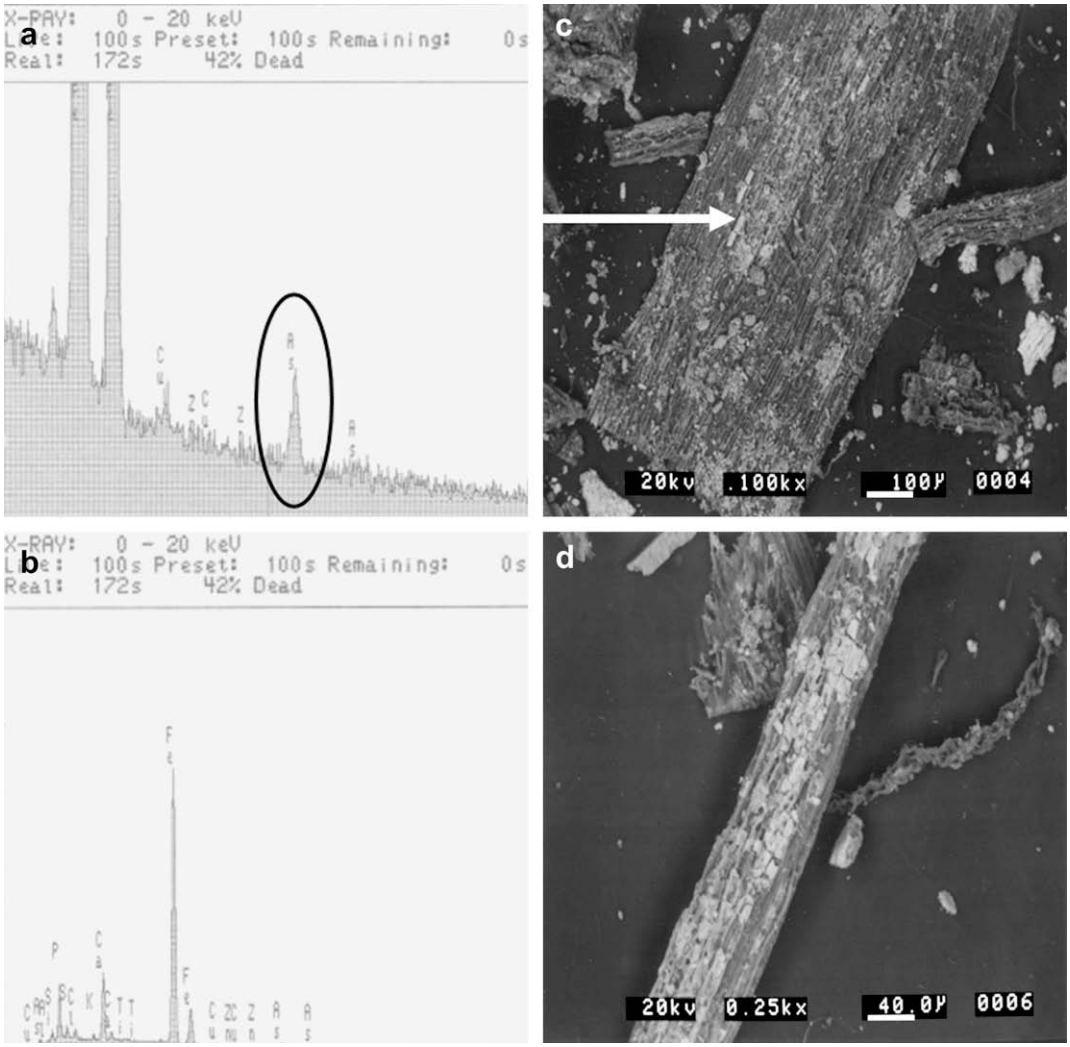

Fig. 3. SEM–EDAX images of macrophyte roots coated in arsenic rich iron plaque. Arrow indicates position of long, bright, thin arsenic rich areas of plaque (estimated to contain >1% arsenic), for which EDAX analysis is shown in (a) and (b). The arsenic peak is highlighted within an oval. The '*y*-axis' on (a) and (b) represents the relative signal intensity for the elements of interest.

