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LA MUSARANYA COMUNA,*Crocidura russula* (Hermann, 1780), COM A BIOINDICADOR EN ESTUDIS ECOTOXICOLÒGICS

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Memòria presentada per
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“*Nada hay más hermoso que la danza de un macizo de bambúes en la brisa. Ninguna coreografía humana tiene la euritmia de una rama que se dibuja sobre el cielo. Llego a preguntarme a veces si las formas superiores de la emoción estética no consistirán, simplemente, en un supremo entendimiento de lo creado. Un día, los hombres descubrirán un alfabeto secreto en los ojos de las calcedonias, en los pardos terciopelos de la falena, y entonces se sabrá con asombro que cada caracol manchado era, desde siempre, un poema*"

Alejo Carpentier

"*You see things; and you say, "Why?" But I dream things that never were; and I say, "Why not?*"

George Bernard Shaw

“…*Iba en mi bicicleta pensando en las musarañas*…”

Zoe Valdés

Després de tants anys des del començament d'aquest projecte, han estat multitud les vivències al llarg de la "fabricació" d'aquesta tesi. Moltes vegades m'he preguntat perquè tant de temps per acabar-la (si ara veient-la no sembla massa complicat fer-la...). Potser una de les principals raons ha estat digerir la informació que tenia i donar-li prou cos com per fer-la interessant pel món científic. Ja deia Confuci que arribar sense meditar és una ocupació inútil. Al llarg del desenvolupament d'un projecte de tesi, que molts cops prenem com "el més important de la nostra vida", et planteges i replanteges cada pas i, un cop acabada una part, la deixis madurar uns dies o setmanes per tornar a agafar-la i recomençar de nou amb alguna "idea nova", "prendrent la llibertat de plantejar qualsevol qüestió, dubtar de qualsevol afirmació i corregir errors " com va molt bé afirmar Oppenheimer. I ara ja està fet i només em cal agrair als que l'han fet possible i m'han acompanyat en aquest llarg i, alguns cops, farragós camí. Hi ha moltes persones a qui he d'agrair el temps, coneixements, suport i paciència dedicats per portar el que era un embrió d'estudi al seu final. Tampoc puc oblidar les entitats que amb el seu finançament han estat cabdals per a poder realitzar aquest treball. Em plantejo uns agraïments *sensu lato*. Espero no deixar-me a ningú.

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Segur que em deixo gent (l'edat no perdona, son milers de neurones que moren cada dia...) però no vull deixar d'agrair als microbiòlegs (a les micobacteriòlogues Marina, Esther, Silvia, Estela i Cris per demostrar-me cada dia que sou bona gent a més d'excel·lents professionals; als "cianobacteriòlegs" Joan, Marina, Mireia i Elia per fer-me riure i pensar i als "cossos-d'inclusiòlegs" Toni, Esther, Elena, Quim i Mònica per ser tant bons usuaris i bona gent), a l'IBB-team (merci Oscar, María, Luíses, Anna, Victor, Victoria, Maria), als veterinaris (gràcies Quim, María, Tufària, Sito, María, Mercedes, i, es clar, a la gran Carolina, por ser tan buena investigadora y cocinera) y a les investigadores de Vall d'Hebron Maite Martin porque nadie sabe como tú ser cínica y sutil y a María, Carmen, Cristina, Ricard, Bea, Cris y Elo por vuestra "intestinable" compañia y buen hacer microscopico. Y claro, a la Patiño y su trouppe de Bio Celular y a Jesús y su trouppe del Cebateg. I a Susanna, Marc, David, Isaac, Maria, Anna, Alba i tota la colla de bioquímics.

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"Fundada sota el signe de la magnanimitat,

la nostra casa será sense cap claudicació

fidel al seu alt destí. I coses glorioses

han estat, són i serán dites de tu, Alma Mater"

Salvador Espriu

En aquesta memòria de tesi es detalla la quantificació de biomarcadors d'exposició (acumulació de Pb, Hg, Cd, Tl, Fe, Mg, Zn, Cu, Mn, Mo, Co, Ni, Cr, Sr, Ba i B a teixits tous i durs) i d'efecte (morfometria, hematologia, activitat enzimàtica, genotoxicitat i histopatologia) en un total de 243 exemplars de musaranya comuna, *Crocidura russula* (Hermann, 1780). En el present estudi, es va tenir en compte la importància de l'edat i el sexe en els paràmetres quantificats ja que són dos factors biòtics que expliquen part de la variabilitat observada. Les zones d'estudi van ser 4 zones contaminades i 4 zones de referència triades per la seva importància tant mediambiental (6 de les 8 zones estudiades tenen estatus de protecció parcial o total) com d'interès per la societat: l'abocador de Garraf, el més gran de la península Ibèrica, la mina abandonada d'Aljustrel a la zona portuguesa de la faixa piritosa ibèrica, el Parc Nacional de Doñana afectat pel vessament de la mina de pirita d'Aznalcóllar, situada a la part espanyola de la faixa piritosa ibèrica i el Delta de l'Ebre afectat diverses activitats humanes incloent caça, industria i residus domèstics. Dels exemplars analitzats, 138 van ser capturats pròpiament per estudis ecotoxicològics a 3 de les zones contaminades (Garraf, Aljustrel i Doñana) i 105 eren mostres de col·lecció procedents d'altres estudis ecològics (Delta de l'Ebre). Especialment en les zones contaminades estudiades, es van observar marcades diferències en la bioacumulació entre elements essencials i no essencials per diferències en biodisponibilitat, toxicitat, i capacitat de transferència i biomagnificació al llarg de les cadenes tròfiques entre ambdós grups d'elements. Els majors augments detectats als teixits de les musaranyes de les zones contaminades es van donar entre els metalls pesants no essencials (Pb, Cd, Hg, Tl i Ni), els nivells tissulars dels quals no estan metabòlicament regulats a mamífers, donant com a resultat alts increments a teixits durs i tous quan augmenta la biodisponibilitat al medi ambient. Dels elements quantificats només Pb, Hg, Cd, i, possiblement Tl, Ni, B, Ba i Sr, van acumular-se fins a nivells que poden ser tòxics en mamífers. Ja que hi ha escassa informació sobre com afecta l'exposició crònica a les poblacions naturals d'insectívors, les dades conjuntes dels biomarcadors d'efecte obtingudes serveixen com valors de referència per a l'espècie i poden contribuir a estimar els efectes tòxics sobre els organismes. Es van detectar disminucions en la massa corporal i l'activitat de GST, i increments de massa i patologies atribuïbles a la contaminació a fetge i ronyó i freqüència de micronuclis en les musaranyes de les zones contaminades. Dels dos factors biòtics estudiats, l'edat va ser el més important ja que 10 dels elements, amb especial rellevància en el cas del Cd, van presentar patrons d'acumulació diferencials entre joves i adults. Per contra, mascles i femelles de musaranya comuna només van presentar petites diferències en l'acumulació de Pb, Hg, Ni, Mo, Mn, Co i Fe. Com amb els marcadors d'exposició, la rellevància de edat en els biomarcadors d'efecte va ser també major que la del sexe per explicar la variabilitat observada. L'ús de diversos biomarcadors d'exposició d'efecte permeten d'una banda obtenir una millor idea del conjunt d'efectes tòxics de la contaminació sobre el normal desenvolupament dels sistemes biològics, així com indiquen la idoneïtat de la musaranya comuna com a bioindicador de contaminació ambiental especialment per l'avaluació de risc ambiental a zones protegides i per les poblacions humanes.

Summary

In this report, a quantification of biomarkers of exposition (accumulation of Pb, Hg, Cd, Tl, Fe, Mg, Zn, Cu, Mn, Mo, Co, Ni, Cr, Sr, Ba and B in soft and hard tissues) and effect (morfometry, haematology, enzymatic activity, genotoxicity and histopathology) in a total of 243 specimens of the greater white-toothed shrew, *Crocidura russula* (Hermann, 1780) was found. In the present study, the age- and sex-dependent variations were also considered in order to explain the variability due to these important biotic factors. The study sites were 4 polluted sites and 4 reference sites selected both for their environmental relevance (6 out the 8 sites have granted partial or total protection status) and for the interest for the society: the landfill of Garraf, the bigger dump site in the Iberian Peninsula; the abandoned mine of Aljustrel in the Portuguese part of the Iberian Pyrite Belt (IPB); the National Park of Doñana affected by the wastes of the mine of Aznalcóllar, sited in the Spanish part of the IPB; and the Ebro Delta disturbed during decades by several human activities including hunting, industries and domestic wastes. A total of 138 shrews were captured for ecotoxicological studies in 3 polluted sites (Garraf, Aljustrel, and Doñana) and 105 specimens were captured for other ecological studies and stored in a zoological collection (Ebro Delta). Especially in the polluted sites studied, there were marked differences between the bioaccumulation patterns of essential and non-essential elements due to differences in the bioavailability, toxicity, and transfer and bioaccumulation throughout the food chains between the two groups of elements. The highest increases in the tissues of shrews from the polluted sites were on non-essential heavy metals (Pb, Cd, Hg, Tl, and Ni), without metabolic regulation of tissular levels in mammals that ends in high burdens in soft and hard tissues when bioavailability increase in the environment. Among elements quantified, only Pb, Hg, Cd, and, probably Tl, Ni, B, Ba and Sr, were accumulated till levels that may produce toxic effects in mammals. The scarce information of the effects of chronic exposure in natural populations of insectivores render more relevant the use of a battery of biomarkers of effect, that serve as reference values for the species and as assessment of toxic effects in organism. Decreases of body mass and GST activity and increases of mass and pathologies attributable to pollution in liver and kidneys were detected in shrews from the polluted sites. Among biotic factors, age was important because 10 of the elements quantified, with special remark on Cd, showed differential bioaccumulation patterns in juveniles and adults. In contrast, only slight differences in bioaccumulation of Pb, Hg, Ni, Mo, Mn, Co, and Fe were found between males and females. The same pattern of importance of age and sex as in the biomarker of exposure was found in the biomarkers of effect. The use of several biomarkers of exposure and effect provide information of the whole toxic effects of pollution in the normal function of biological systems and point out *C. russula* as a suitable bioindicator of environmental pollution, specially to evaluate environmental risk both in protected sites and human populations.

No presente relatório de tese detalha-se a quantificação de biomarcadores de exposição (acumulação de Pb, Hg, Cd, Tl, Fe, Mg, Zn, Cu, Mn, Mo, Co, Ni, Cr, Sr, Ba e B em tecidos molhes e duros) e de efeito (morfometria, hematologia, actividade enzimática, genotoxicidade e histopatologia) num total de 243 exemplares de musaranho comum, *Crocidura russula* (Hermann, 1780). No presente estudo, foi considerada a importância da idade e do sexo nos parâmetros quantificados ja que são dois fatores bióticos que explicam parte da variabilidade observada. As áreas de estudo foram 4 áreas poluidas e 4 de referência, escolhidas pela importância ambiental (6 das 8 áreas estudadas têm um estatuto de protecção parcial ou total) e também pelo interesse para a sociedade: o lixão de Garraf, o maior da Península Ibérica, a mina abandonada de Aljustrel na parte portuguesa da faixa piritosa ibérica, o Parque Nacional de Doñana afetado pelo vertido da mina de pirita de Aznalcóllar, situada na parte espanhola da faixa piritosa ibérica e o Delta do Ebro sob a influência de diversas atividades antropogénicas incluindo caça, indústria e residuos domésticos. Dos exemplares analisados, 138 foram utilizados nos estudos ecotoxicológicos em 3 das áreas poluídas (Garraf, Aljustrel e Doñana) e 105 eram amostras de colecção procedentes de outros estudos ecológicos (Delta do Ebro). Especialment nas áreas poluidas estudadas, observaram-se marcadas diferenças na bioacumulação entre elementos essenciais e não essenciais pelas diferenças na biodisponibilidade, toxicidade, e capacidade de transferencia e biomagnificação ao longo das cadeias tróficas entre os dois grupos de elementos. Os maiores aumentos detetados nos tecidos dos musaranhos das áreas poluidas reportam aos metais pesados não essenciais (Pb, Cd, Hg, Tl i Ni), os níveis tissulares dos quais não estam metabólicamente regulados em mamíferos, dando como resultado altos incrementos en tecidos duros e molhes quando aumenta a biodisponibilidade no meio ambiente. Dos elementos quantificados só Pb, Hg, Cd, e, possivelmente Tl, Ni, B, Ba e Sr, acumularam-se até niveis que podem ser tóxicos em mamíferos. Já que existe pouca informação de como exposição crônica afeta as populações naturais de insectívoros, os dados conjuntos de biomarcadores de efeito obtidos servem como valores de referencia para a espécie e podem contribuir para uma estimativa dos efeitos tóxicos nos organismos. Detectaram-se diminuições de massa corporal e de actividade da GST, e incrementos da massa e patologias atribuíveis à poluição em fígado e rim, bem como umaa presença de micronucleos nos musaranhos das áreas poluídas. Dos factores bióticos estudados, a idade foi a mais importante com especial relevancia do Cd, já que 10 dos elementos, apresentaram padrões de acumulação diferencial entre jovens e adultos. En contraposição, machos e fêmeas de musaranho comum só apresentaram pequenas diferenças na acumulação de Pb, Hg, Ni, Mo, Mn, Co e Fe. Tal como para os marcadores de exposição, a relevância da idade dos indivíduos nos biomarcadores de efeito foi também maior que a do sexo, para explicar a variabilidade observada. O uso de diversos biomarcadores de exposição e de efeito permitem por um lado obter uma melhor ideia do conjunto de efeitos tóxicos da poluição no desenvolvimento normal dos sistemas biológicos, assim como indicam a idoneidade do musaranho comum como bioindicador de poluição ambiental, com especial importância para a avaliação do risco ambiental em áreas protegidas e para as populações humanas.

1. INTRODUCCIÓ GENERAL

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Associats a l'augment de la població humana, degut a la revolució industrial del segle XIX i a l'expansió de la tecnologia al segle XX, s'han produït també efectes negatius, com ara la contaminació que afecta tant al medi ambient en general com a l'espècie humana en particular. Ja que a la contaminació ambiental se la considera responsable directament o indirectament de la mort prematura de fins un 50% dels humans (Pimentel et al., 2007), durant les darreres dècades s'ha intensificat l'avaluació de la toxicitat de milers de compostos que poden representar un important risc mediambiental i per la salut humana (Walker et al., 2004; De Laender et al., 2010) d'entre els, probablement, milions generats per les diverses activitats humanes. Indústries diverses com centrals energètiques, metal·lúrgiques i petroquímiques, activitats com la mineria o la ramaderia, així com la utilització de combustibles fòssils, els dipòsits d'acumulació de residus (domèstics, industrials, radioactius) o la seva incineració, i l'abocament d'aigües residuals, emeten diàriament al medi ambient milions de tones de residus sòlids, líquids i gasosos. Aquests productes contaminen aigües, sòls i aire i poden tenir una gran influència durant llargs períodes a tots nivells sobre la biota, afectant des de nivell molecular fins a individus, poblacions o ecosistemes (Propst et al., 1999; Chassovnikarova et al., 2005).

De tots els contaminants emesos al medi, un dels grups més estudiats per la seva alta toxicitat, persistència al medi i àmplia distribució és el dels metalls, i en especial el dels metalls pesants. Sota aquesta denominació de metall pesant s'associen d'una forma no sempre clara una sèrie d'elements metàl·lics amb pes específic superior a 5 g/cm^3 o que tenen un número atòmic per sobre de 20 i excloent generalment als elements alcalins i alcalinoterris. Entre els metalls pesants es troben elements no essencials per a la vida i altament tòxics pels sistemes biològics com plom (Pb), mercuri (Hg), cadmi (Cd) o tal·li (Tl). Aquests elements, a més de produir efectes tòxics a baixes concentracions, estan àmpliament distribuïts, poden estar sotmesos a fenòmens de biomagnificació al llarg de les cadenes tròfiques (Mason & Wren, 2001). De molts d'ells, com ara el Pb, Hg i Cd, estan ben documentats els casos d'intoxicacions en poblacions humanes (són responsables del saturnisme o plumbisme i de les síndromes de Minamata i Itai Itai, respectivament), que poden ocasionar greus trastorns i fins i tot la mort. En contraposició, hi ha tota una sèrie de metalls pesants i lleugers essencials per al correcte funcionament del metabolisme, com el zenc (Zn), coure (Cu), ferro (Fe), crom (Cr), manganès (Mn) o molibdè (Mo) que, en general, presenten toxicitats menors i la seva concentració als teixits pot ser regulada metabòlicament. A més, alguns metal·loides o semimetalls com bor (B), arsènic (As) i antimòni (Sb) també tenen importància en ecotoxicologia per ser part de diversos processos industrials i poder produir efectes tòxics a baixes concentracions en els organismes. La presència de metalls i metal·loides en el medi ambient va en augment a causa, especialment, d'activitats industrials diverses com les extraccions mineres, les papereres o les metal·lúrgiques. Com a exemple, només la producció minera de 1930 a 1985 va augmentar 7, 4, 2, 5, 8, 2,15, 18 i 35 cops l'entrada al medi ambient de Mg, Zn, Pb, Cu, Mn, Hg, Cd, Cr i Ni (Nriagu, 1988). En general, la capacitat dels metalls de produir efectes tòxics procedeix de la inducció d'estrès oxidatiu per la formació d'espècies reactives d'oxigen (ROS), també anomenades radicals lliures, principalment via reacció de Fenton (Jadhav et al., 2007; Sánchez-Chardi et al., 2008; Włostowski et al., 2008). Les ROS danyen lípids de membrana, proteïnes amb grups tiol i àcids nucleics (Chwełatiuk et al., 2005; Jadhav et al., 2007) i produeixen estrés oxidatiu en les cèl·lules quan els sistemes antioxidants de defensa no poden eliminar-les. Aquests sistemes de defensa, formats per diversos enzims, com glutatió peroxidasa (GPx), glutatió reductasa (GR), glutatió-S-transferasa (GST), superòxid dismutasa (SOD), catalasa i vitamina E, prevenen l'estrès oxidatiu i la seva activitat pot ser alterada per la presència de metalls com Pb, Cd, Cr i Fe (Jadhav et al., 2007). Aquesta producció de ROS pot generar efectes adversos a diversos nivells i afectar virtualment tots els components dels sistemes biològics, p. ex. destrucció de membranes a mitocondris, fenòmens de necrosi i apoptosi (Pereira et al., 2006; Salińska et al., 2012, 2013; Tersago et al., 2004; Tovar-Sánchez et al., 2006, 2012) o de genotoxicitat (Ieradi et al., 1996; Smith & Rongstad, 1982; Topashka-Ancheva et al., 2003), a més d'alteracions en paràmetres morfològics (Laurinolli & Bendell-Young, 1996; Ma, 1989, 1996; Nunes et al., 2001).

Per tal de conèixer els efectes de contaminants persistents i tant àmpliament distribuïts, com els metalls pesants, sobre el medi natural i, molt especialment, sobre la salut humana, en les últimes dècades s'han engegat multitud de treballs ecotoxicològics. Aquests estudis s'han basat en les dades obtingudes en la recerca clàssica de toxicologia amb animals de laboratori, sota condicions controlades i amb individus d'espècies seleccionades (principalment rates i ratolins) en condicions òptimes de temperatura, fotoperíode i alimentació, baixes densitats i amb poca variabilitat genètica. Aquesta situació "ideal" permet obtenir abundants dades de mutagenicitat, teratogenicitat i carcinogènesi, dóna informació sobre el comportament del contaminant en un sistema biològic (dels òrgans i teixits diana i de les rutes metabòliques de transformació o excreció), permet també relacionar l'acumulació del metall amb els seus efectes tòxics (p.ex. per establir el NOAEL: "No Observable Adverse Effects Level" o el LOAEL: "Low Observable Adverse Effects Level"), i ajuda a conèixer els paràmetres marcadors més adients o les diferències en la toxicitat segons variables com l'espècie i el temps (crònic o agut) o la via d'exposició (dèrmica, ingesta, inhalació). Al laboratori, però, es controlen totes les variables menys les estudiades i generalment s'exposa a l'organisme a un sol contaminant (Blanuša et al., 1989; Salińska et al., 2012, 2013; Włostowski et al., 2003, 2008) o, menys freqüentment, s'avaluen dos o més contaminants o barreges complexes de substàncies potencialment tòxiques (Bellés et al., 2002; Jadhav et al., 2007), d'aquesta manera s'obté informació valuosa però parcial sobre els efectes a diferents nivells (de molecular a individual) dels contaminants estudiats. No sempre les dades obtingudes poden ser extrapolables al que succeeix al medi ambient en condicions de "món real" (Klok & Thissen, 2009) ja que en condicions realistes, altament dinàmiques, també s'han d'avaluar altres variables com els efectes sinèrgics o antagònics dels contaminants, a altres nivells d'organització com poblacions i ecosistemes o les variacions temporals i espaials a les zones contaminades. De fet, les dades empíriques obtingudes a laboratori serveixen de base per a la interpretació de les obtingudes en estudis de camp, on els efectes dels contaminants sotmesos a condicions extremadament dinàmiques, com passa als ecosistemes, fan que els estudis experimentals, massa estàtics, siguin una referència i calgui fer seguiments en el temps i en condicions naturals per avaluar els mateixos i altres paràmetres a nivell individual o poblacional, com la densitat i l'estructura poblacionals, o la condició física i l'esperança de vida, així com la variació temporal dels contaminants (Klok & Thissen, 2009; Wijnhoven et al., 2008). En aquestes condicions naturals, on sovint els individus estan exposats a dosis baixes d'una barreja de productes potencialment tòxics durant llargs períodes de la seva vida, es fan imprescindibles tant l'obtenció de valors de referència per diverses espècies i paràmetres com el monitoratge dels contaminants per conèixer el seu comportament al medi natural.

Als ecosistemes, un augment de la disponibilitat del contaminant que es pugui transferir al llarg de la cadena tròfica i acumular-se en plantes i animals generalment provoca l'aparició d'efectes tòxics depenent no només de l'acumulació, sinó també de la seva forma química, de la via i el temps d'exposició, de fenòmens creuats entre diferents compostos degut a interaccions que produeixen antagonisme o sinergisme (Goyer, 1997), o de la tolerància inter- i intra-específica, entre d'altres factors. La toxicitat d'aquests elements a nivell intra-específic pot variar molt degut a la seva biodisponibilitat que vindrà determinada per múltiples factors abiòtics i biòtics. Com a factors abiòtics es poden destacar la forma química en què es troben els metalls (en general, les formes orgàniques són les més tòxiques per ser les que millor s'integren a les xarxes tròfiques) i les característiques físico-químiques dels sòls i les aigües, que determinaran la disponibilitat pels organismes i, per tant, la dispersió al llarg de les cadenes tròfiques. Entre els múltiples factors a nivell biòtic (com estat reproductor, emigració, immigració, predació, estacionalitat), dos dels factors més importants que interfereixen en els patrons d'acumulació i en els efectes tòxics són el sexe i l'edat (Gochfeld, 2007; González et al, 2008; Komarnicki, 2000; Lopes et al., 2002; Pankakoski et al., 1993, 1994; Prospt et al., 1999; Smith & Rongstad 1982; Vahter et al., 2007).

El primer pas per fer un diagnòstic de la qualitat del medi ambient sol ser la realització d'anàlisis físico-químiques d'aigua i sòl (Quevauviller et al., 1989; Mañosa et al., 2001; Turner et al., 2008). Tot i que aquests compartiments abiòtics donen molta informació, aquesta informació no és suficient per saber la difusió dels contaminants al llarg de les cadenes tròfiques, així com els efectes sobre la biota, i per tant, cal treballar amb sistemes complexos com els vertebrats. Per conèixer el comportament dels contaminants i la toxicitat sobre els organismes, cal conèixer quines són les espècies més sensibles (bioindicadors) i quins paràmetres (biomarcadors) es veuran afectats pels contaminants. Quan un organisme d'una població natural es veu exposat a l'estrès produït pels contaminants reacciona de diferents formes intentant protegir-se dels seus efectes tòxics, per exemple immobilitzant, excretant o biotransformant el contaminant. En les últimes dècades, s'han emprat multitud d'espècies de bacteris, fongs, líquens, vegetals o animals com a possibles indicadors de qualitat ambiental. Per tal que una espècie sigui considerada un bon bioindicador de contaminació cal que; (i) subministri un avís ràpid per la resposta natural a les pertorbacions ambientals cròniques o agudes directament (Marshall et al., 1993; Noss, 1990), doncs així s'indica la causa del canvi abans que simplement l'existència del canvi: p.ex. mesurant fecunditat i supervivència més que fer simples mesures d'abundància (Herricks & Schaeffer 1985); (ii) proveeixi una avaluació contínua sobre un ampli rang i intensitat d'estrès que permeti detectar nombrosos impactes a l'ecosistema (O'Connell et al., 1998; Woodley, 1996); i/o (iii) que el cost-benefici de la mesura sigui favorable per poder fer més extensiu els programes de biomonitoratge (Davis, 1989; di Castri et al., 1992; Kriesel, 1984).

Enfront d'un impacte per contaminació, les poblacions silvestres poden desaparèixer, no veure's afectades o sobreviure a aquesta alteració del medi després d'una adaptació més o menys ràpida, depenent tant del tipus i durada de l'exposició com de la sensibilitat de l'espècie enfront els canvis al medi. Entre els organismes emprats com a bioindicadors, els petits mamífers han estat sovint testats en recerca mediambiental com a avaluadors de risc doncs juguen un paper fonamental com a part de diverses cadenes alimentàries, ocupant diferents posicions a l'escala tròfica, i són crucials en la transferència de contaminants persistents cap a ocells i mamífers carnívors dels que són part important de la dieta (Van der Brink et al., 2003; Wijnhoven et al., 2007). Amb més de 370 espècies descrites, l'ordre *Soricomorpha*, antigament inclòs dins d'un ordre més ampli, *Insectivora*, comprèn musaranyes, talps i eriçons i constitueix el tercer grup més nombrós de mamífers, després dels rosegadors i quiròpters. Entre els petits mamífers, els insectívors tenen un risc d'intoxicació per metalls major que els rosegadors (D'Havé et al., 2006; Shore & Douben, 1994; Talmage & Walton, 1991) degut a que es troben més alt a l'escala tròfica i estan exposats als metalls provinents directament de les seves preses (la principal via d'exposició de contaminants a mamífers és a través de la dieta) o bé indirectament per ingestió de sòl (Ma & Talmage, 2001). Específicament, les musaranyes són un model mamífer més proper al model humà que altres grups, i posseeixen característiques que les fan molt adients per a aquest estudis ecotoxicològics (Beardsley et al., 1978; Damek-Poprawa & Sawicka-Kapusta, 2004; D'Havé et al., 2006; Ma & Talmage, 2001; Talmage & Walton, 1991). Cal destacar que en general els insectívors, per una banda, no fan grans migracions i el seu domini vital és relativament petit per la qual cosa poden ser un reflex de la contaminació a nivell local, i, per altra banda, són abundants, àmpliament distribuïts a tota mena d'ambients, fàcilment capturables i identificables. A més, tenen unes característiques fisiològiques, com un metabolisme basal molt alt, que els obliga a ingerir gran quantitat d'aliment, fent-los òptims acumuladors de substàncies potencialment tòxiques presents en poca quantitat al medi ambient. Algunes d'aquestes característiques els fan millors bioindicadors que, p.ex., les aus i els mamífers mitjans i grans, que són menys abundants i tenen un domini vital major i menor taxa metabòlica.

Les musaranyes componen la família dels Sorícids, la més nombrosa de l'ordre, i estan àmpliament distribuïdes a ambients tropicals, temperats i freds de tot l'hemisferi nord (Europa, Àsia, Amèrica del Nord i Central i la part Nord de Sudamèrica) i Àfrica. Les més de 260 espècies descrites es divideixen en dues subfamílies: els soricins i els crocidurins, ambdós grups representats a les nostres latituds. Els soricins, o musaranyes de dents vermelles, són espècies de zones més fredes, de mida petita, estrategs de la R i amb un metabolisme basal i requeriments energètics alts. Contràriament, els crocidurins, o musaranyes de dents blanques, són espècies de zones més càlides, de mida més gran, estrategs de la K (amb menys cries per part i un període reproductor més llarg), solen viure en grups familiars (menys territorials), i si bé tenen un metabolisme basal alt és més baix que el dels soricins. De les espècies de crocidurins existents a la península Ibèrica, la més àmpliament distribuïda per diversos hàbitats i que presenta les més altes densitats és la musaranya comuna, *Crocidura russula* (Hermann, 1780) (Gosálbez, 1987). Al contrari de l'altre gènere de crocidurí present a la península, *Suncus*, *C. russula* és de mida més gran, més fàcilment capturable i relativament abundant ja que habita ambients típicament mediterranis, tant a zones de bosc d'alzines, suredes o pinedes, com a màquies i vores de camps de conreus, i presenta un cert grau d'antropofilia (Figura 1.1). Les altres espècies d'insectívors ibèrics o estan confinades a zones fredes (és el cas dels gèneres *Sorex* i *Neomys*) o són menys abundants (com els gèneres *Talpa, Erinaceus* i *Aethechinus*) que la musaranya comuna. Això fa que *C. russula* sigui, *a priori*, la millor espèciede mamífer insectívor per ser avaluada com a bioindicador. El gènere *Crocidura*, amb més de 100 espècies descrites, és el més abundant i està àmpliament distribuït per Euràsia i Àfrica (Gosálbez, 1987; Ramalhinho et al., 1987; Talmage & Walton, 1991). Tot i aquesta abundància, diversitat i àmplia distribució, hi ha un buit d'informació ja que s'han

Figura 1.1. Imatges d'exemplars adults de musaraña comuna, *Crocidura russula*.

realitzat pocs estudis ecotoxicològics que avaluïn espècies d'aquest grup (Ma & Talmage, 2001). Els treballs realitzats a Europa amb insectívors han inclòs espècies dels gèneres *Talpa*, *Erinaceus* i *Sorex* (D'Have et al., 2006; Komarnicki, 2000; Pankakoski et al., 1993, 1994), però només recentment han avaluat *C. russula* (González et al., 2008; Fernández et al., 2012; Fritsch et al., 2010, 2011; Sánchez-Chardi & Nadal, 2007; Sánchez-Chardi et al., 2007a,b, 2008, 2009a,b). Aquesta espècie, situada al bell mig de la cadena tròfica, pot donar una idea de la biomagnificació que pot estar succeint al medi natural, per la incorporació selectiva de molts compostos a l'organisme i els subseqüents efectes sobre macromolècules, cèl·lules, teixits, individus i poblacions. Aquesta biomagnificació, a més de posar de manifest un tòxic que està en baixes concentracions, pot produir un augment dels efectes negatius sobre els organismes, com pot ser una menor fertilitat o major morbiditat o mortalitat.

El biomonitoratge, o ús d'organismes per a obtenir dades, preferentment quantitatives, de certes característiques de la biosfera (Wolterbeek 2002), proveeix informació ecotoxicològica rellevant dels canvis en paràmetres fisiològics, ecològics, morfològics, genètics, entre d'altres, o en la concentració de substàncies als teixits. Entre la multitud de biomarcadors d'efecte o exposició (Hamers et al., 2002; Peakall, 1992), alguns paràmetres morfomètrics (Laurinolli & Bendell-Young, 1996; Ma, 1989, 1996; Nunes et al., 2001; Tête et al., 2013), hemàtics (Hamers et al., 2002; Nunes et al., 2001; Reynolds et al., 2006), enzimàtics (Lopes et al., 2002; Reynolds et al., 2006; Sánchez-Chardi et al., 2008; Świergosz-Kowalewska et al., 2006), de genotoxicitat (Meier et al., 1999; Prospt et al., 1999; Topashka-Ancheva et al., 2003) i histològics (Damek-Poprawa & Sawicka-Kapusta, 2003, 2004; Pereira et al., 2006; Salińska et al., 2012, 2013; Sánchez-Chardi et al., 2009a,b) han mostrat la seva utilitat en els estudis ecotoxicològics ja que són sensibles als canvis del medi produïts per la contaminació, tant a animals de laboratori com a poblacions silvestres. La quantificació de contaminants com els metalls pesants en teixits és rellevant per conèixer l'exposició i la possible toxicitat (Hamers et al., 2002; D'Havé et al., 2006; Smith & Rongstad, 1982). Un disseny experimental que combini aquests paràmetres i la comparació amb els valors d'acumulació de contaminants, com els metalls pesants, donarà informació valuosa tant dels nivells de contaminants que poden ser potencialment tòxics per l'individu com de la resposta o dany fisiològic que pot afectar el normal desenvolupament de les funcions metabòliques (Chassovnikarova et al., 2005; Hamers et al., 2002; Nunes et al., 2001; Pereira et al., 2006; Reynolds et al., 2006; Topashka-Ancheva et al., 2003).

La present memòria pretén valorar la possible utilització de la musaranya comuna com a bioindicador de contaminació ambiental a clima mediterrani. Per a això s'han analitzat exemplars exposats a 4 tipus diferents de contaminació (Figura 1.2): tres que provenen principalment d'una única font de contaminació (zones de Garraf, Alentejo i Doñana) i un de contaminació deguda a múltiples fonts contaminants (zona del Delta de l'Ebre). S'ha considerat que totes les poblacions de musaranyes estu-

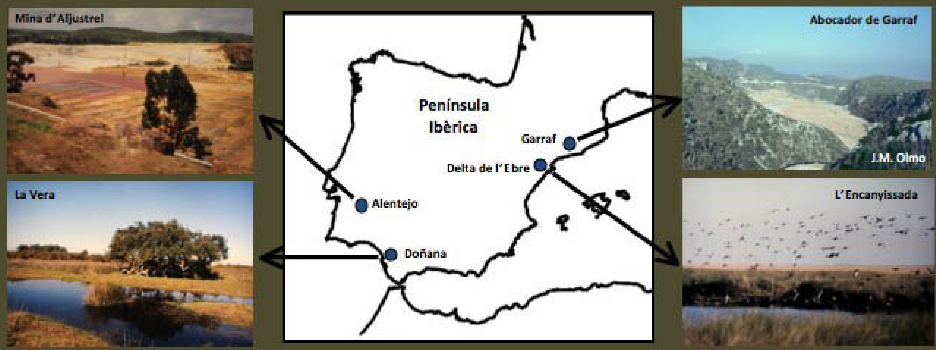

Figura 1.2. Mapa i imatges dels llocs de captura.

diades estaven sotmeses a una exposició crònica als contaminants, des de feia vàries generacions, amb l'excepció de Doñana on un episodi de gran contaminació, assimilable a una exposició aguda, va impactar les poblacions només un any i mig abans de la presa de mostres. En aquest darrer cas, els animals capturats eren tots nascuts després de l'episodi contaminant però pertanyen a les primeres generacions, per la qual cosa el temps des de la gran entrada de metalls es menor que el de les altres zones estudiades i insuficient per aconseguir que els individus i les poblacions s'adaptin a aquests canvis ambientals.

L'abocador de Garraf (Baix Llobregat, Barcelona) és el major abocador espanyol ja que ha estat periòdicament ampliat fins a acumular, segons dades de l'any 2002, més de 22 milions de tones de residus i rep anualment unes 550.000 tones de residus, la gran majoria d'origen domèstic i, en menor mesura, residus industrials i llots de depuradora. Creat al 1972 i clausurat a finals de 2006, l'abocador es troba situat al mig de la zona protegida del massís del Garraf i ocupa una extensió de 64 ha. Els lixiviats produïts son barreges complexes que varien molt segons l'edat i la naturalesa dels residus, entre d'altres, i que contenen una gran quantitat de compostos orgànics i inorgànics potencialment tòxics, com ara ftalats, hidrocarburs aromàtics policíclics (PAH) i alifàtics clorats, fenols i compostos nitrogenats i metalls pesants com Hg, Cd, Cr, Cu, Pb, Ni i Zn (veure referències a Sánchez-Chardi & Nadal, 2007). El potencial de contaminar el medi d'un abocador de residus depèn del procés de degradació de les deixalles (Butt & Oduyemi, 2003). Les dades de *C. russula* de la zona de l'abocador del Garraf es presenten als capítols 4.1 i 4.2. Complementari a aquests treballs, es presenta un estudi ecotoxicològic de ratolí de bosc, *Apodemus sylvaticus*, afectat pels lixiviats del mateix abocador i simpàtrica amb la musaranya comuna objecte d'aquesta tesi (veure Annex 2).

Les mines abandonades, principalment per raons econòmiques, representen una font important de contaminació a àmplies regions ja que els metalls lixivien a l'exterior i persisteixen durant llargs períodes al medi ambient. La mina d'Aljustrel (Alentejo, Portugal) és un bon exemple d'aquesta problemàtica: situada a l'anomenada faixa piritosa ibèrica que comprèn el sud de Portugal i d'Espanya, on algunes mines han estat explotades des del temps dels romans (Tovar-Sánchez et al., 2006), va ser abandonada al 1996 per raons econòmiques i sense cap mesura pal·liativa per reduir o evitar la sortida de metalls al medi. Situada a la mateixa faixa, la mina de pirita d'Aznalcóllar (Sevilla, Espanya), presenta una problemàtica mediambiental diferent. A causa del trencament accidental de la seva bassa de residus, a l'abril de 1998, es van abocar més de 5 milions de metres cúbics de llots carregats de metalls pesants i aigües a pH àcids que van afectar l'àrea protegida de Doñana i va provocar un dels majors desastres ecològics a Europa. Des d'ençà, s`han intensificat els treballs de biomonitoratge de la contaminació a llarg termini a aquesta àrea humida protegida, la primera creada a l'estat espanyol (que actualment inclou 104.970 ha protegides sota les figures de Parque Nacional (amb la Reserva Biológica) i Parque Natural de Doñana i una de les més importants del sud-est europeu, que conserva una gran diversitat de biòtops i importants poblacions d'espècies emblemàtiques i en perill d'extinció, com el linx ibèric (*Lynx pardina*) i l'àliga imperial (*Aquila adalberti*). Les dades de *C. russula* de la zona de la mina d'Aljustrel es presenten als capítols 4.3 i 4.4 i les de la zona de Doñana es presenten als capítols 4.5 i 4.6. Complementari a aquests treballs, es presenta un estudi ecotoxicològic de musaranya comuna afectada per la mina abandonada de plom i zenc de Preguiça a Portugal (veure Annex 2).

Tal com a d'altres zones deltaiques de països desenvolupats, a la zona del Delta de l'Ebre s'ha produït una reducció important de superfície deltaica i dels hàbitats típics, així com dessecament de zones humides i contaminació antròpica de diverses fonts. Aquest delta va ser declarat parcialment protegit (Parc Natural del Delta de l'Ebre) l'any 1983 i ampliat posteriorment al 1986 fins els 320 km^2 actuals, constituint la més important zona d'aquest tipus a Catalunya i, junt amb Doñana i La Camarga, una de les més importants del sud-est europeu. Com moltes zones humides, el Delta de l'Ebre ha estat tradicionalment molt transformat per l'home, amb la utilització dels sòls per conreus (principalment arròs), de l'aigua per rec i abastament humà i dels recursos piscícoles. A més, indústries de tot tipus (incloent una planta de clor i indústries petroquímiques) aboquen les seves aigües residuals al llarg del riu Ebre, que es sumen als abocaments d'aigües residuals urbanes. La caça ha estat també font important de pressió humana al Delta de l'Ebre, el que ha significat l'acumulació de perdigons de plom que, en algunes zones com l'Estany de l'Encanyissada, ha arribat a quantitats de 266 perdigons/m^2 (Mateo et al., 1997; Mañosa et al., 2001), una de les més altes a nivell mundial. Es calcula que al Delta de l'Ebre moren anualment milers d'aus a causa del plumbisme (Mateo et al., 1998).). Les dades de *C. russula* de la zona del Delta de l'Ebre es presenten als capítols 4.7 i 4.8.

2. OBJECTIUS

2. OBJECTIUS

Els objectius principals del present treball són:

1. Quantificar l'acumulació de metalls i metal·loides a musaranya comuna, *C. russula,* com a biomarcadors d'exposició per conèixer la qualitat del medi ambient i la disponibilitat d'aquests elements potencialment tòxics

2. Quantificar diversos paràmetres morfomètrics, histològics, hemàtics, enzimàtics i de genotoxicitat a la mateixa espècie com a biomarcadors d'efecte per conèixer la seva idoneïtat en estudis ecotoxicològics

3. Avaluar la contribució de dos importants factors biòtics (sexe i edat) en la variabilitat observada en aquests paràmetres.

4. Avaluar si la musaranya comuna pot ser una bona bioindicadora de contaminació ambiental, especialment a zones protegides de clima Mediterrani.

3. INFORME DELS DIRECTORS

3. INFORME DELS DIRECTORS

El doctorand Alejandro Sánchez-Chardi presenta a la seva tesi doctoral un total de 8 articles com a primer autor i 2 més inclosos a annexos (un d'ells també com a primer autor) publicats en revistes científiques internacionals incloses al primer quartil de *Environmental Sciences* al *Science Citation Index* (*Environmental Pollution*, *Chemosphere*, *Science of the Total Environment* i *Environmental Research*) que acumulen actualment al voltant de 90 cites sense incloure autocites. Passem a detallar la informació dels 8 articles presentats com a cos de la tesi amb el factor d'impacte de les revistes en els anys de publicació segons el *Thompson Institute for Scientific Information*, així com la contribució científica del doctorand en cadascun dels 7 articles en coautoria.