Table 1
Comparison between extended X-ray absorption fine structure (EXAFS) results and crystallography parameters for models.

Crystallography				EXAFS		
	Type	CN	r_{cr} (Å)	r_{ex} (Å)[e]	$2\sigma^2$ (Å^2)[f]	*R* factor
Sodium arsenate[a]	O	4	1.688 av	1.68	0.004	25.9
Arsenopyrite[b]	S	1	2.332 av	2.26	0.003	47.4
	Fe	3	2.369 av	2.33	0.010	
	As	5	3.247 av	3.32	0.017	
	S	6	3.242 av	3.31	0.019	
	Fe	4	3.738 av	3.72	0.012	
Sodium arsenite[c]	O	3	1.794 av	1.79	0.013	30.2
	As	2	3.224 av	3.23	0.018	
As:glutathione 1:3[d]	S	3	2.252 av	2.26	0.006	28.8

[a] Crystal structure of the analogous $Cs_2Na(AsO_4)$ taken from Schneidersmann and Hoppe (1991).
[b] Crystal structure of FeAsS taken from Morimoto and Clark (1961).
[c] Crystal structure of sodium arsenite hydrate, $NaAsO_2(H_2O)_4$ taken from Sheldrick and Häusler (1987).
[d] Crystal structure of the analogous (i.e. As(III) coordinated by three sulphur-ligands) tris(tri-*t*-butoxysilanethiolato)As(III) taken from Peters et al. (1997).
[e] Error ± 0.02 Å for inner shells, ± 0.05 Å for outer shells.
[f] Error ± 25%.

phase with a high level of sorbed surface As. These particles form distinct parts (Fig. 3c) of a more extensive iron plaque on roots (Fig. 3d), but appear to be geochemically dissimilar to the bulk plaque since areas high in As also tended to be higher in phosphorous (and to a lesser extent calcium). Hansel et al. (2002) also found "hot-spots" of As, within, and on the surface of macrophyte roots. The physical form of the particles shown in Fig. 3c (long, thin, and parallel to the root growth direction), may suggest that they have precipitated/formed within the root, in a cell or vacuole, and then, as the outer root surface has decayed, the precipitate has been exposed. Notably, Fig. 3b also shows lower, but significant signals for Cu and Zn, highlighting that plaque can simultaneously play a role in controlling the fate of a range of potentially toxic metals in saturated sediments.

The XAS data for the first four root samples shown in Table 2 are consistent with the mechanism of iron plaque formation on macrophyte roots, as it is understood. This is, that within relatively reduced sediments, oxygen diffuses from macrophyte roots, promotes oxidation of reduced Fe and As phases, Fe and As in solution diffuses towards/is drawn by root uptake to the root, and at the root surface, these (co)-precipitate/become sorbed in an oxidised form, in this case, as arsenate. Arsenic is known to have a high affinity for iron oxides in soils/sediments, and very stable

Table 2
X-ray absorption near edge structure (XANES) and extended X-ray absorption fine structure (EXAFS) data for Aznalcóllar mine spill sludge, contaminated Doñana sediment, *S. maritimus* and *T. dominguensis* roots.

	XANES						EXAFS				
	n	Arsenate (%)	FeAsS (%)	Arsenite (%)	As:glutathione	Fit	Scatterer	No. of atoms	*r* (Å)[a]	$2\sigma^2$ (Å^2)[b]	*R* factor[c]
Sludge	4	85	15	–	–	3.0	O	2.9	1.69	0.005	28.5
							S	0.3	2.30	0.003	
							Fe	0.8	2.34	0.006	
							As	1.3	3.21	0.008	
							S	1.6	3.12	0.013	
							Fe	1.1	4.01	0.027	
								(72% arsenate; 28% FeAsS)			
Sediment	2	85	15	–	–	1.9	O	3.0	1.69	0.007	34.3
							S	0.2	2.26	0.002	
							Fe	0.7	2.33	0.005	
							As	1.2	3.20	0.010	
							S	1.5	3.11	0.023	
							Fe	1.0	4.00	0.009	
								(75% arsenate; 25% FeAsS)			
Scirpus root	4	100	–	–	–	3.5	O	4.0 (100% arsenate)	1.68	0.015	69.0
Scirpus root	4	100	–	–	–	4.4	O	4.0 (100% arsenate)	1.69	0.002	28.1
Scirpus root	2	100	–	–	–	17	O	4.0 (100% arsenate)	1.67	0.017	66.8
Typha root	4	100	–	–	–	6.7	O	4.0 (100% arsenate)	1.68	0.017	53.4
Typha root	3	50	–	50	–	2.0	O	3.5 (100% arsenate/arsenite)	1.71	0.009	55.0

Fit (index) of the calculated XANES spectra, with experimental XANES spectra, is defined as $\Sigma[(I_{obs} - I_{calc})^2]/n$, where *n* is the number of points in each spectrum (lower index = better fit). For EXAFS, $2\sigma^2$ is the Debye–Waller type factor, and the *R* factor indicates fit.
[a] Error ± 0.02 Å for inner shells, ± 0.05 Å for outer shells.
[b] Error ± 25%.
[c] Residual, as defined in Binsted et al. (1992); values of above 50% indicate noisy data. Note that the EXAFS data quality did not allow resolution between arsenate and arsenite As–O distances, and therefore it is assumed that an As–O distance less than 1.70 Å is mainly arsenate, a distance of greater than 1.76 Å is mainly arsenite, and anything in between represents a mixture of the two.

complexes are commonly formed (Sun and Doner, 1996; Sadiq, 1997; Manning et al., 1998; Raven et al., 1998; Sherman and Randall, 2003). Likewise, iron plaque on macrophyte roots also has a high affinity for As, tending to have a higher affinity for arsenate than arsenite (Chen et al., 2005; Liu et al., 2005). The fifth root sample shown in Table 2 suggests that arsenite was however present on some root surfaces. This particular root had a very high density of secondary lateral roots. In the field, it was growing in a particularly anoxic sediment, dark in colour and rich (as apparent from its odour) in reduced forms of sulphur. Increased secondary root growth (to enhance oxygen diffusion from the roots) was perhaps promoted in this plant as a response to prevent sulphide toxicity or the internal precipitation of iron (hydr-)oxides in the roots. Increased external plaque precipitation may also, in itself, have decreased the plants ability to take up nutrients and hence promoted root growth. The roots may simply have been in increased contact with reduced forms of As in the sediment (which have not been removed by washing), or alternatively, prevailing anoxic conditions around less active/dead roots may have allowed arsenate associated with plaque to be reduced to arsenite, a process perhaps driven by microbial activity (Emerson et al., 1999; King and Garey, 1999; Jones et al., 2003; Weiss et al., 2003). However, as in Hansel et al. (2002), no evidence of organic As species on root iron plaque has been found here.

4.1. Ecotoxicological implications

Little is known about the food chain transfer of metals from above ground parts of potentially metal-accumulating macrophytes to herbivores (Jackson, 1998). Even less is known about the pathway suggested here, where below ground macrophyte material (roots, bulbs, rhizomes) pose the greater, less obvious ecotoxicological risk. Historically, although accumulation of metals on macrophyte roots with iron plaque has been noted to occur in a range of wetland environments, the assumption has perhaps been that since this process occurs sub-surface, and roots are not commonly a major food source, no ecotoxicological threat exists.

In Doñana, residual contamination in sediments is likely to undergo weathering and redistribution in the future, and one possible sink for As and a range of other metals is in association with macrophyte root iron plaque. The surfaces of bulbs and rhizomes, and the rhizosphere sediment, are also likely to attract elevated metal levels. Certain herbivores in Doñana feed extensively on macrophyte rhizomes and bulbs, and will inevitably consume a certain amount of root tissue and rhizosphere sediment in association with such food items. It should also be noted that the root As levels presented here (up to 1089 mg kg^{-1} DW at Aznalcazar) are extremely conservative, as these roots underwent several washing and freeze–thaw cycles prior to analysis. The data presented may greatly underestimate the degree and level of As exposure to herbivorous grazers who may actually spend little time washing their food. Water may not even be available to wash such items in certain scenarios.