Sánchez-Chardi A, Nadal J, 2007. Bioaccumulatiorn of metals and effects of a landfill in small mammals. Part I. The greater white-toothed shrew, *Crocidura russula*. Chemosphere 68(4), 703-711. IF (2007): 2.739

Disseny del treball: **ASC**, JN
Mostreig i anàlisi de mostres: **ASC**
Anàlisi de resultats: **ASC**
Redacció del manuscrit: **ASC**

Sánchez-Chardi A, Peñarroja-Matutano C, Borrás M, Nadal J, 2009. Bioaccumulation of metals and effects of a landfill in small mammals. Part III. Structural alterations. Environmental Research 109(8), 960-967. IF (2009): 3.237

Disseny del treball: **ASC**, JN
Mostreig i anàlisi de mostres: **ASC**, CPM
Anàlisi de resultats: **ASC**
Redacció del manuscrit: **ASC**

Sánchez-Chardi A, Marques CC, Nadal J, Mathias ML, 2007. Metal bioaccumulation in the greater white-toothed shrew, *Crocidura russula*, inhabiting an abandoned pyrite mine site. Chemosphere 67 (1), 121-130. IF (2007): 2.739

Disseny del treball: **ASC**, JN, MLM
Mostreig i anàlisi de mostres: **ASC**, CCM
Anàlisi de resultats: **ASC**
Redacció del manuscrit: **ASC**, MLM, CCM

Sánchez-Chardi A, Marques CC, Gabriel SI, Capela-Silva F, Cabrita AS, López-Fuster MJ, Nadal J, Mathias ML, 2008. Haematology, genotoxicity, enzymatic activity and histopathology as biomarkers of metal pollution in the *shrew Crocidura russula*. Environmental Pollution 156(3), 1332-1339. IF (2008): 3.135

Disseny del treball: **ASC**, JN, MLM

Mostreig i anàlisi de mostres: **ASC**, CCM, SIG, FCS, ASC

Anàlisi de resultats: **ASC**, SIG, CCM

Redacció del manuscrit: **ASC**, SIG, MLM, MJLF

Sánchez-Chardi A, 2007. Tissue, age, and sex distribution of thallium in shrews from Doñana, a protected area in SW Spain. Science of the Total Environment 383(1-3): 237-240. IF (2007): 2.182

Sánchez-Chardi A, Oliveira Ribeiro CA, Nadal J, 2009. Metals in liver and kidneys and the effects of chronic exposure to pyrite mine pollution in the shrew *Crocidura russula* inhabiting the protected wetland of Doñana. Chemosphere 76(3), 387-394. IF (2009): 3.253

Disseny del treball: **ASC**, JN

Mostreig i anàlisi de mostres: **ASC**, CAOR

Anàlisi de resultats: **ASC**, CAOR

Redacció del manuscrit: **ASC**, CAOR

Sánchez-Chardi A, López-Fuster MJ, Nadal J, 2007. Bioaccumulation of lead, mercury, and cadmium in the greater white-toothed shrew, *Crocidura russula*, in the Ebro Delta (NE Spain): sex- and age-dependent variation. Environmental Pollution 145(1), 7-14. IF (2007): 3.135

Disseny del treball: **ASC**, MJLF, JN

Mostreig i anàlisi de mostres: **ASC**, MJLF

Anàlisi de resultats: **ASC**, MJLF

Redacció del manuscrit: **ASC**, MJLF

Sánchez-Chardi A, López-Fuster MJ, 2009. Metal and metalloid accumulation in shrews (Soricomorpha, Mammalia) from two protected Mediterranean coastal sites. Environmental Pollution 157(4), 1243-1248. IF (2009): 3.426

Disseny del treball: **ASC**, MJLF

Mostreig i anàlisi de mostres: **ASC**, MJLF

Anàlisi de resultats: **ASC**, MJLF

Redacció del manuscrit: **ASC**, MJLF

Igualment informem que els dos articles de la mina de pirita de Aljustrel (Capítols 4.3 i 4.4) han estat emprats sense autorització explícita a la tesi doctoral de C.C. Marques, mentre que els altres 6 treballs no han estat emprats implícitament o explícitament per cap dels coautors en l'elaboració de la seva pròpia tesi doctoral.

Barcelona, a 20 de Setembre de 2013

Director	Directora
Jacint Nadal Puigdefábregas	**María José López Fuster**
Universitat de Barcelona	Universitat de Barcelona

4. CAPÍTOLS

4.1. GARRAF I

Bioaccumulation, morphometry and genotoxicity in the shrew *Crocidura russula* exposed to landfill leachates

Bioaccumulation of metals and effects of a landfill in small mammals. Part I. The greater white-toothed shrew, *Crocidura russula*

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Sánchez-Chardi A, Nadal J, 2007. Bioaccumulatiorn of metals and effects of a landfill in small mammals. Part I. The greater white-toothed shrew, *Crocidura russula*. Chemosphere 68(4), 703-711.

Abstract

Here we quantified the bioaccumulation of metals (lead, mercury, cadmium, iron, magnesium, zinc, copper, manganese, molybdenum, and chromium) and assessed several morphometrical (RI, relative weights) and genotoxic (MNT) parameters as biomarkers of pollution from the landfill of Garraf (Barcelona, NE Spain). Specimens of *C. russula*, (Soricomorpha, Mammalia) from the landfill site showed increased Pb, Cd, Mg, Zn, Cu, and Cr concentrations in their tissues. Levels of mercury were below detection limits. Cd, Pb and Cr varied significantly by age, whereas no significant differences were found in metal bioaccumulation between males and females. While no differences were found in morphometrical parameters between shrews from the two sites, those from the polluted one showed more micronuclei in blood than those from reference site (1.786±0.272 *vs* 0.088±0.045 ‰; U=46.000, p<0.001). The considerable amounts of potentially toxic metals (Pb till 59.71 and Cd till 56.57 µg/g DW in kidneys) and the genotoxic effects indicate the harmful effect on biota. We consider necessary biomonitoring this landfill sited in a partially protected area.

4.1.1. Introduction

Landfills are the main form of solid waste accumulations in several regions, including Mediterranean countries (Loukidou and Zouboulis, 2001). However, if these sites are not adequately controlled they may have a severe environmental impact. Gaseous compounds, mainly methane and carbon dioxide, as well as volatile organic compounds (VOCs) are common pollutants of landfills (Christensen et al., 2001; Li et al., 2006; Wichmann et al, 2006). Also, liquid effluents named leachates are produced by the decomposition of wastes or by interaction between wastes and rain water, and are often a considerable source of contamination for groundwater aquifers as well as for adjacent soil and surface waters. Leachate composition, volume and toxicity vary depending on the nature and age of wastes, method of disposal, dump depth and climatic factors (De Rosa et al., 1996; Li et al., 2006; Thomas et al., 2009). These effluents often contain a wide variety of organic and inorganic pollutants including metals such as Pb, Cd, Mg, Fe, Zn, Cu, Mn, Mo, and Cr (Christensen et al., 2001; De Rosa et al., 1996; Johnson et al., 1996; Ragle et al., 1995; Slack et al., 2005). These mixtures are highly toxic for biota (e.g. Cabrera and Rodriguez, 1999; Cheung et al., 1993; Sang and Li, 2004), however, information on the effects of landfill leachates on wildlife health is scarce. Genotoxic effects such as increases in micronuclei (MN), in chromosomal aberrations, and in abnormal sperm morphology frequencies, have been reported in laboratory rodents exposed to landfill leachates (Bakare et al., 2005; Li et al., 2006) and in wild mice from a hazardous waste site (e.g. Tull-Singleton et al., 1994).

Morphometrical parameters are used as markers of physiological alterations in wild populations of small mammals exposed to some kinds of pollution (e.g. Ma, 1996; Ma and Talmage, 2001; Sánchez-Chardi et al., 2007a,b). In addition, some heavy metals have varied genotoxic effects because they may damage and induce mutations in DNA (Eisler, 1985, 1986; Palus et al., 2003; Seoane and Dulout, 2001). The micronucleus test (MNT) is an easy and quick method to obtain information about the clastogenic effect of xenobiotics and other environmental pollutants on wild mammals (e.g. Ieradi et al., 1996; Meier et al, 1999).

Like other insectivorous species (e.g. Komarnicki, 2000; Talmage and Walton, 1991), the greater white-toothed shrew, *Crocidura russula*, has been used as bioindicator of the bioaccumulation of metals and the effects of pollution (Sánchez-Chardi et al., 2007a,b). To our knowledge, the study presented here is the first to assess the accumulation of metals and effects of landfill pollution on insectivorous mammals.

The objectives of this study were: i) to quantify the concentrations of heavy metals in *C. russula* exposed to leachates from a landfill; ii) to evaluate the effects of this kind of pollution on the basis of several morphometrical and genotoxic parameters; iii) to identify the influence of sex and age as source of intrapopulation variation; and iv) to assess the environmental consequences of landfill pollution particularly in protected areas.

4.1.2. Material and Methods

4.1.2.1. *Study sites*

Opened in 1974, each year the landfill of Garraf receives more than 850,000 t of solid wastes mainly of domestic origin but also significant amounts of industrial wastes and sewage sludge from sewage treatment plants. With a capacity of 17 M/m^3, the landfill is located on the Garraf massif, a karstic area characterized by Mediterranean climate and xerophytic vegetation. The chemical characterization of landfill leachates from the year 2001 was provided by the Metropolitan Environmental Authority and is shown in Table 4.1.1. Unfortunately, to our knowledge there are no data available for the same parameters corresponding to the year of captures. The reference site has similar vegetation and climate conditions to the polluted site, and is not affected by anthropogenic activities (Figure 4.1.1). Moreover, since 1986 an area of 12,376 ha, including both study sites, enjoys protected status as the “Parc del Garraf”.

From February to April 1998, n= 55 greater white-toothed shrews were collected with Sherman traps in the polluted site (n=21), downstream of the leachate pool and affected by the landfill (Vall d’En Joan) and n=34 from the reference site (Olesa de Bonesvalls). The total capture effort was 1600 traps per night (TN). Specimens were transported to the laboratory and treated following legal and ethical procedures. The body weight (BW) to the nearest 0.01 g and body length (BL) to the nearest 0.01 mm of all specimens were measured. The shrews were lightly anaesthetized and killed by cervical dislocation. Liver and kidneys were immediately removed, weighted, and frozen at -20ºC prior to chemical analyses. Relative age and sex were determined for all individuals as described in Sánchez-Chardi et al. (2007a,b).

4.1.2.2. *Morphometric parameters*

The residual index (RI) was calculated following Jacob et al. (1996) as a regression of BL and BW. Specimens with positive RI values are considered to be in better condition than predicted for their weight and length. Consequently, negative values, down to the linear regression, are attributed to animals with lower body condition than expected. The relative hepatic and renal weight ratios were calculated as a percent ratio of somatic tissue (100x tissue weight/body weight). All morphometric parameters are presented as arithmetic mean ± standard error of the mean (M±SEM) on a wet weight (WW) basis.

Table 4.1.1. Detection limit, mean ± standard deviation (M±SD), and range of metals (in mg/kg), other compounds (in mg/l: Cyanide, Nitrites, Nitrates, Phenols; in mg/kg: Hydrocarbons), and pH in leachates from the Garraf landfill (n.d.: non detected values) (after EM, 2001).

	Detection Limit	M±SD	Range
Iron	0.1	6.38±1.84	3.70-7.60
Magnesium	10	86.75±16.34	77.00-111.00
Lead	0.5	n.d.	
Mercury	0.01	0.05±0.01	n.d.-0.02
Cadmium	0.1	0.62±0.10	n.d.-0.14
Zinc	0.1	1.15±0.60	0.60-2.00
Copper	0.1	0.88±0.21	0.70-1.10
Manganese	1	n.d.	n.d.
Chromium	0.1	0.62±0.10	0.51-0.80
Nickel	0.1	0.36±0.05	0.30-0.40
Cyanide	0.01	0.11±0.09	n.d.-0.13
Nitrates	100	n.d.	
Nitrites	0.5	n.d.	
Phenols	0.2	3.47±2.97	0.70-8.00
Hydrocarbons	5	29.05±17.35	7.20-43.00
pH		8.41±0.12	8.28-8.79

4.1.2.3. *Chemical analyses*

The material used for the digestion was thoroughly acid-rinsed. The tissues were dried at 60ºC till constant weight (48h). From 50 to 100 mg of dry sample was digested by 5 ml of nitric acid (Instra, Baker Analyzed) and 2 ml of perchloric acid (Instra, Baker Analyzed), in open tubs in a Prolabo Microdigest A301 microwave placed in a clean room. Mg and Fe concentrations were determined by a Perkin-Elmer OPTIMA-3200RL Inductively Coupled Plasma Optical Spectrometer (ICP-OES), while Pb, Hg, Cd, Zn, Cu, Mn, Mo, and Cr were measured by a Perkin-Elmer ELAN-6000 Inductively Coupled Plasma Mass Spectrometer (ICP-MS) as described in Sánchez-Chardi et al. (2007a,b). For the purpose of statistical analyses, non-detected values were replaced by the value

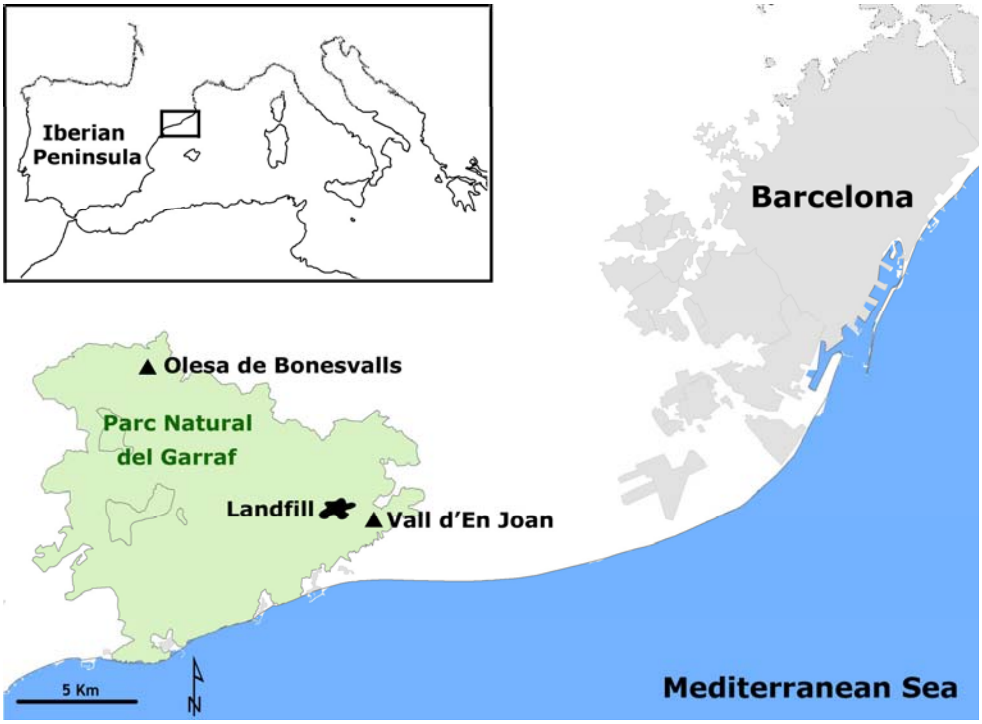

Figure 4.1.1. Map showing the geographical location of the sites studied (▲).

equal to half the detection limit. The metal concentrations are presented as M±SEM in μg/g on dry weight (DW) basis.

4.1.2.4. *Genotoxicity: The micronucleus test (MNT)*

Peripheral blood was collected by cardiac punction into a heparinized syringe. Duplicate smears were made for each specimen on pre-cleaned microscope slides, they were then fixed with heat, and stained with conventional May-Grünwald Giemsa. For each individual, MN frequency was scored on 2,000 erythrocytes through an oil immersion objective (x100) on a Leica Leitz DMRB microscope. The results of the MNT are presented as M±SEM.

4.1.2.5. *Statistical analyses*

Data were log transformed and tested for normal distribution (Shapiro-Wilk test) and for homogeneity of variance (Levene test). From each tissue, the effect of sex, age, and site in metal concentration was obtained by three-way multivariate analysis of variance (MANOVA). Intra- and inter-population comparisons of metals and divergences in RI and relative somatic ratios were evaluated by Student's *t*-tests (*t*), whereas differences in MN frequencies were assessed with Mann-Whitney test (*U*). To establish the relations between metal concentrations and MN frequency, Spearman correlation coefficients (*r*) were calculated in both liver and kidneys. Significant differences were accepted at $p<0.05$. For all sequential tests, *p*-values were corrected by the Bonferroni adjustment (Rice, 1989). All statistical procedures were performed with SPSS (version 11.5 for Windows, SPSS Inc.).

4.1.3. Results

Captures of shrews were more abundant in the reference site (34 shrews in 550 TN) than in the landfill site (21 shrews in 1050 TN). In both sites, a large percentage of shrews were adults (30 out of 34 in the reference site and 14 out of 21 in the polluted site). A high number of males were captured in the reference site compared with the landfill site (Table 4.1.2). No differences in the morphometrical parameters measured were detected between sites (Table 4.1.3).

Table 4.1.2. Number of animals captured in the reference and landfill sites by sex and age.

Site	Sex	Age		
		Juveniles	**Adults**	**Total**
Reference	**Males**	3	23	26
	Females	1	7	8
Landfill	**Males**	4	9	13
	Females	3	5	8

Table 4.1.3. Mean ± SEM of morphometrical parameters in shrews from the reference and landfill sites.

	Reference site (*n*=34)	**Landfill site (*n*=21)**
BW (g)	7.64 ± 0.18	7.29 ± 0.34
BL (mm)	70.68 ± 0.61	69.02 ± 1.17
RI	0.357 ± 0.532	-0.578 ± 0.888
Liver (g)	0.521 ± 0.016	0.492 ± 0.029
% Liver	6.83 ± 0.16	6.72 ± 0.17
Kidneys (g)	0.116 ± 0.006	0.113 ± 0.005
% Kidneys	1.54 ± 0.08	1.61 ± 0.10

All the elements quantified in this study were detected in all samples of all specimens captured at either site, with two exceptions. Hg was not detected in any sample, and Cr was not detected in the kidneys of shrews from the reference site. MANOVA for all data showed that the importance of parameters in liver was: site (F=12.812; p<0.001) > age (F=3.010; p=0.005) > sex (F=1.365; p=0.229). Metal bioaccumulation in kidneys showed a similar pattern, where site (F=7.526; p<0.001), age (F=3.052; p=0.006), and their interaction (F=2.546; p=0.017) were the main parameters influencing bioaccumulation, and sex (F=0.873; p=0.581) was the least important factor in variance.

No significant difference between males and females was found for any of the metals quantified. Adult shrews from both sites showed significantly higher Pb and Cd in liver (Landfill: t=-2.121, p=0.05 and t=-5.002, p<0.001, respectively; Reference: Cd: t=-6.650, p<0.001) and kidneys (Landfill: t=-2.245, p=0.039 and t=-3133, p=0.006, respectively) and lower Cr concentrations in liver (Reference: t=3.679, p=0.010) and kidneys (Landfill: t=2.319, p=0.035). Moreover, levels of Fe, Mo, and Cu tended to increase with age in shrews from the two sites (Figure 4.1.2).

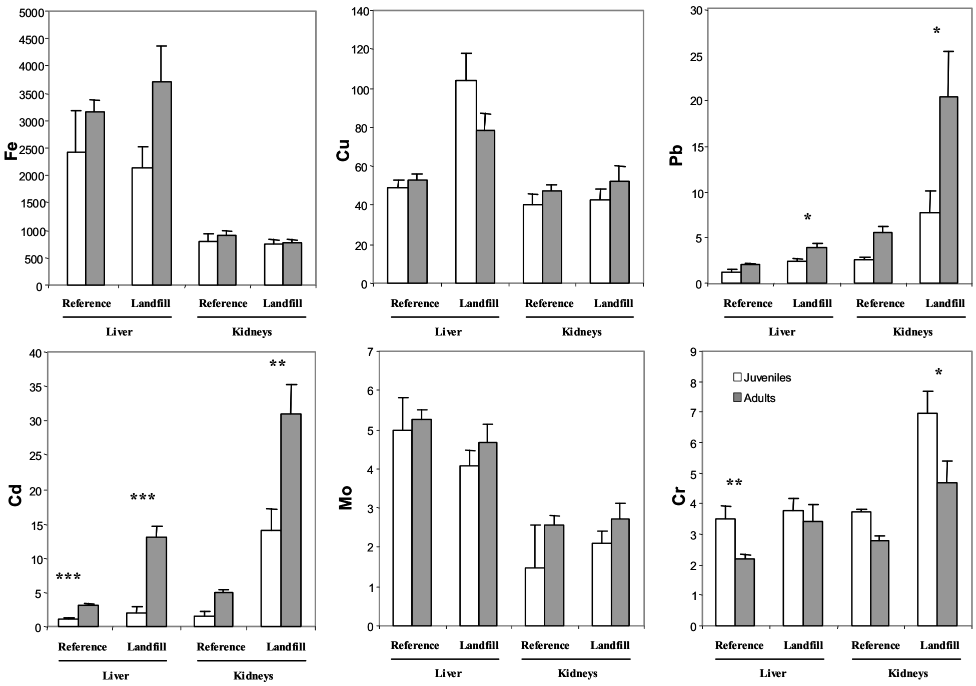

Figure 4.1.2. Mean ± SEM values for metals (Fe, Pb, Cd, Cu, Mo, and Cr) in *C. russula* by tissue, site and age (in µg/g DW) (* $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$).

Despite the age-dependent variation found in Pb, Cd and Cr, in order to increase sample size the age groups were pooled for comparisons of these elements by site. Specimens from the landfill site showed significantly more Pb, Cd, Zn, Mg, Cu, and Cr in liver and Pb, Cd, and Cr in kidneys compared with reference specimens (Table 4.1.4).

In both study sites, the liver was the main accumulator of Fe (Landfill: t=8.098, p<0.001; Reference: t=11.437, p<0.001), Mn (Landfill: t=14.851, p<0.001; Reference: t=15.845, p<0.001), and Mo (Landfill: t=4.680, p<0.001; Reference: t=7.283, p<0.001), whereas the kidneys bioaccumulated the highest concentrations of Pb (Landfill: t=7.382, p<0.001; Reference: t=-6.482, p<0.001), and Cd (Landfill: t=-3.756, p<0.001; Reference: t=-2.614, p=0.011). Mg (t=-2.702, p=0.009), Zn (t=2.803,

Table 4.1.4. Mean ± SEM values for metals in *C. russula* by tissue and site (in µg/g DW).

	Liver				Kidneys			
	Reference site (n=34)	**Landfill site (n=19)**	*t*	*p*	**Reference site (n=31)**	**Landfill site (n=19)**	*t*	*p*
Fe	3085.27 ± 230.29	3304.48 ± 502.00	--	--	895.76 ± 74.02	755.00 ± 39.33	--	--
Mg	1392.74 ± 182.09	1608.63 ± 76.49	3.145	0.003	1490.34 ± 20.93	1455.26 ± 37.42	--	--
Pb	1.93 ± 0.20	3.40 ± 0.49	3.263	0.002	5.37 ± 0.69	16.36 ± 3.78	3.213	0.003
Cd	3.03 ± 0.26	10.28 ± 1.63	3.202	0.004	4.65 ± 0.48	25.59 ± 3.58	9.129	< 0.001
Zn	199.80 ± 6.80	232.26 ± 11.14	2.650	0.011	209.87 ± 6.23	194.65 ± 8.79	--	--
Cu	52.47 ± 3.03	84.90 ± 7.84	4.511	< 0.001	46.97 ± 2.73	49.47 ± 5.42	--	--
Mn	37.42 ± 1.37	42.61 ± 2.20	--	--	16.52 ± 0.56	16.44 ± 0.69	--	--
Mo	5.24 ± 0.22	4.50 ± 0.38	--	--	2.49 ± 0.24	2.53 ± 0.29	--	--
Cr	2.31 ± 0.16	3.49 ± 0.45	2.864	0.006	n.d.	5.40 ± 0.59	3.611	0.001

Table 4.1.5. Spearman coefficients (*r*) and *p*-values between MN frequencies and metals in liver and kidneys of *C. russula*.

	Liver		Kidneys	
	r	*p*	*r*	*P*
Pb	0.388	0.004	0.254	- -
Cd	0.454	0.001	0.709	< 0.001
Cr	0.104	- -	0.317	0.025

p=0.008), and Cu (*t*=4.300, *p*<0.001) showed significantly high levels in livers of shrews from the polluted site. Moreover, in this polluted area Cr was significantly higher in kidneys compared with liver (*t*=-2.658, *p*=0.011).

The shrews from the landfill site showed a significant increase in MN compared with those from the reference site (1.786±0.272 *vs* 0.088±0.045 ‰; *U*=46.000, *p*<0.001). No difference was found by age or sex for this parameter at either site. Moreover, significant correlations were found between MN frequencies and Pb, Cd, and Cr for hepatic and renal tissues (Table 4.1.5).

4.1.4. Discussion

Although landfills are common in several countries, few ecotoxicological studies have addressed this kind of pollution. Leachate has been described as a complex mixture of organic compounds and metals (e.g. De Rosa et al., 1996; Gagnon and Saulnier, 2003; Wichmann et al, 2006). This chemical characterization is only the first step for a meaningful environmental impact (Pohland and Harber, 1986) and alone cannot generate sufficient information on impact because the absolute metal concentration alone does not reflect the degree to which these compounds affect the environment (Cheung et al., 1993). Data on the bioaccumulation and effects of leachates on wild populations are essential to assess the environmental impact of these landfills.

4.1.4.1. *Metal bioaccumulation by site*

Our results are consistent with those reported in liver and kidneys of insectivorous mammals (e.g. Hamers et al., 2006; Komarnicki, 2000; Pankakoski et al., 1993, 1994; Talmage and Walton, 1991). Nevertheless, concentrations of essential metals were slightly higher than data obtained for the same species (Sánchez-Chardi et al., 2007b), probably because of the particular conditions (alkaline pH, calcareous soil, dry climate) of the study sites.

When compared with reference specimens, the shrews from the landfill site showed more Pb, Cd, Mg, Zn, Cu, and Cr in their tissues. Our results on bioaccumulation of Pb and Cd are concordant but higher than those reported by Torres et al. (2006) in the wood mouse, *Apodemus sylvaticus*, from the same landfill site. In fact, insectivores are suitable bioindicators of these non-essential elements because they ingest and/or bioaccumulate more Pb and Cd than sympatric species of rodents (e.g. Dodds-Smith et al., 1992; Ma and Talmage, 2001; Talmage and Walton, 1991). In the shrews from the landfill site, Hg concentrations were under detection limits (0.20 µg/kg in the diluted solution, approximately 0.40 µg/g DW), which is in agreement with low levels in leachates of Garraf (Table 4.1.1) and other landfills (revision in Christensen et al., 2001).

Magnesium is an abundant cation and a main constituent of the colloidal mass in leachates (Gounaris et al., 1993) and it can reach high concentrations in soils near landfill sites. However, because of its low toxicity, few ecotoxicological studies have measured. An increase in this element was also reported in the Algerian mouse, *Mus spretus*, and the rat, *Rattus rattus*, from a pyrite mine site (Pereira et al., 2006).

The increase in Zn and Cu concentrations in liver of shrews from the landfill site may be related, at least partially, to protective and/or detoxification regulation. These elements have a strong physiological regulation in mammals and a high increase in concentrations in mammalian tissues has been reported only in cases of very high intake or disrupted metal metabolism (e.g. Goyer, 1997; Ma and Talmage, 2001). However, lower increases, as found in the present study, have been reported in small mammals exposed to heavy metals (e.g. Ieradi et al., 1996; Pankakoski et al., 1993; Pereira et al., 2006; Talmage and Walton, 1991). Zinc and copper interact with many chemicals and participate in detoxification processes, as part of the enzymes of the antioxidant systems, such as superoxide dismutase (SOD), and in metallothioneins (MT).

Chromium is also a common metal in leachates and landfill composts. This metal leaches rapidly and is often organically complexed in these effluents, thereby becoming more bioavailable and/or mobile through the soil (De Rosa et al., 1996; Gagnon and Saulnier, 2003; Gounaris et al., 1993; Outridge and Scheuhammer, 1993). Chromium concentrations in shrews from landfill site are among the highest found in liver and kidneys of small mammals (Eisler, 1986; Pankakoski et al., 1993, 1994; Talmage and Walton, 1991). This observation indicates the increased bioavailability of this metal in the polluted site.

Manganese and iron are redox-sensitive elements that generate hydroxides after oxygenation of leachates, thereby becoming important carriers of trace elements (e.g. Ragle et al., 1995). No significant difference between sites found in our study indicates a low bioavailability and/or a proper physiological regulation of these elements in shrews. Moreover, no differences in Mo concentrations between sites may be explained by low levels of this element derived from food consumption (Gagnon and Saulnier, 2003).

4.1.4.2. *Metal bioaccumulation by age and sex*

A clear bioaccumulation of Cd by age was observed in shrews from the two sites, in concordance with data reported in insectivores (e.g. Pankakoski et al., 1993, 1994; Sánchez-Chardi et al., 2007b). In particular, in the renal tissue the increase in concentration was up 6-fold between juveniles and adults from the landfill site. This bioaccumulation is related to the formation of stable cadmium-metallothionein complexes as a detoxification mechanism to reduce toxic effects (e.g. Ma and Talmage, 2001). The increase of Pb with age was also reported in *C. russula* bones from a polluted wetland (Sánchez-Chardi et al., 2007a), but was not demonstrated in soft tissues of this species inhabiting a pyrite mine site (Sánchez-Chardi et al., 2007b). In fact, adult shrews from this polluted mining site showed an increase of Pb in liver compared with juveniles (4.81±0.97 *vs* 5.81±1.20 µg/g, respectively), but this pattern was not observed in the reference site. This result may be attributed to the differences in exposure levels and/or chemical forms of Pb as well as to inter-population variation in metal bioaccumulation. A decrease of Cr with age has also been reported for the same species and other insectivores (Pankakoski et al., 1993, 1994; Sánchez-Chardi et al., 2007b). This decrease was attributed to a high digestive absorption rate in juveniles and, therefore, a poor intestinal absorption in adults (Eisler, 1986; Outridge and Scheuhammer, 1993). Despite no statistically significant differences for Fe, Cu, and Mo, we observed a general tendency of increase concentrations of these metals in adults, as reported for *C. russula* (Sánchez-Chardi et al., 2007b). High concentrations of Cu in the liver of juveniles of several mammalian species in polluted sites have been reported, including *C. russula* inhabiting a pyrite mine (Sánchez-Chardi et al., 2007b). Because of the high metabolic rates during this growth period, young animals may are highly exposed to xenobiotics taken up mainly through diet. Cu has important roles in detoxifying function in Cu-containing metallothioneins, which may be found in high concentrations in livers of young mammals, and in protective systems against oxidative stress as part of enzymes such as CuZnSOD. These physiological functions of Cu may explain, at least in part, these increases found in juvenile shrews inhabiting metal-polluted sites.

In the present study, we did not detect any significant differences in metal concentrations between males and females. However, females showed more Mo than males, especially in the liver of shrews from the polluted site (5.24±0.79 *vs* 4.07±0.36 μg/g), in concordance with data obtained for the same species (Sánchez-Chardi et al., 2007b). On the whole, given the asymmetric number of captures by age and sex (see Table 4.1.2) as well as the high variation inherent in wild populations, as shown for the common shrew, *Sorex araneus*, and the mole, *Talpa europaea* (e.g. Dodds-Smith et al., 1992; Komarnicki, 2000; Talmage and Walton, 1991), we consider that more studies are required to confirm these results.

4.1.4.3. *Metal bioaccumulation by tissue*

Similar results on tissue distribution of Fe, Mg, Cd, Zn, Cu, Mn, and Mo were found in the same species inhabiting a pyrite mine site (Sánchez-Chardi et al., 2007b). In this previous article, Cr did not reach significantly high concentrations in livers, whereas in the present study significantly high concentrations were found in kidneys of shrews from the polluted site. Also, Pb distribution reached higher concentrations in kidneys when compared with liver in the shrews from the Garraf area. This distribution is not consistent with previous data reported for *Crocidura* species (Sánchez-Chardi et al., 2007b; Topashka-Ancheva et al., 1999) but agrees with main results for small mammals (e.g. Milton et al., 2003; Pankakoski et al., 1994; Talmage and Walton, 1991). Although tissue distribution of metals is usually constant within specific target organs, differences in bioaccumulation patterns have been reported for *S. araneus* and *T. europaea* (Dodds-Smith et al, 1992; Komarnicki 2000; Świergosz-Kowalewska et al., 2005; Talmage and Walton, 1991). This phenomenon may be related to physiological mechanisms to decrease toxicity, type and time of exposure, and/or the half-life of metals in soft tissues.

4.1.4.4. *Captures and morphometric parameters*

Distribution of captures by age and sex agrees with data reported by López-Fuster (1985) for this species. More males were captured because at the start of the breeding period (February-March) they are more active on the ground surface and wander in search of females (Churchfield, 1990).

Moreover, specimens from the Garraf landfill had a lower body condition than reference shrews (see Table 4.1.3). The lower percentage of captures of *C. russula* in the former may indicate a fall in shrew population. In fact, an increase in bird and rodent populations and a decrease in captures of shrews in zones near landfill sites have been reported (Elliott et al., 2006; Gabrey, 1997; Schroder and Hulse, 1979). Although data are scarce, these observations indicate that landfill sites are not suitable habitats for these mammals. This apparent decrease in insectivorous populations may be a result of the specific toxicity of leachates in shrews and/or in their prey or of competition with rats and other rodents, which are abundant in waste disposal sites.

Our results for morphometrical parameters are similar to those reported for the same species (e.g. Bartels et al., 1979; Sánchez-Chardi et al., 2007a,b) and for other small mammals from polluted and reference sites (Ma, 1996; Milton et al., 2003; Pereira et al., 2006). No differences in these parameters were found between sites, indicating similar general health conditions in the two sites.

4.1.4.5. *Genotoxicity*

Our data are consistent with available literature reporting background mean MN frequencies of 2.43, 0.92, 0.1, and 0.00-1.74‰ in least shrew, *Cryptotis parva*, hedgedog, *Erinaceus europaeus*, bat *Artibeus jamaicencia*, and a few rodent species, respectively (Ieradi et al., 1996; Meier et al., 1999; Zúñiga-González et al., 2000). Leachates contain a large variety of compounds, and some are mutagens that may induce genotoxic effects in biota (Cabrera and Rodriguez, 1999; Sang and Li, 2004). In fact, the increase in MN frequencies and other genotoxic damage in laboratory mice exposed to landfill leachates may be induced by free radicals produced by oxidative stress in response to metal exposure (see Li et al., 2006). Moreover, Pb, Cr and Cd have clastogenic and/or genotoxic effects (e.g. Eisler, 1985, 1986; Tull-Singleton et al., 1994) and the MN frequencies increase after exposure to these elements (Ieradi et al., 1996; Seoane and Dulout, 2001; Palus et al., 2003). In agreement with these previous data, the shrews examined in our study showed high correlations between MN and Pb, Cr and Cd concentrations. However, wild populations from polluted sites are rarely exposed to a single compound and landfill sites are no an exception. Leachates are complex mixtures containing a wide range of organic and inorganic compounds that are potentially toxic. Despite the high metal-MN correlations detected, the influence of other potentially genotoxic compounds from leachates, such as several hydrocarbons and nitrogen-, chlorine- and phosphorous-containing compounds, on the increase in MN frequencies cannot be ruled out.

4.1.4.6. *Toxicity effects*

Landfill leachates form a widely variable and complex mixture of toxic compounds. A low pH is crucial to increase mobility and/or bioavailability of several metals, consequently a reduction of this transfer across the food-chain may be expected in the calcareous area of Garraf. In contrast, in these leachates Zn, Cu, and Cr, among other metals, are often associated with organic matter and colloids that increase their lability and enhance their dispersion in soils and waters (Gounaris et al., 1993; Slack et al., 2005; Yin et al., 2002). The high metal concentrations observed in shrews from the landfill site suggest that a high exchangeability of metals from leachates produces high bioavailability of metals at levels that are potentially toxic for biota (Cabrera and Rodriguez, 1999; Cheung et al., 1993; Li et al., 2006). Although insectivores show high tolerance to metal pollution (Talmage and Walton, 1991; Ma, 1996; Ma and Talmage, 2001), the Pb and Cd concentrations reported in the specimens from the landfill site may reach toxic levels. The mean Pb concentration in kidneys of shrews from this site (16.36 ± 3.78 µg/g DW) was slightly higher than critical renal Pb level of 15 µg/g DW (Ma, 1996) considered a chemical biomarker of toxic exposure to this metal in mammals. In our study, 7 out of 19 shrews in the Garraf landfill site showed more than 15 µg/g of this metal in renal tissue (maximum of 59.71 µg/g DW). Among small mammals, insectivores are more tolerant to Cd than rodents. Liver and kidney lesions in common shrews have been associated with high Cd concentrations of 577 and 253 ug/g DW, respectively (revision in Ma and Talmage 2001), whereas concentrations of 110-260 µg/g produce a nephrotoxic effect in mice (Pankakoski et al., 1993), and bank voles, *Myodes glareolus (*formerly *Clethrionomys glareolus*), show hepatic and renal alterations with Cd concentrations of 15.1 and 17.0 µg/g DW, respectively (Włostowski et al., 2003). The Cd concentrations observed in shrews from the landfill site were higher than the no-observable-adverse-effects-level (NOAEL) in rodents (maximum in liver and kidneys of 20.32 and 56.57 µg/g DW, respectively) indicating a considerable environmental pollution. The concentrations for the remaining elements do not indicate a risk of poisoning or toxic effects on mammals. Furthermore, cumulative effects and/or interactions between potentially toxic metals and with other compounds cannot be disregarded.

4.1.4.7. *Pollution in protected areas*

Environmental legislation is abundant and in some cases very restrictive in developed countries. In fact, areas with partial or complete protection status are becoming increasingly more common, although in some cases protection status is established after a pollution event, as occurred in Garraf. The “Parc del Garraf” reached a partial protection status 12 years after the opening of the largest landfill site in Spain. To improve the management of this natural interesting area, a plan was designed to close the landfill at the end of 2006 and restore the site. Disturbances that occur outside a protected area can affect biota. However, events within a protected area may alter the health status of animals by reducing life expectancy because specimens are more exposed to predators, by impairing reproduction, and/or by affecting population dynamics. Moreover, when natural communities are subjected to severe disturbances such as a landfill, an increase in the population density of opportunistic species may occur (Elliott et al., 2006; Gabrey, 1997; Schroder and Hulse, 1979) since these sites provide abundant nutrients (solid wastes and leachates with nitrogen and phosphorous). However, the landfill area may also become less suitable for wildlife due to these pollution sources often lead to increased environmental concentrations of toxic compounds, which stress wild populations, even if acute poisoning effects are not observed (Pankakoski et al, 1994). To our knowledge, the literature on ecotoxicological data in wild populations and terrestrial ecosystems exposed to landfill pollution is scarce. We therefore consider our data of particular relevance for the management of this protected area and for the biomonitoring of this source of pollution after the closure of the site.

Leachates contain compounds, such as metals, that may be long-term highly bioavailable for biota living some distance from the pollution source (e.g. Gagnon and Saulnier, 2003). In fact, the landfill of Garraf pollutes groundwater, a very persistent pollution. Our results show that this site also has a considerable impact (bioaccumulation of metals at toxic levels and genotoxic damage) on wild populations of insectivorous mammals. However, the extent of the area affected by the dispersion of leachates and of the impact in individuals, communities and ecosystems is unknown.

4.1.5. Conclusions

The leachates from the Garraf landfill are a considerable source of metals. They produce an increase in Pb, Cd, Zn, Cu, and Cr in tissues of the shrew *Crocidura russula*. Age-dependent was greater than sex-dependent variation on the intrapopulation variance of metal levels. Leachate pollution also produced genetic damage to shrews, which demonstrate the effects of this kind of pollution on health. This study also shows that the greater white-toothed shrew, *Crocidura russula*, is a suitable species for biomonitoring this kind of pollution.

These findings are relevant for the management of wastes and for the evaluation of the hazardous effects of landfill leachates on biota. Moreover, to efficiently manage areas of ecological interest, we propose a continuous biomonitoring study to assess the effects of the Garraf landfill in terrestrial ecosystems.

4.1.6. References

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4.2. GARRAF II

Histopathology in the shrew *Crocidura russula* exposed to landfill leachates

Bioaccumulation of metals and effects of a landfill in small mammals. Part III. Structural alterations

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Abstract

The leachates from the Garraf landfill located in a protected site (NE Spain) contain several potentially toxic substances such as heavy metals. Here we report the histopathological alterations produced by this pollution in wild specimens of an insectivorous species, the greater white-toothed shrew, *Crocidura russula*. Hepatic tissue presented the most severe alterations in the species, namely cell cycle arrest (apoptosis and necrosis), inflammation, vacuolation and microsteatosis. The kidneys were less altered, presenting tubular necrosis and dilatation, inflammation, and cylinders, suggesting that different metabolic pathways render renal tissue more tolerant to toxicity induced by pollutants. No pollution-related alterations were observed in lung, spleen, pancreas, gonads, oesophagus, intestine, or adrenals. We conclude that this species could be used in conjunction with others as bioindicators to assess the effects of environmental pollution at distinct trophic levels.

4.2.1. Introduction

Wild populations inhabiting polluted sites are often exposed to a mixture of chemical pollutants which are mainly taken in the food. These pollutants results in multiple stresses and affect biological systems at virtually all levels, from molecules to ecosystems. The assessment of the risks of the diversity of pollutants to natural populations is difficult as this depends on a variety of abiotic (e.g. temperature, pluviosity) and biotic (e.g. age, gender, species) factors. However, studies under field conditions provide crucial ecotoxicological data on bioindicator species that can provide information on the quality of the environment (e.g. Pereira et al., 2006) and how to manage protected areas (e.g. Sánchez-Chardi and Nadal, 2007; Sánchez-Chardi et al., 2007). The species used for monitoring purposes should cover various levels of the food chain, like primary (herbivorous) and secondary (carnivorous) consumers. Thus, terrestrial small mammals (rodents and insectivores) have often been used as bioindicators of physical and chemical pollution (revisions in Ma and Talmage, 2001; Sheffield et al., 2001; Talmage and Walton, 1991). Whereas abundant information is available in rodent species, similar information on the effects of chronic pollution in shrews is scarce. The greater white-toothed shrew, *Crocidura russula* (Soricomorpha, Mammalia), is a relatively common secondary consumer in South Europe, predating on arthropods, molluscs and earthworms. It has recently been reported as a suitable species for ecotoxicological studies (González et al., 2008; Sánchez-Chardi and Nadal, 2007; Sánchez-Chardi et al., 2007, 2008; Wijnhoven et al., 2007). The use of wild specimens in ecotoxicological studies includes aspects of bioavailability, toxicity and detoxification mechanisms, and specific or individual exposure and susceptibility as determining factors for environmental risk under natural conditions. However, most information concerning the toxic effects of pollutants, such as heavy metals, on the structure and function of organs have derived from laboratory experiments on animals exposed to a single compound (Damek-Poprawa and Sawicka-Kapusta, 2004; Hoffmann et al., 1975; Koyu et al., 2006; Włostowski et al., 2000) and, less frequently, to multiple compounds (Jadhav et al., 2007; Silva de Assis et al., 2005) or complex mixtures such as landfill leachates (Li et al., 2006; Talorete et al., 2008). This controlled assessment of individual health status is the basis for further assessments of the true effects of pollutants on wild populations and dynamic natural systems.

Landfills are a considerable source of pollution in Mediterranean countries (Loukidou and Zouboulis, 2001), including Spain. The decomposition of disposed waste or the interaction between water and waste leads to the formation of liquid effluents named leachates. If landfills are not adequately controlled and sealed, leachates enter natural systems and cause toxic effects in plants and animals (Li et al., 2006a,b; Wilke et al., 2008). The quantity, composition and toxicity of leachates vary depending on the nature and age of wastes, the method of disposal, dump depth and climatic factors (see references in Sánchez-Chardi et al., 2007). Landfill effluents often contain a wide variety of organic and inorganic pollutants, including potentially toxic metals such as Pb, Cd, Fe, Zn, Cu, Mn, Mo, and Cr (see references in Sánchez-Chardi et al., 2007). These elements may be responsible for oxidative stress and a number of genotoxic effects reported in culture cells and laboratory rodents (Bakare et al., 2005; Li et al., 2006a,b; Talorete et al., 2008; Thomas et al., 2009) and wild mice from a waste site (Tull-Singleton et al., 1994). The induction of oxidative stress as a result of the production of reactive oxygen species (ROS) can damage lipids, thiol-proteins and nucleic acids (Jadhav et al., 2007 and references herein) thereby producing large alterations to tissues and ending in cell cycle arrest (Damek-Poprawa and Sawicka-Kapusta, 2003, 2004; Goyer et al., 1997; Sánchez-Chardi et al., 2008). Although hepatic and renal tissues are the primary targets of pollutants ingested with the diet (e.g. Koyu et al., 2006; Włostowski et al., 2000), metals can also affect other organs and tissues, such as blood, gonads, spleen, lung, brain and bones (Damek-Poprawa and Sawicka-Kapusta, 2003; Jadhav et al., 2007; Li et al., 2006a,b), as reported in histopathological evaluations for wild rodents inhabiting near metallurgical industries (Damek-Poprawa and Sawicka-Kapusta, 2004) and abandoned mines (Pereira et al., 2006). To our knowledge, the present study is the first to assess the histopathological alterations caused by chronic exposure to environmental pollution in a crocidurine species (the greater white-toothed shrew). Despite the scarce information on structural alterations in wild mammals exposed to landfill leachates, previous studies in the Garraf landfill (NE Spain) reported the bioaccumulation of highly toxic metals such as Cd, Zn, Cu, and Cr and also morphological, biochemical and genotoxic alterations in the wood mouse, *Apodemus sylvaticus*, and *C. russula* exposed to leachates (Sánchez-Chardi and Nadal, 2007; Sánchez-Chardi et al., 2007). From 1974 to 2006, the Garraf landfill served as a disposal site for domestic and industrial wastes and solid sludge from the metropole of Barcelona accumulating about 25 million t of waste. In 1986, the karstic area of Garraf granted protection status in recognition of its singular habitats and the endangered species found there. Therefore, the need to biomonitor of the landfill site increased.

The main objectives of this study are: i) to qualify and quantify histological alterations in target tissues of an insectivore exposed to landfill leachates; ii) to identify the influence of sex and age as source of intrapopulational variation; iii) to correlate histological alterations with metal bioaccumulation and other biomarkers; and iv) to assess the environmental consequences of landfill pollution particularly in protected areas.

4.2.2. Material and Methods

4.2.2.1. *Study sites*

The two study sites are located on the karstic massif of Garraf (NE Spain), a coastal system formed by hills of about 700 m, traversed by several valleys and covered by Mediterranean xerophytic vegetation. The area is located 30 km south of Barcelona city, and has granted partial protection as “Parc del Garraf”. Using Sherman live traps, 28 specimens of greater white-toothed shrew, *Crocidura russula*, were trapped at two sites from February to May 1998. One polluted site called “Vall d’En Joan” is in the vicinity of the pool of the leachates placed at the lower end of a valley. The reference site (“Olesa de Bonesvalls”) is also a valley close to the landfill. No sources of pollution are known for this area.

Table 4.2.1. Number of shrews captured and used in histological assessment in the reference and landfill site by site, age and sex (In brackets: males, females).

Site	Age		Total
	Juveniles	Adults	
Reference	1 (1,0)	15 (11,4)	16 (12,4)
Landfill	3 (1,2)	9 (9,0)	12 (10,2)

Specimens were transported to the laboratory for dissection, following all ethical procedures for experimental animals. Sex was determined during dissection and relative age was determined on the basis of the tooth wear (see references in Sánchez-Chardi and Nadal, 2007; Sánchez-Chardi et al., 2007). The number of specimens by capture site, sex and age is described in Table 4.2.1.

4.2.2.2. *Histopathological evaluation*

After dissection, the gonads and the left kidney and a small portion of liver, lung, spleen, pancreas, oesophagus, intestine, or adrenals of all specimens were immediately fixed in 10% neutral-buffered formaldehyde. Samples of all lesions observed macroscopically were also taken and fixed. Tissues were individually dehydrated in ethanol series, cleared in toluene and embedded in Paraplast Histocomp (Vogel). Sections of 5-10 µm were stained with conventional hematoxylin and eosin and mounted in DPX. A Leitz DMRB light microscope with a camera Leica DC 500 was used for observations.

The incidence of alterations was reported in a qualitative evaluation. For statistical purposes, the alterations were measured on a semi-quantitative scale. A global score for each tissue was assigned to each specimen in a conventional scale on the basis of the severity and/or extent of lesions: without alterations (-), slightly altered (+), intermediately altered (++), and strongly altered (+++). A global score was given to each sample (0, 1, 2, 3). All results are expressed as arithmetic mean and standard error of the mean (M±SEM).

4.2.2.3. *Statistical analyses*

For each tissue, the results of the semi-quantitative assessment were compared by site, age, and sex using Mann-Whitney tests (*U*). To detect relations between histopathology and other biomarkers, Spearman's correlation coefficients (*r*) were calculated between the histopathological evaluation and the bioaccumulation of metals and morphometric, plasmatic, and genotoxic parameters for each species. Significant differences were accepted at $p < 0.05$. For all sequential tests, *p*-values were corrected using the Bonferroni adjustment. All statistical procedures were performed with the SPSS package (version 15.0 for Windows, SPSS Inc.).

4.2.3. Results

In general, the organs and cells of specimens of *C. russula* from the reference site showed normal aspect. Livers had a compact structure and hepatocytes had a normal shape (Figure 4.2.1A). Kidneys had a well developed cortex and medulla (Figure 4.2.2A). Nine out of 12 shrews of the landfill site showed hepatic alterations related to pollution; apoptosis, necrosis, periportal and perivascular lymphocite infiltration and signs of regenerating activity, vacuolation, and microsteatosis (Figure 4.2.1B-E). Thirteen out of 16 animals from the reference site showed healthy tissue structure. No severe pathologies were observed in kidneys, although some cases of focal tubular dilatation, tubular necrosis, hyaline cylinders (formed by proteic casts and/or cellular debris) in tubular lumina and inflammatory focusses were seen. The mean scores of alterations in livers and kidneys for polluted and reference site were 0.92 ± 0.28 *vs* 0.06 ± 0.05 (U = 48,000; p = 0.008) and 0.15 ± 0.10 *vs* 0.05 ± 0.04 (U = 100,000; p = 0.680), respectively. No lesions related to pollution were observed in the other tissues evaluated. However, severe acute and chronic inflammatory reactions related to the presence of helminths, with presence of plasma cells, neutrophils and a high number of eosinophils, were found in airways, spleen, and digestive tract of several shrews from both sites (data not shown). These alterations not related to pollution were not considered in the present histopathological evaluation. Severity and frequency of histological pathologies related with pollution is summarized in Table 4.2.2.

No significant relations were found between sex or age and structural alterations. Few

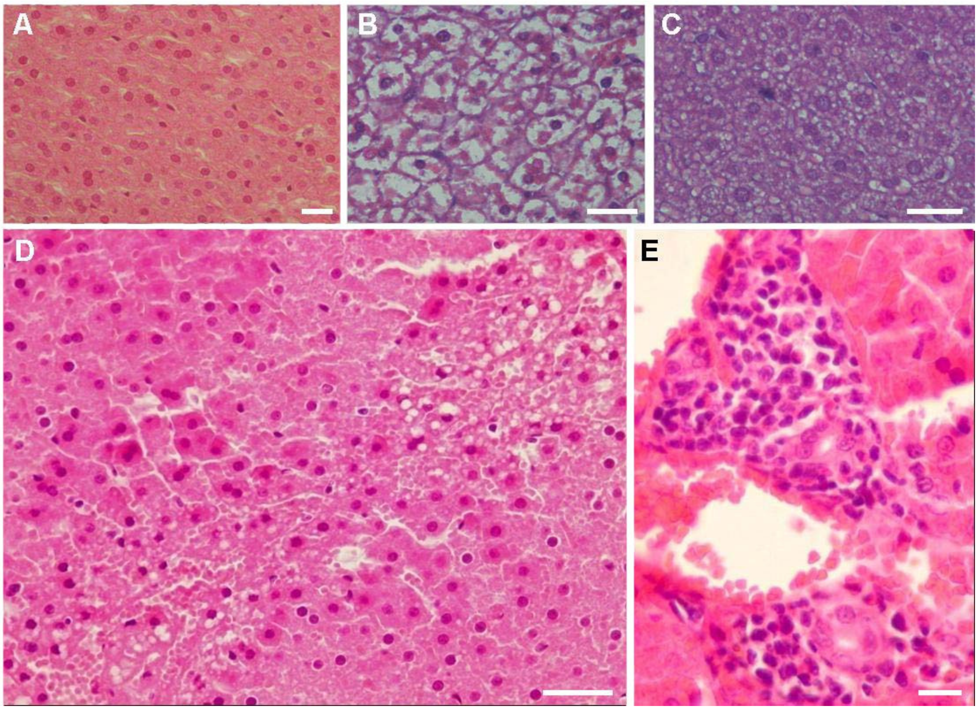

Figure 4.2.1. Representative micrographs of hepatic sections from *C. russula* showing: (A) non-altered tissue with hepatocytes with normal shape; (B) highly vacuolated hepatocytes; (C) steatosis; (D) extensive necrotic areas surrounded vacuolated areas; (E) inflammations (bar size corresponding to 50 µm (A,D) or 25 µm (B,C,E)).

significant correlations were detected between hepatic histopathology and other parameters previously reported. Correlations were found between hepatic histopathology and liver weight ratio ($r = 0.396$, $p = 0.041$) and bioaccumulation of Mn in kidneys ($r = 0.405$, $p = 0.045$).

Table 4.2.2. Frequency and intensity of histological alterations by site and tissue in greater white-toothed shrews (*C. russula*) (slightly altered (+), intermediately altered (++), strongly altered (+++)).

		Reference				Landfill			
		-	+	++	+++	-	+	++	+++
Liver	**Apoptosis**	16	-	-	-	8	2	2	-
	Necrosis	15	1	-	-	6	3	1	2
	Vacuolation-Microsteatosis	15	1	-	-	7	1	3	1
	Inflammation	15	1	-	-	9	3	-	-
	Preneoplasic nodules	16	-	-	-	12	-	-	-
Kidneys	**Cylinders**	14	1	-	-	9	1	-	-
	Tubular necrosis	15	-	-	-	9	-	1	-
	Inflammation	15	-	-	-	8	1	1	-
	Tubular dilatation	15	-	-	-	7	2	1	-

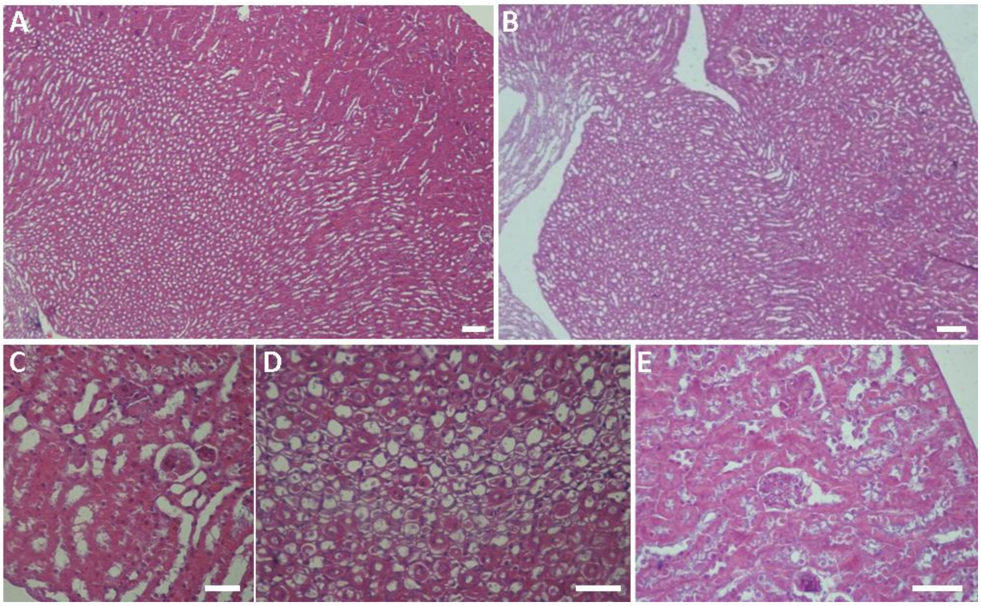

Figure 4.2.2. Representative micrographs of renal sections from *C. russula* showing: (A) normal structure of cortex and medulla; (B) tubular dilatation; (C) tubular dilatation in cortex; (D) tubular dilatation in medulla and cylinders; and (E) tubular necrosis. (bar size corresponding to 100 µm (A,B) or 50 µm (C,D,E)).

4.2.4. Discussion

4.2.4.1. *Structural alterations by tissue, site, age and sex*

Given the concern about the environmental consequences of pollution, several approaches have been proposed for the assessment and prediction of pollutant behaviour and the effects of these substances on ecosystems. Laboratory studies under controlled conditions (Jadhav et al., 2007; Koyu et al., 2006; Zhang et al., 2009), models to predict toxicity or dispersion of pollutants (Kools et al., 2009) or the biomonitoring of abiotic or biotic components as well as several biomarkers in wild biota (Pereira et al., 2006; Scheirs et al., 2006; Tersago et al., 2004) attempt to address these issues. However, the exposure to mixtures of pollutants that interact among themselves or with abiotic and biotic components, the differential exposure or toxicity related to several biotic factors (nutritional status, age or gender among others) and other non-controlled variables of dynamic environments that alter bioavailability of pollutants are just some of the factors that hinder research to effects of mixtures in the field. Here we addressed the effects of landfill pollution under natural conditions by performing a study of structural alterations in tissues of *C. russula* in a protected area. We propose that our approach can be a suitable as a biomarker to detect toxicity and pollution effects caused by chronic or acute exposure of wildlife more sensitive than other methods. The alterations observed in specimens from the polluted site are compatible with exposure to pollutants and, more specifically to landfill leachates. In general, hepatic and renal toxicity effects appear when primary or acquired tolerance of cells and tissues are overloaded. The main exposure route to metals for mammals is though the diet and hepatic and renal tissues are the primary targets (Clark et al., 1992; Koyu et al., 2006; Larregle et al., 2008; Pereira et al., 2006; Włostowski et al., 2008). In fact, liver was the organ in which most histological alterations caused by exposure to landfill pollution were observed and significant correlations between histopathological alterations in liver and liver weight reinforce this observation. The damage to this organ may be related to its metabolic functions in toxic transformation, bioaccumulation and excretion. In contrast to shrews, mice from the landfill site showed considerable alterations in kidneys, thereby indicating inter-specific differences in the response to toxicity. Kidney lesions concordant with our results have been reported in wild rodents from polluted areas (Stansley and Roscoe, 1996) and in laboratory shrews exposed to Pb (Pankakoski et al., 1994).

Leachates are inductors of oxidative stress that injure cells and tissues (e.g. Li et al., 2006a,b). Small mammals from the landfill site of Garraf accumulate heavy metals up to toxic concentrations in their tissues (Sánchez-Chardi and Nadal, 2007; Sánchez-Chardi et al., 2007). This might indicates the important contribution of these elements to the toxic effects reported. Although the precise molecular mechanism or mechanisms by which metals cause this damage remain unclear, the production of ROS is believed to be involved (Chwełatiuk et al., 2005; Włostowski et al., 2008). Depending on the nature of the injury, the generation of this oxidative stressor damages DNA, thereby producing apoptosis, necrosis or carcinogenic processes and altering membranes and lipid and protein metabolism (Jadhav et al., 2007; Larregle et al., 2008; Mena et al., 2009; Pereira et al., 2006). For example, the hepatotoxicity *in vivo* induced by metals such as Cd seems to be produced as ischemia caused by damage to endothelial cells and activation of Kupffer cells (Pagliara et al., 2003). A cascade of events involving inflammatory and cytotoxic mediators (Koyu et al., 2006) results in hepatocytes damage. Exposure to several pollutants, including heavy metals or mixtures as leachates has shown to poduce apoptosis and necrosis in laboratory studies (Larregle et al., 2008; Talorete et al., 2008) and in wild small mammals (Clark et al., 1992; Damek-Propawa and Sawicka-Kapusta, 2003; Sánchez-Chardi et al., 2008; Stansley and Roscoe, 1996; Tersago et al., 2004).

Shrews from the polluted site showed inflammatory processes similar to those reported for wild small mammals (Clark et al., 1992; Sánchez-Chardi et al., 2008) and laboratory rodents (Larregle et al., 2008; Włostowski et al., 2004) in other studies. Hepatotoxicity caused by metal exposure includes the involvement of proinflammatory cytokines and chemokines, Kupffer cell activation and neutrophil infiltration (Koyu et al., 2006 and references herein) producing inflammatory focuses such as observed in the present study. Nephritis was also reported in wild rodents exposed to environmental pollution (Clark et al., 1992). Chronic inflammation such as that produced by exposure to landfill leachates has been described as an inductor of carcinogenic processes (Talorete et al., 2008). Carcinogenesis was observed in a specimen of wood mouse from the same landfill site (data not shown). It is known that metals such as cadmium have carcinogenic activity (Damek-Poprawa and Sawicka-Kapusta, 2003).

Hepatocyte vacuolation and microsteatosis can be the result of lipids, glycogen or water accumulation, which are indicative of great metabolic disturbances (Pereira et al., 2006) such as autophagic processes. Lipophilic pollutants can also be stored in these droplets (Włostowski et al., 2008), thereby being less available, which reduces the oxidative stress in cell components. We observed this alteration more frequently in livers of shrews from the polluted site. This is consistent with previous studies on wild and laboratory small mammals exposed to metals and organic xenobiotics (Clark et al., 1992; Damek-Poprawa and Sawicka-Kapusta, 2004; Pereira et al., 2006; Sánchez-Chardi et al., 2008; Włostowski et al., 2008). In *C. russula* from Garraf, the vacuolated areas were often surrounded by necrotic zones (Figure 4.2.1D), thereby indicating extensive damage in especially the liver such as occurs with steatohepatosis.

Renal tissue is the main target for bioaccumulation and effects of several pollutants, including metals such as Cd (Chmielnicka et al., 1989; Prozialeck et al., 2009). Tubular dilatation and proteic cylinders in kidneys were also reported in common shrews, *Sorex araneus*, experimentally exposed to Pb (Pankakoski et al., 1994) and in laboratory rodents exposed to essential and non-essential metals (Cerejeira Matos et al., 2009; Chmielnicka et al., 1989). Moreover, nephrotoxicity induced by metals includes cell death necrosis and apoptosis in tubules (references in Prozialeck et al., 2009). In general, these renal alterations may reflect a later phase of pollutant-induced hyperfiltration caused by chronic exposure to landfill leachates. Laboratory studies showed an increase in urinary output in rats exposed to metals (e.g. Chmielnicka et al., 1989; Prozialeck et al., 2009). In the present study, several renal alterations were found together in the specimens analyzed. These results suggest that dilatation may be a compensatory mechanism after the loss of renal excretory function of nephrons by tubular necrosis and cylinders.

In addition to hepatic and renal tissues, pollutants can specifically affect other organs and tissues, such as blood, gonads, spleen, lung, brain and bones (e.g. Damek-Poprawa and Sawicka-Kapusta, 2003, 2004; Jadhav et al., 2007; Li et al., 2006a,b; Pereira et al., 2006). The spleen may be a suitable tissue to detect immune-depression effects as a response to pollution (Pereira et al., 2006;

Tersago et al., 2004). In our case, the interference of parasitosis in spleen and intestine hindered the histopathological evaluation. The general health status may be decreased in animals heavily parasitized in digestive tract, spleen and lungs. Nevertheless, immuno-depression in stressed specimens by pollution exposure (Tersago et al., 2004) can result on an increase of parasites. These observations suggest further research focussing on quantitative studies and specific biomarkers for these tissues.

No sex- or gender-dependent variation was observed in the structural alterations detected in shrews. Although gender and age-related toxicity rates have been found elsewhere (e.g. Gochfeld, 2007; Scheirs et al., 2006; Pereira et al., 2006), our result are consistent with previous data on *C. russula* (Sánchez-Chardi et al., 2008) and are indicative of similar toxic effects in all classes (juveniles, adults, males and females) of individuals.

4.2.4.2. *Structural alterations as biomarker of pollution in protected sites*

We analysed the structural effects of pollution in *A. sylvaticus* and *C. russula* (Sánchez-Chardi et al., 2009). This comparison of toxic effects between sympatric species in a distinct trophic compartment provides useful information on the effects of landfill pollution on terrestrial food chains. In general, our histopathological findings are compatible with morphological, hematological and biochemical results previously reported in the same specimens (Sánchez-Chardi and Nadal, 2007; Sánchez-Chardi et al., 2007). In agreement with our data, histological evaluations of wild mammals from polluted sites often describe a variety and severity of lesions, from unaltered to highly damaged tissues, as well as alterations not related to pollution in both control specimens and in animals exposed to pollution. Although significant age- or sex-dependent variation was not observed in the present study, the differential exposure under natural conditions and/or the physiological response of each specimen to environmental disturbances is influenced by the genetic pool but differs depending on abiotic and biotic factors, including the photoperiod, temperature, and sex, age, or general health condition (e.g. Włostowski et al., 2008). This wide intra-species variability implies that histopathological assessments in ecotoxicology should be quantitative or semi-quantitative in order to detect statistical significances or tendencies regarding histotoxic effects. Although mice and shrews showed histological alterations, the inter-specific differences in severity and frequency of the pathologies indicate the differences in sensibility of these animals to chronic pollution. In general, insectivores are considered more tolerant to toxicants than rodents as reported in non-observable-adverse-effects-level (NOAEL) for metals (Ma and Talmage, 2001; Sheffield et al., 2001; Talmage and Walton, 1991). However, the comparisons between small mammal species are usually made between rodents and soricine species (e.g. Hunter et al., 1987) and scarce comparative information is available on crocidurine species (González et al., 2008; Wijnhoven et al., 2007). Our results indicate similar toxic effects in liver in both mice and shrews but slightly lower toxicity rate in kidneys of the latter. Wood mice from the Garraf landfill showed several differences in morphological, plasmatic and genotoxic parameters while shrews showed differences in genotoxicity (Sánchez-Chardi et al., 2007). In contrast, the highest levels of toxic metals in tissues have been reported in *C. russula* (Sánchez-Chardi and Nadal, 2007). This observation is attributed to the fact that carnivores are usually more exposed to metals and accumulate more of these elements than omnivores and herbivores (Hunter et al., 1987; Ma and Talmage, 2001; Pankakoski et al., 1994; Talmage and Walton, 1991). Sympatric species of small mammals may differ dramatically in metal bioaccumulation in the absence of differences in external bioavailability mainly by dietary uptake and ingestion of contaminated sediments. Thus environmental quality management programs should compare species with different trophic position.

Metabolic alterations caused by chronic exposure to pollutants can affect the basic functions of biota such as reproduction or life expectancy. Therefore, the assessment of environmental quality through the evaluation of structural alterations caused by pollution is of particular relevance in protected areas such as the Parc of Garraf. Although the environmental quality of such zones is often high, long-term pollutant activities, such as landfilling, may disturb, modify or destroy ecosystems, thereby making them less suitable for wildlife. Therefore, continuous biomonitoring of pollutants by means of selected species is required. In this context, our results corroborate other studies that reported the wood mouse and the greater white-toothed shrew as effective bioindicators of non-essential metals and the effects of environmental pollution (González et al., 2008; Sánchez-Chardi and Nadal, 2007; Sánchez-Chardi et al., 2007; Wijnhoven et al., 2007). Moreover, further studies are necessary to elucidate more exactly the inter-specific differences in tolerance of environmental pollution between rodents and crocidurine species. These studies may include an ultrastructural evaluation or molecular studies to quantify genetic or metabolic alterations. With all this in mind and given the inter-specific differences between mice and shrews, we propose the use of both species in biomonitoring programs.

4.2.5. Conclusions

Histopathology of the major organs involved in metabolism and excretion of xenobiotics, as the liver and the kidney, is an useful biomarker of the effect of pollution in wild small mammals collected near the landfill of Garraf. Specimens of *C. russula* analysed showed toxic effects that could be attributed to direct action (cell cycle arrest, inflammation, pre-neoplasic nodules), storage (vacuolation, microsteatosis) and excretion (tubular dilatation, cylinders) of pollutants. The wood mice showed considerable alterations in liver and kidneys as a result of chronic exposure to leachates while the shrews showed main alterations in the liver. This result contrasts with higher bioaccumulation of metals in the shrews than in the mice. On the basis of our findings, we conclude that the comparison of several biomarkers in sympatric species of small mammals at different trophic levels provides suitable ecotoxicological data for a complete assessment of environmental pollution. Moreover, given the protected status of the Garraf area, the use of bioindicator species provides useful information for environmental quality management and to improve our understanding of the response capacity of natural populations to pollution in this area and in other areas with similar characteristics.

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4.3. ALENTEJO I

Bioaccumulation and morphometry in the shrew *Crocidura russula* inhabiting an abandoned pyrite mine

Metal bioaccumulation in the greater white-toothed shrew, *Crocidura russula*, inhabiting an abandoned pyrite mine site

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Abstract

Hepatic and renal concentrations of iron, magnesium, zinc, lead, copper, manganese, mercury, cadmium, molybdenum, chromium, and nickel were quantified in shrews (*Crocidura russula*) inhabiting a pyrite mine site in Portugal. Several morphometrical parameters (body weight, residual index, and relative weights) were also examined to clarify the physiological effects of pollution. Shrews from the mine showed increased bioavailability of Fe, Pb, Hg, Cd, Mo, and Ni in comparison with reference specimens. Adult shrews had the highest Cd levels while Cr and Ni concentrations diminished. Intersexual differences were found for Mo and Ni. As a consequence of metal pollution, the relative hepatic weight was higher in shrews from the mine site when compared with reference specimens. These data indicate that *C. russula* is a good bioindicator of metal pollution. We also evaluated the toxic effects of Pb, Hg, Cd, and Ni, because several shrews from the polluted site showed high concentrations of these metals. To approximate at the real biological impact of abandoned mines, after this first step it is necessary to associate the bioaccumulation levels and morphometrical effects with other physiological, ecological and genetical biomarkers.

4.3.1. Introduction

Heavy metals are common environmental pollutants that have increased their concentration and bioavailability by biota because of industrial activities such as mining. In fact, between 1930 and 1985 the mine production of Mg, Zn, Pb, Cu, Mn, Hg, Cd, Cr, and Ni alone increased 7-, 4-, 2-, 5-, 8-, 2-, 15-, 18-, and 35-fold respectively, with the subsequent release of these potentially toxic elements into the environment (Nriagu, 1988). Mines are closed down when they become economically non viable and usually become a large and uncontrolled source of metal pollution. In the case of pyrite deposits, natural weathering interaction generates large amounts of effluents named “acid mine drainage” (AMD), characterized by the presence of toxic metals such as Zn, Pb, Cu, Mn, Cd, Mo, Cr, and Ni in low pH solution (Quevauviller et al., 1989; Santos Oliveira et al., 2002). These chemical conditions often increase the bioavailability of metals at potentially toxic levels (Lacal et al., 2003; Scheuhammer, 1991) and, consequently, the environmental risk of these deposits remain long after mines have been abandoned.

Several studies have reported the bioaccumulation of heavy metals in tissues of terrestrial small mammals inhabiting abandoned mines, mainly in rodent than in insectivorous species probably because mice and voles are, in general, more abundant and rapidly trapped than shrews. However, when comparing sympatric small mammal species, carnivores showed greater bioaccumulation than omnivores and herbivores, which is explained by their high metabolic rate, high food consumption rate, and position at the top of the food-chain (Andrews et al., 1984; Hunter et al., 1989; Ma and Talmage, 2001; Shore, 1995). In fact, a range of species of Talpidae and soricine has been demonstrated as good bioindicators of metal pollution (e.g. Pankakoski et al., 1994; Komarnicki 2000; Talmage and Walton, 1991). However, crocidurine species have rarely been used as models in ecotoxicological studies on metal accumulation (Sánchez-Chardi et al., 2007; Topasha-Ancheva and Metcheva, 1999). The greater white-toothed shrew, *Crocidura russula* (Soricomorpha, Mammalia), inhabits the south-western Europe and is the most abundant and widely distributed shrew in the Iberian Peninsula.

Larger variation in the concentration of metals in the same population may be explained mostly by biotic and abiotic parameters. In fact, bioaccumulation patterns are dependent on age and sex, as well as physiological status and diet, which are subjected to seasonal differences (e.g. Cloutier et al., 1986; Lopes et al., 2002). However, few studies have addressed the variation in metal concentrations produced by these three parameters in insectivore populations (Komarnicki, 2000; Ma and Talmage, 2001; Smith and Rongstad, 1982).

In addition to the bioaccumulation levels, it is necessary to assess the physiological effects of chronic exposure to metals in order to evaluate the impact of pollution in "real world" conditions. Information on these effects can be partially attained by morphometrical parameters based on weight and/or length of animals. In fact, poisoning by some metals may result in a decrease in total body weight or an increase in relative organ weight, which may be indicative of histopathological alterations (e.g. Ma and Talmage, 2001). The wide range of responses shown by small mammals to heavy metal pollution suggests that natural populations are very tolerant, adapt to low quality environments and that only high metal exposure alter these morphometrical parameters (Ma 1989; Ma and Talmage, 2001; Milton et al., 2003; Roberts and Johnson, 1978; Stansley and Roscoe, 1996).

Here we: i) quantified the heavy metal concentrations in *C. russula* inhabiting an area near a pyrite mine, ii) examined the seasonal, sexual, and age bioaccumulation patterns in this species, and iii) assessed the toxic effects of these abandoned mines on small mammals.

4.3.2. Material and Methods

In the Baixo Alentejo region (Southern Portugal) several mines have been closed down because of economic reasons and with no previous planning for environmental recovery. Located in the Iberian Pyrite Belt, the open pit and underground mine of Aljustrel was worked from 1867 to 1996. From 1900 to 1991 about 16,000,000 t of metals were mined, out of which 1,150,000 t from 1991 to 1996, including Fe=1,000 t, Cu=6,300 t, Zn=27,500 t, and Pb=4,400 t (Instituto Geológico e Mineiro, SIORMINP, internal report). The polluted site of Aljustrel mine chosen for this study (37º53'08''N; 08º08'32''W) is affected by the AMD from the pyrite mine. A second area (Moura) where no sources of heavy metals have been reported was used as a reference. The Moura site is located 69 km northeast from Aljustrel mine (38º11'13''N; 07º24'34''W) (Figure 4.3.1).

In spring and autumn 2003, a total of 54 greater white-toothed shrews were collected in the polluted (n=32) and in the reference (n=22) site. The total capture effort was 1250 traps per night. Shrews were live-trapped and transported to the laboratory, where they were lightly anaesthetized and killed by cervical dislocation. The body weight (BW) to the nearest 0.0001 g and body length (BL) to the nearest 0.01 mm of all shrews were measured. The liver, kidneys and spleen were removed and weighted. The residual index (RI) indicating body condition index was calculated following Jacob et al. (1996) as a regression of BL and BW. The liver:body, kidneys:body and spleen:body weight ratios were calculated as a percent ratio of somatic tissue (100x tissue wet weight/body wet weight). The results of these morphometric parameters are presented as arithmetic mean ± standard error of the

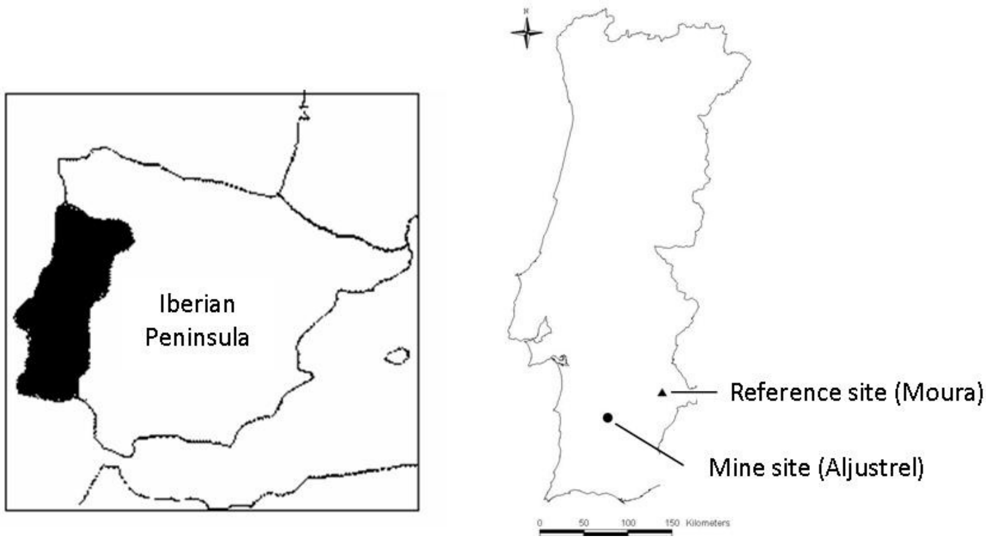


Figure 4.3.1. Localization of the study areas in south of Portugal.

mean (M±SEM). Sex was determined during dissection. For statistical analyses, animals were distributed into two relative age-classes (juveniles; adults) according to the degree of toothwear (Vesmanis and Vesmanis, 1979).

The material used for the digestion process was acid-rinsed. The liver and the right kidney from all shrews were acid digested in Teflon vessels following the methodology described in Sánchez-Chardi et al. (2007). Mg concentration was determined by a Perkin-Elmer OPTIMA-3200RL Inductively Coupled Plasma Optical Spectrometer (ICP-OES), while Pb, Hg, Cd, Cu, Zn, Mn, Mo, Cr and Ni were measured by a Perkin-Elmer ELAN-6000 Inductively Coupled Plasma Mass Spectrometer (ICP-MS). Two replicate subsamples and a standard reference material (Bovine Liver SRM-1577a) certified by the National Bureau of Standards (NBS) were included in the analyses. To obtain the concentration of elements, the mean values from 20 blanks were subtracted from each sample. The results of the metal analyses are presented as M±SEM in µg/g on dry weight (DW) basis.

Data were log transformed and tested for normal distribution (Shapiro-Wilk test) and for homogeneity of variance (Levene, *F*-test). From each tissue, an overall measure of the effect of season, sex, age, and site was obtained by four-way multivariate analysis of variance (MANOVA). Seasonal, sexual and age variation for each site and tissue were calculated by Student's t-tests (*t*). The divergences in site, tissue, RI and relative somatic ratios were also calculated by Student´s *t*-tests. Pearson's correlation coefficients (*r*) were calculated in liver and kidneys, in order to establish the relations between elements in the polluted site. Significant differences were accepted at $p<0.05$. For all sequential tests, *p*-values were corrected by the Bonferroni adjustment (Rice, 1989). All statistical procedures were performed with SPSS (version 11.5 for Windows, SPSS Inc.).

4.3.3. Results

Metal concentrations in all the samples analysed were above the detection threshold for all individuals (in µg/kg, Fe:10; Mg:50; Zn:0.50; Pb:0.05; Cu:0.10; Mn:0.10; Hg:0.20; Cd:0.05; Mo:0.05; Cr:0.50; Ni:0.20). In both sites, a large percentage of shrews caught in spring were juveniles, whereas adults were more abundant in autumn. A similar number of males and females were captured in the two sites (Table 4.3.1).

Table 4.3.1. Number of animals captured in the reference and mine site by season and age (in brackets: males, females).

Site	Season	Age		Total
		Juveniles	Adults	
Moura	**Spring**	14 (8,6)	6 (3,3)	20 (11,9)
	Autumn	3 (2,1)	9 (2,7)	12 (4,8)
Mine site	**Spring**	7 (2,5)	3 (2,1)	10 (4,6)
	Autumn	1 (0,1)	11 (6,5)	12 (6,6)

Table 4.3.2. Mean ± SEM of morphometrical parameters in shrews from the reference and mine site.

	Reference site (n=17)	Mine site (n=18)
BW (g)	7.48 ± 0.99	7.20 ± 0.94
BL (mm)	70.60 ± 3.59	68.84 ± 5.49
RI	-0.215 ± 4.187	0.266 ± 0.899
Liver (g)	0.418 ± 0.088	0.456 ± 0.107
% Liver	5.56 ± 0.16	6.27 ± 0.22
Kidneys (g)	0.125 ± 0.018	0.119 ± 0.016
% Kidneys	1.68 ± 0.03	1.66 ± 0.04
Spleen (g)	0.030 ± 0.008	0.031 ± 0.014
% Spleen	0.41 ± 0.03	0.44 ± 0.43

All morphometric parameters were calculated on wet weight (WW) basis. In comparison with specimens from the reference site, the shrews from the polluted site showed a significant increase in the hepatic ratio (t=2.529; p=0.016). No difference was detected between sites for the remaining morphometrical parameters (Table 4.3.2).

MANOVA for all data showed that the importance of parameters in liver was: site (F=8.600; p<0.001) > season (F=5.715; p<0.001) > age (F=3.614; p=0.003) > sex (F=2.451; p=0.027), and all factors were statistically significant. In kidneys, season (F=8.619; p<0.001), site (F=6.657; p<0.001) and age (F=3.449; p=0.004) were the main parameters influencing metal bioaccumulation, and sex (F=2.247; p=0.041) keeping as the less important in variance.