In previous work (Mateo et al., 2006) we showed, using invasive and non-invasive techniques, that greylag geese feeding in the Entremuros are exposed to higher amounts of metals than geese feeding in areas unaffected by the Aznalcóllar spill. Levels of Pb, Zn, Cu, and As were all higher in feces of birds feeding in the Entremuros than in birds feeding elsewhere, and As and Zn concentrations were up to 14.5 and 840 mg kg^{-1}, respectively. Further, levels of As in feces were correlated with increased excretion of coproporphyrin isomer I, indicating subclinical effects may be occurring. If these As data for feces are plotted against Fe and Al (Fig. 4; a reanalysis of data from Mateo et al., 2006), levels are tightly correlated ($r^2 = 0.835$ for Fe and 0.839 for Al, both at $p < 0.001$). The close relationship between As and Al is a strong indicator that the As source is a soil based one, rather than originating from above ground plant material that has accumulated unusual levels of metals. Although soil in the Entremuros contains elevated As, it has been remediated, and the maximum level found here was 32.64 mg kg^{-1}, similar to the maximum level noted previously (31.7 mg kg^{-1}; Taggart et al., 2005). Mateo et al. (2006) calculated the estimated percentage of ingested soil (%EIS; calculated as in Beyer et al., 1994) for the feces presented in Fig. 4, and also estimated the amount of

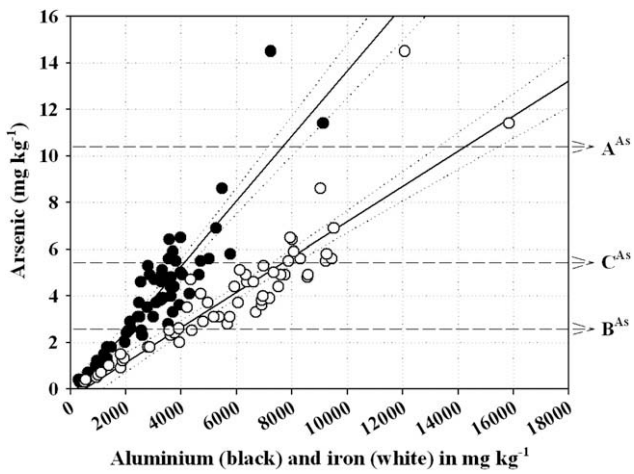

Fig. 4. Iron (white dots) and aluminium (black dots) against arsenic in feces of greylag geese collected from the Entremuros in 2001–2002. Control lines A–C show the modelled expected concentration of arsenic given that 63% of plant material is indigestible, and that in A, the soil concentration is maximal at 32.64 mg kg^{-1} and the % estimated ingested soil (% EIS) is 22.64%. In B, the soil concentration and % EIS is based on a mean value of 16.1 mg kg^{-1}, and 10.9%, whilst in C we use mean plus one standard deviation, i.e. 22.5 mg kg^{-1} As in soil, and a % EIS of 16.6%.

indigestible plant material being consumed in this area at 63%. Given these figures, we can calculate the concentration of As expected in feces of geese using:

$$\text{Total \% indigestible (feces)} = \%\text{EIS} + ((\text{\% plant ingested} \div 100) \times \text{\% plant which is indigestible})$$

Thus:

$$\text{Expected feces As concentration} = \text{bulk soil concentration} \div (\text{total \% indigestible} \div \text{\% EIS})$$

Worked example (using %EIS of 22.64% and figures noted in paragraph above):

$$\text{Total \% indigestible} = 22.64 + ((77.36 \div 100) \times 63) = 71.38\%$$

$$\text{Feces As concentration} = 32.64 \div (71.38 \div 22.64) = 10.35 \text{ mg kg}^{-1}$$