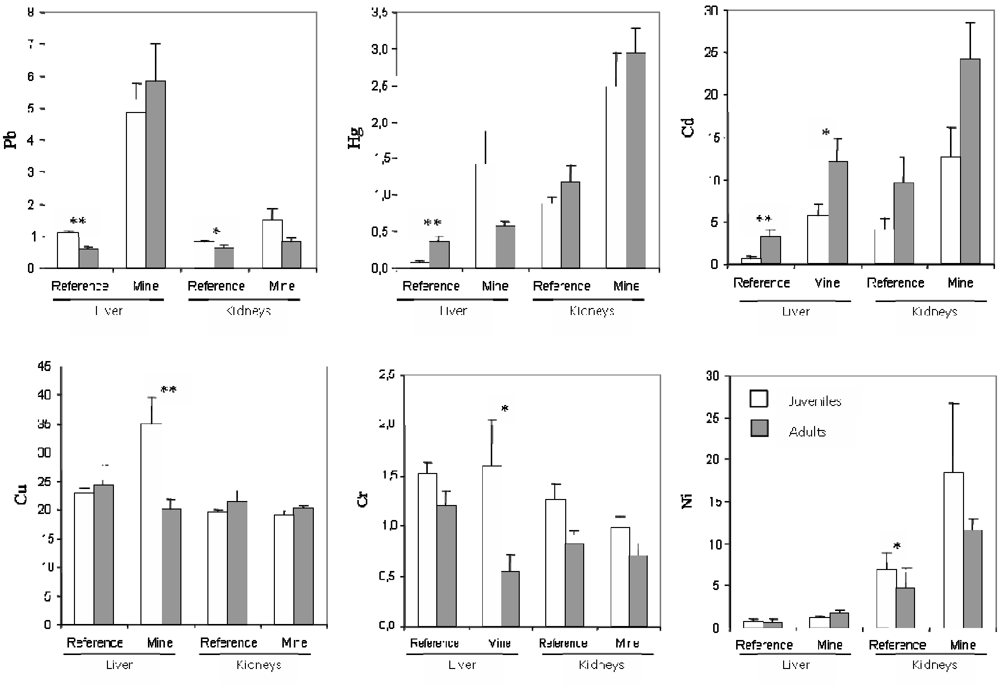
The accumulation pattern of metals by tissues was similar in the two sites. The liver was the main target organ for Fe, Zn, Cu, Mn, and Mo, whereas the kidneys bioaccumulate the highest

Table 4.3.3. Results of the *t*-Student tests for variation in metal concentrations according to tissue, for each site. *p* values corrected by the Bonferroni adjustment.

	Reference site		Mine site	
	t	*p*	*t*	*p*
Pb	--	--	7.382	< 0.001
Hg	-6.009	< 0.001	-5.541	< 0.001
Cd	-4.386	< 0.001	--	--
Fe	8.918	< 0.001	15.391	< 0.001
Mg	--	--	3.786	< 0.001
Zn	4.183	< 0.001	3.434	< 0.001
Cu	--	--	--	--
Mn	13.226	< 0.001	9.401	< 0.001
Mo	7.208	< 0.001	9.851	< 0.001
Cr	--	--	--	--
Ni	--	--	-8.210	< 0.001

concentrations of Hg, Cd and Ni. Cr has similar concentrations in these two organs analysed. Moreover, in the polluted area Mg and Pb were significantly higher in liver when compared with kidneys (Table 4.3.3). Copper and chromium showed not significant differences between tissues in either study sites.

The shrews from Aljustrel mine showed significantly high levels of Pb, Hg, and Ni in hepatic and renal tissues (see Table 4.3.4). Moreover, hepatic levels of Fe, Cd, and Mo were higher and Cr lower in shrews from the mine compared with reference specimens. The hepatic and renal values of the other elements (Mg, Zn, Cu, and Mn) remained statistically invariant between sites. These results indicate that the liver was the tissue with most significant differences in metal bioaccumulation between sites.



Figures 4.3.2-4.3.7. Mean ± SEM values for metals in *C. russula* by tissue, site and age (in µg/g DW) (* p ≤ 0.05; ** p ≤ 0.01; *** p ≤ 0.001).

The bioaccumulation of several elements in *C. russula* tissues showed seasonal differences, and in some cases bioaccumulation patterns varied between tissues and sites of capture (see Table 4.3.5). In shrews from Aljustrel mine, liver showed higher concentrations of Mg and Cu in spring in comparison to autumn, whereas Pb and Ni increased in autumn. In shrews from the reference site,

Table 4.3.4. Mean ± SEM values for metals in *C. russula* by tissue and site (in µg/g DW).

	Liver				Kidneys			
	Reference site (n=22)	**Mine site (n=31)**	*t*	*p*	**Reference site (n=21)**	**Mine site (n=32)**	*t*	*p*
Fe	1257.36 ± 160.03	2958.45 ± 395.21	5.054	< 0.001	323.01 ± 22.99	381.02 ± 30.52	--	--
Mg	899.81 ± 27.51	987.32 ± 83.84	--	--	803.33 ± 45.56	746.87 ± 32.09	--	--
Pb	0.77 ± 0.09	5.26 ± 0.75	7.733	< 0.001	0.70 ± 0.05	1.19 ± 0.21	2.503	0.016
Hg	0.26 ± 0.05	1.03 ± 0.26	5.004	< 0.001	1.08 ± 0.16	2.70 ± 0.29	4.363	< 0.001
Cd	2.27 ± 0.65	8.61 ± 1.47	4.428	< 0.001	7.83 ± 2.06	18.17 ± 2.80	--	--
Zn	145.23 ± 7.27	167.26 ± 19.18	--	--	107.07 ± 7.97	109.46 ± 8.96	--	--
Cu	23.83 ± 10.31	28.31 ± 16.17	--	--	20.82 ± 6.23	19.67 ± 2.69	--	--
Mn	17.56 ± 0.74	17.26 ± 1.42	--	--	5.84 ± 0.46	5.69 ± 0.52	--	--
Mo	3.38 ± 0.24	5.30 ± 0.52	3.252	0.002	1.73 ± 0.08	1.79 ± 0.10	--	--
Cr	1.32 ± 0.11	1.12 ± 0.28	- 3.360	0.002	0.97 ± 0.12	0.86 ± 0.08	--	--
Ni	0.67 ± 0.24	1.48 ± 0.17	4.114	< 0.001	5.40 ± 1.80	15.28 ± 4.39	3.398	0.002

hepatic tissue showed significantly high levels of Mg and Pb in spring, whereas Hg decreased. Finally, chromium showed a significant decrease in autumn in the two sites and tissues studied. Similar pattern was revealed for manganese as a tendency without significant differences.

A clear pattern of age-dependent increase was detected in Cd levels in *C. russula*. In contrast, a decrease with age was observed for Cr and Ni. Moreover, Pb, Cu, and Hg also showed significant differences with age (Figure 4.3.2). The female shrews from the two sites showed higher mean Mo and Ni concentrations in liver and kidneys than males. In the shrews from Aljustrel these sexual differences were significant in liver for Mo in autumn (2.76±1.43 *vs* 6.04±1.09 µg/g; t=-2.876, p=0.017) and in renal tissue for Mo (1.34±1.05 *vs* 1.90±1.08 µg/g; t=-3.439, p=0.011) and Ni (0.34±3.67 *vs* 7.40±1.39 µg/g; t=-2.555, p=0.038) in autumn control shrews. No sex-dependent variation was detected in the concentrations of the other metals at the two sites.

Table 4.3.5. Mean ± SEM values for metals in *C. russula* by site and season (in µg/g DW).

		Reference site				**Mine site**			
		Spring (n=10)	**Autumn (n=12)**	*t*	*p*	**Spring (n=19)**	**Autumn (n=12)**	*t*	*p*
Liver	**Mg**	991.82 ± 39.48	823.14 ± 20.28	4.031	0.001	1118.18 ± 128.75	780.14 ± 14.63	3.359	0.002
	Pb	1.07 ± 0.09	0.52 ± 0.10	3.466	0.002	4.24 ± 0.95	6.88 ± 1.12	--	--
	Hg	0.05 ± 0.01	0.43 ± 0.05	-6.932	< 0.001	1.32 ± 0.41	0.58 ± 0.78	--	--
	Cu	25.51 ± 3.84	22.43 ± 2.54	--	--	33.66 ± 3.81	19.83 ± 3.35	2.290	0.007
	Mn	19.34 ± 1.04	16.08 ± 0.86	--	--	19.31 ± 2.12	14.01 ± 1.05	--	--
	Cr	1.47 ± 0.11	1.19 ± 0.17	--	--	1.65 ± 0.41	0.29 ± 0.09	3.189	0.003
	Ni	0.72 ± 0.25	0.63 ± 0.40	--	--	0.95 ± 0.15	2.32 ± 0.19	-5.646	< 0.001
Kidneys	**Mg**	766.80 ± 15.70	830.72 ± 79.45	--	--	697.43 ± 12.94	829.26 ± 79.19	--	--
	Pb	0.78 ± 0.04	0.64 ± 0.07	--	--	1.38 ± 0.33	0.88 ± 0.13	--	--
	Hg	0.93 ± 0.05	1.19 ± 0.28	--	--	2.71 ± 0.39	2.68 ± 0.45	--	--
	Cu	19.07 ± 0.27	22.13 ± 2.34	--	--	19.50 ± 0.38	19.96 ± 1.13	--	--
	Mn	6.34 ± 0.33	5.46 ± 0.77	--	--	6.23 ± 0.61	4.78 ± 0.93	--	--
	Cr	1.45 ± 0.12	0.60 ± 0.08	5.407	< 0.001	1.16 ± 0.054	0.36 ± 0.08	8.959	< 0.001
	Ni	5.33 ± 1.79	5.45 ± 2.92	--	--	18.13 ± 6.96	10.55 ± 1.45	--	--

Significant correlations between metal bioaccumulation in shrews caught in Aljustrel are shown in Table 4.3.6.

Table 4.3.6. Significant Pearson coefficients and *p*-values between elements in liver and kidneys of the *C. russula* from the mine site.

Liver			Kidneys		
	r	*p*		*r*	*p*
Cu/Mg	0.578	< 0.001	**Cu/Fe**	0.619	< 0.001
Cu/Zn	0.653	< 0.001	**Cu/Mg**	0.818	< 0.001
Cu/Mn	0.411	0.002	**Cu/Zn**	0.752	< 0.001
Pb/Fe	0.665	< 0.001	**Cu/Mn**	0.591	< 0.001
Pb/Hg	0.373	0.006	**Cu/Mo**	0.385	0.004
Pb/Cd	0.374	0.006	**Fe/Mg**	0.484	< 0.001
Pb/Mo	0.556	< 0.001	**Fe/Zn**	0.407	0.003
Pb/Cr	-0.498	< 0.001	**Fe/Mn**	0.440	< 0.001
Pb/Ni	0.523	< 0.001	**Mg/Zn**	0.610	< 0.001
Fe/Hg	0.500	< 0.001	**Mg/Mn**	0.457	< 0.001
Fe/Cd	0.429	< 0.001	**Mg/Mo**	0.374	0.006
Fe/Mo	0.790	< 0.001	**Mn/Cd**	0.470	< 0.001
Fe/Cr	-0.381	0.005	**Mn/Mo**	0.570	< 0.001
Mg/Zn	0.694	< 0.001	**Mn/Cr**	0.510	< 0.001
Mg/Mn	0.712	< 0.001	**Hg/Cd**	0.431	< 0.001
Mg/Mo	0.384	0.005	**Cd/Mo**	0.493	< 0.001
Zn/Mg	0.694	< 0.001	**Mo/Cr**	0.380	0.005
Zn/Mn	0.499	< 0.001			
Mn/Mo	0.523	< 0.001			
Mn/Cr	0.395	0.003			
Hg/Cd	0.525	< 0.001			
Hg/Mo	0.507	< 0.001			
Cd/Mo	0.590	< 0.001			

4.3.4. Discussion

Studies on environmental pollution produced by mining activities in Portugal have reported metal increases in sediments, waters, plants, fish, and mammals (Lopes et al., 2001, 2002; Nunes et al., 2001a, 2001b; Quevauviller et al., 1989; Pereira et al., 2006; Viegas-Crespo et al., 2003). These findings indicate that mines are an important source of metal pollution in this country. The pyrite mine of Aljustrel is located in the Iberian Pyrite Belt, a metalogenic province that crosses the

Southeast of the Iberian Peninsula, which includes the mines of St. Domingos (Portugal) and Aznalcóllar (Spain), two sites known for environmental contaminants. The bioaccumulation and effects of toxic metals has been reported in rodents captured in St. Domingos (Pereira et al., 2006), while the Aznalcollar mine spill of 4.5 millions cubic meters of mine sludge in 1998 affecting the protected area of Doñana. This ecological disaster serves to highlight the great environmental risk of pyrite mines.

4.3.4.1. *Metal bioaccumulation by site*

The mine at Aljustrel releases large amounts of effluents loaded with potentially toxic metals into the environment (Quevauviller et al., 1989; Santos-Oliveira et al., 2002). These metals may bioaccumulated and be biomagnified along the food-chain (Farag et al., 1998) long after mine closure. The main increases of metal concentrations in shrews from the mine site were observed for highly toxic non-essential elements (Pb, Hg, and Cd), as well as two essential elements (Fe and Mo). Nickel, an ubiquitous element that essentiality in mammals is discussed (e.g. Eisler, 1998; Talmage and Walton 1991) and known to be carcinogen, teratogenic, genotoxic and hepatotoxic both to humans and animals (e.g. Kasprzak et al., 2003; Punshon et al., 2003), also increased in these shrews. Essential and non-essential metals displayed contrasting behaviour in their food-chain mobility within soil, plants, invertebrates, and shrews. Non-essential metals are associated with metal ores, pollute soils and waters, may be mobilized by several biological and chemical mechanisms and are highly transferred from soils and AMD across food-chains, and therefore bioaccumulated in small mammals (e.g. Johnson et al., 1978; Lacal et al., 2003; Ma and Talmage, 2001; Milton et al., 2004). Contaminated preys and soil ingest are the main routes of metal intake in mammals (Stansley and Roscoe 1996; Talmage and Walton, 1991) and shrews ingest soil-residing organisms with relatively high bioaccumulation factors such as earthworms. The increase in Pb, Hg, Cd, and Ni in shrews from Aljustrel agrees with previous studies reporting significant amounts of these elements in AMD from pyrite mines in Portugal (Santos-Oliveira et al., 2002), in soils from Aljustrel (Quevauviller et al., 1989), and in tissues of rodents from the St. Domingos pyrite mine (Pereira et al., 2006). Moreover, Pb, Cd, and Ni were accumulated in shrews inhabiting mine sites (Johnson et al., 1978; Smith and Rongstad, 1982; Świergosz-Kowalewska et al., 2005). To our knowledge, mercury is not reported as a common pollutant of pyrite mine tailings and data on the bioaccumulation in shrews from mine sites are lacking. Mercury concentrations in *C. russula* from mine site are higher than those recorded in other insectivores from polluted sites (Alleva et al., 2006; Pankakoski et al., 1993, 1994). This observation supports the hypothesis of a high transfer of this element in the Aljustrel site probably because of the high mercury bioavailability in acidic conditions (Scheuhammer, 1991) such as AMD.

The high amounts of iron found in AMD and in mine soils (Quevauviller et al., 1989; Santos-Oliveira et al., 2002) seem to be responsible for high dietary levels and consequent bioaccumulation in small mammals in pyrite mines (Pereira et al., 2006). Compared with highly toxic metals, environmental and ecotoxicological information on molybdenum is limited and levels of this metal in tissues of insectivorous are not commonly quantified. Pankakoski et al. (1993) reported lower liver Mo concentration in the mole, *Talpa europaea* (M=1.58-1.65 μg/g) that recorded in our study. The increased concentrations of this metal in shrews from Aljustrel might be related to physiological interaction between metals (Pankakoski et al., 1993). Not significant differences between sites were found for the remaining essential elements quantified and their levels in shrew tissues did not appear to be related to increased environmental levels. Thus, in mammalian systems, it is likely that the uptake, bioaccumulation and excretion of these essential elements are effectively controlled physiologically and that they bioaccumulated only in cases of extremely high intake or disrupted metal metabolism (e.g. Goyer, 1997; Ma and Talmage, 2001).

Quantification of metals from reference sites is crucial to determine the current levels of clean sites and the loss of environmental quality in polluted zones. On a general trend, the metal concentrations in *C. russula* from our reference site were similar or lower than those reported in other insectivores from clean sites (Komarnicki, 2000; Ma and Talmage, 2001; Pankakoski et al., 1993, 1994; Shore, 1995; Talmage and Walton, 1991). Only Mo and Ni in kidneys and Mn in liver were among the highest reported in small mammal species. The characteristic species-dependent pattern of essential elements such Mn, the general high baseline metal values such Ni in insectivores, and the scarce information on levels of these three elements (Andrews et al., 1984; Eisler, 1998; Hunter et al., 1989; Lopes et al., 2002; Ma and Talmage, 2001) can explain these high concentrations.

4.3.4.2. *Metal bioaccumulation by season, age, and sex*

The wide range of variation observed in polluted areas may be partially explained by factors such as age and sex or by seasonal differences in diet and, therefore, by the individual response of uptake and bioaccumulation of metals. Few studies have addressed the seasonal changes in intake and bioaccumulation of metals in wild populations of mammals, but similar results were found for essential metals in the Algerian mouse, *Mus spretus*, and for non-essential metals in the Brown hare, *Lepus europaeus (*Massányi et al., 2003; Viegas-Crespo et al., 2003). In the case of variations in the concentration of essential metals, these may be related to life cycle (growth needs, gonad maturation, and reproduction). Moreover, seasonal differences may arise because of the indirect effects of pollution that affect food diversity and bioavailability and the consequent changes in exposure to essential and non-essential elements (e.g. Cloutier et al., 1986; Hunter and Johnson, 1982; Hunter et al., 1989; Lopes et al., 2002; Massányi et al., 2003).

The adult shrews from the two study areas showed about a two-fold increase in Cd concentrations compared with juveniles. This metal bioaccumulates with age in insectivores (Pankakoski et al., 1993, 1994; Read and Martin, 1993) and rodents from mine sites (Milton et al., 2003; Smith and Rongstad, 1982). This age-bioaccumulation in renal tissue is associated with the formation of a stable cadmium-metallothionein complex as a detoxification mechanism to prevent toxic effects (Johnson et al., 1978; Ma and Talmage, 2001; Świergosz-Kowalewska et al., 2006). The increase in Hg with age also occurs in *C. russula* from a polluted wetland (Sánchez-Chardi et al., 2007). Although the livers from shrews caught in Aljustrel showed an increase in Pb with age, this metal did not show a clear bioaccumulation pattern with this factor. This observation contrasts with results from bones from the same species (Sánchez-Chardi et al., 2007). This discordant result could be attributed to the differences between hard and soft tissues bioaccumulation and/or by differences in exposure levels or chemical forms of Pb. The half-life of Pb in soft tissues is in the order of days or weeks and the level of this element indicates a relatively recent exposure, whereas the main Pb body burden in mammals is the bones, where it is stored in a lower toxic form. In contrast, Cr and Ni decrease with age in *C. russula*. Similar data for Cr was also reported in the common shrew, *Sorex araneus*, and *T. europaea* (Pankakoski et al., 1993, 1994) and for Ni in the meadow vole, *Microtus pennsylvanicus* (Smith and Rongstad, 1982). These observations may be explained by an analogous metabolic mechanism for Cr and Ni, and/or a higher digestive absorption rate in juveniles and, therefore, poor intestinal absorption of these elements in adults (Bonda et al., 2004; Eisler, 1984; Outridge and Scheuhammer, 1993; Pereira et al., 2006). While Fe, and Mn bioaccumulated with age in small mammals from polluted sites (e.g. Stansley and Roscoe 1996; Talmage and Walton, 1991), we did not observe this trend in *C. russula* from study sites.

When compared with the other parameters, sex remains as less importance in bioaccumulation patterns. Metal bioaccumulation in mammals is related to reproduction and hormonal status. During pregnancy and/or lactation females may mobilized metals and transfer them across the placenta to the foetus or via milk to sucklings. Therefore, *a priori* a reduction in the concentration of toxic metals in females might be expected; however, contradictory results have been reported (e.g. Komarnicki, 2000; Lopes et al., 2002; Pankakoski et al., 1993; Smith and Rongstad, 1982; Stansley and Roscoe 1996). The high concentrations of Mo and Ni in female shrews may be due to nutritional status as well as to differences in dietary intake and uptake of metals.

4.3.4.3. *Metal bioaccumulation by tissue*

The liver and kidneys are the main tissues involved in metabolic processes of toxic heavy metals such as biotransformation, bioaccumulation, detoxification and excretion. Our results on metal distribution in *C. russula* are consistent with those reported for other small mammal species (e.g. D'Havé et al., 2006; Eisler, 1998; Ma and Talmage, 2001; Pankakoski et al, 1993, 1994; Talmage and Walton, 1991). The liver was the main accumulator of Zn, Cu, Fe, Mg, Mn, and Mo, whereas Hg, Cd, and Ni were higher in kidneys and Cr showed similar mean values in both tissues and study sites. In contrast, lead exhibited distinct tissue pattern between sites: while Pb average values were similar between tissues in the reference site, the liver of shrews from Aljustrel showed higher concentrations. This latter distribution corroborates results for the bicoloured white-toothed shrew, *Crocidura leucodon,* and for the European hedgehog, *Erinaceus europaeus* (D'Havé et al., 2006; Topashka-Ancheva and Metcheva, 1999) and is discordant with data for other small mammal species that show higher renal than hepatic Pb concentrations (e.g. Pankakoski et al., 1994; Talmage and Walton, 1991). Although non-essential metals typically show accumulations within specific target organs, a change in tissue distribution has been reported in *S. araneus*, and *T. europaea* in relation with pollution (Andrews et al., 1984; Komarnicki 2000; Talmage and Walton, 1991). This phenomenon seems related to physiological mechanisms to decrease toxicity, but the role of chronic exposure and half-life of Pb in soft tissues, as well as particular tissue distribution in *C. russula* must not be disregarded.

4.3.4.4. *Morphometric parameters*

Given the scarce data on morphometrical parameters in insectivorous mammals, our results contribute to estimating the effects of pollution on wildlife. The results obtained for RI and relative weights in the two study sites were similar to those reported by other authors for small mammals inhabiting polluted and reference sites (Bartels et al., 1979; Ma, 1989; Milton et al., 2003; Pereira et al., 2006; Sánchez-Chardi et al., 2007). We found no differences in RI between the two sites, indicating similar fitness in the two areas; however, we did detect an increase in relative hepatic weight in shrews from mine site which may be indicative of hepatic edema (Milton et al., 2003). In fact, while body condition index is a measure of energy reserves and environmental quality, changes in the relative weight of somatic tissues may indicate metal exposure at toxic levels (Ma, 1989; Ma and Talmage, 2001). The increase in relative weight has been reported in kidneys of common shrews with extremely high Pb levels (Ma, 1989; Ma and Talmage, 2001) and in livers of bank voles, *Myodes glareolus* (formerly *Clethrionomys glareolus*), with similar Pb levels (8.0±0.8 μg/g DW; Milton et al., 2003) to those recorded in shrews from the Aljustrel mine site. These large differences between rodents and insectivores indicate that although these alterations are associated with Pb levels, they may also depends on other factors such as the interspecific tolerance of metals at toxic levels, exposure route and chemical form of the metal (Farag et al., 1998; Ma, 1989; Ma and Talmage, 2001).

4.3.4.5. *Toxicity effects*

Environmental pollution may stress animal populations, even if acute poisoning effects or levels are not observed (Pankakoski et al, 1994). From an ecotoxicological point of view, high metal exposure may produce toxic effects on wild specimens reducing reproduction or life expectancy because of animals may be more exposed to predators. Although insectivores show high tolerance to metal pollution (Ma, 1989, 1996; Ma and Talmage, 2001; Shore, 1995; Talmage and Walton, 1991), the Pb, Hg, and Cd concentrations reported in specimens from Aljustrel may reach toxic levels. These non-essential elements have been associated with a wide range of toxicological responses in liver and kidneys of small mammals; these include oxidative stress, acute injury, apoptosis, and necrosis (e.g. Milton et al., 2003; Pankakoski et al., 1993; Stanley and Roscoe, 1996). In these organs, a wide range of critical Pb levels varying from 70 µg/g DW to 5-10 µg/g DW (Ma, 1996; Scheuhammer 1991) is considered a chemical biomarker of Pb toxic exposure in mammals. In our study, 11 out of 31 shrews in Aljustrel showed more than 5 µg/g of Pb in liver (maximum of 15.99 µg/g DW). Twenty-four out 32 specimens the mine site showed Hg concentrations above 1.1 µg/g in kidneys (maximum of 5.65 µg/g DW) which is considered indicative of mercury pollution (Eisler 1987). In contrast with rodents, insectivores are very tolerant to Cd as demonstrated in laboratory and field studies. While liver and kidney lesions in common shrews were associated with Cd concentrations of 577 and 253 ug/g DW respectively, and 350 µg/g DW is reported as indicative of harmful effects in insectivores (Hunter et al., 1989; Ma and Talmage 2001), renal concentrations of about 110-260 µg/g produce nephrotoxic effect in mice (Pankakoski et al., 1993). Moreover, hepatic and renal alterations have been reported in bank voles with 15.1 µg/g DW Cd in liver and 17.0 in kidneys (Włostowski et al., 2003). The cadmium concentrations observed in shrews from the mine site did not reach toxic levels for these animals, but were higher than the no-observable-adverse-effects-level (NOAEL) in rodents and may indicate a considerable environmental pollution. Scarce information is available about food-chain transfer and uptake and tissue concentration of Ni (Andrews et al., 1984; Eisler, 1998; Hunter et al., 1989); however the high concentrations found in *C. russula* require more toxicological information that allows the assessment of their environmental consequences.

4.3.4.6. *Correlation between metals*

In spite of these toxic levels of single elements, the interaction and aggregative effect of metal mixture must be considered to determine subclinical environmental problems because these mixtures may increase toxic effects by synergism (Bellés et al., 2002). In fact, shrews from mining areas are, in general, exposed to a mixture of metals from mineral ores and associated inclusions (e.g. Johnson et al., 1978). Our data agree with relations reported for Fe, Zn and Cu with Pb, Cd, and Hg (e.g. Bonda et al., 2004, Johnson et al., 1978; Pereira et al., 2006; Włostowski et al., 2003). On the whole, the role of the liver in homeostatic mechanisms and the kidneys in excretion processes may explain the correlations observed. Interaction between non-essential metals and essential metals is attributed mainly to competition in intestinal absorption, to competition mechanisms regarding metal enzymes complexes, and/or to the protective effects in front of oxidative processes produced by metal exposure. Despite of this, our results require confirmation due to the lack of information about these interactions in wild populations.

4.3.5. Conclusions

The increase of metal in shrews due to their great feeding requirements of shrews may reveal low or current levels of pollution by bioaccumulation at detectable levels. Here we report that an abandoned pyrite mine produces a considerable increase in the bioavailability and bioaccumulation of potentially toxic metals (Pb, Hg, Cd, and Ni) in shrews. In fact, these elements might represent an environmental risk in these abandoned mines. Our results also indicate that season-, age- and sex-dependent variations must be considered when interpreting exposure, bioaccumulation and effects of metals in wild mammals. The trophic position and physiological characteristics of insectivores make them suitable bioindicators in the assessment of environmental quality and bioaccumulation in other species such as protected carnivorous mammals.

4.3.6. References

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4.4. ALENTEJO II

Haematology, genotoxicity,
enzymaticactivity and histopathology
in the shrew *Crocidura russula*
inhabiting an abandoned pyrite mine

Haematology, genotoxicity, enzymatic activity and histopathology as biomarkers of metal pollution in the shrew *Crocidura russula*

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Abstract

Haematological (WBC, RBC, Hgb, Hct) and genotoxicity (MNT) parameters, hepatic enzymatic activities (GST, GPx, GR), and a histopathological evaluation of liver, kidneys and gonads were assessed as general biomarkers of metal pollution in the shrew Crocidura russula inhabiting a pyrite mining area. Specimens exposed to metals presented a few significant alterations when compared with reference animals: GST activity decreased; micronuclei increased; and evident liver alterations related to metal exposure were observed. Haematological parameters also showed a tendency to increase in shrews from the polluted site. On the basis of all the parameters studied, age was an important factor that partly explained the observed variation, whereas sex was the least important factor. Significant correlations were also found between heavy metal concentrations and biomarkers evaluated, demonstrating the great influence of these metals in the metabolic alterations. To the best of our knowledge, these data constitute the first measurements of a battery of biomarkers in shrews from a mine site and are among the few available for insectivorous mammals.

4.4.1. Introduction

Abandoned mines constitute a serious environmental problem throughout the world. These sites represent a potential threat to human and ecosystems health and cause important impacts on wide areas over long periods if nothing is done towards pollution mitigation. The mine of Aljustrel is a good example of this situation. This mine site is one of the biggest deactivated pyrite mines in Portugal and spills out great amounts of liquid effluents, in what is known as acid mine drainage (AMD), characterized by high metal contents and low pH. In terrestrial food webs, toxic levels of some metals are easily reached in abandoned mining sites due to the high bioavailability of metals found in these areas. In fact, previous studies have reported the accumulation of several metals in soils, plants, and animals as well as their physiological effects in areas affected by deactivated mines in this country (e.g. Gerhardt el al., 2005; Quevauviller et al., 1989; Pereira et al., 2006; Sánchez-Chardi et al., 2007b). In spite of this high risk, scarce data is available concerning the influence of metal pollution on the health of species belonging to higher trophic levels, such as shrews. Almost no data was found on the effects of contamination in carnivorous small mammals inhabiting abandoned pyrite mining areas, Marques et al. (2007) quantify few haematological and enzymatic parameters in greater white-toothed shrews, *Crocidura russula*, from a Pb/Zn mine site. To our knowledge, the present study measures, for the first time, a battery of biomarkers in *C. russula*.

The assessment of physiological effects of chronic exposure to metals using biomarkers of sublethal toxicity is necessary in order to evaluate the impact of pollution under realistic conditions. These effects include genotoxic (Ieradi et al., 1996; Sánchez-Chardi and Nadal, 2007), enzymatic (Lopes et al., 2002; Świergosz-Kowalewska et al., 2006), haematological (Nunes et al., 2001; Reynolds et al., 2006; Rogival et al., 2006), and histological alterations (Clark Jr. et al., 1992; Damek-Poprawa and Sawicka-Kapusta, 2004; Pereira et al., 2006; Stansley and Roscoe, 1996) that in general only occur when substantial concentrations of metals are present in the tissues. In fact, the combined use of biomarkers with bioaccumulation data provides a suitable measure of health status, physiological condition, and response of terrestrial small mammal populations to pollution (e.g. Świergosz et al., 1998; Walker, 1998).

Environmental control should be multidirectional, but one of the most essential criteria of analysis is related to observations carried out on wild animals. Small mammals are ideal for monitoring environmental pollution as well as for evaluating the risk for human populations living in polluted areas such as abandoned metalliferous mining sites (e.g. Pereira et al., 2006; Sánchez-Chardi et al., 2007b; Talmage and Walton, 1991; Walker, 1998).

Here we quantify haematological and genotoxic parameters, enzymatic activities, and histopathological alterations in shrews inhabiting an abandoned pyrite mining site. Moreover, the effect of age and sex was also measured and correlations between metal bioaccumulation and biomarkers were determined in order to explain the variation found in these parameters. Finally, with all this information in mind, we discuss the risk involved with this kind of environmental pollution and the suitability of insectivorous mammals as bioindicators.

4.4.2. Material and Methods

4.4.2.1. *Study sites*

This study was carried out in the vicinity of the abandoned pyrite mine of Aljustrel, located in the Baixo Alentejo province (southern Portugal). Deactivated about a decade ago, approximately 50,000,000 t of target metals were extracted from this mine during its 130 years of exploitation. The sampled area in Aljustrel (37º53'08''N; 08º08'32''W) is located along the "Água forte" riverside, where the stored acidic effluents are discharged during rainy periods. For comparative purposes, an area with similar relief, climate and vegetation, located 69 Km northeast of the Aljustrel mine (38º11'13´´N; 07º24'34´´W) was selected as a reference, considering that no exogenous sources of heavy metals are known. The climate of the region where both sampling areas are located is characterized by hot dry summers and mild winters. The vegetation mainly consists of sparsely scattered specimens of *Quercus rotundifolia*, as well as several shrub and herbaceous species (*Rubus ulmifolius*, *Nerium oleander*, *Echium plantagineum*, *Bromus rigidus*, *Vulpia myunos*, and *Phleum phleoides*).

In 2003, a total of 34 specimens of the greater white-toothed shrew were live-trapped using Longworth® and Sherman® traps. Hepatic metal concentrations of these shrews were reported in a previous study (Sánchez-Chardi et al., 2007b). Distribution of captures by site, sex, and age is showed in Table 4.4.1. All captured animals appeared to be in good physical condition with no major signs of illness or deformity. Animals were sexed, weighed and transported to the laboratory. After a slight anaesthesia, specimens were sacrificed by cervical dislocation in strict accordance with ethical directives on the protection of animals.

Table 4.4.1. Distribution of specimens by site, sex and age.

Site	Sex	Age		
		Juveniles	**Adults**	**Total**
Reference	**Males**	6	4	10
	Females	5	3	8
Mine	**Males**	4	4	8
	Females	4	4	8

4.4.2.2. *Haematological and genotoxic parameters*

Peripheral blood was collected by cardiac punction using heparinized syringes. White Blood Cells count (WBC, $\times 10^3$/mm), Red Blood Cells count (RBC, $\times 10^6$/mm), Haemoglobin concentration (Hgb, g/dl), and Haematocrit (Hct, %) were quantified in a Coulter Counter Analyser (Beckman Coulter, USA).

For the micronucleus test, duplicate blood smears were made for each specimen on pre-cleaned microscope slides, fixed with heat, and stained with conventional May-Grünwald Giemsa stain. For each individual, MN frequency was scored on 2,000 blood erythrocytes through an oil immersion objective (x100) on a Leica Leitz DMRB microscope.

4.4.2.3. *Enzymatic parameters*

A liver portion of about 300 mg was rinsed in an ice-cold 0.154 mM KCl solution immediately after dissection. Glutathione S-transferase (GST), glutathione peroxidase (GPx) and glutathione reductase (GR) activities were assessed according to Habig et al. (1974), Paglia and Valentine (1967) and Calberg and Mannervik (1985) respectively. Total protein contents were determined according to the Biuret method (Gornall et al., 1949) using Bovine Serum Albumin (Sigma, Spain) as standard. Additional methodological descriptions may be found in Marques et al. (2007).

4.4.2.4. *Histopathological evaluation*

A small part of the liver, the left kidney and the gonads were fixed in 10% neutral-buffered formalin, dehydrated in ethanol, cleared in xylene and embedded in paraffin wax. Sections of 5 µm thick were stained with conventional hematoxylin and eosin and the slides were examined by light microscopy. The incidence of alterations was reported in a qualitative evaluation. Moreover, for statistical purposes the severity of alterations was measured on a semi-quantitative scale scored in four categories according to the intensity of alterations: without alteration (0), slightly altered (1), intermediately altered (2), and strongly altered (3).

4.4.2.5. *Statistical analyses*

All data were expressed as arithmetic mean ± standard error of the mean (M±SEM). When necessary, data were log transformed and then tested for normal distribution (Shapiro-Wilk test) and homogeneity of variance (Levene, *F*-test). Comparisons of haematological and enzymatic parameters by age, sex, and site were performed with Student's tests (*t*). Significant genotoxic and histopathological divergences for these three factors were assessed using Mann-Whitney tests (*U*). Moreover, for each of these factors, data were pooled in order to obtain an appropriate sample size for statistical analyses. To investigate associations between bioaccumulation and its effects, Spearman or Pearson correlation coefficients (*r*) were calculated between hepatic metal concentrations and enzymatic, haematological, histopathological, and genotoxic parameters for both study sites. All statistical analyses were performed with SPSS 11.5 software for Windows.

4.4.3. Results

4.4.3.1. *Haematological and genotoxic parameters*

Descriptive statistics for haematological parameters are shown in Table 4.4.2. Though no significant differences were found by study site, season, age, or sex in these parameters, a tendency toward increase was detected in specimens from the polluted site. An age-dependent increase in both studied sites was also observed (Table 4.4.2). Moreover, at the polluted site, significant correlations were observed between RBC and Pb (r=-0.609, p=0.009), RBC and Cr (r=0.618, p=0.008), Hct and Pb (r=-0.510, p=0.036), and Hgb and Cu (r=0.525, p=0.031).

Table 4.4.2. Haematological parameters in *C. russula* by age and site.

	Reference site			**Mine site**		
	Juveniles	**Adults**	**Total**	**Juveniles**	**Adults**	**Total**
	n=8	n=8	n=16	n=5	n=10	n=15
WBC (x 10^3/mm)	3.150±0.327	4.625±0.740	3.887±0.435	3.840±0.819	4.060±0.749	3.987±0.553
RBC (x 10^6/mm)	5.997±0.917	8.147±0.664	7.072±0.613	7.507±0.468	8.544±0.526	7.853±0.371
Hgb (g/dl)	12.212±0.861	13.887±0.487	13.050±0.524	13.300±0.595	14.130±0.427	13.853±0.350
Hct (%)	25.012±3.816	34.700±2.887	29.856±2.628	34.920±1.970	33.330±2.226	33.860±1.592

Shrews collected on the polluted site exhibited significantly higher micronuclei mean frequencies than specimens from the reference site (1.00±0.15 *vs* 0.14±0.09‰, respectively; U=4.195, p=0.001). No relationships were found in MN frequencies with age or sex; whereas significant correlations were detected between MN and several metals, namely Fe (r=0.722, p=0.002), Mn (r=-0.592, p=0.020), Cu (r=-0.565, p=0.028), Pb (r=0.524, p=0.045), Cd (r=0.537, p=0.022), and Cr (r=-0.744, p=0.001).

4.4.3.2. *Enzymatic parameters*

Specimens from the abandoned mine site showed a significant decrease in GST activity (t=-3.422; p=0.002). No site variation was found in the activity of the other quantified enzymes (Table 4.4.3). With age, a general tendency toward a decrease in GST activity was detected, while GPx (Reference site: t=-2.336, p=0.034) and GR tended to increase (Table 4.4.3). No sex-dependent variation was observed in enzymatic activities of *C. russula* from both study sites. Moreover, in the specimens from the polluted area, significant positive correlations were identified between GPx activity and hepatic concentrations of Pb (r=-0.587, p=0.013), Cu (r=0.791, p<0.001), Mn (r=0.651, p=0.005), and Cr (r=0.536, p=0.027).

Table 4.4.3. Enzymes activities (µmol/min/g protein) in the liver of *C. russula* by age and site.

	Reference site			**Mine site**		
	Juveniles	**Adults**	**Total**	**Juveniles**	**Adults**	**Total**
	n=8	n=9	n=17	n=6	n=11	n=17
GST	1094.01±96.99	891.44±67.65	986.76±61.54	791.57±74.71	628.36±89.37	685.96±65.01
GPx	113.20±19.93	161.57±11.34	139.24±12.63	128.55±10.45	143.25±16.60	133.14±8.73
GR	66.25±3.61	68.11±3.12	67.24±2.31	64.67±4.24	76.00±5.67	71.75±4.04

4.4.3.3. *Histopathological evaluation*

Specimens exposed to metal pollution in the mining area showed a significant increase in number and severity of pathological alterations, namely foci of cell necrosis, apoptosis, and cytoplasmic vacuolization in the hepatic tissue, when compared with reference shrews (U=17.500; p<0.001). In fact, 86% of the analysed specimens from the mine site showed evidences of necrosis and 79% of vacuolization. On the other hand, 8% of the specimens from the reference site showed evidences of necrosis and 58% presented signs of vacuolization. Moreover, a specimen from the polluted site showed evidences of apoptosis in the hepatic tissue with nuclei fragmentation and picnotic nuclei. This was confirmed with an immunohistochemistry analysis, namely the detection of cysteine protease Caspase-3 (data not shown). Table 4.4.4 reports a semi-quantitative severity scale of alterations in the liver and Figure 4.4.1 shows examples of control and altered tissues. No significant age- or sex-dependent variation was detected in these pathologies for either of the studied sites. There were also no significant relationships between pathologies and hepatic metal concentrations. Moreover, no alterations related with metal pollution were observed in the kidneys, testes or ovaries of any of the analysed shrews.

Table 4.4.4. Frequency and severity of histological alterations in livers of *C. russula* (without alteration (-), slightly altered (+), intermediately altered (++), strongly altered (+++)).

	Reference site (n=12)				**Mine site** (n=14)			
	-	+	++	+++	-	+	++	+++
Necrosis and Degeneration	11	1	0	0	2	8	4	0
Apoptosis	12	0	0	0	13	0	0	1
Vacuolization	5	4	2	1	3	1	4	6

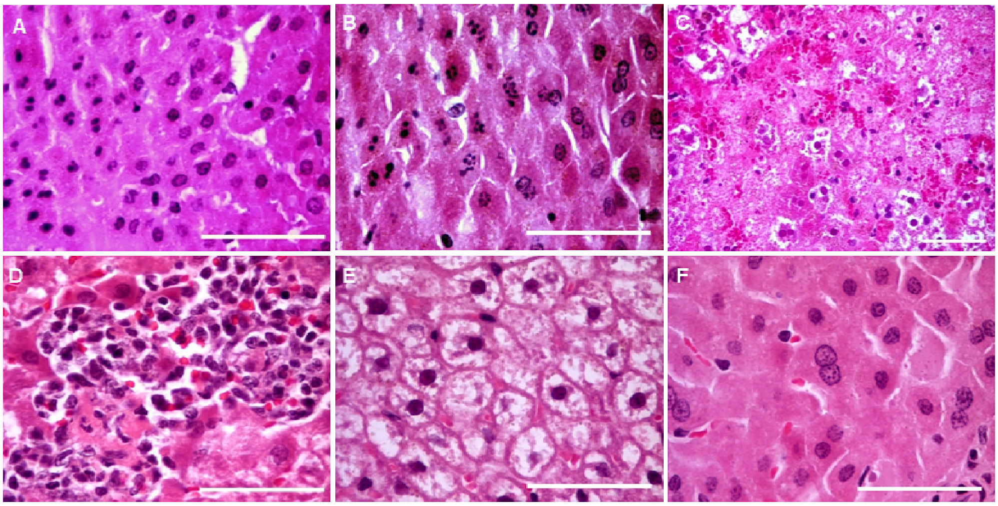

Figure 4.4.1. Sections of hepatic tissue of *C. russula* showing: A-B) apoptosis with nuclei fragmentation; C-D) degeneration and necrosis with lymphoid elements; E) more rounded cells, swollen parenchyma with vacuolization; and F) normal hepatic tissue. (bar size: 50 µm).