This concentration is annotated as line A (Fig. 4). This is, however, based on the maximum soil level in the Entremuros, the highest value for %EIS plotted in Mateo et al. (2006), and assumes no uptake of As from soil in the digestive tract (contrary to evidence from the coproporphyrin work presented in Mateo et al., 2006). Line B, Fig. 4, uses the mean soil As level in the Entremuros (16.1 mg kg^{-1}) and the mean %EIS reported (10.9%), whilst line C utilises these mean values plus one standard deviation (22.5 mg kg^{-1} and 16.6%). The maximal As level in feces of 14.5 mg kg^{-1} corresponds to feces which had just 12.8% EIS. Given this value, even assuming the level in soil was at its maximum, such feces should only be expected to contain 6.2 mg kg^{-1} As. Since the concentration of As can be very high (perhaps >1%) in a small particle of iron plaque, consumption of only a small amount of plaque could potentially generate results with high As, Fe and Al, but apparently low soil ingestion levels, as we see here.

5. Conclusions

There is increasing evidence that the clean-up following the Aznalcóllar spill has been somewhat ineffective in relation to As (Simón et al., 2008). At the same time, Doñana is one of the most important protected areas for conservation and wildlife within Europe and the long-term impact of residual contamination here is yet to be fully addressed. Herein, we show that levels of As in soils/sediments in Natural Park areas (specifically the Entremuros) are <50 mg kg^{-1} at 0–5 cm depth. Given the limits for As levels in soil recently proposed by the USEPA (2005) in relation to protecting certain avian species (avian ground insectivores, 43 mg kg^{-1}; avian herbivores, 67 mg kg^{-1}), this is encouraging. However, areas north of the Entremuros, in protected "Green Corridor" zones designed to provide habitat for wildlife to reside in and migrate through, are polluted to levels above these thresholds. Likewise, the risk toward herbivores may not be solely or simply limited to bulk soil ingestion or ingestion of plants that have accumulated metals in above ground parts. Around 50% of roots analysed here, within and beyond Natural Park areas, had >50 mg kg^{-1} As associated with them. Levels reached >1000 mg kg^{-1} in thoroughly washed root samples and some of the As detected was in the form of arsenite. It is inevitable that certain species in this area will be exposed to this material as they feed.

Whilst more work is needed to investigate the potential food chain transfer of As (and a range of other metals) via iron plaque to herbivores, this could be ecotoxicologically significant in any wetland worldwide, where biogeochemical conditions favour the accumulation of iron plaque on macrophyte roots. The range of environments, and the range of species exposed to dietary root iron plaque is potentially quite broad. Cranes (Ma et al., 2003), snow geese (Belanger and Bedard, 1995), canvasbacks (Hohman et al., 1990), redheads (Mitchell et al., 1994), Bewick swans (Nolet, 2004), tundra swans (Nolet et al., 2001), trumpeter swans (LaMontagne et al., 2003), northern pintails (Ballard et al., 2004), Canada geese and common goldeneye (Seymour et al., 2002), and helmeted guinea fowl (Njiforti et al., 1998) are just some of the avian species which will feed on rhizome or bulb material in wetlands, as do the greylag geese and purple gallinule discussed herein (Viellard, 1974; Amat, 1995; Esselink et al., 1997; Madejón et al., 2006). Beyond avian species, red swamp crayfish (Gutierrez-Yurrita et al., 1998), muskrats (Campbell et al., 1998), black-tailed deer (Gillingham et al., 2000), and pre-historic (Kubiak-Martens, 2002) and modern humans (Sundriyal et al., 2004) are also known to consume this material. Perhaps certain species have adapted to consuming small volumes of concentrated metals associated with iron plaque in areas where sediments are not affected by anthropogenic metal pollution, but, where sediments are additionally polluted, the levels of metals accumulating on iron plaques may become extremely high, and this exposure route could be as yet unrecognised, but ecotoxicologically significant.

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