4.4.4. Discussion

Several pyrite mines have been closed in southern Portugal in the past few decades, mainly due to economic reasons. Most of them were abandoned without any previous environmental recovery plan, and thus have continued to be an environmental threat long after mining activities have ceased. Harmful effects to biota include deep changes in cells, tissues, individuals, populations, and ecosystems. However, scarce information is available on effects on higher trophic level species such as insectivorous mammals.

4.4.4.1. *Haematological and genotoxic parameters*

Registered values for haematological parameters are in agreement with available literature on small mammal species (see revision in Table 4.4.5). No significant differences were found between study sites in spite of the higher mean values detected in the polluted area. Such a result could, at least partially, be due to the great dispersion of data that may mask any significant difference. In fact, homeostasis of mammalian systems under chronic exposure to environmental pollution may decrease and/or attenuate differences in wild populations, in contrast with laboratory animals that often showed significant differences when acutely exposed to metals (e.g. Jadhav et al., 2007; Medina et al., 2007; Włostowski et al., 2003). Several studies have reported alterations of haematological parameters in Algerian mice, *Mus spretus*, house mice, *Mus musculus*, wood mice, *Apodemus sylvaticus*, and northern pocket gophers, *Thomomys talpoides*, inhabiting polluted sites (see Table 4.4.5). In our study, this tendency toward an increase of haematological values might be related with protective effects of essential metals such as iron or with interactions of toxic metals with oxygen transport, as suggested by the low level of efficiency of their mitochondria in the elimination of reactive oxygen species (ROS) and/or immunotoxic effects of pollutants (e.g. Stewart et al., 2005; Tersago et al., 2004). Moreover, the higher levels of toxic metals in adults as compared with juveniles and the alterations of metabolism in old animals could explain the tendency toward an increase of some haematological parameters with age. In accordance with our results, no sex-dependent variation was showed for the same analysed parameters in *M. spretus* and *A. sylvaticus* (Nunes et al., 2001; Rogival et al., 2006).

The micronuclei frequencies obtained for the reference area are in agreement with previously published data of background levels in *C. russula* and other small mammal species collected from different reference sites (see references in Sánchez-Chardi and Nadal, 2007). The significant positive correlation found between MN frequencies and non-essential metal concentrations in shrews from the pyrite mining site may be indicative of clastogenic effects of these elements in wild specimens (e.g. Ieradi et al., 1996; Sánchez-Chardi and Nadal, 2007; Topashka-Ancheva et al., 2003; Tull-Singleton et al., 1994). Correlations between MN and essential elements could be partly explained by the protective and/or antagonistic effects between elements in specimens exposed to high concentrations of toxic metals.

4.4.4.2. *Enzymatic parameters*

It is well documented that heavy metal exposure alters the normal activity of antioxidant enzymes, as reported in laboratory and wild mammals (e.g. Li et al., 2006; Lopes et al., 2002;

Table 4.4.5. Haematological parameters and hepatic enzymes activities (mean, mean ±SD, mean±SEM or mean and range) reported for wild small mammals from polluted (P) and reference (R) sites. References: 1. Marques et al., 2007; 2. Bartels et al., 1979; 3. Gorriz et al., 1996; 4. Lopes et al., 2002; 5. Tersago et al., 2004; 6. Topashka-Ancheva et al., 2003; 7. Świergosz-Kowalewska et al., 2006; 8. Knopper and Mineau, 2004; 9. Nunes et al., 2001; 10. Viegas-Crespo et al., 2003; 11. Stansley and Roscoe, 1996; 12. Kucera, 1988; 13. Reynolds et al., 2006.

			Haematological parameters				Enzymes activities					
Species		n	WBC x10^3 (mm^{-3})	RBC x10^6 (mm^{-3})	Hgb (g l^{-1})	Hct (%)	GST	GPx	GR	Units	Statistics	Refs.
Crocidura	P	16	4.80	8.13	14.0	35.7	1.18	0.128	0.067	uM/mg prot/min	Median (range)	1
russula	R	17	3.30	6.64	13.2	29.3	0.933	0.168	0.067			
	R	6		10.8±0.5	15.6±1.7	44.3±3.0					M±SD	2
Suncus	R	3		18.3±0.3	17.4±0.8	50.3±1.5						
etruscus												
Apodemus	P	25	1.42±2.25	8.20±1.66	16.00±1.53	45.83±3.24					M±SD	3
sylvaticus	P	30	1.21±1.43	8.43±1.42	16.59±1.59	47.40±4.43						
	R	27	1.51±1.36	8.57±1.78	16.69±1.61	46.77±4.06						
	R	10-15					96.8±31.1 - 89.2±32.7			uM/g WW liver/ min	M±SD	4
	P	6	1.34±0.15	7.10±0.05							M±SEM	5
	P	7-8	1.56±0.23	8.92±0.03								
	R	7-9	2.03±0.24	8.94±0.04								
Apodemus	P	8	3.68±1.26	8.16±0.88	18.44±0.79	54.4±4.08					M±SEM	6
flavicollis	P	12	3.17±1.33	7.8±3.2	18.40±1.8	56.2±2.7						
Myodes	P	6					0.921±0.292	21.622±6.950	0.053±0.009	uM/mg prot/min	M±SEM	7
glareolus	P	5					0.334±0.057	5.642±1.017	0.134±0.016			
	R	10					0.558±0.113	27.134±9.124	0.196±0.042			

Table 4.4.5. Continued.

Species		n	Haematological parameters				Enzymes activities				Statistics	Refs.
			WBC x10^3 (mm-^{3})	RBC x10^6 (mm-^{3})	Hgb (g l^{-1})	Hct (%)	GST	GPx	GR	Units		
M. glareolus	P	9	3.73±8.58	8.25±1.00	17.82±1.8	53.92±3.39					M±SEM	6
(continued)	P	8	3.15±1.25		17.80±1.0	54±0.8						
	R	11	2.83±0.75	9.97±1.56	20.30±1.3	55.31±4.75						
Chionomys	P	5	3.62±1.16	8.92±1.81	14.8±2.34	49.95±4.87					M±SEM	6
nivalis	R	4		10.96±1.36	16.85±2.1	46.15±3.43						
Microtus	P	23	4.4±0.5								M±SEM	8
pennsylvanicus	P	27	5.2±0.6									
	P	5	4.6±1.1									
	P	11	4.5±0.5									
	R	10	5.2±0.6									
Mus	P	60-80	7.92±5.81	8.23±1.57	15.6±2.6	37.7±7.9					M±SD	9
spretus	R	36-46	6.83±4.35	8.49±0.84	15.1±1.7	39.1±4.6						
	R	16-14					162.8±93.8 - 129.5±37.1			uM/g WW liver/ min	M±SD	4
	P	49					187.8±56.9			uM/g WW liver/ min	M±SD	10
	R	30					147.3±73.9					
Peromyscus	P	11			14.0 (11.5-16.0)	47 (37-55)					Mean (range)	11
leucopus	R	12			15.0 (12.5-17.0)	49 (42-56)						

Table 4.4.5. Continued.

Species		n	Haematological parameters				Enzymes activities					
			WBC x10^3 (mm^{-3})	RBC x10^6 (mm^{-3})	Hgb (g l^{-1})	Hct (%)	GST	GPx	GR	Units	Statistics	Refs.
Peromyscus	P	19			14.6	44.3					M	12
maniculatus	P	34			15.2	48.5						
	R	44			12.4	40.7						
	R	44			12.7	41.4						
Thomomys	P	10	2.71±0.44								M±SEM	13
talpoides	P	9	5.29±1.17									
	R	10	4.81±0.78									

Pinheiro et al., 2001; Reynolds et al., 2006; Viegas-Crespo et al., 2003). However, data with measurements on these parameters is quite scarce for shrews (Hamers et al., 2006; Marques et al., 2007). Some shrew species are among the smallest mammals and in order to maintain their body temperature, they have extremely high food requirements due to their high metabolic rate, which is higher than predicted by allometric scaling (e.g. Stewart et al., 2005). These atypical physiological conditions also affect enzyme activities in detoxification systems, as reported for the short-tailed shrew, *Blarina brevicauda* (Stewart et al., 2005). In the present study, though no significant differences were found for GPx and GR between study areas, a decrease of GST activity was registered in shrews from Aljustrel. GST catalyzes the conjugation of various substances with glutathione, playing an important role against oxidative stress. This result agrees with data provided by Swiergosz-Kowalewska et al. (2006) for bank voles, *Myodes glareolus* (formerly *Clethrionomys glareolus*), exposed to metals from a zinc-lead smelter. Similar levels of Cd and Pb bioaccumulation in renal tissue (maximum 47 µg/g and 20 µg/g, respectively) were reported in shrews from the Aljustrel mining site (Sánchez-Chardi et al., 2007b). In general, the non-significant differences detected in GPx and GR activities between study sites can possibly be related to an adaptation or increasing tolerance of specimens to chronic metal exposure.

The free radical theory proposed by Harman (1992) postulates an increase of ROS with age and, therefore, a consequent increase of antioxidant defences. Moreover, age-dependent variations were reported in antioxidant activity of rats (e.g. Gupta et al., 1991) and in the activity of hepatic cytochrome P450 in common shrews, *Sorex araneus* (Hamers et al., 2006). Since toxic metals such as Cd, Pb, and Hg reached significantly higher levels in adult shrews as compared with juveniles collected in Aljustrel (Sánchez-Chardi et al., 2007b), the correlation found between enzyme activities and age might be indicative of metal toxicity when a certain level of bioaccumulation is reached, as well as a decline in cellular response to oxidative stress with increasing age (e.g. Holbrook and Ikeyama, 2002). In fact, several studies have showed that metal exposure can cause alterations of GPx, GST, and GR activities, preventing some adverse effects of oxidative stress induced by the production of ROS species (e.g. Cnubben et al., 2001; Jadhav et al., 2007).

In rodents (Fouchécourt and Rivière, 1995; Li et al., 2006) and insectivores (Hamers et al., 2006) sex-dependent variation has been previously reported in enzymes from detoxification systems. Nonetheless, no differences were found in *C. russula* in the present study, in agreement with other surveys using wild populations of small mammals (Lopes et al., 2002; Viegas-Crespo et al., 2003). These divergent results may be indicative of inter-species and/or inter-populations differences. Due to the scarce information available on biotic parameters obtained from wild populations of small mammals exposed to metal contamination, it is essential that more studies dealing with these aspects be carried out.

4.4.4.3. *Histopathological evaluation*

One of the goals of this study was to assess, for the first time, histological alterations related to metal exposure in livers of wild shrews. As far as we know, available data on the histopathological effects of metal pollution in wild insectivorous mammals is very scarce and limited to renal lesions (e.g. Stansley and Roscoe, 1996). Histopathological evaluation of target tissues is a suitable biomarker that provides important qualitative and quantitative information about acute or chronic effects of toxic compounds, sometimes not so finely predicted by other parameters (e.g. Jadhav et al., 2007; Reynolds et al., 2006; Thijssen et al., 2007). This approach is commonly used on laboratory animals but seldom on wild populations of insectivorous mammals. Several other studies have used the liver as the main target organ for the assessment of histopathological alterations, examining various rodent species exposed to non-essential metals on polluted sites (Clark Jr. et al., 1992; Damek-Poprawa and Sawicka-Kapusta, 2004; Pereira et al., 2006).

In the present study, shrews from the mining area presented an increase in the number and severity of pathological alterations in the liver attributed to the effects of bioaccumulation of toxic compounds in this organ. In fact, the relative liver weight and hepatic levels of Pb, Hg, and Cd in these specimens were above the no-observed-adverse-effects-level (NOAEL) and, therefore, susceptible to toxic effects (Sánchez-Chardi et al., 2007a,b). Among tissues and cellular alterations, chronic exposure to these elements induces hepatic cell necrosis and apoptosis, and produces cytoplasmic vacuolization in hepatocytes, showing metals toxicity and carcinogenicity (e.g. Damek-Poprawa and Sawicka-Kapusta, 2004; Jadhav et al., 2007; Świergosz et al., 1998). However, it was impossible to determine which metal caused which specific lesion, due to wild populations are often exposed to a mixture of toxics. These signs of hepatic damage may be due to oxygen deficiency and/ or the presence of ROS induced by metal exposure. This can often occur when detoxifying systems such as metallothioneins are not responding efficiently enough to bind all metal ions (Jadhav et al., 2007; Świergosz et al., 1998; Włostowski et al., 2003).

Despite the abundance of data relating toxic effects with age and gender, no dependent variation was found in *C. russula* for the two parameters. The absence of histological changes in the kidneys, testes or ovaries of any of the analysed shrews may reflect these tissues higher tolerance to heavy metals. This physiological adaptation to chronic exposure is probably connected with efficient detoxification systems acting in these tissues or low exposure to toxic species of metals due to the important role of the liver in detoxification of xenobiotics uptaked in food.

4.4.4.4. *Small mammals as bioindicators*

Small mammals are often considered to represent an intermediate stage between low and high trophic levels, since they constitute important items in the diet of carnivorous birds and mammals. However, shrews are insectivorous and can be considered predators with a high position in the food-chain. In fact, they usually accumulate larger amounts of toxic pollutants than rodents, which makes these insectivores suitable bioindicators of environmental contamination (e.g. Talmage and Walton, 1991).

Wild mammals from polluted environments can uptake metals until toxic levels are reached. The assessment of this potential risk can be measured by daily intake, metal bioaccumulation in tissues, or through biomarkers that report toxic effects at different levels. Daily metal intake in shrews from the pyrite mine of Aljustrel remains unknown, but considering the high concentrations of non-essential metals found in tissues of the analysed specimens (Sánchez-Chardi et al., 2007b) it is obvious that it exceeds the threshold of toxicity. Even though biomarkers can be influenced by a multiplicity of factors under wild conditions, they provide suitable information on animals' health status when exposed to some sort of pollution in their natural habitat. In fact, the assessment of environmental pollution effects in wild biota has been a vital challenge for ecotoxicologists in spite of evident difficulties caused by constantly changing environments and high intra-specific variability.

Organelles, cells, tissues, organs, individuals, populations, communities, or ecosystems can suffer the deleterious consequences of toxic substances. An important and complicated issue is to understand precisely how pollutants affect each of these organizational levels. Here we have quantified several parameters in order to better understand possible deleterious effects of non-essential metals exposure from the sub-cellular to the individual and population levels. As a result, we reported significant genotoxic, enzymatic and histological alterations, as well as a tendency toward an increase in haematological parameters in shrews chronically exposed to metals in an abandoned mining area.

Several authors have suggested that wild small mammals inhabiting polluted sites are more sensitive than animals that are the subjects of laboratory experiments (e.g. Reynolds et al., 2006). Laboratory animals are usually under strict, controlled conditions, with minimal variation in abiotic (temperature, humidity, photoperiod, etc.) and biotic factors (such as gender or age). In addition, controlled contamination protocols usually involve animals uniformly exposed to a single toxic compound at a known and constant concentration. Conversely, specimens from natural populations live under numerous (and often uncontrolled) circumstances, such as parasitosis or reduced food availability, that can contribute to a decline in their health status. Thus the amount of energy available for essential activities and defence against toxic effects of metal exposure is considerably diminished. Moreover, in the wild, metal exposure frequently includes a combination of potentially toxic compounds that are not distributed constantly in time and space. These factors can lead to intra-specific variation, differential exposure and response (Talmage and Walton, 1991), contributing to the great variability observed in some physiological parameters in polluted areas (e.g. Marques et al., 2007; Sánchez-Chardi et al., 2007a). Pollutants may also be transformed before exposure, producing cumulative effects and/or undergoing interactions between them. So wild populations chronically exposed to pollution apparently make use of adaptive processes to better tolerate toxicants in a changing environment (e.g. Marques et al., 2007; Medina et al., 2007). All these factors can complicate the interpretation of ecotoxicological data as some parameters often do not show statistical significance, but only increasing/decreasing tendencies. A multidirectional approach such as the one described in the present study is extremely important for a wider and more accurate view of the effects of environmental pollution.

4.4.5. Conclusions

Shrews from the abandoned pyrite mine of Aljustrel not only showed significant heavy metals accumulation in their tissues (above toxic levels) but also presented physiological alterations. Age was a factor that particularly contributed to the observed variability, whereas sex was the one that contributed the least to the variation of the quantified parameters. Statistically significant correlations were reported between biomarkers and heavy metal contents. In spite of these relationships, none of the analysed metals can be pointed to as being chiefly responsible for the variation of the quantified biomarkers. A number of other metals, as well as other xenobiotics often present in wild conditions, may have also contributed to these results.

The combination of multiple biomarkers at different levels of organization can contribute to an integrative view of overall effects of environmental pollution at realistic conditions in wild specimens and populations. Here we have showed that non-destructive biomarkers such as MNT and haematological parameters provide suitable information, but histopathological evaluation of target tissues has revealed itself to be an important, specific, and sensitive tool for ecotoxicological assessment.

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4.5. DOÑANA I

Thallium bioaccumulation
in the shrew *Crocidura russula*
inhabiting the protected
wetland of Doñana

Tissue, age, and sex distribution of thallium in shrews from Doñana, a protected area in SW Spain

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Abstract

In 1998, the protected area of Doñana, an important natural region in SW Europe, was affected with great amount of acidic waters and sludge from a pyrite mine loaded with toxic metals such as thallium (Tl). Since this ecological catastrophe, several studies have addressed the effects of this pollution on the flora and fauna in this protected area. However, in contrast to other non-essential metals, scarce information on Tl was available after this disaster, especially in terrestrial environments. Here I reported a 3- and 10-fold increase in Tl in liver and kidneys, respectively, of the greater white-toothed shrew, *Crocidura russula*, in the polluted site in comparison with reference animals. Kidneys showed the highest concentrations of this metal in the polluted site, whereas both organs analysed have similar concentrations in the reference site. Although no significant age-dependent variation was found, adults had higher concentrations than juveniles. Moreover, females showed higher concentrations than males. These results demonstrate the high entrance and transfer of Tl in terrestrial food-chains. To the best of my knowledge, these data constitute the first measurements of Tl in mammals from the protected area of Doñana and are among the few available for insectivorous mammals.

4.5.1. Introduction

When the tailing dam at the Los Frailes Mine in Aznalcóllar (SW Spain) broke in April 1998, a total of 5Hm^3 of acidic waters and sludge from mining activities was released into the Guadiamar river affecting the protected area of Doñana. This spill included high amounts of toxic metals such as lead (Pb), mercury (Hg), cadmium (Cd), zinc (Zn), copper (Cu), and thallium (Tl). Since this accident, several studies have measured these elements and their effects in Doñana. However, while abundant information is available for Pb, Hg, Cd, Zn and/or Cu in biotic and abiotic compartments, little attention has been devoted to Tl (e.g. Prat et al., 1999; Simón et al., 1999; Madejón et al., 2003, 2006, 2007; Solà and Prat, 2006). To my knowledge, no data are available on the concentrations this metal in terrestrial small mammals in Doñana. In fact, although wild rodents and insectivorous mammals are often used as bioindicator of pollution (Talmage and Walton, 1991; Ma and Talmage, 2001), data on the concentrations of Tl in this group are scarce. The only report available was made by Dmowski et al. (1998), who described extremely high Tl bioaccumulation in three rodent species from a polluted site and low concentrations in the common shrew, *Sorex araneus*, from a reference site.

Tl is a non-essential heavy metal used in several industrial activities. It is found in sulfite deposits such as those in the Aznalcóllar mine, worked since Roman times (e.g. Grimalt et al., 1999; Kazantzis, 2000). Although this metal is not as widely distributed as other heavy metals, it has recently been examined in ecotoxicological studies as it is more toxic for organisms than other metals (Nriagu, 1998; Lin et al., 2001; Ma and Talmage, 2001; Rocha et al., 2004; Wierzbicka et al., 2004; Gao et al., 2007). In fact, chronic exposure to and poisoning by thallium has been reported in humans (Léonard and Gerber, 1997; John Peter and Viraraghavan, 2005) and the increase in levels and bioavailability of this element in the environment presents a risk for human and environment.

Here I provided the first data on thallium bioaccumulation in an insectivorous mammal, the greater white-toothed shrew, *Crocidura russula*, from a polluted site and analyse the tissue distribution and age- and sex-dependent variation of this non-essential element in this species. We also discuss the implications of thallium pollution for environmental risk and management of protected areas.

4.5.2. Material and Methods

In November and December 1999, it was captured 29 shrews in two sites in the protected area of Doñana (Figure 4.5.1). The first is an area affected by the spill from the Aznalcóllar mine (“Entremuros, Parque Natural”, 37º01'12"N, 6º16'38"W) and the second a reference site not affected by this pollution (“La Vera, Reserva Biológica”, 36º59'25"N, 6º26'41"W). Specimens were transported to the laboratory and killed by cervical dislocation following ethical procedures. Sex was determined during dissection. Shrews were classified as juveniles or adults on the basis of the degree of tooth wear.

Liver and right kidneys were dissected, dried and digested in Teflon vessels with 2 ml of nitric acid and 1 ml of hydrogen peroxide (Instra, Baker Analyzed), as described in Sánchez-Chardi et al. (2007). Tl concentrations were measured in diluted samples using rhodium as internal standard in a Perkin-Elmer ELAN-6000 Inductively Coupled Plasma Mass Spectrometer (ICP-MS). Two replicate subsamples were analysed and a reference material (Bovine Liver SRM-1577a) certificated by the National Bureau of Standards was included in the analysis. To calculate the concentration of

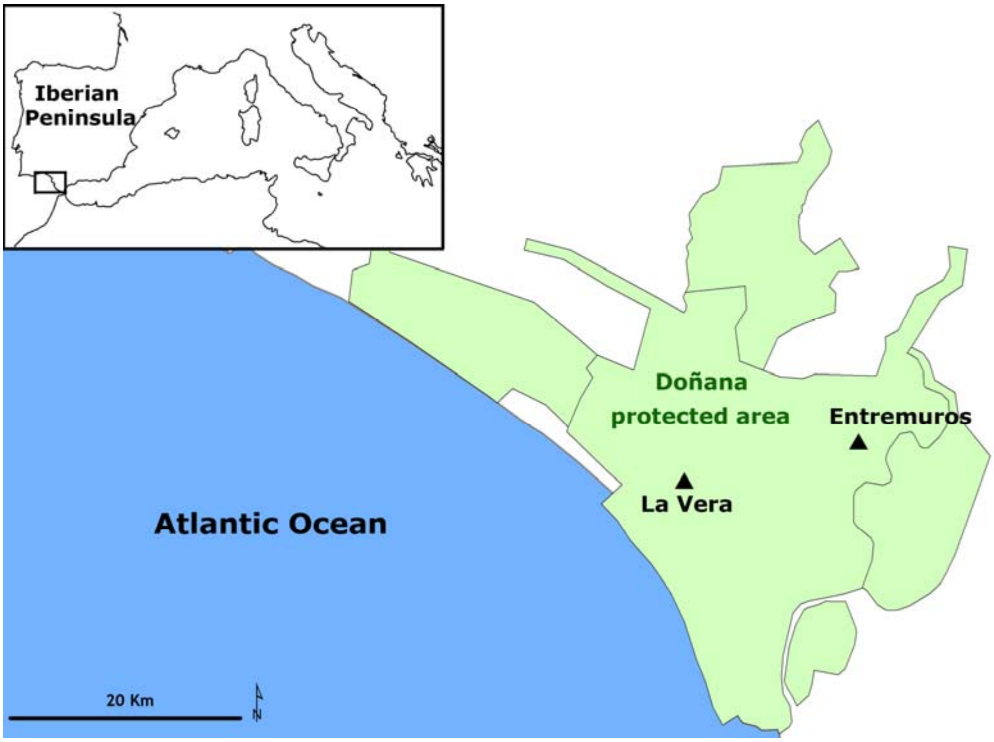

Figure 4.5.1. Map showing the geographical location of the sites studied (▲).

elements, the mean values from 10 blanks were subtracted from each sample. Results are expressed as arithmetic mean ± standard error of the mean (M±SEM) in µg/kg on a dry weight basis (DW). Chemical analyses were performed at the Elemental Analysis Facility of the Scientific-Technical Services at the University of Barcelona.

After log-transformation and test for normal distribution (Shapiro-Wilk test) and for homogeneity of variance (Levene, F-test), differences in Tl concentrations by site, sex and age were analysed by Student tests (t) using SPSS (version 11.5 for Windows, SPSS Inc.). Significant differences were assumed at $p<0.05$. Details of the procedures can be found in Sánchez-Chardi et al. (2007).

4.5.3. Results and Discussion

Tl concentrations in all the samples were above the detection limit (0.01 µg/kg). Compared with reference specimens, the shrews from the polluted site showed a significant increase in Tl concentrations in liver (147.69±44.47 *vs* 52.68±7.76 µg/kg; $t=2.717$, $p=0.012$) and kidneys (325.27±33.59 *vs* 33.32±7.63 µg/kg; $t=8.477$, $p<0.001$). These results indicate high bioavailability of Tl in shrews collected near the river, in an area with tidal fluctuation. In fact, when present in the environment, this metal is often dissolved and may be adsorbed onto clay minerals and, particularly in wet soils, may be highly transferred to plants and animals (Dmowski et al., 1998; Gao et al., 2007; Madejón et al., 2007). This great availability of Tl has also been reported in plants, periphyton, plankton, and macroinvertebrates collected in the Guadiamar river (Prat et al., 1999; Madejón et al., 2003, 2006, 2007; Solà and Prat, 2006). In contrast, low concentrations of this element have been reported in blood from birds captured in Doñana (Benito et al., 1999). These low levels could be explained by the short half-life of Tl in blood or to differences in metal intake or turnover between birds and mammals.

Compared with previous studies in wild mammals, the Tl concentrations registered in *C. russula* from the reference site were lower or similar to those reported in liver and kidneys of the Japan mongoose, *Herpestes javanicus*, the common shrew and three rodent species, and in the liver of Amami rabbit, *Pentalagus furnessi* (Dmowski et al., 1998; Horai et al., 2006).

Although mean values of Tl were high in the liver (95.97±47.37 *vs* 168.04±52.65 µg/kg) and kidneys (310.59±35.77 *vs* 353.03±35.77 µg/kg) of adults from the polluted site, no significant age-dependent variation was found in specimens from this site. No age comparisons were possible in shrews from the reference site because all the specimens captured were adults. Laboratory data indicate an increase in Tl with age as a result of decreased excretion by kidneys in adult rats was observed in relation to juveniles (Fleck and Appenroth, 1996). Moreover, although field studies show the same increase with age in polluted areas (e.g. Lin et al., 2001), the high variability in wild specimens reduces statistical significance, as occurs in this study. I consider that more studies are required to elucidate this question.

Females of *C. russula* tended to accumulate more Tl than males (Figure 4.5.2). This increase was significant in the reference site (Liver: t=2.554; p=0.038; Kidneys: t=-3.705, p=0.014). Although a decrease in Tl is expected in females because this metal is transferred across the placenta to the foetus (Dmowski et al., 1998; Nriagu, 1998), similar results have been reported for several metals in small mammals (see references in Sánchez-Chardi et al., 2007). These findings may be explained, at least in part, by differences in metal intake, uptake and/or turnover because metal bioaccumulation in mammals is related to reproduction and hormonal status. However, specific studies are necessary to identify the mechanisms that might explain these gender differences in Tl accumulation.

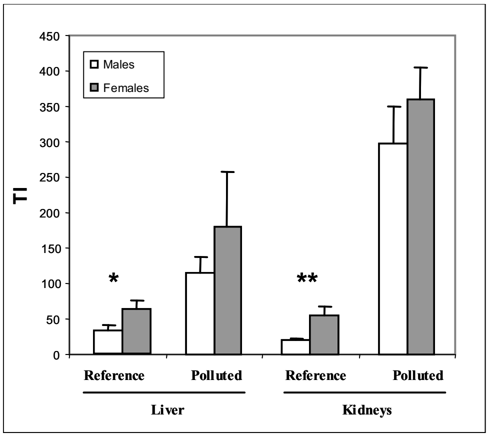

Figure 4.5.2. M±SEM values for thallium in *C. russula,* by tissue, sex and site (in µg/kg DW). (* $p \leq 0.038$; ** $p \leq 0.014$)

The tissue distribution pattern of Tl differed between specimens from the polluted and reference sites. While significantly high Tl concentrations were found in kidneys when compared with liver in animals from the polluted site (t=-3.002, p<0.001), no statistical differences were detected between tissues from specimens in the reference site. Background concentrations of this metal with low toxic interference could explain the data in the former. High bioaccumulation of Tl in renal tissue could be attributed to considerable exposure to this metal in polluted sites. This element is rapidly absorbed through the gastrointestinal tract and through skin. It circulates in intra- and extra-cellular fluid as a monovalent cation, mimics potassium cations, and binds with sulfhydryl groups of proteins at the mitochondrial membrane, as its toxicity seems related with K-dependent processes, i.e. substituting this element in Na^+/K^+-ATPase (Nriagu, 1998; Rocha et al., 2004; Wierzbicka et al., 2004). These observations may explain why the highest concentrations of Tl in mammals have been reported in renal medulla, where it interferes with filtration and excretion processes and produces serious toxic effects (Fleck and Appenroth, 1996).

Tl is a strong oxidant with high bioavailability. It is soluble in physiological conditions and has mutagenic, carcinogenic, and teratogenic effects in mammals (Léonard and Gerber, 1997; Nriagu, 1998; Rocha et al., 2004; Wierzbicka et al., 2004). The exact mechanism of toxicity is unclear (John Peter and Viraraghavan, 2005) and critical tissue levels in mammals are unknown. However, there is a clear dose-response relationship between low mean Tl concentrations (5.2±8.3 µg/l) in urine and adverse effects in humans (Kazantzis, 2000). Moreover, its average dietary intake in natural unpolluted environments is usually less than 5 µg/day and soils with concentrations ranging from 1 to 500 mg/kg produce toxic effects on biota (Heim et al., 2002; Wierzbicka et al., 2004). Concentrations found in the present study indicate a higher daily Tl intake and therefore reason of concern.

The protected area of Doñana is of considerable ecological importance. This area is one of the largest and most conserved areas in SW Europe. However, during centuries, pyrite mines have continuously released heavy metals into the Guadiana river, thereby producing chronic exposure that may affect individuals, communities and ecosystems (Grimalt et al., 1999; Simón et al., 1999; Prat et al., 1999). A mining accident, such the one in Aznalcóllar, intensifies these chronic effects and can increase the morbidity and mortality of specimens, reduce population viability, and deeply alter or even destroy entire habitats. Once deposited, Tl persists in soils for long periods (Kazantzis, 2000; Rocha et al., 2004). Because of this extended bioavailability, it is taken up by plants and biota. Soil bioavailability and persistence increase in clay-rich and non-acidic soils such as in the polluted site examined in the present study (Madejón et al., 2006). Moreover, small mammals are common prey for endangered carnivorous birds and mammals found in Doñana. Given that the maximum admissible daily dose of Tl for humans is 15.4 µg/kg DW (see references in Kazantzis, 2000), these results indicate a greater increase in Tl intake for carnivores that feed on these small mammals and, therefore, bioaccumulation to toxic concentrations at high trophic levels. It is highly recommendable that the management of the protected area of Doñana include the biomonitoring of persistent and highly toxic metals such as Tl.

4.5.4. Conclusions

The spillage from the Aznalcóllar mine leads to the bioaccumulation of harmful concentrations of Tl in kidneys of shrews in the protected area of Doñana. Moreover, biotic factors (age and sex) determine Tl bioaccumulation patterns.

Given the high concentrations of Tl found in shrew tissues as well as the serious long-term toxic effects and persistence of this element in the environment, a biomonitoring programme is required to assess levels and sublethal effects in individuals, populations, and ecosystems in this protected area.

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4.6. DOÑANA II

Bioaccumulation, morphometry, genotoxicity and histopathology in the shrew *Crocidura russula* inhabiting the protected wetland of Doñana

Metals in liver and kidneys and the effects of chronic exposure to pyrite mine pollution in the shrew *Crocidura russula* inhabiting the protected wetland of Doñana

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Abstract

Historically impacted by anthropogenic activities, the nature reserve of Doñana (SW Spain) was affected by an unprecedented spillage of mud and acidic water from the Aznalcóllar pyrite mine in April 1998. Although several studies have addressed the influence of this spill on soils, water, and biota, there is little information on mammals, especially carnivorous species. We measured the concentrations of Fe, Mg, Pb, Hg, Cd, Zn, Cu, Mn, Mo, Co, and Cr in specimens of the greater white-toothed shrew, *Crocidura russula*, inhabiting the protected area affected by the mine spillage. We also examined other parameters to approach at the physiological effects of pollution. We found an increase in non-essential metals (Pb, Cd, Hg), and morphometric, histologic, and genotoxic alterations. Age and gender were two significant factors explaining metal bioaccumulation: adults had higher Hg and Cd levels than juveniles, whereas males bioaccumulated more Pb and Co and less Mo than females. The micronucleus frequencies in blood erythrocytes were significantly higher in specimens from the polluted site than animals from the control site. Shrews from the impacted area also had hepatic alterations, namely increased liver-body ratio, focal necrosis, and signs of apoptosis in hepatocytes. Due to the relevance of small mammals in the diet of endangered species such as carnivorous birds and mammals, the findings of our study are of practical use for the management of the Doñana wildlife reserve and other protected Mediterranean wetlands.

4.6.1. Introduction

Doñana wildlife reserve is inhabited by several protected and endangered species, especially birds and mammals. On 25 April 1998, an incident at the pyrite mine "Los Frailes" in Aznalcóllar discharged about 5 Hm^3 of acidic waters and toxic mud carrying heavy metals such as Pb, Cd, Hg, Zn, Sb, and Tl into the Agrio river, thereby affecting this protected wetland. Since this event, several studies have provided abundant information on metal pollution in sediments and waters as well as on bioaccumulation and effects on plants, invertebrates, fish, reptiles, birds, rodents, carnivorous mammals, and human populations (e.g. Bonilla-Valverde et al., 2004; Fletcher et al., 2006; Gil et al., 2006; Madejón et al., 2007; Márquez-Ferrando et al., 2009; Millán et al., 2008; Smits et al., 2007; Turner et al., 2008). However, information on metal concentrations and their effects on insectivorous mammals is scarce, with only one study reporting a great increase in Tl in shrew tissues (Sánchez-Chardi, 2007).

Several heavy metals lead to clastogenic effects as a result of DNA breakage and can induce the generation of reactive oxygen species (ROS), which can lead to cell damage or death. Depending on the nature of the injury, the damage to the DNA may be repaired, or it may induce mutation or lead to apoptosis (Bragadin et al., 2003; Leonard et al., 2004), necrosis (Jadhav et al., 2007; Pereira et al., 2006) or other important cell alterations in somatic and germinative tissues (e.g. Damek-Poprawa and Sawicka-Kapusta, 2004; Nordberg et al., 2007). Effects are generally assessed or modelled in laboratory animals or culture cells in controlled conditions for a single compound or a few metals. However, biota is often exposed to a mixture of several chemical pollutants (Bellés et al., 2002). Field studies on ecotoxicological effects are useful to identify bioindicator species and assess the quality of the environment. Insectivorous mammals (shrews, hedgehogs, and moles) have been widely used as site-specific bioindicators of anthropogenic pollution, including heavy metals (e.g. D'Have et al., 2006; Ma and Talmage, 2001; Talmage and Walton, 1991). When non-degradable pollutants are released into the environment, they can be taken up and transferred through food chains and may accumulate in predators such as the greater white-toothed shrew *Crocidura russula* (Alleva et al., 2006; Wijnhoven et al., 2007; González et al., 2008). In particular, this species have small home ranges, and show high food requirements and a high metabolic rate (e.g Ma and Talmage, 2001; Pankakoski et al., 1994; Stewart et al., 2005; Talmage and Walton, 1991).

Here we examine the effect of a recent large increase in the environmental concentration of heavy metals to *C. russula* from the protected reserve of Doñana. We measure the bioaccumulation of Fe, Mg, Pb, Cd, Hg, Zn, Cu, Mn, Mo, Co, and Cr, and assessed several morphometric, genotoxic, and histopathological parameters as biomarkers of environmental metal pollution in shrews. Moreover, the relation between age and sex and the bioaccumulation patterns was evaluated as inherent sources of variability.

4.6.2. Material and Methods

4.6.2.1. *Study areas*

During November and December 1999, 29 specimens of the greater white-toothed shrew, *Crocidura russula* were trapped. Of these, 19 were caught in an area affected by the spill from the Aznalcóllar mine ("Entremuros, Parque Nacional", 37º01'12"N, 6º16'38"W) and 10 were collected at a reference site not affected by this pollution ("La Vera, Reserva Biológica", 36º59'25"N, 6º26'41"W). Traps were placed 5-8 m apart for about 400 m in a transect running along the intertidal zone. Both sites are situated in the protected reserve of Doñana (Figure 4.6.1), an extensive ecological coastal wetland in SW Spain inhabited by several protected species. The total capture effort was 1950

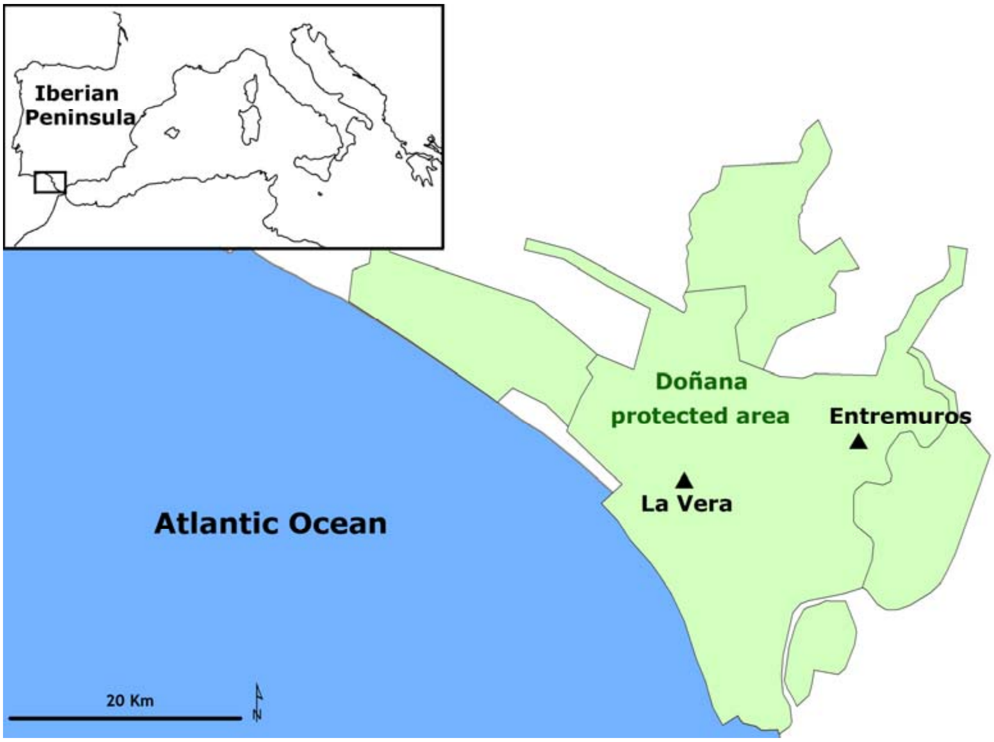

Figure 4.6.1. Map showing the geographical location of the sites studied (▲).

traps per night. Shrews were transported to the laboratory and killed by cervical dislocation. The study was conducted in accordance with the European and Spanish legislation for laboratory animals (Guideline of the Council of the European Communities 86/609/EEC of 24/11/1986 and Real Ordinance 1201/2005 of 10/10/2005). Sex was determined during dissection. Specimens were classified in two relative age classes (juveniles, adults) on the basis of the degree of tooth wear (Vesmanis and Vesmanis, 1979). In the polluted area, three females were pregnant and another two more lactating, whereas only three males showed signs of sexual activity. In the reference site, one male presented sexual activity and one female was lactating.

4.6.2.2. *Morphometric parameters*

The body weight (BW) to the nearest 0.01 g and head and body length (BL) to the nearest 0.01 mm of all specimens were measured during dissection. The residual index (RI) is calculated by the regression of BW and BL (Jakob et al., 1996). Positive values are an indication for specimens with relative higher body condition than expected for their weight and length. Animals with negative values have a relative lower body condition than predicted by the BW to BL ratio. Hepatic and renal weight to the nearest 0.001 g was measured on a wet weight basis (WW). The relative weights were calculated as a percent ratio of somatic tissue (100x tissue weight/ body weight).

4.6.2.3. *Chemical analyses*

About 300-500 mg of the liver and right kidney of each specimen were dissected, dried to constant weight (48h, 60ºC), weighed, and then digested in Teflon vessels with 2 ml of nitric acid and 1 ml of hydrogen peroxide (Instra, Baker Analized). Duplicate subsamples diluted (1:5), with rhodium as internal standard, were measured for Pb, Hg, Cd, Zn, Cu, Mn, Mo, Co, and Cr using a Perkin-Elmer ELAN-6000 Inductively Coupled Plasma Mass Spectrometer (ICP-MS) and for Fe and Mg by a Perkin-Elmer OPTIMA-3200RL Inductively Coupled Plasma Optical Spectrometer (ICP-OES). Moreover, standard reference material (Bovine Liver SRM-1577a) and 6 blanks were included in the analyses. Metal concentrations were expressed in µg/g on a dry weight basis (DW). All chemical analyses were performed at the Elemental Analysis Facility of the Scientific-Technical Services at the University of Barcelona.

4.6.2.4. *Micronucleus test (MNT)*

Blood samples are obtained by cardiac punction with a heparinized syringe. For each specimen, duplicate smears were made on pre-cleaned microscope slides, fixed with heat and stained with conventional May Grünwald-Giemsa staining. Micronuclei (MN) frequencies were scored by two observers on 2,000 blood erythrocytes for each specimen through an oil immersion objective (100x) on a Leica Leitz DMRB microscope. The frequencies were expressed as arithmetic mean and standard error of the mean (M±SEM).

4.6.2.5. *Histopathology*

A fraction of liver and the left kidney of 6 adult shrews from the polluted site and 8 from the reference site were fixed by 10% neutral-buffered formaldehyde and prepared for histological studies following conventional procedures, as described in Sánchez-Chardi et al. (2008). For the qualitative analysis, the stained sections were analysed under a Leica Leitz DMRB light microscope using a Sony Cyber-Shot, 7.2 mega pixels, to capture the images. Four images from each slide were captured under a 40x objective. These images were then processed by Image Tools software for quantitative analysis. The endpoints used were the presence of necrosis areas or apoptosis, inflammatory response, fibrosis, neoplasia, pre-neoplasic focuses and cytoplasmic vacuolization. For the quantitative analyses, we measured the perimeter, elongation, roundness, and compactness of a total of 1588 cells.

4.6.2.6. *Statistical analyses*

After log-transformation and test for normal distribution (Shapiro-Wilk test) and for homogeneity of variance (Levene, *F*-test), a three-way multivariate analysis of variance (MANOVA) was performed to obtain an overall estimation of the effects of on the parameters evaluated of site, age, and sex, together with their interactions. In order to increase sample size, the data were pooled and the effects of age and sex on metal bioaccumulation were analysed for each site. The differences in metal concentrations and morphometric parameters were analysed for site, age and gender classes with Student's tests (*t*). The same comparisons of MNT and histopathology were performed with Mann-Whitney tests (*U*). In order to increase sample size, sexual activity was not considered as factor since significant differences had not been found in previous analyses. Significant differences were assumed at $p<0.05$.

4.6.3. Results

Among the morphometric parameters, liver weight and relative liver weight were significantly higher in specimens from the polluted area than reference specimens ($t=2.544$, $p=0.017$; $t=3.233$,

Table 4.6.1. Mean ± SEM values for morphometric parameters of *C. russula* by site (* p ≤ 0.05; ** *p* ≤ 0.01).

	Reference site (n=10)	Polluted site (n=19)
BW (g)	6.57 ± 0.18	6.14 ± 0.20
BL (mm)	66.80 ± 0.47	64.88 ± 0.73
RI	-0.559 ± 0.246	0.265 ± 0.226
Liver (g)	0.263 ± 0.016	0.324 ± 0.019 *
% Liver	4.02 ± 0.28	5.47 ± 0.27 **
Kidneys (g)	0.102 ± 0.005	0.115 ± 0.009
% Kidneys	1.58 ± 0.09	1.87 ± 0.15

p=0.003, respectively). Moreover, the former tend to have greater relative renal weights and lower body weight than reference shrews (Table 4.6.1).

In liver, significant differences in metal levels between sites (F=33.421, p<0.001), ages (F=48.191, p<0.001), and sexes (F=52.930, p<0.001) were found. Hepatic mean concentrations of Hg and Cd were significantly higher in shrews from the polluted area, while more Cr was found in high concentrations in specimens from the reference site (Table 4.6.2). Adults from the polluted site had more Hg (t=3.240; p=0.005) and Cd (t=3.252; p=0.005) than the juveniles (Figure 4.6.2A). Moreover, levels of Pb, Mo and Fe tend to increase and Cu and Cr decrease in adults. Among gender differences, males had significantly high mean values of Pb and Co at the polluted site (t=2.520; p=0.023, t=2.358; p=0.031, respectively), whereas females bioaccumulated more Mo (t=-3.119; p=0.017) at the reference site (Table 4.6.3).

In renal tissue, the importance of the factors regarding metal differences were more site related (F=3.624 p=0.024), than sex related (F=1.315, p=0.329) and to a lesser extent age related (F=1.133,

Table 4.6.2. Mean ± SEM values for several metals (Fe, Mg, Pb, Hg, Cd, Zn, Cu, Mn, Mo, Co, and Cr) in tissues of *C. russula* compared by site (in µg/g DW).

	Liver				**Kidneys**			
	Reference site (n=10)	**Polluted site** (n=19)	*t*	*p*	**Reference site** (n=9)	**Polluted site** (n=19)	*t*	*p*
Fe	732.16 ± 230.29	952.37 ± 170.16	--	--	351.36 ± 15.08	362.49 ± 12.99	--	--
Mg	390.17 ± 20.92	394.14 ± 46.08	--	--	239.52 ± 21.19	313.83 ± 9.83	3.635	0.001
Pb	2.16 ± 0.39	2.79 ± 0.37	--	--	2.09 ± 0.19	2.43 ± 0.19	--	--
Hg	0.53 ± 0.12	1.28 ± 0.13	4.167	< 0.001	1.08 ± 0.54	3.04 ± 0.24	3.976	< 0.001
Cd	1.57 ± 0.21	5.56 ± 1.04	4.008	0.001	4.66 ± 1.01	16.74 ± 2.76	2.670	0.014
Zn	52.18 ± 3.52	49.34 ± 5.92	--	--	160.10 ± 11.34	177.12 ± 6.44	--	--
Cu	51.97 ± 10.30	56.16 ± 7.87	--	--	37.63 ± 4.98	48.95 ± 1.78	2.720	0.012
Mn	36.78 ± 1.83	38.27 ± 7.31	--	--	17.81 ± 2.23	20.02 ± 0.89	--	--
Mo	4.27 ± 1.22	6.81 ± 0.91	--	--	3.32 ± 0.58	3.75 ± 0.24	--	--
Co	0.64 ± 0.11	0.54 ± 0.10	--	--	0.62 ± 0.12	1.09 ± 0.10	2.589	0.016
Cr	3.00 ± 0.48	1.74 ± 0.60	-2.955	0.006	2.07 ± 0.27	1.91 ± 0.19	--	--

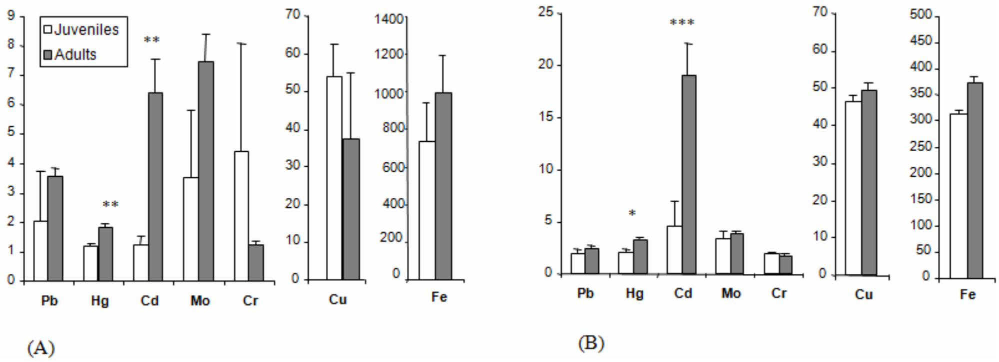

Figure 4.6.2. Mean ± SEM values for Pb, Hg, Cd, Mo, Cr, Cu and Fe in *C. russula* collected at the polluted site, by age in liver (A) and kidneys (B) (in µg g^{-1} DW), (*$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$).

p=0.415). In a similar pattern to that of hepatic tissue, the shrews from the polluted site had elevated Mg, Hg, Cd, Cu, and Co concentrations (Table 4.6.2). Moreover, adults from the polluted site had higher Hg (t=3.068; p=0.018) and Cd (t=3.886; p=0.001) concentrations than juveniles (Figure 4.6.2B). No significant sex-dependent variation was detected in kidneys; however, males and females showed a similar pattern of metal bioaccumulation as that detected in livers (Table 4.6.3).

The specimens from the polluted site showed a significant increase in micronuclei frequencies in peripheral erythrocytes compared with those from the reference site (1.222±0.169 *vs.* 0.250±0.119 ‰; U=1.500, p<0.001).

Table 4.6.3. Mean ± SEM values for metals in *C. russula* by tissue, site and sex (in µg/g DW), (* $p \leq 0.05$).

		Reference site		**Polluted site**	
		Males (n=6)	**Females** (n=3)	**Males** (n=9)	**Females** (n=10)
Liver	**Fe**	803.27 ± 223.87	589.92 ± 248.45	1086.3 ± 361.3	845.2 ± 119.8
	Pb	2.88 ± 0.53	2.52 ± 0.56	3.20 ± 0.69	1.56 ± 0.18 *
	Hg	0.83 ± 0.07	0.38 ± 0.14	1.49 ± 0.15	1.11 ± 0.20
	Zn	24.54 ± 2.41	63.94 ± 22.96	67.93 ± 22.37	77.85 ± 15.71
	Mo	2.41 ± 0.22	8.00 ± 2.69 *	6.07 ± 1.30	7.74 ± 1.25
	Co	0.63 ± 0.08	0.67 ± 0.25	0.63 ± 0.19	0.29 ± 0.05 *
Kidneys	**Fe**	162.30 ± 19.67	136.77 ± 19.67	161.41 ± 14.92	163.36 ± 20.86
	Pb	2.40 ± 0.33	2.14 ± 0.70	2.36 ± 0.21	2.52 ± 0.27
	Hg	2.57 ± 0.56	1.00 ± 0.51	3.09 ± 0.29	3.03 ± 0.37
	Zn	157.31 ± 7.89	163.81 ± 0.51	175.78 ± 11.81	178.19 ± 7.34
	Mo	3.44 ± 0.62	3.27 ± 1.26	3.37 ± 0.38	4.05 ± 0.27
	Co	0.64 ± 0.09	0.59 ± 0.27	0.86 ± 0.09	1.26 ± 0.14

The histopathological findings did not reveal the lesions that are commonly described in animals exposed to toxic metals or other pollutants; lesions such as fibrosis, vacuolization of the cytoplasm, inflammatory response, or neoplasic focus. However, our data showed some hepatic alterations in specimens from the polluted area, namely the occurrence of necrotic areas in 4 out of 6 individuals (Figure 4.6.3) and high incidence of apoptotic figures (Figure 4.6.3). When alterations were observed in hepatocyte nuclei, 1036 cells from specimens from the polluted site were compared by image analysis with 552 cells from specimens from the reference site. There was no significant difference in these parameters. However, shrews exposed to metals showed a tendency to increase the cellular perimeter when compared with reference shrews (7.531±0.356 *vs* 6.914±0.222 µm), whereas the elongation, roundness, and compactness remained invariant between sites (M±SEM: 1.190±0.020 *vs* 1.193±0.021 µm; 0.724±0.021 *vs* 0.755±0.010 µm; 0.912±0.009 *vs* 0.917±0.007 µm, respectively). These parameters were significantly correlated with Pb, Mn and Cr contents in liver. The perimeter was correlated with Pb and Cr (r=-0.665, p=0.018; r=-0.597, p=0.041, respectively), the roundness with Pb (r=-0.597, p=0.041), Mn (r=-0.597, p=0.041), and Cr (r=-0.597, p=0.041), and compactness with Mn (r=-0.597, p=0.041). Moreover, no alterations related to toxic metals were detected in renal tissue. Given the small number of animals examined, micronucleus and histopathological data were not analysed to assess the effect of age or gender or correlations with metal bioaccumulation.

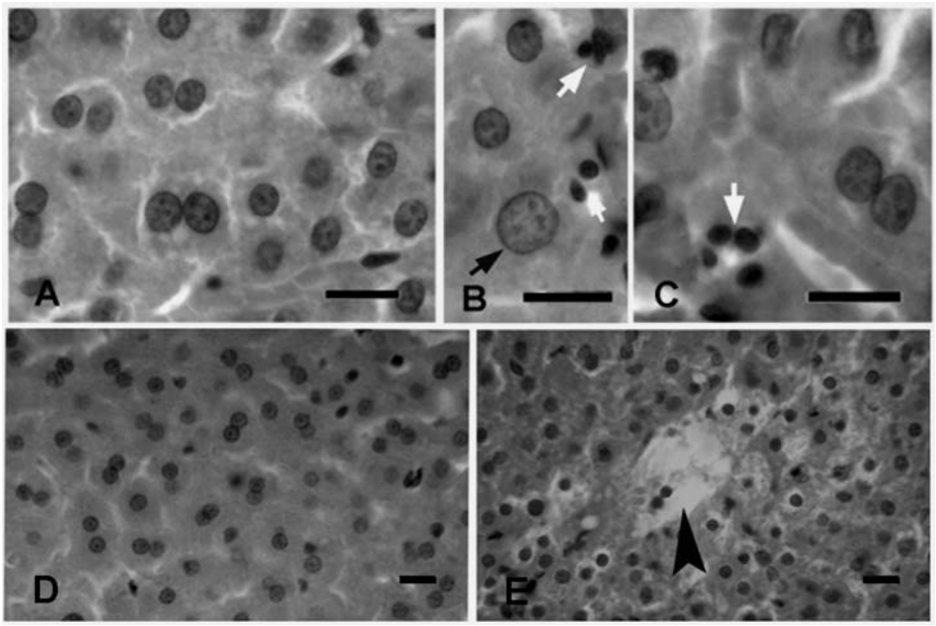

Figure 4.6.2. Hepatic sections showing normal and altered tissue in *C. russula*. (A,D) Healthy livers from shrews collected at the reference site; (B,C,E) Livers from shrews collected at the polluted site: Observe the enlarged nuclei (black arrow) (B), the apoptotic figure (white arrow) (B,C), and the foci of cell necrosis (black arrow) (E). Haematoxilin and eosin stains. (Scale bars: A,D,E = 50 mm; B,C = 20 mm).

4.6.4. Discussion

4.6.4.1. *Bioaccumulation of metals*

Since the Aznalcóllar mine spillage, several studies have reported an increase in heavy metal concentrations in soil and water (Tovar-Sanchez et al., 2006) which lead to toxic effects for wildlife of Doñana (e.g. Blasco et al., 1999; Bonilla-Valverde et al., 2004; Sánchez-Chardi, 2007). After this ecological disaster, most of the sludge from the floodplains was removed to minimize pollution effects. However, even after this remediation measure, later studies reported the continued presence of metals in the environment (e.g. Fletcher et al., 2006; Márquez-Ferrando et al., 2009; Millán et al., 2008; Smits et al., 2007; Tovar-Sanchez et al., 2006).

Eighteen months after this environmental disaster, the availability of potentially toxic metals such as Cd and Hg in the impacted site was still higher than in the reference site and they were accumulated in the tissues of shrews. In general, these non-essential elements are available to biota over a long time and are usually strongly accumulated in soft tissues of mammals recently exposed to contaminated air, food or water. Cd is a highly bioavailable metal and is mobile in less acidic soils such as those found in the Doñana area. It is retained with Fe oxides (Kraus and Wiegand, 2006) and is one of the main pollutants of mine residues. Mercury was also present in considerable amounts in the residues (e.g. Bonilla-Valverde et al., 2004). The low increase in Pb concentrations detected in shrews from the polluted area could be explained by low bioavailability several months after the spillage, probably because this metal is immobile in less acidic soils (Kraus and Wiegand, 2006). Zn, Cu, Co, Mn, and Mo were abundant in waters and slurry spilled from the mine and remained in high concentrations in the water and soil of the polluted area several years after the incident (Tovar-Sanchez et al., 2006).

Essential metals had similar concentrations in tissues from shrews from the polluted and reference sites, as previously reported in the Algerian mouse *Mus spretus* (Bonilla-Valverde et al., 2004). This is a result of metabolic regulation that prevents disruption of metabolic turnover and high bioaccumulation in mammalian tissues despite substantially elevated dietary intake (e.g. Goyer, 1997; Ma and Talmage, 2001; Talmage and Walton, 1991). In contrast, the higher mean values of Cr, another essential element, in specimens from the reference site, could be partially explained by the effect of fertilizers, which load the waters and soils of the reference area with metals (Tovar-Sanchez et al., 2006). The Doñana area has been exposed to agricultural practices and mining extraction for centuries, which contributed metals to the environment before the spillage of 1998 (Arambarri et al., 1996; Fletcher et al., 2006).

The adult shrews from Doñana had higher Cd accumulation than juveniles, which is consistent with previous reports in soft tissues of *C. russula* (Sánchez-Chardi and Nadal, 2007; Sánchez-Chardi et al., 2007b) and other small mammals species exposed to mine pollution (Bonilla-Valverde et al., 2004; Smith and Rongstad, 1982). This age-dependent bioaccumulation is associated with detoxification mechanisms, namely the formation of cadmium-metallothionein in liver, which is transported by blood and then stored mainly in the renal cortex (e.g. Bonilla-Valverde et al., 2004; Sánchez-Chardi et al., 2007b). A similar mechanism could explain the age-dependent accumulation of Hg and Pb, as also reported for other small mammals species (e.g. Damek-Poprawa and Sawicka-Kapusta, 2004; Pankakoski et al., 1993, 1994; Stansley and Roscoe, 1996). The tendency of essential elements that are physiologically well regulated in mammals to increase with age (Figure 4.6.2) could be related to the metabolic requirements of adult specimens as well as to interferences with non-essential elements (Bellés et al., 2002; Goyer, 1997; López Alonso et al., 2004; Pankakoski et al., 1994). The decrease in Cr detected is concordant with a lower intestinal absorption of this essential metal in adults (Outridge and Scheuhammer, 1993; Sánchez-Chardi et al., 2007a).

Gender differences in the bioaccumulation pattern of non-essential metals have been reported in several species of mammals including human (Clark et al., 2007; Komarnicki, 2000; Smith and Rongstad, 1982; Vahter et al., 2007). Low Hg and Pb levels in females could be related mainly to the reduction of metal burden mobilized and transferred during gestation to the foetus and/or during lactation to the young (see references in Sánchez-Chardi et al., 2007b). No sex-dependent variation was reported for Mo in *T. europaea* (Pankakoski et al., 1993). In contrast, the females of *C. russula* collected in a pyrite mine site and in a landfill site had higher Mo concentrations than males (Sánchez-Chardi and Nadal, 2007; Sánchez-Chardi et al., 2007a). More Fe was detected in males of different species including human (Clark et al., 2007; Khan et al., 1995). Zn also tends to increase in soft tissues in the females of some mammals such as the mole (Komarnicki, 2000). More specifically, steroid hormone receptors are Zn-finger proteins and Zn is also part of several enzymes, including some related to antioxidant systems such as cytosolic superoxide dismutase (Lopes et al., 2002). Generally speaking, the gender differences of these essential elements (Co, Fe, Zn, and Mo) may be associated with differences in the metabolic profile of metals involved in the activity of sexual hormones, the intake or uptake of metals, nutritional requirements or interactions between elements (Bellés et al., 2002; Chmielnicka and Sowa, 2000; Goyer, 1997; Lopes et al., 2002; López Alonso et al., 2004; Vahter et al., 2007).

4.6.4.2. *Genotoxicity*

The micronucleus numbers found in shrews from the central part of the Doñana reserve ("La

Vera") could be considered references for genotoxicity rates and are in concordance with data previously reported (Festa et al., 2003; Mateos et al., 2008; Tanzanella et al., 2001). The MN frequencies were higher in shrews exposed to metals from the pyrite mine, thereby indicating the clastogenic effects of this kind of pollution, which is bioavailability to biota over a long period of time. Similar results were reported by Festa et al. (2003) and Tanzanella et al. (2001) in a survey of the Algerian mouse, *Mus spretus*, collected at the same sites during the same period. These authors speculate on the contribution of the chronic pollution that occurred before the mine accident to genotoxicity. In fact, the Guadiamar River was severely affected by anthropogenic activities before this accident (some mines have been operating since Roman times), thereby causing increased levels of toxic substances in the area (e.g. Arrambari et al., 1996; Fletcher et al., 2006). However, the significant effect of the spillage in 1998 on the bioavailability of toxic metals and on the genotoxic damage to the Doñana reserve is evident, as shown by the increase in metal concentrations in soil, water, plants and animals (e.g. Blasco et al, 1999; Bonilla-Valverde et al., 2004; Fletcher et al., 2006; Gil et al., 2006; Kraus and Wiegand, 2006; Madejón et al., 2007; Smits et al., 2007; Tovar-Sanchez et al., 2006).

4.6.4.3. *Morphometric parameters*

In shrews from the polluted site, the increase in liver weight and the tendency to show increased renal weight and decreased body weight may be indicative of considerable physiological and histological alterations (Sánchez-Chardi et al., 2007b, 2008; Shore and Douben, 1994). The presence we detected of both histological alterations in the livers and genotoxic effects in the blood of shrews from the polluted area are consistent with these morphological findings and could be explained by exposure to toxic levels of non-essential metals. Similar results of morphometric parameters were previously obtained for small mammals exposed to metals (Ma and Talmage, 2001; Pereira et al., 2006; Sánchez-Chardi et al., 2007a,b). In mammals, the main exposure route to pollutants is through diet. The liver is the most important detoxifying tissue. This organ plays a crucial role in food conversion, biotransformation of xenobiotics, and vitellogenesis for reproduction purposes. Consequently, the impairments of hepatic function have a number of negative consequences on growth, health, life expectancy and reproductive success of individuals and may therefore adversely affect whole populations.

4.6.4.4. *Histopathology*

Histopathological endpoints could also contribute to the sensitivity of organs to heavy metal pollution and could provide information on the mechanism of action of pollutants and on target organs (Wester et al., 2002). Cellular biomarkers act as early warning signals of stress by organisms exposed to contamination. They may provide information on the level of the developing stress, ranging from initial biological effects to the impact on cell physiology. There are wide descriptions that exposure to heavy metals generates ROS via Fenton-type or Haber-Weiss-type reactions. Heavy metals such as Tl, Cd, Co, Fe, Pb, and Cr also react directly with cell molecules within the cytosol and cause a wide range of cellular responses such as apoptosis and finally necrosis (e.g. Bragadin et al., 2003; Chia et al., 2005; Leonard et al., 2004; Oliveira Ribeiro et al., 2005), depending on the level of oxidative stress generated. The occurrence of cell cycle arrest (necrosis and apoptotic figures) in liver of *C. russula* chronically exposed to high concentrations of Tl, Pb, Cd, and Hg corroborates the above effects reported in the laboratory. In addition, the nuclear alterations described here are also additional evidence that the uptake and high bioaccumulation of toxic metals in livers of *C. russula* are related to these findings. Theses alterations may represent chromatin disorganization and have serious consequences on gene expression by altering the transmission of information from the nucleus to the cytoplasm and outside the cell. In fact, the presence of distinct nuclear forms reported here provides evidence that the toxic mechanism of metals also disturbs the DNA and protein array within the nucleus.

A few studies have described renal alterations (see references in Sánchez-Chardi et al., 2008) in soricine species of shrews. However, the response of *C. russula*, a crocidurine species, to metal pollution is more similar to that shown by rodents (Damek-Poprawa and Sawicka-Kapusta, 2004; Pereira et al., 2006). Given their particular metabolic characteristics (e.g. Stewart et al., 2005) and differences in phylogenic origin and ecological strategy, further studies including biochemical, physiological or morphological parameters, are required to explain the differences observed between groups of shrews in response to exposure to heavy metals.

4.6.4.5. *Toxicity effects*

Several studies with laboratory specimens have reported toxic effects of a single metal under controlled conditions. However, data on wild populations of species of higher trophic levels are scarce. The effects of heavy metals considered alone as well as cumulative toxicity rates or interactions such as synergism could explain the increase in genotoxicity and histological alterations observed in shrews from the Doñana reserve. No complete guideline on the toxic levels of the heavy metals addressed in this study is currently available for shrews. However, considered alone, the levels found for essential metals and Pb do not appear to be of toxicological hazard in the specimens collected in Doñana. In contrast, Cd and Hg levels in renal and hepatic tissues of shrews from the polluted area merit concern. The mean values of Hg higher than 1.1 ug/g should be regarded as presumptive evidence of an environmental mercury problem in wild mammals (Eisler, 1987). In our study, 8 out of 19 shrews in the polluted site showed more than 15 µg/g of this metal in renal tissue (maximum of 42.74 µg/g DW). Altough insectivores seem more tolerant to Cd than rodents, the Cd concentrations observed in shrews from the polluted site were higher than the no-observable-adverse-effects-level (NOAEL) in rodents ((Włostowski et al., 2003) indicating a considerable environmental pollution. In fact, wild specimens with high loads of non-essential metals may suffer toxic effects such as impairment of reproduction or a decrease in life expectancy. When assessing the status of wild animals, the toxic effects of environmental pollution could be biased because of the limited number of animals studied as well as by the intra-specific variation and selection in natural populations, which may reduce the metal load or eliminate impacted animals that are easier preys to predators. These features may remove extreme values that can be measured in laboratory studies. In fact, laboratory rodents showed alterations such as necrosis, apoptosis, vacuolization and fibrosis when chronically exposed to realistic concentrations of metals (e.g. Jadhav et al., 2007).

4.6.4.6. *Environmental quality assessment in protected sites*

Inhabited by several endangered vertebrate species, including carnivorous birds and mammals, the area of Doñana is one of largest protected coastal sites and one of the last great wildernesses in Europe. These protected species feed on small mammals and one of the most abundant in Doñana is the greater white-toothed shrew (Cagnin et al., 1998). Could *C. russula* serve as a bioindicator to assess environmental quality? Given that this species has a high metabolic rate, food requirements and tolerance to toxins, it reacts to pollution by bioaccumulating higher concentrations of heavy metals than sympatric rodents (Alleva et al., 2006; Sánchez-Chardi and Nadal, 2007; Wijnhoven et al., 2007; González et al., 2008), as occurs with other insectivores (Ma and Talmage, 2001; Talmage and Walton, 1991). These metabolic characteristics as well as the ecological interest of abundant species of non-migratory and easily available carnivores make shrews as suitable biomonitoring species to detect very finely an increase of xenobiotics (Komarnicki, 2000; Ma and Talmage, 2001; Shore and Douben, 1994; Talmage and Walton, 1991). In contrast, the shrews appear to be more tolerant to pollutants than rodents as shown by less alteration of physiological parameters such as biochemical biomarkers (e.g. Ma and Talmage, 2001; Sánchez-Chardi et al., 2008). Comparative studies of sympatric species are required for correct assessment of environmental quality and risk in order to determine inter-specific sensitivity to pollutants and detect the most sensitive species. Thus, it may be possible to assess the critical loads, namely highest levels of pollutants without toxic effects, for wild populations and ecosystems. Moreover, a database for heavy metal concentrations in autochthonous biota with diverse trophic strategies is required to allow studies on the bioavailability, transfer and behaviour of chemicals through a variety of protected ecosystems. The study of temporal variation of metals throughout the ecosystems could also be important for these protected areas. In fact, our samples were collected a few months after the spillage from the pyrite mine. Later studies in Doñana showed differences in bioavailability of metals in soils, waters and plants. However, to our knowledge, terrestrial mammals at high trophic position have not been biomonitored. We consider that *C. russula* is a suitable species to assess environmental quality especially for bioaccumulable pollutants and recommend that it be included as part of management programmes for protected sites.

4.6.5. Conclusions

Here we have identified Hg and Cd as toxic elements that are highly bioaccumulated in terrestrial mammals in Doñana. We have also reported age and sex as two relevant factors to explain variability in bioaccumulation patterns in wild populations recently exposed to extremely high amounts of metals. Moreover, we have found morphometric, genotoxic, and histological effects of metal pollution in shrews from the area affected by the mine spillage. These effects and the considerable concentrations of non-essential metals in tissues of shrews from this protected area also indicate the need for frequent sampling to evaluate the food chain transfer of these long-term persistent pollutants. The high trophic position of shrews as well as their abundance makes them a suitable species for biomonitoring programmes especially in areas of ecologic value such as Doñana.

4.6.6. References

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4.7. EBRE I

Bioaccumulation of non-essential metals and morphometry in the shrew *Crocidura russula* from two protected Mediterranean coastal sites

Bioaccumulation of lead, mercury, and cadmium in the greater white-toothed shrew, *Crocidura russula*, from the Ebro Delta (NE Spain): sex– and age-dependent variation

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Abstract

We quantified bioaccumulation of lead, mercury, and cadmium in bones from 105 greater white-toothed shrews (*Crocidura russula*) collected at the Ebro Delta, a polluted area, and the Medas Islands, a control site. Lead and mercury levels varied with site, age, and sex, although statistical significances depended on each factor. Globally, shrews from the polluted area exhibited significantly higher concentrations of Pb and Hg. Increment of Pb with age was particularly remarkable in wetland animals and was interpreted in relation to human activities, namely hunting. Unlike males, females from the Ebro Delta maintained low Hg levels, which were associated with gestation and lactation. Cadmium levels did not differ between sites, sexes, or ages. This study provides the first data on heavy metals in mammals from this wetland and suggests that *C. russula* is a good bioindicator of metal pollution. We concluded that sex and age may represent an important source of variation in the bioaccumulation of these metals in wild populations.

4.7.1. Introduction

Anthropogenic activities may generate metal pollution in air, soil, and water. Over the last decades, the production of lead (Pb), mercury (Hg), and cadmium (Cd) from mining alone increased 2-, 2- and 15-fold, respectively, and the subsequent release of these metals into the environment is of some concern (Nriagu, 1988). The ecosystem seems to offer an effective filter, retaining contaminants in soil profiles, transferring them into aquatic (Négrel and Roy, 2002) and/or terrestrial systems, and thereby increasing the bioavailability and poisoning risk both to humans and the environment. Lead, Hg, and Cd, three non-essential elements widely distributed, are well known for their highly toxic effects on biological systems (e.g. Lewis et al., 2001; Wolfe et al., 1998). In countries where lead additive is prohibited, the use of lead shot pellets, which are composed by 98% Pb and by traces of other metals such as Cd (Mozafar et al., 2002), may become the primary source of environmental lead pollution (Ma, 1989). Although mercury is not an abundant element in the environment, it is widely distributed due to various industrial and agricultural practices. The source of cadmium may be natural or anthropogenic resulting among other activities from the use of superphosphate fertilizers or the spill from industrial and domestic sewage.

The pollution status at the Ebro Delta, a partially protected area in NE Spain, is well documented, particularly for certain heavy metals present in freshwater and marine habitats. In this wetland, fertilizers, mainly employed in rice farming, and especially the lead shot pellets from hunting activities remain the primary sources of in situ metal pollution (Mañosa et al., 2001). Up to 4–9 t of these pellets are spread across rice fields and lagoons each year and even 2.5 x 10^6 pellets/ha were found in sediments (Mateo et al., 1997). Direct lead poisoning by shot is very common among granivorous waterbirds, but it has also been described in several species of birds of prey, which may feed on game birds and mammals (Mateo and Guitart, 2003; Mateo et al., 1997, 2003). Moreover, industrial and domestic effluents transported by the Ebro River are also responsible for ex situ pollution, spreading large amounts of Pb, Hg, and Cd in this protected area (Lacorte et al., 2006; Lavado et al., 2006; Mañosa et al., 2001; Schuhmacher et al., 1994). As a consequence, considerable amounts of Pb and Hg have been detected in the river water, sediments, earthworms, fish, and marine organisms close to this deltaic area (Lavado et al., 2006; Morera et al., 1997; Ramos et al., 1999; Schuhmacher et al., 1990, 1993). In the Ebro Delta, Cd concentration was high in sediments, but low in soil, water, rice, and seabird eggs (Mañosa et al., 2001; Morera et al., 1997; Ramos et al., 1999), suggesting that terrestrial environments may be less affected by Cd pollution.

Although the intake and bioaccumulation of pollutants by mammals is known to occur (Komarnicki, 2000; Shore, 1995; revision in Talmage and Walton, 1991), to our knowledge, metal concentrations in mammals from the Ebro Delta have never been examined. Several studies have shown that insectivores are mammals suitable for toxicological research, especially because of their high trophic chain position and metabolic rate, as well as their demanding feeding requirements (see revision in Ma and Talmage, 2001). They accumulate more potentially harmful metals than other small mammal species, and their exposure to pollutants tends to exceed other mammals with lower metabolic rates (Ma, 1989; Ma and Talmage, 2001; Talmage and Walton, 1991). Moreover, consumers at higher trophic levels in terrestrial ecosystems may be useful in predict risks to human health (Komarnicki, 2000). For the present work, we used the greater white-toothed shrew, C*rocidura russula* (Hermann 1780), an insectivore species widely distributed throughout south-western Europe (Ramalhinho et al., 1999) and very common to the Mediterranean region under study.

In general, the biological effects of pollutants on individuals and populations are assessed by means of a variety of factors, including several genetic, morphometric, and/or biochemical parameters (Ma and Talmage, 2001; Talmage and Walton, 1991; Topashka-Ancheva and Metcheva, 1999). Some criteria regarding physiology, such as a body condition index, can be used as rapid and easy measures of adaptability in wild mammals in polluted environments (Ma, 1989; Jakob et al., 1996; Nunes et al., 2001a).

With all this in mind, the main goals of this study were: i) to quantify for the first time the bioaccumulation of non-essential metals in the greater white-toothed shrew, *C. russula*, from NE Spain; ii) to analyse patterns of variation based on sex and age; iii) to assess the impact of metal pollution from human activities on a protected Mediterranean area; and iv) to evaluate the use of osteological material drawn from biological collections as a source of ecotoxicological information on mammalian populations.

4.7.2. Material and Methods

We used large bones from 105 greater white-toothed shrews, *C. russula*, originally collected during the period 1976-1981 from two partially protected coastal areas in north-eastern Spain (Figure 4.7.1): the Ebro Delta (40°43'N, 00°40E) and the Medas Islands (42º20’N, 03º13’E). The Ebro Delta, the second most important wetland in the Iberian Peninsula, has an area of 32,000 ha, of which 7,802 ha correspond to a Natural Park. In this site, specimens (n=73) were captured at L’Encanyissada lagoon, characterized by helophytic vegetation (*Phragmitetea*). This zone is polluted by Pb shot pellets into the soil, as well as by Hg and Cd from industrial, domestic, and agricultural activities (mainly rice farming). With an area of 21.5 ha and about 1 mile off the nearest mainland (L’Estartit),

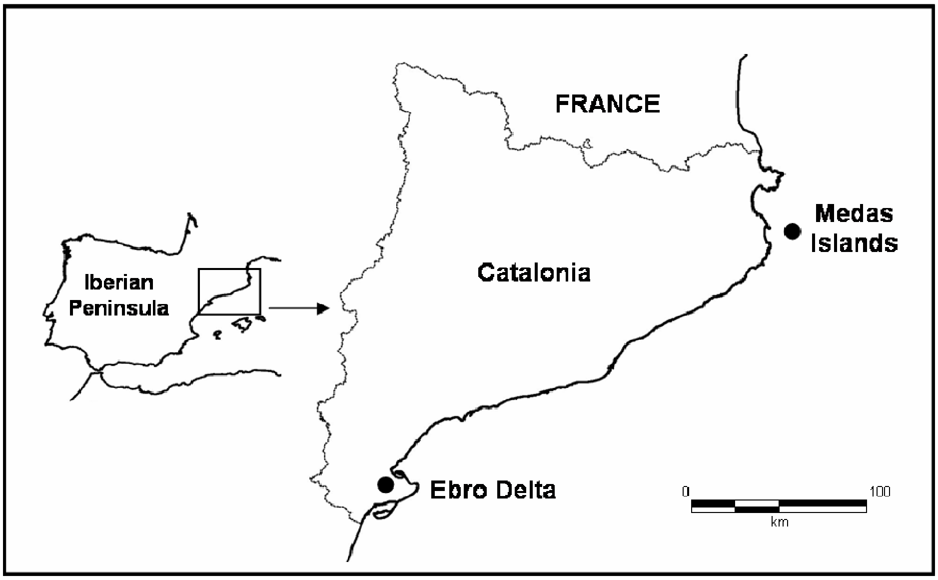

Figure 4.7.1. Map showing the geographical location of the samples analysed.

the Medas Islands constitute a small archipelago formed by seven isles. In this clean and uninhabited area, which is not affected by human activities, animals (n=32) were caught on the Meda Gran Island in halophilous vegetation (*Carpobrotetosum*).

For each animal, body mass (BM, in g) and body length (BL, in mm) were measured; in pregnant females the weight of the embryos were subtracted. To evaluate the general health status of animals, we used the residual index (RI), as a body condition index, in which BM is regressed on BL after the data were appropriately transformed to meet the assumptions of regression. The residual distances of individual points from this regression line were then used as the estimator of condition (see e.g. Jakob *et al*., 1996). The results of these morphometric parameters were expressed as arithmetic mean ± standard error of the mean (M±SEM). For statistical analyses, animals were distributed into three relative age-classes (1: juveniles; 2: adults; 3: seniles), based on the degree of toothwear (Vesmanis and Vesmanis, 1979) and reproductive status (López-Fuster et al., 1985). The bones of each animal were kept in single metal-free paperbags and stored in the zoological collection of the Animal Biology Department (University of Barcelona) until the chemical analyses were performed.

Materials used in the digestion process were thoroughly acid-rinsed. The bones were then washed with high purity grade water (Milli-Q© system, 18.2 MΩ·cm^2) and dried until constant weight was attained (50ºC, 48h). Eighty to 100 µg of tissue from each animal were placed on Teflon vessels and digested (120ºC, 12h) with 2 ml of nitric acid 70% (Instra, Baker Analyzed) and 1 ml hydrogen peroxide 30% (Instra, Baker Analyzed). Samples were diluted 1:5 in Milli-Q© water with 1% HNO_3 and Rhodium as an internal standard. Concentrations of Pb, Hg, and Cd were determined by Inductively Coupled Plasma Mass Spectrometer Perkin-Elmer ELAN-6000. Two replicate subsamples were analysed and a standard reference material (Bovine Liver SRM-1577a) certificated by the National Bureau of Standards was included in the analysis. The results regarding heavy metals were expressed as M±SEM in microgram/gram (μg/g) on a dry weight basis.

Data were log transformed and tested for both normal distribution (Kolmogorov–Smirnov) and homogeneity of variance (Levene, F-test). Initially, an overall measure of the effects of sex, age group, and site was obtained by a three-way multivariate analysis of variance (MANOVA). Intersexual differences for each age-class and site were calculated by Student's t-tests. For each site and sex, divergences in the concentration of elements by age were performed using one-way analyses of variance (ANOVAs), and pairwise comparisons of sample means were conducted by Scheffe's method. Interpopulation differences were assessed by the Student's t-test for each sex and age group. To minimize seasonal, reproductive, and growth variations, only non-reproductive adults from winter were used to calculate the RI; comparison between sites was evaluated by a Student t-test. For all sequential tests, p-values were corrected by the Bonferroni adjustment (Rice, 1989). All statistical procedures were performed using SPSS 11.5 software (SPSS Inc., Chicago, IL).

4.7.3. Results

Lead, mercury, and cadmium concentrations in the bones analysed were above the detection threshold for all animals (in μg/kg, Pb: 0.05; Hg: 0.20; Cd: 0.05). MANOVA revealed significant differences by sex (F=3.799, p=0.013), age (F=9.611, p=0.001) and site (F=24.502, p=0.001). Therefore, basic descriptive statistics (sample size, arithmetic mean, standard error, and range) of heavy metal concentrations are shown for each factor (Table 4.7.1).

Intersexual differences were observed for Pb and Hg, although bioaccumulation patterns varied according to the age and capture site. Thus, while in the Ebro Delta significant sexual differences in the concentration of Hg were detected for adults (p=0.017) and seniles (p= 0.001), in the control area these differences corresponded to juveniles (p=0.008) and adults (p=0.024). Moreover, in the latter area Pb accumulation also differed significantly between the sexes for both adults (p=0.013) and seniles (p=0.021). The bioaccumulation of Cd did not exhibit significant sex-dependent variation in any of the studied sites.

Table 4.7.1. Descriptive statistics of metal concentrations (μg/g, in dry weight) in the samples analysed according to sex and age.

Ebro Delta

		Males					**Females**				
	Age	**n**	**Mea n**	**SEM**	**Min.**	**Max.**	**n**	***Mean***	**SEM**	**Min.**	**Max.**
Pb	**1**	14	4.88	1.05	0.13	12.47	13	2.56	0.79	0.11	9.30
	2	6	6.14	1.97	0.30	12.98	6	6.47	2.20	0.30	15.18
	3	16	13.03	1.04	6.09	19.60	18	14.69	0.80	10.09	23.00
Hg	**1**	14	1.79	0.27	0.27	3.73	13	1.21	0.16	0.56	2.30
	2	6	1.93	0.59	0.55	3.99	6	0.55	0.12	0.19	1.00
	3	16	5.53	0.82	0.61	12.63	18	1.09	0.19	0.12	3.09
Cd	**1**	14	0.19	0.03	0.08	0.47	13	0.16	0.02	0.06	0.34
	2	6	0.09	0.01	0.06	0.13	6	0.13	0.02	0.08	0.22
	3	16	0.20	0.05	0.04	0.77	18	0.22	0.04	0.07	0.85

Medas Islands

		Males					**Females**				
	Age	**N**	**Mea n**	**SEM**	**Min.**	**Max.**	**n**	**Mean**	**SEM**	**Min.**	**Max.**
Pb	**1**	3	0.49	0.09	0.35	0.67	6	0.97	0.25	0.07	1.68
	2	5	0.73	0.15	0.32	1.21	7	1.46	0.20	1.01	2.46
	3	7	1.70	0.24	1.04	2.80	4	2.50	0.10	2.30	2.76
Hg	**1**	3	1.04	0.03	0.97	1.08	6	0.66	0.06	0.47	0.88
	2	5	0.68	0.08	0.38	0.85	7	1.01	0.08	0.64	1.34
	3	7	0.95	0.18	0.47	1.61	4	1.42	0.22	0.79	1.74
Cd	**1**	3	0.09	0.01	0.07	0.11	6	0.10	0.02	0.05	0.18
	2	5	0.15	0.03	0.10	0.23	7	0.15	0.02	0.06	0.25
	3	7	0.14	0.01	0.11	0.17	4	0.14	0.02	0.10	0.20

Table 4.7.2. Results of the one-way ANOVA for variation in metal concentrations according to age, for each site and sex. *P*-values corrected by the Bonferroni adjustment.

Site	Sex	Pb			Hg		Cd	
		d.f.	*F*	*p*	*F*	*p*	*F*	*p*
Ebro Delta	**males**	2, 33	8.64	0.001	10.22	0.001	1.27	--
	females	2, 34	24.80	0.001	3.33	--	1.34	--
Medas Islands	**males**	2, 12	11.60	0.002	1.40	--	4.24	--
	females	2, 14	3.75	--	10.12	0.002	1.45	--

Age-dependent statistical signification in the metal concentrations for each sex and site are shown in Table 4.7.2. Pairwise comparisons between sample means revealed that in the Ebro Delta, Pb concentration was significantly higher in seniles than in juveniles, both in males (p=0.002) and females (p=0.001). As for Hg, concentration increased significantly in senile males with respect to juveniles (p=0.001) and adults (p=0.014), whereas it did not vary in females. In the Medas Islands, increase in Pb was significant in senile males with respect to juveniles (p=0.004) and adults (p=0.012), while for Hg significantly greater values were found only between senile and juvenile females (p=0.02). No variation of Cd concentration with age was observed either in the polluted or the control site.

Globally, shrews from the polluted site showed a significant increase in Pb (8.91±0.72 µg/g *vs* 1.34±0.13 µg/g) and Hg (2.24±0.29 µg/g *vs* 0.93±0.66 µg/g) than individuals from the control site. In males, significantly higher values were found in the Ebro Delta compared with the Medas Islands for Pb (juveniles, p=0.015; seniles, p=0.001) and Hg (seniles, p=0.001). In females, concentrations of Pb and Hg were significantly higher in seniles (p=0.001) and juveniles (p=0.005) from the Ebro Delta, respectively. No difference in the concentration of Cd was observed between the two sites (0.18±0.02 µg/g *vs* 0.13±0.01 µg/g). Likewise, a comparison of residual indexes between the sites (Ebro: 0.242±0.134, n=23; Medas: -0.253±0.260, n=22) did not show any statistical difference (t=1.713, p=0.094).

4.7.4. Discussion

4.7.4.1. *Bioaccumulation by site, age, and sex*

Our results revealed that in the shrews analysed Pb and Hg bioaccumulation varied according to site, age, and sex, arranged in ascending order of importance, whereas Cd remained statistically invariable for all three factors. The high Pb and Hg levels found in the shrews from the Ebro Delta can be easily explained as result of many decades of hunting, and of intense industrial and agricultural activities carried out in this area (Mateo and Guitart, 2003, Mateo et al., 1997, 2003; Mañosa et al., 2001; Schuhmacher et al., 1990). Such anthropogenic actions have led to an increase in the bioavailability of non-essential heavy metals by biota, and consequently to chronic exposure for wild mammals inhabiting the Ebro Delta. Because of Pb exposure, shrews from the polluted site experienced a 6-fold increase in their Pb levels compared with control animals. Values obtained in the shrews from the Ebro Delta are comparable to those reported for the bi-coloured white-toothed shrew, *C. leucodon* (Topashka-Ancheva and Metcheva, 1999). In contrast, Pb concentrations in the femur of the common shrew, *Sorex araneus*, were considerably higher, ranging from 134 to 1469 µg/g (Ma, 1989; see Table 4.7.3). The difference in Pb concentration between crocidurinae and soricinae may be particularly linked to their metabolic rates (*C. russula*: 2.45 ml O_2/g·h; *S. araneus*: 7.74 ml O_2/g·h; Churchfield, 1990). In fact, Ma and Talmage (2001) noted that the crocidurine species exhibited bioaccumulation levels similar to rodents and lower than those of the soricine species. Additionally, these authors stated that the exposure of insectivores depended strongly on the soil conditions governing the bioavailability of Pb intake by preys, especially earthworms. For instance, it has been pointed out that a higher shot pellet corrosion rate occurs in acidic soils, demonstrating that soil pH can affect Pb bioavailability (Ma, 1989, 1996; Shore, 1995). Thus, the alkaline character of the Ebro sediments (8.03$\pm$0.32; Ramos et al., 1999) may also partially explain the lower Pb values observed in *C. russula* with respect to *S. araneus* collected in acidic soils (Ma, 1989). Other conditional factors, such as interspecific diet variation and/or intraspecific seasonal differences in prey items, cannot be disregarded.

Bones are considered a useful indicator of cumulative lead exposure, since most of the Pb intake via ingestion (90%) is transferred to them, where it has a low excretion rate and is stored in a relatively stable chemical form (Ma, 1989; Scheuhammer, 1991; Shore and Douben, 1994). This fact may explain the increase in lead burden that occurs with aging and which was observed in *C. russula* from the Ebro Delta, a finding similarly reported for other insectivores such as the common shrew, the pygmy shrew, *S. minutus*, and the common mole, *Talpa europaea* (Komarnicki, 2000; Pankakoski et al., 1993, 1994; Read and Martin, 1993; Stanley and Roscoe, 1996; Talmage and Walton, 1991).

Although mercury and cadmium accumulate mainly in soft tissues, it is well known that they directly affect the physiology of bone cells. These heavy metals interfere with calcium metabolism and bone remodelling, suppress bone cell activities, inhibit osteoclastic activities, and generate hypocalcemia and such histopathological changes as osteopenia and osteomalacia (Gdula-Argasinska

Table 4.7.3. Lead and cadmium concentrations (Mean, Mean ± SD, Mean ± SEM or Mean and Range) reported for bones of several insectivore species from polluted (P) and control (C) sites. Values in dry weight, except for *Myosorex*. References: 1. Getz et al., 1977; 2. Stansley and Roscoe, 1996; 3. Topashka-Ancheva and Metcheva, 1999; 4. Reinecke et al., 2000; 5. Chmiel and Harrison, 1981; 6. Hunter and Johnson, 1982; 7. Andrews et al., 1984; 8. Andrews et al., 1989; 9. Hunter et al., 1989; 10. Ma, 1989; 11. Komarnicki, 2000.

Species	Site	N	Pb	Cd	References
Blarina brevicauda	P	49	67.1±53.0 SD		1
	P	67	19.9±21.0 SD		
	P	32	12.2±8.3 SD		
Blarina brevicauda	P	1	437		2
	C	4	12.3 (6.27-16.4)		
Crocidura leucodon	P	3	89.6±25 SD	0.95±0 SD	3
Myosorex varius	P	3	372.4±171.6 SEM		4
Sorex araneus	P	20	193.0		5
	C	6	41.0		
Sorex araneus	P	7		3.00±0.65 SEM	6
	P	8		3.30±0.28 SEM	
	C	8		3.65±0.16 SEM	
Sorex araneus	P	17		1.89±0.19 SEM	7
	C	13		1.00±0.10 SEM	
Sorex araneus	P	23	610±53.06 SEM		8
	C	18	55.3±11.89 SEM		
Sorex araneus	P	16		1.83±0.23 SEM	9
	P	20		2.84±0.51 SEM	
	P	25		4.25±0.86 SEM	
	C	21		2.04±0.22 SEM	
Sorex araneus	P	12	550 (134-1469)		10
	C	10	53.7 (20.0-102)		
Talpa europaea	P	7	42.4±33.3 SD	0.032±0.017 SD	11
	P	5	24.8±15.6 SD	0.038±0.018 SD	
	P	9	22.0±11.1 SD	0.039±0.019 SD	

et al., 2004; Goyer, 1997; Suzuki et al., 2004). Mercury pollution is of crucial concern in aquatic ecosystems, where inorganic mercury is highly transformed into methylmercury and bioaccumulates in biota (Schuhmacher et al., 1993). In fact, fish and seafood are not only the main culprits in mercury poisoning incidents, but are also one of the main dietary exposure routes for humans (López-Alonso et al., 2003). The available information on mercury concentrations in terrestrial biota is scarce, the Ebro Delta being no exception. Indeed regarding this region, evaluations of the effect this metal exerts have mainly focused on freshwater and marine ecosystems (Lacorte et al., 2006; Lavado et al., 2006; Mañosa et al., 2001; Schuhmacher et al., 1993). Our study provides the first data on Hg accumulation in a terrestrial mammal from this wetland and shows that in this area a significant increase of about 2.5–fold in mercury levels was produced compared with the control site. The relatively high values recorded in the shrews from the Ebro Delta indicate that there is some transfer of this metal from aquatic to terrestrial environments. Similar high Hg levels have also been reported in the bones of other terrestrial mammals (see Talmage and Walton, 1991 for a revision) and humans (e.g. Lindh et al., 1980) inhabiting polluted sites. Furthermore, different patterns of mercury bioaccumulation were observed between sexes in *C. russula* from the Ebro Delta: males exhibited a significant increase as they aged, whereas Hg levels remained unvarying throughout the life of females. This finding suggests that the mercury burden in females is reduced by transfer to the foetus via placenta and to offspring via milk, as widely described for other mammals (see e.g. Frodello et al., 2000; Yoshida et al., 1994). In the control area no significant sexual variation was observed, consistent with observations made at other sites with low mercury levels, in which the normal excretion rate is high enough to prevent bioaccumulation (López-Alonso et al., 2003; Talmage and Walton, 1993).

Although sediments carried by the river and fertilizers from agriculture activities are sources of cadmium in the Ebro Delta (Lavado et al., 2006; Ramos et al., 1999; Schuhmacher et al., 1990), several studies have shown that levels of this metal remain low or moderate (revision in Mañosa et al. 2001). This agrees with our own findings, which demonstrate that Cd concentrations in shrews from this delta did not differ from those obtained in Medas' animals. In fact, the values achieved from both sites were similar or lower to those recorded in the bones of other insectivore species from unpolluted sites (Table 4.7.3). Nevertheless, it must be taken into account that since cadmium is excreted via osteoclasts (Suzuki et al., 2004), it does not appear to bioaccumulate in hard tissues in polluted areas. Thus, for example, when comparing *S. araneus* from control and polluted sites, levels of this metal in liver and kidney increased until 82- and 33-fold respectively, whereas in femur it hardly varied (Andrews et al., 1984; Hunter and Johnson, 1982). Therefore, considering that insectivores are effective bioindicators of this metal in polluted areas (e.g. Komarnicki, 2000; Ma and Talmage, 2001; Talmage and Walton 1991), we conclude that an additional examination of the soft tissues of *C.*

russula is needed to further elucidate the entry of Cd into the biota of this wetland.

4.7.4.2. *Toxicity effects*

Our results showed that, in comparison with the control site, *C. russula* from the Ebro Delta bioaccumulated lead and mercury in levels that *a priori* were expected to have adverse effects. Ma (1996) found concentrations of Pb above 10 µg/g in the liver or 25 µg/g in the kidney, to be harmful to the common shrew. Assuming Pb ratios of 1:2 to 1:6 for kidney:femur and 1:30 for liver:femur from Ma's data on *S. araneus* (1989), values obtained in the present study seem far below the risk threshold for acute poisoning postulated in *C. russula*. Nonetheless, neither subclinical exposure nor direct mortality can be assessed by our data, and therefore cannot be disregarded. In the case of mercury, levels at which it may prove toxic vary greatly in wildlife. Based on the available literature, Eisler (1987) deduced that Hg concentrations greater than 1.1 µg/g for liver and kidney, should be regarded as presumptive evidence of an environmental mercury problem in wild mammals. Moreover, 30 µg/g for hepatic and renal tissues is considered the intoxication threshold for mammals, with levels of 13 to 69 µg/g reported in the kidneys of wild and laboratory mammals whose deaths had been attributed to mercury poisoning (Lord et al., 2002; Wren, 1986). As all of these Hg levels were expressed in terms of wet weight, a ratio of 2:1 must be applied to obtain comparable dry weight concentration since the water contained in bones constitutes approximately 50% of total weight. While Hg ratio between bone and soft tissues is not generally provided, using the data published by Jefferies and French (1976) for the wood mouse, *Apodemus sylvaticus*, we have considered an approximate Hg ratio of 1:4 for bone:kidney and bone:liver. The mercury concentrations detected in shrews from the Ebro Delta (up to 12.44 µg/g; i.e. approximately 24.88 µg/g wet weight in soft tissues) would indicate that mercury pollution affects this protected area.

4.7.4.3. *Morphometric parameters*

Several studies have shown that small mammals exposed to heavy metals may lose body mass and/or may experience relative weight alterations in some tissues (e.g. Ma, 1989, 1996; Nunes et al., 2001a). Body weight variation is reflected in the body condition index, a parameter that has been used to correlate individual responses and physiological alterations in animals inhabiting environments of diminished quality, for example by metal exposure. Although shrews from the Ebro Delta present high levels of lead and mercury, their fitness did not differ from that of control animals as can be deduced from their body condition index. This result is in agreement with those observed in seabirds and waterfowls from the Ebro Delta, where metal pollution was not associated with impairment of reproduction or body condition (Mateo et al., 1997; Morera et al., 1997). As previously reported (Ma, 1989; Milton et al., 2003; Nunes et al., 2001a), our results indicate that *C. russula* exhibits a high tolerance to metal pollution, similar to other small mammals. Body condition among shrews varied greatly in the two study sites suggesting that it is not driven by metal pollution but rather by particular conditions, such as habitat characteristics, food availability, and predation, among others. Thus, as has already been suggested in other studies (e.g. Nunes et al., 2001a, b), genetic, haematological, biochemical and developmental parameters should be used in conjunction with morphometrical criteria, to obtain a more suitable measure for assessing the response of terrestrial small mammals inhabiting polluted areas.

4.7.4.4. *Biomonitoring environmental quality in protected wetlands*

As can be deduced from published data, the range of variation in metal concentration is generally wider in polluted areas than in clean sites (Table 4.7.3). This observation was noted in the Ebro Delta, not only in *C. russula* but also in several bird (Mateo et al., 1997, 2003; Sanpera et al., 2000) and invertebrate species (Schuhmacher et al., 1990, 1994). This circumstance may indicate an individual response due to a different metal exposure and/or to particular ecological, genetic, and physiological factors in chronically exposed animals (Talmage and Walton, 1991). Additionally, the tissular turnover that occurs in bones causes changes in metal concentration, particularly in lead, over the life of an animal, which may also partially explain the great variation observed at the population level. In contrast, in non-polluted sites, animals do not need detoxicant mechanisms to control the intake of metals and to prevent toxic effects to their organs.

Because anthropogenic contamination frequently often ends up in wetlands, the degradation of deltaic areas is a common phenomenon in most developed countries, despite increased environmental legislation and protection. Specifically in the Ebro Delta, hunting, land use, and other anthropogenic activities have considerably increased non-essential heavy metal exposure, diminishing the environmental quality. Our results not only corroborate previous and recent studies reporting increased environmental levels of lead and mercury in this protected wetland (e.g. Lacorte et al., 2006; Lavado et al., 2006; Mañosa et al., 2001), but also analyse mammal data, which allows improving the assessment of environmental risk in this particular habitat. Furthermore, this study demonstrates that osteological material from mammal collections offers an effective tool for ecotoxicological studies, constituting a non invasive method since it does not involve unnecessary animal captures. Moreover, it provides specific reference values to better assess the time variation of pollutants in wild populations in future research, particularly when relevant toxicological episodes occur. In fact, our samples were collected over 25 years ago, when environmental legislation in Spain was scarce. Nowadays, the use of lead shot pellets is forbidden and industrial and domestic wastewaters are more controlled and/or are treated in order to reduce river pollution. Consequently, a decrease in metal input in the Ebro Delta may be expected. Nevertheless, given the long environmental persistence of Pb and Hg, further studies are required to evaluate temporal variation in the levels of these metals and the efficacy of the recent legislation to improve the environmental quality of this wetland.

4.7.5. Conclusions

We conclude that terrestrial wildlife is exposed to non-essential heavy metals (Pb and Hg) in the protected area of the Ebro Delta. The lead from shot pellets and the mercury from anthropogenic activities deposited in the sediments from terrestrial environments and transported by water may become highly bioavailable to biota in this region. In contrast, cadmium does not appear to bioaccumulate in shrews. Sex and age are two important factors to be considered when conducting ecotoxicological studies, since they may represent a source of variability in wild populations. Apparently, the exposure to environmental pollutants does not affect the fitness of the shrews from the Ebro Delta, suggesting a high tolerance to toxic heavy metals. The greater white-toothed shrew may be regarded as a good indicator of Pb and Hg accumulation in the Mediterranean climate. In addition, bones from collections are a suitable source for assessing contamination levels in mammals. Further studies combining several criteria (including morphometrical, genetic, behavioural, physiological or developmental parameters) are needed in order to obtain additional information on the exposure, dietary uptake, pollutant bioavailability, and environmental quality of this protected area.

4.7.6. References

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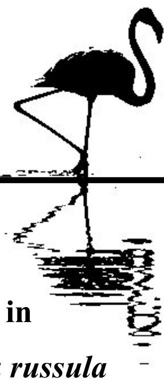
4.8. EBRE II



Bioaccumulation of metals and metalloids in the shrew *Crocidura russula* from two protected Mediterranean coastal sites

Metal and metalloid accumulation in shrews (Soricomorpha, Mammalia) from two protected Mediterranean coastal sites

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Abstract

Although ecotoxicological data on heavy metals are abundant, information on other potentially toxic elements with attributed deficiency and/or toxic disturbances is scarce. Here we quantify zinc, copper, iron, manganese, chromium, molybdenum, strontium, barium, and boron in bones of greater white-toothed shrews, *Crocidura russula*, inhabiting two protected Mediterranean coastal sites: the Ebro Delta, a wetland impacted by human activities, and the Medas Islands, a reference site. Natural and anthropogenic inputs increase significantly Fe, Mn, Mo, Sr, Ba, and B in specimens from the Ebro Delta, whereas Cu and Cr were higher in Medas' shrews. Principal component analysis allowed complete separation between sites along the first two axes particularly due to B, Sr, and Cu. This study provides metal reference values in bones of insectivores, explores deeply in their variability and bioaccumulation patterns, and assesses the potential environmental risk and toxicity for biota exposed to the above elements.

4.8.1. Introduction

Non-essential metals such as lead (Pb), mercury (Hg), and cadmium (Cd), and essential elements, such as zinc (Zn) and copper (Cu) are common pollutants widely distributed throughout the ecosystems. They have been extensively quantified and their toxic effects for biota are well-known (e.g. Goyer, 1997; Mañosa et al., 2001; Nriagu, 1988; Sánchez-Chardi et al., 2007a). In contrast, environmental information concerning other metals and metalloids, like iron (Fe), manganese (Mn), molybdenum (Mo), strontium (Sr), barium (Ba), and boron (B) is less readily available. All these elements are natural constituents of soils and sediments (Fernández-Turiel et al., 2003; He et al., 2005; Nriagu, 1988; West et al., 2001) and their levels in mammalian tissues may depend on season, age and/or sex, as well as physiological, pathological, and ecological conditions (Komarnicki, 2000; Lopes et al., 2002; Pankakoski et al., 1993, 1994; Sánchez-Chardi and Nadal, 2007; Sánchez-Chardi et al., 2007a,b). Moreover, their background levels and/or bioavailability may increase due to industrial, agricultural, and domestic pollution, and other human activities such as hunting. Thus, lead shot pellets may contain traces of Zn and Cu (Mozafar et al., 2002), fertilizers are a source of Zn, Cu, Fe, Mn, Cr, Mo, and B (He et al., 2005, Otero et al., 2005), and plaguicides may have traces of Zn, Cu, Fe, Mn, Ba, and B (He et al., 2005; Mañosa et al., 2001). Additionally, effluents and atmospheric deposition from industries may contain virtually all elements, and domestic effluents may be an important input of Zn, Cu, Fe, Mn, Cr, and B (e.g. Lucho-Constantino et al., 2005; Outridge and Scheuhammer, 1993). Generally, data on some of these metals and metalloids in wild populations are scarce or absent probably due to their low toxicity compared with those of widely distributed non-essential elements and the complexity of behaviour of metal mixtures at real conditions. This lack of data hinders our understanding of metal and metalloid migration through food chains.

Deltaic environments are fragile ecosystems often affected by heavy metals and other pollutants, especially in developed countries (e.g. Oliveira Ribeiro et al., 2005; Sánchez-Chardi et al., 2007a) and one of the main goals when analysing heavy metal concentrations is to distinguish between natural background levels and those originating from anthropogenic contaminant sources (Rodríguez Martín et al., 2006). For many decades, the Ebro Delta has been impacted by severalhuman activities such as lead shot pellets from hunting, fertilizers and pesticides from agricultural processes, industrial poles, and domestic sewage (Lacorte et al., 2006; Lavado et al., 2006; Mañosa et al., 2001; Ocampo-Duque et al., 2008; Schuhmacher et al., 1993). However, most studies focusing on the Ebro area have reported fragmentary, not always coincident, information about elements at both biotic and abiotic levels (e.g. Grimalt and Albaigés, 1990; Lavado et al., 2006; Mañosa et al., 2001; Navas and Machín, 2002; Ocampo-Duque et al., 2008). The Medas Islands are a nature reserve that is barely impacted by pollution sources and considered a pristine site with background levels of toxic metals and other pollutants (Pastor et al., 1995; Sánchez-Chardi et al., 2007a).

Small mammals, especially shrews, are reliable bioindicators of environmental pollution (bioaccumulation of metals and physiological effects) because they show high food requirements and their metabolic rate is high (e.g. Komarnicki, 2000; Pankakoski et al., 1993, 1994; Sánchez-Chardi et al., 2007a,b; Talmage and Walton, 1991). Likewise, bones accumulate several metals, especially bivalent elements that mimic calcium (e.g. EPA, 1998; Nielsen, 2004). In addition, bones from zoological collections have been used successfully to study contamination levels in small mammals (e.g. Sánchez-Chardi et al., 2007a). However, there is little information on the accumulation of several elements in the hard tissues in wild mammals.

With the above considerations, the aims of this study were: i) to determine levels of those elements scarcely quantified in previous ecotoxicological studies; ii) to analyse site, age-, and sex-dependent variation in the bioaccumulation patterns; iii) to provide reference values for metal and metalloid contents in hard tissues of shrews; and iv) to assess the use of these parameters as biomarkers in Mediterranean sites.

4.8.2. Material and Methods

We analysed the element content in large bones of 105 greater white-toothed shrews, *C. russula*. Animals were collected from 1976 to 1981 in the Ebro Delta (n=73) and the Medas Islands (n=32), two partially protected coastal areas in north-eastern Spain. Specimens were sexed and aged (1-Juveniles: immature shrews in their first year of life; 2-Adults: mature shrews in their first year of life; 3-Seniles: shrews in their second year of life) according to toothwear (Vesmanis and Vesmanis, 1979) and reproductive condition (López-Fuster et al., 1985). Bones were cleaned by exposure to dermestid larvae (Dermestidae, Coleoptera), kept in single metal-free paperbags and stored in the zoological collection of the Animal Biology Department (University of Barcelona). Initially, this material constituted the basis of several investigations on morphometric and biological aspects of the species (López-Fuster, 1985; López-Fuster and Ventura, 1992; López-Fuster et al., 1985, 1986).

For chemical quantification, dried samples were placed in Teflon vessels and digested by nitric acid and hydrogen peroxide (Instra, Baker Analyzed). Samples were diluted 1:5 in deionized (Milli-Q©) water with 1% nitric acid. Rhodium was used as internal standard and Bovine Liver (SRM-

1577a), certificated by the National Bureau of Standards, was included as reference material in the analysis. Concentrations of Fe were quantified by a Perkin-Elmer OPTIMA-3200RL Inductively Coupled Plasma Optical Spectrometer (ICP-OES) whereas the rest of the metals (Zn, Cu, Mn, Cr, Mo, Sr, Ba, Ni, and Co) and a metalloid (B) were determined by a Perkin-Elmer ELAN-6000 Inductively Coupled Plasma Mass Spectrometer (ICP-MS). The results of element concentrations were expressed as mean ± standard error of the mean (M±SEM) in microgram/gram (µg/g) on a dry weight basis. Detailed methodological information is described by Sánchez-Chardi et al. (2007a,b).

Normal distribution and homogeneity of variance of the log transformed data were assessed by the Kolmogorov–Smirnov test and the Levene, *F*-test, respectively. Initially, a three-way multivariate analysis of variance (MANOVA) was performed to obtain an overall estimation of the effects of site, age, and sex and their interactions on the element levels. When element concentrations did not differ according to age and/or sex, data were combined to increase the sample size of each site. Depending on this previous procedure, intra- and interpopulational comparisons were evaluated either by Student's *t*-test or by one-way analysis of variance (ANOVA). When ANOVA was applied, pair-wise comparisons of sample means were conducted by Scheffe's method. In an attempt to visualize the degree of divergence between both populations considering all elements analysed, a principal component analysis was performed. This statistical technique reduces multidimensional data sets to lower dimensions while retaining those characteristics of the data set that contribute most to its variance. After factor extraction, a Varimax rotation was employed to aid interpretability of the low-variance principal components. Pearson's correlation coefficients were calculated to evaluate the relationship between the elements analysed in shrews from the polluted site. For all sequential tests, *p*-values were corrected by the Bonferroni adjustment (Rice, 1989), as modified by Chandler (1995). For all statistical analyses, SPSS 14.0 (2005) was used.

4.8.3. Results

Nickel and cobalt concentrations were under threshold limit of ICP-MS (in µg/kg in diluted acidic solution, Ni: 0.20; Co: 0.05) and therefore were not included in the analyses. The rest of elements were above the detection threshold in all specimens (in µg/kg in diluted acidic solution, Fe: 10; Zn: 0.50; Cu: 0.10; Mn: 0.10; Cr: 0.50; Mo: 0.05; Sr: 0.10; Ba: 0.05; B: 0.05).

Table 4.8.1. (next page) Descriptive statistics (sample size (n), arithmetic mean (M), standard error of the mean (SEM), minimum (Min) and maximum (Max)) of element concentrations (µg/g, in dry weight) in the samples analysed according to site and age (1: juveniles; 2: adults; 3: seniles).

	Age	Ebro Delta					Medas Islands				
		n	**M**	**SEM**	**Min.**	**Max.**	**n**	**M**	**SEM**	**Min.**	**Max.**
Zn	**1**	27	213.09	13.63	111.21	358.44	9	203.52	3.52	191.95	217.85
	2	12	227.73	16.39	126.78	345.61	12	228.15	4.54	202.00	252.11
	3	34	238.09	8.68	81.12	360.31	11	232.94	6.78	187.99	265.96
	Total		227.14	7.03				222.87	3.69		
Cu	**1**	27	4.67	0.50	2.31	15.73	9	5.68	0.63	3.70	9.65
	2	12	3.51	0.29	2.12	5.17	12	5.09	0.42	2.63	8.14
	3	34	4.78	0.48	1.02	13.93	11	6.11	0.41	4.33	8.39
	Total		4.53	0.29				5.61	0.28		
Fe	**1**	27	258.93	18.55	145.85	501.96	9	154.03	11.69	112.11	209.52
	2	12	220.41	13.78	153.43	324.34	12	158.35	8.32	122.45	206.77
	3	34	211.86	18.34	117.55	602.00	11	176.57	4.80	152.23	199.88
	Total		230.67	11.36				163.39	4.97		
Mn	**1**	27	7.45	0.69	1.94	16.01	9	4.06	0.20	2.83	4.79
	2	12	10.81	1.32	5.24	18.82	12	4.89	0.25	3.78	6.77
	3	34	7.02	0.57	3.65	15.47	11	4.66	0.26	3.64	6.35
	Total		7.80	0.45				4.58	0.15		
Cr	**1**	27	2.28	0.16	1.06	5.47	9	1.84	0.12	1.44	2.67
	2	12	1.81	0.17	1.18	3.02	12	2.07	0.17	1.31	3.61
	3	34	1.78	0.17	0.75	5.97	11	1.91	0.11	1.36	2.72
	Total		1.97	0.11				1.95	0.08		
Mo	**1**	27	0.18	0.02	0.08	0.57	9	0.11	0.01	0.09	0.14
	2	12	0.13	0.01	0.08	0.24	12	0.11	0.01	0.09	0.16
	3	34	0.20	0.06	0.07	2.16	11	0.12	0.01	0.09	0.15
	Total		0.18	0.25				0.11	0.02		
Sr	**1**	27	250.65	16.61	126.69	474.88	9	68.41	1.81	59.46	74.93
	2	12	228.79	17.32	100.32	321.30	12	73.27	2.58	61.89	91.03
	3	34	246.93	20.80	55.87	680.58	11	78.28	2.82	58.38	91.57
	Total		245.32	11.73				73.63	1.58		
Ba	**1**	27	17.64	2.06	9.48	53.17	9	8.96	1.08	5.14	14.41
	2	12	13.70	0.98	8.65	18.29	12	10.05	0.67	7.15	15.48
	3	34	18.19	1.87	2.73	64.77	11	12.63	1.33	9.29	24.26
	Total		17.25	1.17				10.63	0.64		
B	**1**	27	143.99	6.49	75.58	209.22	9	28.44	8.54	7.73	86.97
	2	12	136.05	7.59	102.34	174.68	12	11.93	1.81	5.87	22.48
	3	34	169.01	18.38	39.42	445.11	11	26.92	3.92	7.46	52.54
	Total		154.34	9.05				21.73	3.05		

MANOVA showed significant differences in the element concentrations by site (F=103.487, p=0.001), age (F=2.528, p=0.001), and their interaction (F=2.434, p=0.002). Since sexual divergence was not observed, sexes were pooled in subsequent analyses. Descriptive statistics of the elements quantified according to site and age are shown in Table 4.8.1.

Significant age-dependent variation was only observed in Zn levels in the Medas Islands (F=8.226, p<0.001), with statistical divergences between juveniles and both adults (p=0.009) and seniles (p=0.003). Mean values of this metal increased with age in the two capture sites although in the Ebro Delta differences were not significant.

Element concentrations varied significantly between sites (in all cases p<0.001), except for Zn (differences evaluated for each age-class) and Cr (Table 4.8.1). Globally, shrews from the polluted area showed the highest mean values, with the exception of Cu.

Principal component analysis was performed on the log transformed data excluded Zn due to the age-dependent variation observed in the Medas'animals and the absence of interpopulational differences. Two rotated principal components with eigenvalues greater than 1 were extracted, which accounted for 61.46% of the total variance. Variables with higher positive loadings were B and Sr on the first factor (PC I) and Cu on the second factor (PC II; Table 4.8.2). Projection of the specimens

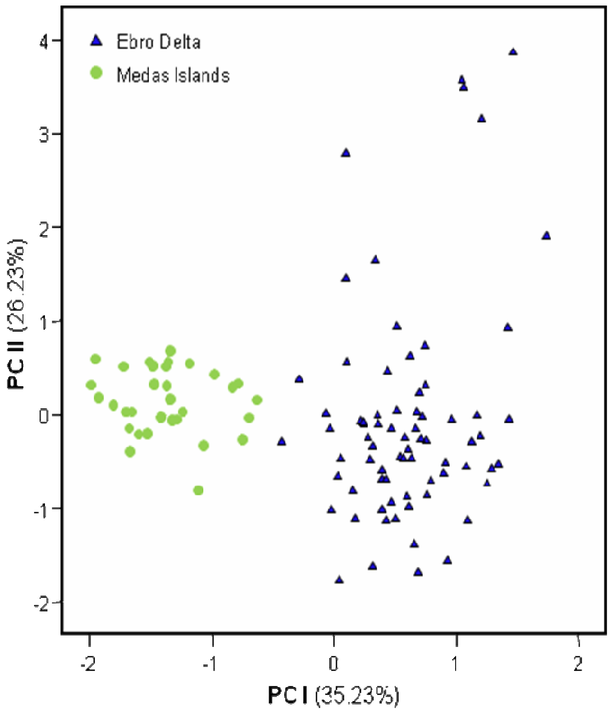

Table 4.8.2. Component correlations obtained after applying a PCA with Varimax rotation to the metals and metalloids in bones of shrews.

	Components	
	PC I	**PC II**
B	0.854	-0.026
Fe	0.555	0.612
Ba	0.579	0.351
Sr	0.898	0.180
Cu	-0.382	0.735
Mn	0.552	0.446
Mo	0.345	0.649
Cr	-0.265	-0.639

Figure 4.8.1. Biplot of the individual scores onto the first two component axes according to site.

onto the first two axes showed a complete separation along PC I, due to the lower scores exhibited by the Medas'shrews (Figure 4.8.1).

Significant Pearson's correlation coefficients and the corresponding *p*-values evaluated in shrews from the Ebro Delta are shown in Table 4.8.3. In general, the highest number of correlations appeared between Cu and Fe and the rest of elements (7 and 6 cases, respectively), whereas Cr correlated with none of the elements analysed.

Table 4.8.3. Significant Pearson correlation coefficients (*r*) and *p*-values (*p*) between metals analysed in shrews from the polluted site.

	r	*p*		*r*	*p*
Zn/Cu	0.263	0.025	**Cu/B**	0.266	0.023
Zn/Fe	0.520	< 0.001	**Fe/Mn**	0.600	< 0.001
Zn/Mn	0.409	< 0.001	**Fe/Mo**	0.322	0.005
Zn/Mo	0.246	0.036	**Fe/Sr**	0.492	< 0.001
Zn/Sr	0.424	< 0.001	**Fe/Ba**	0.302	0.009
Zn/Ba	0.211	0.073	**Mn/Sr**	0.333	0.004
Cu/Fe	0.329	0.004	**Mn/Ba**	0.235	0.045
Cu/Mn	0.332	0.004	**Mo/Ba**	0.096	< 0.001
Cu/Mo	0.405	< 0.001	**Mo/B**	0.439	< 0.001
Cu/Sr	0.449	< 0.001	**Sr/Ba**	0.721	< 0.001
Cu/Ba	0.382	0.001			

4.8.4. Discussion

4.8.4.1. *Bioaccumulation by site, age, and sex*

Levels of Zn found in *C. russula* agreed with those reported in the bones of other Soricomorpha (Andrews et al., 1989; Komarnicki, 2000; Topashka-Ancheva and Metcheva, 1999). Similar concentrations observed between the sites studied might be mainly due to the good homeostatic mechanisms of this metal in mammalian tissues (Goyer, 1997).

Copper levels found in bones of *C. russula* from Medas Islands were in agreement with data reported for the common shrew, *Sorex araneus*, from reference sites and lower than those observed in *S. araneus* and *C. leucodon* from polluted sites (Hunter and Johnson, 1982; Hunter et al., 1989; Topashka-Ancheva and Metcheva, 1999). In the Delta shrews, Cu levels were significantly low, a circumstance that has also been reported in fish and eggs of seabirds from the same area (Lavado et al., 2006; Morera et al., 1997; Sanpera et al., 1997). Although high percentage of sand in Ebro Delta soils reduces Cu availability for biota (Grimalt and Albaigés, 1990), low values of essential elements in polluted areas are often related to the interaction between essential and non-essential metals. In mammalian tissues, this metal is effectively controlled by homeostatic mechanisms (Goyer, 1997; Hunter et al., 1989; Johnson et al., 1978; López Alonso et al., 2004) that may be disrupted by high exposure to Pb and Hg (Blanuša et al., 1989; Lavado et al., 2006; Pankakoski et al., 1994), heavy metals that appeared in high concentrations in the specimens from the Ebro Delta (Sánchez-Chardi et al., 2007a).

Iron concentrations found in *C. russula* did not differ from those previously obtained in the bones of bank voles, *Myodes glareolus* (Sawicka-Kapusta et al., 1990; Damek-Poprawa and Sawicka-Kapusta, 2004). The increase in shrews from the Ebro Delta may be explained by the protective effects of iron, aspreviously observed in this species (Sánchez-Chardi et al., 2007b) and in several tissues of other species, for example bone medule (see e.g. López Alonso et al., 2004; Pereira et al., 2006; Włostowski et al., 2003). There are several cellular detoxification mechanisms involving iron, such as the induction of synthesis of proteins like cytochromes and ferritin, that protect mammalian cells from the toxic effects of both essential and non-essential metals (Lopes et al., 2002; Pereira et al., 2006), which might explain our findings.

Manganese levels were low and similar to those reported for the bank vole, the field vole, *Microtus agrestis*, and the wood mouse, *Apodemus sylvaticus* (Beardsley et al., 1978; Gdula-Argasinska et al., 2004; Gorriz, 1996). The levels of this metal are physiologically regulated in mammals and environmental increases are not generally related to high levels in tissues (Beardsley et al., 1978). As with iron, the higher levels observed in the Ebro Delta shrews in relation to the Medas' specimens might be related to detoxification mechanisms and/or interaction between elements (see references in Lopes et al., 2002; Sánchez-Chardi et al., 2008).

Previous studies on the Ebro Delta indicate high variability in the concentration of chromium (Ocampo-Duque et al., 2008) with no clear pattern of bioaccumulation (Schuhmacher et al., 1993, 1994, 1995). As occurs in other small mammals (Pereira et al., 2006; Talmage and Walton, 1991), we found the highest chromium concentrations in shrews from the reference site. Chromium levels are mainly influenced by natural content of parent rocks on a regional scale (Rodríguez Martin et al., 2006). Consequently, and considering that Cr was not significantly correlated with the rest of the elements analysed, our results might indicate a low bioavailability of this metal in the Ebro Delta rather than competitive mechanisms between essential and non-essential elements as suggested in previous studies (e.g. Pereira et al., 2006).

The molybdenum levels found in specimens from the Ebro Delta may be related to the high redox potential of deltaic soils that act as a net to catch this element as well as these alkaline lime-rich environments also facilitate Mo solubilization and mobilization (Fernández-Turiel, personal communication; Frank, 2004). Although molybdenum is known to be a copper antagonist (Pankakoski et al., 1993) due to the formation of a sulphur-molybdenum complex that binds Cu, we found significant correlation between both elements, as has also been reported in other mammals (Blanco-Penedo et al., 2006; López-Alonso et al., 2004).

Strontium and barium are widely distributed non-essential elements that do not biomagnify throughout the trophic chain, and calcium mimics in biological systems (EPA, 1998; Malina, 2004; Nielsen, 2004; Purdey, 2004). Whereas Sr levels found in *C. russula* from Medas Islands did not differ from those found in other natural environments, levels in the Ebro Delta specimens were significantly higher than those reported in the whole body, soft and hard tissues, of other small mammals inhabiting polluted and reference sites (Appleton et al., 2000; Cloutier et al., 1986; Seifert et al., 1999). Strontium is considered toxic only at high concentrations (Nielsen 2004), producing hypocalcaemia in exposed animals. Nevertheless, in the present study neither calcium decrease (M±SEM: Ebro Delta: 224.01±3.02 mg/g; Medas Islands: 229.65±1.98 mg/g) nor body weight loss (Sánchez-Chardi et al., 2007a) were observed in shrews from the Ebro Delta. The input of Sr in this area may be partially explained by the geochemistry of river sediments and soils in the Ebro Basin (with high evaporitic rock content) and the seawater entrance of the Encanyissada lagoon that are rich in this element (Fernández-Turiel et al., 2003), although, the importance of anthropogenic sources of Sr cannot be disregarded. Conversely, although the uptake and accumulation of Ba in bones of mammals is higher than their associates (EPA, 1998; Nielsen, 2004), the low Ba concentrations found in shrews may be due, at least partially, to its tendency to precipitate as $BaCO_3$ when there is a high $CaCO_3$ content. Additionally, Ba has a short half-time in biological systems, as it is excreted in faeces and urine, reducing body burden in mammals (EPA, 1998; Malina, 2004).

Boron is a metalloid naturally occurring throughout the environment (Malina 2004) and its essentiality for animals is discussed (Chapin et al., 1997). Low levels found in shrews from the reference site agreed with data obtained in bones of laboratory mammals (Chapin et al., 1997) and could be considered as background concentrations. The high B levels observed in shrews from the Ebro Delta may be due to anthropogenic activities, as occurs in other areas (Lucho-Constantino et al., 2005; Powell et al., 1997). Moreover, limited water circulation in the lagoons and soils with high clay content in the Ebro Delta (Lacorte et al., 2006) might increase boron bioavailability (e.g. Lucho-Constantino et al., 2005). Boron affects weight and size of bones, as well as their essential elements and fat content (Chapin et al., 1997). Considering the data reported by these authors for laboratory rodents, a very high B intake, up to levels related to bone alteration, would be expected in shrews from the Ebro Delta. However, this metalloid showed a wide range of non observable adverse effects level (NOAEL) depending on the species and parameters tested (revision in Eisler, 1990).

As for age is concerned, bones of adult shrews tended to have higher Zn concentrations than those of juveniles, as has also been reported for *S. araneus*, the meadow vole, *Microtus pennsylvanicus*, and the wood mouse (Cloutier et al., 1986; Pankakoski et al., 1993, 1994; Read and Martin, 1993; Tersago et al., 2004). However, this pattern has not been observed in soft tissues of *C. russula* (Sánchez-Chardi et al., 2007b; Sánchez-Chardi and Nadal, 2007), probably because bone acts as a storage tissue for excess zinc (where it accumulates as metabolically inactive) and/or due to interaction with other elements (Johnson et al., 1978; Pankakoski et al., 1993, 1994; Talmage and Walton, 1991).

Among parameters analysed in the present study, gender remained the least important factor, as reported for the common shrew and the lesser shrew, *S. minutus*, (Pankakoski et al., 1994). In *C. russula*, this circumstance also applies to heavy metal bioaccumulation (Sánchez-Chardi et al., 2007a,b). Although sexual differences were not significant, females showed higher levels of Mn, Mo, and Fe than males (data not show). A similar trend has also been reported for the species and other wild mammals (Lopes et al., 2002; Pankakoski et al., 1993; Sánchez-Chardi et al., 2007a,b), although gestation and lactation are expected to reduce metal body burden. Nutritional requirements, different metabolic profiles of metals related to sexual hormones, and/or interaction between elements (see references in Goyer, 1997; Lopes et al., 2002; Pankakoski et al., 1993) may be responsible for the results obtained concerning these essential metals.

4.8.4.2. *Biomarkers and bioindicators of environmental quality*

Usually a species is considered a suitable bioindicator when it accumulates high concentrations of toxic heavy metals and reacts to the entrance of xenobiotics into the environment. For this purpose, small mammals in general and shrews in particular have proved as suitable bioindicators of pollution because they constitute an intermediate step between top predators and invertebrates. Earthworms, snails, slugs, and arthropods are common shrew preys, some of them well-known as metal accumulators (Hunter and Johnson, 1982; West et al., 2001), and diet is the main source of metals in mammals (e.g. Johnson et al., 1978; Appleton et al., 2000; Torres and Johnson, 2001). Apart from the direct accumulation of metal content through the preys, the shrews also intake xenobiotics indirectly through soil ingestion (Stansley and Roscoe, 1996). These feeding characteristics as well as high metabolic rate and tolerance to toxic compounds make shrews highly accumulators of several elements (e.g. Komarnicki, 2000; Pankakoski et al., 1993, 1994; Talmage and Walton, 1991).

In the search for suitable parameters (i.e. biomarkers) to assess environmental quality, metal and metalloid concentrations may be used. In general, the adequate tracer element may depend on the kind of pollution, the physicochemical conditions of the soil and water, or the species studied, among other factors. Moreover, the combined data of several elements and their interactions may provide useful information for ecotoxicologists. Nevertheless, the interaction of elements, as well as the information derived from analysis of essential metals and other elements is often forgotten in ecotoxicological studies. We propose the use of the whole data set provided by several elements as a valuable information source on environmental quality. Principal component analysis performed on these data allowed us to obtain a good separation of the specimens according to the study sites. Thus, whereas specimens from the reference area appeared as a homogeneous group, shrews from the polluted zone exhibited more dispersed scores. This increase of variability in the accumulation of pollutants has also been reported in altered areas as a consequence of intraspecific variation and individual exposure and response to pollutants (e.g. Sánchez-Chardi et al., 2007a and references therein). Similar methodology has been previously used with several kinds of samples from reference and polluted areas but not, to our knowledge, with bones of shrews. Moreover, our results with PCA must be checked in other polluted areas and with other species in order to use hard tissues as a suitable source of ecotoxicological data.

As has been demonstrated in the case of highly toxic metals (Sánchez-Chardi et al., 2007a), examination of bones from zoological collections is a reliable and non-invasive method of studying elements with ecotoxicological interest. The samples used in this study were collected 30 years ago and since then environmental conditions have been strongly modified. On the one hand, environmental laws have protected places of ecological significance, such as the Ebro Delta, reducing and controlling human impact. Since a reduction in industrial and domestic untreated wastewaters is expected by this restrictive environmental legislation, metal entrance into the Ebro River should have diminished throughout this time. On the other hand, the increase in human population and its pollutant activities (traffic, industries and domestic wastes) may increase metal pollution in the Ebro Delta. Therefore, having demonstrated the bioaccumulation of toxic metals in biota of the Ebro Delta, we consider that it would be interesting to undertake further analysis based on recent material to assess current levels, effects, and sources of potentially toxic elements in protected areas.

4.8.5. Conclusions

This study constitutes the first quantification of several metals and metalloids in the bones of shrews. A few of these elements may be toxic for biota and may be of special concern in protected areas. Main entrances of these elements seem to occur naturally, but further analyses are needed to quantify anthropogenic inputs.

Like other insectivorous species, the greater white-toothed shrew, *Crocidura russula*, seems to be a suitable bioindicator of metals and boron because it is a strong bioaccumulator and the element concentrations in their tissues vary in relation to environmental changes. Moreover, the information obtained may be useful to know the distribution and mobility of metals and metalloids within ecosystems and to predict the risk of environmental pollution in protected Mediterranean sites.

4.8.6. References

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5. DISCUSSIÓ GENERAL

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Les zones contaminades d'estudi van ser triades d'acord amb la significació del tipus de contaminació que les afecta: i) el més gran abocador de residus urbans i industrials de la península Ibèrica a Garraf; ii) residus de la mineria de pirita d'una gran mina abandonada a Aljustrel i els seus efectes sobre Doñana, l'aiguamoll més important d'Espanya; i iii) la barreja de residus agrícoles, caça, abocaments industrials i domèstics que afecten el Delta de l'Ebre, la més important zona deltaica de Catalunya. També es va tenir en compte el seu interès ecològic atès que 6 de les 8 zones estudiades tenen estatus de protecció parcial (Parc o Parc Natural) o total (Parc Nacional o Reserva Biològica). Aquests aspectes han fet que les zones triades hagin estat objecte de diversos estudis ecotoxicològics previs basats en els nivells de contaminació a sòls i aigües (Quevauviller et al., 1989; Mañosa et al., 2001; Turner et al., 2008) o emprant diverses espècies autòctones de plantes, invertebrats, peixos, amfibis rèptils i ocells com a bioindicadors (García-Barrera et al., 2012; García-Sevillano et al., 2012, en premsa; Lopes et al., 2002; Nunes et al., 2001; Torres et al., 2006). No obstant, en aquestes àrees s'han realitzat pocs estudis ecotoxicològics amb petits mamífers terrestres en general (Bonilla-Valverde et al., 2004; Torres et al., 2006) i amb musaranyes en particular, malgrat la gran importància ecològica dels rosegadors i dels insectívors en la transferència de contaminants al medi, ja que ocupen diferents posicions a les xarxes tròfiques i són sensibles als canvis al medi, pel qual se'ls considera com a excel·lents bioindicadors (Talmage & Watson, 1991). En la present memòria es comparen per primer cop els efectes a nivell subcel·lular, cel·lular, tissular i individual de diversos tipus de contaminació a poblacions naturals de musaranya comuna *C. russula* de Garraf (Barcelona, Catalunya, NE Espanya), Aljustrel (Alentejo, SE Portugal) Doñana (Huelva, Andalusia, SO Espanya), i Delta de l'Ebre (Tarragona, Catalunya, NE Espanya). S'han emprat biomarcadors d'exposició (acumulació d'elements en teixits diana) i d'efecte (morfometria, hematologia, activitat enzimàtica, genotoxicitat i histopatologia). La recopilació de totes aquestes dades permetrà emmarcar la idoneïtat de l'espècie estudiada com a bioindicador de contaminació ambiental en general i de metalls en particular.

Els nivells de metalls i metal·loides als teixits tous (fetge i ronyó) i durs (ossos llargs) a les musaranyes de les zones de referència van ser similars o menors als que han reportat altres autors en els mateixos teixits a poblacions naturals de rosegadors i insectívors, incloent *C. russula* (p.ex. Beardsley et al., 1978; Damek-Poprawa & Sawicka-Kapusta, 2003, 2004; D'Havé et al., 2006; Fritsch et al., 2010, 2011; González et al., 2008; Komarnicki, 2000; Ma, 1989; Ma & Talmage, 2001; Pereira et al., 2006; Sawicka-Kapusta et al., 1990; Talmage & Walton, 1991; Topashka-Ancheva et al., 2003;). Especialment a les zones contaminades estudiades, els elements essencials quantificats (Mg, Fe, Cu, Zn, Mn, Mo, Co, Cr, Fe i Zn) presenten marcades diferències en la bioacumulació en relació als no essencials (Pb, Cd, Hg, Tl, Ni, B, Ba i Sr), el que està relacionat amb diferències en quant a biodisponibilitat, toxicitat, i capacitat de transferència i biomagnificació entre ambdós grups d'elements. Els majors augments detectats als teixits de les musaranyes de les zones contaminades es van donar entre els metalls pesants no essencials (Pb, Cd, Hg, Tl i Ni), els nivells tissulars dels quals no estan metabòlicament regulats a mamífers, donant com a resultat alts increments a teixits durs i tous quan augmenta la biodisponibilitat al medi ambient (Shore & Douben, 1994; Shore, 1995; Talmage & Walton, 1991). A aquestes zones d'estudi, tal com passa molt sovint a les zones naturals contaminades per l'acció de l'home, els sòls són els compartiments més afectats per la contaminació, els que regulen la seva dispersió en funció de les característiques fisicoquímiques (T^a, pH, matèria orgànica, composició) i els que determinen la biodisponibilitat dels contaminants al llarg de les xarxes tròfiques (Farag et al., 1998), inclús molt de temps després del cessament de l'activitat contaminant. La principal via d'entrada dels metalls a mamífers de zones contaminades és directament via ingesta de preses com els cucs de terra, grans acumuladors de metalls (Ma, 1989; Shore, 1995; Torres & Johnson, 2001) o indirectament per l'ingesta de terra (Doyle et al., 2010; Pascoe et al., 1994; Pelfrene et al., 2013; Roussel et al., 2010; Stansley & Roscoe, 1996; Talmage & Walton, 1991). Un cop ingerits, aquests metalls són difícilment excretats, provocant grans increments als teixits diana (p.ex. Shore, 1995; Shore & Douben, 1994; Talmage & Walton, 1991) i produint efectes tòxics al superar determinats nivells tissulars (da Silva Junior et al., 2013; Ma, 1996; Shore & Douben, 1994). Entre els metalls més tòxics, el Cd és el que va presentar majors concentracions a les zones contaminades estudiades de Garraf, Aljustrel i Doñana, amb augments entre el 200% i més de 500% en relació als nivells de les zones control, degut en gran part a que és un dels pocs metalls pesants altament biodisponible a pHs bàsics, com els dels sòls de la península Ibérica (Kraus & Wiegand, 2006). Especialment significatius són els valors obtinguts per a aquest metall als exemplars de la zona afectada pels lixiviats de l'abocador del Garraf, amb un augment mig de més del 300% a fetge (3,03 ug/g *vs* 10,28 ug/g) i més de 500% a ronyó (4,65 ug/g *vs* 25,59 ug/g). Malgrat la baixa disponibilitat del Pb a sòls bàsics o neutres (Kraus & Wiegand, 2006), els exemplars de l'abocador, amb un augment mig de gairebé el 200% a fetge (1,93 ug/g *vs* 3,40 ug/g) i més del 300% a ronyó (5,37 ug/g *vs* 16,36 ug/g), són els que van presentar les majors concentracions tissulars, el que indica l'alta disponibilitat d'aquest metall en aquesta zona càrstica. Per la seva banda, les musaranyes d'Aljustrel van ser les que presentaren els valors més elevats de Pb, amb un augment a fetge de gairebé el 700% (0,77 ug/g *vs* 5,26 ug/g), mentre que, un any i mig després del vessament, els valors de Pb van ser inesperadament baixos a Doñana. En quant al Hg, els majors valors van ser quantificats als exemplars impactats per les mines de pirita d'Aljustrel i d'Aznalcóllar, amb increments mitjos entre el 200% i gairebé el 400%, concentracions majors que les detectades a teixits tous d'insectívors (Pankakoski et al., 1993, 1994), ja que el mercuri està present a la pirita i als residus àcids resultants de l'extracció d'aquest mineral (Scheuhammer, 1991; Quevauviller et al., 1992; Tovar-Sánchez et al., 2006; Turner et al., 2008). Altament lipofílic, el Hg s'acumula a greixos i s'acomplexa amb els residus cisteïna de les proteïnes, incloent el glutatió (GSH) del sistema antioxidant mitjançant ponts sulfhidril, per la qual cosa els principals òrgans diana d'aquest metall són els teixits tous amb alt contingut en lípids com el sistema nerviós, teixit adipós, fetge i ronyó. Tot i aquest fet, en el present estudi també es van detectar alts valors de mercuri als óssos dels exemplars de la zona contaminada del Delta de l'Ebre, atribuïbles a les grans quantitats de Hg que transporta el riu Ebre degut principalment a diverses activitats industrials i que s'havien quantificat a diverses espècies aquàtiques però no a les de medi terrestre (Cid et al., 2010; Mateo et al., 1997; Mañosa et al., 2001: Ochoa et al., 2012, 2013; Schuhmacher et al., 1990, 1993, 1994). Globalment, aquests resultats indiquen clarament, per una banda, un augment de la biodisponibilitat i l'entrada a les xarxes tròfiques d'aquests elements no essencials, alguns d'ells altament biomagnificables, i, per una altra, que l'espècie emprada, a l'igual que altres insectívors, pot ser considerada un bon bioindicador d'aquests metalls ja que respon amb un augment dels nivells als seus teixits quan hi ha un increment de metall disponible al medi. A més d'aquests tres metalls, a algunes de les zones estudiades es van quantificar altres elements no essencials potencialment tòxics presents als residus contaminants. Són els casos del Tl a Doñana, Ni a Aljustrel i B, Ba i Sr al Delta de l'Ebre. Tot i que caldria disposar de dades d'altres zones, els alts nivells d'aquests elements als exemplars de les zones contaminades, alguns d'ells entre els més alts reportats a petits mamífers (Dmowski et al., 1998; Heim et al., 2002), fa pensar que l'espècie podria ser també un bon bioindicador de contaminació per Tl, Ni, B, Ba i Sr.

Contràriament als metalls no essencials, els essencials són comuns i abundants tant a les àrees control com a les contaminades, són necessaris pels organismes ja que participen en funcions metabòliques bàsiques, estan presents en els teixits de mamífers on les seves concentracions estan ben regulades homeostàticament i generalment presenten una baixa toxicitat (Goyer, 1997; Hunter et al., 1989; Johnson et al., 1978; López-Alonso et al., 2004; Ma & Talmage, 2001; Talmage & Walton, 1991). Malgrat les altes concentracions d'alguns d'ells a les zones contaminades estudiades (Tovar-Sánchez et al., 2006), les dades obtingudes no mostren grans increments de les concentracions a teixits de musaranya comuna que poguessin indicar una disrupció metabòlica (Goyer, 1997; Ma & Talmage, 2001). Només es van detectar augments moderats, entre un 10-50% dels valors mitjos en les zones de referència, però significatius de Mg, Cu, Co, Fe, Cr, Mo i Mn als exemplars d'alguna de les zones contaminades. Aquests lleugers increments es poden atribuir principalment a un augment de la ingesta de metalls per l'augment de la biodisponibilitat a zones alterades (Lopes et al., 2002; Tovar-Sánchez et al., 2006), a les interaccions entre elements com pels fenòmens de sinergisme o antagonisme (Goyer, 1997; López-Alonso et al., 2003, 2004) i/o a les funcions metabòliques en les que participen, entre les que hi ha formar part dels sistemes de detoxificació (Lopes et al., 2002), com els enzims antioxidants que tenen un paper fonamental en la protecció de les estructures cel·lulars enfront l'exposició a contaminants com alguns metalls.

Les diferències entre no essencials i essencials indiquen clarament que el primer grup inclou elements que es troben més biodisponibles per *C. russula* a les zones alterades per les activitats humanes, acumulant-se en els teixits de musaranya comuna fins concentracions per sobre els llindars que poden significar un risc pel medi ambient i per l'home. L'impacte de les activitats humanes produeix canvis en els cicles biogeoquímics de metalls i metal·loides, sovint traduint-se en un augment de la biodisponibilitat d'elements altament tòxics que poden així entrar a les xarxes tròfiques (Fritsch et al., 2010, 2011; Iavicolli et al., 2009). Moltes d'aquestes substàncies que entren al medi ambient per diverses activitats humanes tenen un potencial d'afectar la biota, incloent l'home, a diferents nivells, des d'alterar molècules fins destruir ecosistemes sencers. Tot i que la toxicitat depèn de molts factors, incloent temps i via d'exposició, factors biòtics com edat, sexe, forma química del metall, i resposta biològica que depèn tant de l'espècie com de l'individu, l'acumulació a teixits diana dona molta informació sobre possibles efectes tòxics a l'organisme i sobre els elements que poden representar un risc (Eisler, 1985, 1986, 1987; Ma, 1996; Shore & Douben, 1994; Wren, 1986). Tenint en compte individualment cadascuna de les concentracions dels metalls i metal·loides quantificats, només Pb, Hg, i Cd en teixits tous i Pb i Hg a teixits durs semblen significar algun risc de toxicitat per la seva elevada concentració als exemplars de les zones contaminades estudiades. De fet, un alt percentatge d'individus de les zones contaminades presentaren concentracions d'un o varis d'aquests elements per sobre dels llindars de toxicitat per Cd (per sobre el NOAEL a rosegadors de 15µg/g a fetge i 105µg/g a ronyó; Shore & Douben, 1994), Pb (per sobre dels 5-10 ug/g a fetge i 15 ug/g a ronyó, considerat marcador d'exposició tòxica a mamífers; Ma, 1989, 1996) i Hg (per sobre d'1.1 ug/g (pes fresc) a teixits tous de mamífers, considerat indicador de problema ambiental; Eisler, 1987). A més d'aquests elements les altes concentracions de tissulars de Tl a Doñana i de B i Sr al Delta de l'Ebre van ser de les més altes reportades en teixits de petits mamífers, però caldrien més estudis al laboratori i al camp per conèixer la toxicitat d'aquests elements i la tolerància de la musaranya comuna. La resta d'elements quantificats, per si sòls, no assoleixen concentracions que puguin considerar-se tòxiques, encara que altres aspectes, com els efectes subclínics de l'exposició crònica a la contaminació ambiental durant generacions, la interacció entre metalls i els efectes tòxics acumulatius haurien de tenir-se en compte en estudis específics amb diverses espècies per conèixer la seva tolerància (Pankakoski et al., 1993; Wlostowski et al., 2003).

El fetge i ronyó juguen papers metabòlics crucials en la detoxificació dels metalls ingerits amb la dieta i absorbits via intestinal. El fetge és un òrgan diana en l'acumulació de tòxics degut a que és el principal òrgan segrestador de metalls immobilitzant-los via unió amb metalotionines, biotransformant-los en espècies químiques menys tòxiques o compartimentant-los on no puguin danyar les estructures cel·lulars (Jaeschke et al., 2002). Per aquesta capacitat de metabolitzar els productes de l'absorció intestinal, el fetge ha estat el major acumulador de Pb, Fe, Mg, Zn, Cu, Mn i Mo a *C. russula*. Per la seva part, el ronyó pot filtrar diferents contaminants de la sang i acumular-los o pot excretar-los per reduir l'acumulació als teixits i prevenir toxicitats. A més, té una capacitat d'acumular metalls similar a la del fetge, presentant altes concentracions de Hg, Cd i Cr a *C. russula*. Aquesta distribució de metalls per teixit a les zones estudiades correspon en general a l'observada a altres poblacions de *C. russula* i a altres espècies de petits mamífers, on acostuma a seguir un patró constant entre poblacions de la mateixa espècie i molt similar entre espècies properes (Fritsch et al., 2010, 2011; González et al., 2008; Ma, 1989) En el present estudi, però, es van detectar diferències puntuals entre poblacions de *C. russula*, sent la més significativa les majors concentracions de Pb a ronyó enlloc de a fetge als exemplars de Garraf. Similars diferències interpoblacionals en aquest metall ja s'han descrit en algunes espècies de petits mamífers, incloent la musaranya cuaquadrada, *Sorex araneus*, i el talp, *Talpa europaea*, de zones contaminades. Aquestes diferències s'han relacionat tant amb el temps i via d'exposició i concentració del contaminant, com amb un mecanisme per disminuir tant la toxicitat com el temps mig del tòxic als teixits (Fowler et al., 1980; Marcheselli et al., 2010; Scheuhammer, 1991).

L'edat i el sexe són dos dels factors biòtics amb major influència als patrons d'acumulació de metalls i metal·loides en teixits de mamífers, l'espècie humana inclosa (Komarnicki, 2000; Lopes et al., 2002; López-Alonso et al., 2004; Pankakoski et al., 1993, 1994; Vahter et al., 2007). En estudis ecotoxicològics, aquests factors poden explicar bona part de la variabilitat observada en l'acumulació de contaminants i els seus efectes en poblacions silvestres de petits mamífers, incloent la musaranya comuna (Fritsch et al, 2010, 2011; González et al., 2008; Komarnicki, 2000). D'aquests dos factors, l'edat va ser el més important a *C. russula* ja que 10 dels elements quantificats (Cd, Pb, Hg, Tl, Ni. Cu, Zn, Fe, Mo, Cr) van presentar patrons d'acumulació diferencials entre joves i adults en una o varies de les zones estudiades. És especialment rellevant el cas del Cd, que va augmentar les seves concentracions amb l'edat als teixits tous dels exemplars de totes les àrees estudiades, arribant a assolir fins a 6 cops més de concentració mitjana als adults respecte als juvenils al teixit renal en els exemplars de les zones contaminades. Aquest fenomen, ja descrit a altres mamífers a àrees contaminades amb metalls (Bonilla-Valverde et al., 2004; Komarnicki, 2000; Pankakoski et al., 1993; Smith & Rongstad, 1982), estaria relacionat amb la baixa taxa d'eliminació del Cd i la formació de complexos estables Cd-metalotionina com a mecanisme per reduir la toxicitat als teixits en exposicions cròniques (Fritsch et al., 2010). Tant el Pb com el Hg són també difícilment eliminats dels teixits de mamífers i poden així mateix fer complexos amb metalotionines, en el cas del Pb, o lligar-se a lípids, en el cas del Hg, augmentant les concentracions a adults, tal com s'ha detectat a algunes de les zones estudiades, així com a estudis previs amb petits mamífers (Damek-Poprawa & Sawicka-Kapusta, 2004; Pankakoski et al., 1993, 1994; Stansley & Roscoe, 1996). Aquests mecanismes podrien explicar també els augments de Tl i Ni detectats a les zones afectades per la mineria de pirita, tot i que hi ha encara poca informació ecotoxicològica sobre cóm es comporten en els teixits de mamífers. La tendència a l'augment de Fe, Cu i Mo amb l'edat està probablement relacionada amb diferències metabòliques entre juvenils i adults i/o amb mecanismes protectors o de detoxificació (Goyer, 1997; Lopes et al., 2002). Per altra banda, el decrement de Cr als adults observat a totes les zones estudiades és un fenomen comú descrit a vàries espècies que està relacionat amb l'alta taxa d'absorció intestinal als juvenils i la menor absorció als adults (Eisler, 1986).

Tal com amb l'edat, els patrons d'acumulació de metalls i metal·loides dependents del gènere han estat descrits en diverses espècies de mamífers (Komarnicki, 2000; Pankakoski et al., 1993; Smith & Rongstad, 1982) incloent l'home (Vahter et al., 2007). A vàries de les poblacions estudiades, mascles i femelles de musaranya comuna van presentar petites diferències en l'acumulació de metalls no essencials (Pb, Hg i Ni) i essencials (Mo, Mn, Co, Fe), tot i que la importància del sexe va ser menor que la de l'edat per explicar la variabilitat en l'acumulació d'elements. Es van quantificar concentracions menors de Pb i Hg a femelles a l'Ebre i Doñana, una tendència que podria estar relacionat amb la reducció de metalls mobilitzats i transferits cap al fetus durant la gestació i a les cries lactants via llet materna (Eisler, 1987; Yoshida et al., 1994, 2002). Aquest fenomen es va observar majoritàriament a les zones contaminades però no a les controls on els valors baixos d'aquests elements no essencials farien que la taxa d'excreció fos suficient per prevenir la bioacumulació (Eisler, 1987; López-Alonso et al., 2003; Talmage & Walton, 1991). Les diferències sexuals en l'acumulació de Ni i Tl podrien ser explicades pels mateixos mecanismes, però caldria més informació toxicològica per poder donar suport a aquesta afirmació. En els metalls essencials, les majors concentracions de Mo i Mn a femelles i de Fe i Co a mascles poden ésser associades a les diferències metabòliques relacionades amb els requeriments nutricionals, la ingesta i/o captació de metalls o amb l'activitat d'hormones sexuals que poden influir en les interaccions i l'acumulació dels elements (Lopes et al., 2002).

A més de l'avaluació efectuada a partir de l'acumulació de metalls, els resultats obtinguts sobre diversos paràmetres morfològics, hemàtics, bioquímics, histològics i de genotoxicitat van mostrar els efectes tòxics de l'exposició crònica de poblacions naturals de *C. russula* a la contaminació ambiental, en general, i a metalls, en particular. Però, tot i que es coneixen efectes tòxics a diferents nivells de molts contaminants ambientals, inclosos metalls i metal·loides (Eisler, 1985, 1986, 1987; Iavicolli et al., 2009; Kasprak et al., 2003; Punshon et al., 2003), hi ha poques dades sobre aquests efectes sobre la biota de les zones de clima mediterrani. En la present memòria, la bateria de biomarcadors testats ha permès conèixer la idoneïtat de cadascun d'ells per aplicar-los a futurs estudis ecotoxicològics.

Les dades obtingudes referents als paràmetres morfològics (longitud del cos, massa corporal, massa total i relativa d'òrgans diana, índex de condició corporal) mostren només lleugeres diferències entre zones contaminades i control. A les primeres, disminueix el pes del cos (Garraf, Aljustrel, Doñana) i incrementa el pes del fetge (Aljustrel, Doñana) i ronyó (Garraf, Doñana), mentre que l'índex residual (RI) no varia significativament. Aquest últim resultat mostra que es manté un bon estat de salut general als individus de les zones contaminades, mentre que l'increment de pes relatiu de fetge i ronyons és indicatiu d'edema o altres greus alteracions metabòliques per l'exposició crònica a metalls pesants (Ma, 1989; Ma & Talmage, 2001; Shore & Douben, 1994). Aquests metalls, especialment els no essencials, són altament tòxics fins i tot a baixes concentracions, com han fet referència altres autors en poblacions de petits mamífers (Ma, 1989; Ma & Talmage, 2001; Pereira et al., 2006) i el seu principal òrgan diana és el fetge, degut al seu paper principal en la detoxificació dels xenobiòtics que entren via absorció intestinal, tal com passa a la majoria d'exposicions de mamífers a contaminants, incloses les poblacions estudiades. L'alteració de les funcions hepàtiques pot produir un alt nombre de conseqüències negatives a nivell individual en el creixement, salut, esperança de vida i reproducció en les poblacions exposades a tòxics, el que pot repercutir en canvis en la composició de les comunitats de petits mamífers (p.ex. Mukhacheva et al., 2010). Ja que hi ha escassa informació sobre com afecten els metalls als paràmetres morfològics en poblacions silvestres d'insectívors, les dades obtingudes en *C. russula* poden contribuir a estimar aquests efectes. Caldrien, però, estudis en profunditat d'altres paràmetres ecològics que poden afectar la morfologia en zones contaminades, com ara els canvis en la composició de fauna que poden variar la competència interespecífica o, a nivell intraespecífic, els canvis individuals o poblacionals relacionats amb la toxicitat crònica dels contaminants sobre les musaranyes. El test de micronuclis a sang circulant com a mesura per avaluar la genotoxicitat a cèl·lules somàtiques va demostrar ser un bon indicador de clastogenicitat a *C. russula,* ja que la freqüència de micronuclis va augmentar significativament als exemplars de les zones contaminades del Garraf, d'Aljustrel i de Doñana en relació als espècimens control.

El test de micronuclis a sang circulant com a mesura per avaluar la genotoxicitat a cèl·lules somàtiques va demostrar ser un bon marcador de clastogenicitat a *C. russula,* ja que la freqüència de micronuclis va augmentar significativament als exemplars de les zones contaminades del Garraf, d'Aljustrel i de Doñana en relació als espècimens control. Diversos estudis han mostrat increments de clastogenicitat (Tanzanella et al., 2001; Festa et al., 2003) i altres efectes genotòxics (Topaska-Ancheva et al., 2003) a poblacions silvestres de petits mamífers exposats crònicament a contaminants ambientals persistents, degut a la capacitat de molts d'ells, inclosos alguns metalls com Pb, Cd, Cr o Ni, d'induir alteracions en el material genètic a nivells genòmic, proteomic o metabolòmic, tant a cèl·lules somàtiques com germinals (Eisler, 1985, 1986; García-Barrera et al., 2012; García-Sevillano et al., 2012, en premsa; González-Fernández et al., 2013). Malgrat haver estat poc estudiada en poblacions naturals, aquesta quantificació de la genoxicitat ambiental resulta de vital importància per conèixer l'estat de salut i l'èxit reproductor dels individus que són bàsics per avaluar la viabilitat de les poblacions.

Els paràmetres hemàtics i enzimàtics mesurats als exemplars d'Aljustrel són interessants, per una banda, com a valors de referència per a l'espècie, ja que es disposa de poques dades en poblacions silvestres de musaranyes degut principalment a la dificultat per obtenir-los, i per altra, com a biomarcadors de l'exposició crònica a metalls, tal i com s'ha demostrat a estudis de camp i experimentals (Nunes et al., 2001; Reynolds et al., 2006; Rogival et al., 2006; Stansley & Roscoe, 1996; Zúñiga-González et al., 2000). En el present estudi no es van observar grans variacions entre els exemplars de la zona contaminada i els de la zona control, detectant-se només una tendència a augmentar alguns paràmetres hemàtics i disminuir l'activitat de la GST degut a la contaminació. Això pot indicar que cal emprar aquests paràmetres amb mostres més grans i/o, com es va fer en el present estudi, en conjunció amb altres variables per obtenir una millor idea del conjunt d'efectes de la contaminació sobre els paràmetres fisiològics.

L'anàlisi histopatològica dels teixits hepàtic i renal, els dos principals teixits diana per contaminació per via oral, va mostrar augments de la freqüència i severitat de patologies atribuïbles a l'exposició a metalls a les zones contaminades de Garraf, Doñana i Aljustrel. Al fetge, l'òrgan més afectat per la contaminació, els exemplars de les zones contaminades van presentar diverses alteracions estructurals atribuïbles a la contaminació, principalment mort cel·lular (necrosi i apoptosi), inflamació, vacuolització i focus pre-neoplàsics. Tal com s'ha comentat anteriorment, aquest òrgan juga un paper crucial en moltes funcions vitals, incloent un paper predominant en l'acumulació, transformació i excreció de contaminants ingerits. Les alteracions histopatològiques detectades van ser similars a les 3 zones contaminades estudiades, encara que la freqüència i severitat de les lesions va ser major a la zona de Garraf. De fet, els exemplars afectats per aquest abocador van presentar també danys a ronyó, bàsicament necrosi, dilatació i cilindres hialins als túbuls, i inflamació a còrtex i medul·la. L'exposició a una barreja complexa de tòxics orgànics i inorgànics com els efluents d'un gran abocador com el del Garraf, sembla més tòxica que l'exposició a altes concentracions principalment de metalls, com era d'esperar a Doñana i Aljustrel, afectades per la mineria de pirita. Aquesta alta toxicitat dels lixiviats dels abocadors ja s'havia mostrat a estudis experimentals amb cultius cel·lulars i animals de laboratori (Bakare et al., 2005; Cabrera & Rodriguez, 1999; Li et al., 2006; Sang & Li, 2004).

Un aspecte important del present estudi és la utilització d'aquestes dades per l'avaluació de risc ambiental tant de zones protegides com per les poblacions humanes. Els especímens de *C. russula* mostraren l'entrada de metalls i efectes tòxics a diferents nivells d'organització, el que seria compatible amb un augment del risc ambiental. Cal recordar que al voltant de les zones d'estudi hi ha importants poblacions humanes que poden estar exposats directament a la contaminació i que aquesta, indirectament, pot afectar altres poblacions humanes, per exemple pel consum de productes contaminats que entrin a la cadena alimentària. A més, l'ingesta accidental de sòls contaminants és una font important d'exposició a metalls en humans, especialment en infants , que augmenta el risc a patir efectes tòxics per l'exposició a contaminants (Doyle et al., 2010; Pelfrene et al., 2013; Roussel et al., 2010). Tampoc cal oblidar que de les 8 zones estudiades, 6 tenen protecció per ser espais singulars i per la seva riquesa ecològica. L'estudi de la qualitat del medi ambient en aquestes zones és crucial per la conservació dels espais protegits i per la viabilitat de les poblacions que hi habiten.

Una visió en conjunt dels biomarcadors d'exposició i d'efecte quantificats indica un increment en la bioacumulació de metalls i metal·loides fins a concentracions que poden ser tòxiques a les zones contaminades estudiades, així com diversos efectes per l'exposició a aquests contaminants. Les concentracions potencialment tòxiques assolides per alguns metalls als teixits de *C. russula* demostren que la espècie estudiada pot ser considerada un bon bioindicador per aquests elements altament tòxics i àmpliament distribuïts que afecten el normal funcionament dels sistemes biològics (Bonilla-Valverde et al., 2004; Choudburi et al., 2010; Ercal et al., 2001; García-Sevillano et al., 2012, en premsa; García-Barrera et al., 2012; González-Fernández et al., 2013; Iavicoli et al., 2009; Liu et al., 2010; Pereira et al., 2006; Topashka-Ancheva et al., 2003). L'espècie estudiada també s'ha mostrat sensible a les pertorbacions degudes a la contaminació ambiental ja que diversos biomarcadors d'efecte han presentat diferències significatives entre els individus de les zones contaminades i controls, la qual cosa remarca el seu potencial com a bioindicador. En les últimes dècades, vàries espècies de petits mamífers terrestres han estat emprades com a bioindicadors de contaminació, principalment mesurant biomarcadors d'exposició, com l'acumulació de metalls (Dodds-Smith et al., 1992; González et al., 2008; Fernández et al., 2012; Fritsch et al., 2010, 2011; Read et al., 1993; Pankakoski et al., 1993; Shore, 1995; Talmage & Walton, 1991; Wijnhoven et al., 2007), de pesticides (Dell'Omo & Shore, 1996; Forsyth & Peterle, 1984; Hendriks et al., 1995), d'altres compostos sintètics (Fanelli et al., 1980; Meharg et al., 1997) i de radionuclèids (Cloutier et al., 1986). Aquests estudis, junt amb els que mesuren biomarcadors d'efecte, com diversos paràmetres morfomètrics, bioquímics, hematològics, histològics, o genotòxics (Ieradi et al., 1998; McBee & Bickham, 1988; McBee et al., 1987; Pankakoski, 1994; Pereira et al., 2006; Tull-Singleton et al., 1994), permeten avaluar la incidència dels contaminants al medi i, fins a un cert punt l'augment de biodisponibilitat i els efectes tòxics als individus i poblacions (Fritsch et al., 2010, 2011; González et al., 2008; Janiga et al., 2012; Ma & Talmage, 2001; Nunes et al., 2001; Shore & Douben, 1994; Topashka-Ancheva et al., 2003; Wijnhoven et al., 2007). Entre les espècies d'insectívors emprades, hi ha abundant informació ecotoxicològica sobre els soricins, més abundants al centre d'Europa i a Amèrica del Nord, que demostra que les musaranyes són excel·lents bioindicadors (revisions a Ma & Talmage, 2001; Talmage & Walton, 1991). En canvi, les espècies de crocidurins, més abundants a les zones de clima mediterrani del sud d'Europa, han estat poc estudiades. De fet, només recentment s'han publicat les primeres dades ecotoxicològiques de *C. russula*, normalment acompanyant altres espècies simpàtriques (González et al., 2008; Fernández et al., 2012; Fritsch et al., 2010, 2011; Wijnhoven et al., 2007). Aquests estudis indiquen, a l'igual que els nostres, que cal tenir aquesta espècie en compte per el seu potencial com a biodindicador de contaminació ambiental tant a nivell de biomarcadors d'exposició com de diversos biomarcadors d'efecte. Resta, però, molt per conèixer sobre com afecten a diferents nivells els diversos tipus de contaminants a aquesta espècie i a altres abundants a la zones de clima mediterrani. Futurs treballs incorporant factors com les variacions temporals, biomonitoratges més extensos en temps i espai, o avaluacions d'altres paràmetres, com biomarcadors metabòlics o genètics per avaluar els efectes del metalls i metal·loides, la quantificació de contaminants orgànics o la combinació d'estudis de camp amb altres de laboratori ajudarien a definir millor a nivell ecotoxicològic el potencial de l'espècie com a bioindicador.

6. CONCLUSIONS

Les principals conclusions del present estudi van ser:

1. La quantificació de metalls i metal·loides va mostrar que l'espècie estudiada, la musaranya comuna, *Crocidura russula*, és un bon bioindicador d'elements no essencials pels mamífers, especialment de Pb, Hg i Cd, els quals varen presentar altes concentracions als espècimens de les àrees contaminades, en alguns casos amb nivells d'acumulació per sobre dels nivells de toxicitat a fetge i ronyó.

2. La quantificació de diferents biomarcadors d'efecte va mostrar que els paràmetres morfomètrics, histopatològics i de genotoxicitat van ser els més informatius de l'estrés causat per l'exposició crònica a la contaminació ambiental en l'espècie estudiada.

3. Els dos factors biòtics avaluats van ser rellevants per explicar la variabilitat tant dels biomarcadors d'exposició com dels d'efecte a *Crocidura russula*, essent les majors diferències entre joves i adults.

4. La musaranya comuna pot ser considerada com un bon bioindicador de contaminació ambiental ja que l'exposició crònica a un conjunt de substàncies potencialment tòxiques, especialment metalls pesants, va produir una resposta quantificable no només a l'acumulació sinó també als paràmetres marcadors de possibles disfuncions metabòliques.

The main conclusions of the present study were:

1. The quantification of metals and metaloids showed that the greater white-toothed shrew, *Crocidura russula*, is a suitable bioindicator of non-essential elements for mammals, especially of Pb, Hg and Cd, that reached high concentrations in the specimens of the polluted sites, in few cases with accumulation levels up to the toxicity thresholds in liver and kidneys.

2. The quantification of few biomarkers of effect showed that the morphometric, histopathological and genotoxicity parameters were among the most interesting to reveal the stress caused by the chronic exposure to environmental pollution in the studied species.

3. The two biotic factors studied were relevant to explain the variability of both exposure and effect biomarkers in *Crocidura russula*. The main differences were found between juveniles and adults.

4. The greater white-toothed shrew may be considered as a suitable bioindicator of environmental pollution since the chronic exposure to a mixture of potentially toxic pollutants, especially heavy metals, produced a quantificable response in the bioaccumuation and in biomarkers of effect that are indicative of possible metabolic disfunctions.

As conclusões do presente estudo foran:

1. A quantificação de metais e metaloides revelou a espécies estudada, o musaranho de dentes brancos, *Crocidura russula*, como um bom bioindicador de elementos não essenciais para os mamíferos, especialmente de Pb, Hg e Cd, os quais apresentaram altas concentrações nos animais das áreas poluídas, em alguns casos com níveis de acumulação acima dos níveis de toxicidade em fígado e rim.

2. A quantificação de diferentes biomarcadores d'efeito mostrou que especialmente os parâmetros morfométricos, histopatológicos e de genotoxicidade foram os mais informativos do stress causado pela poluição crónica do meio ambiente na espécie estudada.

3. Os dois fatores bióticos avaliados foram relevantes para explicar a variabilidade tanto dos biomarcadores de exposição como dos de efeito em *Crocidura russula*, sendo que as maiores diferenças encontram-se entre jovens e adultos

4. O musaranho de dentes brancos pode ser considerado um bom bioindicador de poluição ambiental ja que a exposição crónica a um conjunto de substâncias potencialmente tóxicas, especialmente metais pesados, produziu uma resposta quantificavel não só na acumulação mas também nos parâmetros marcadores de possivel perturbação das funções metabólicas.

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8. RESUM DELS CAPÍTOLS

Article 1: Bioaccumulatiorn of metals and effects of a landfill in small mammals. Part I. The greater white-toothed shrew, *Crocidura russula*.

Resum

S'ha quantificat l'acumulació de plom (Pb), mercuri (Hg), cadmi (Cd), ferro (Fe), magnesi (Mg), zenc (Zn), coure (Cu), manganès (Mn), molibdè (Mo) i crom (Cr), a més d'alguns paràmetres morfològics (BL, BM, RI, pesos relatius de diversos òrgans) i de genotoxicitat (Test de micronuclis) com a biomarcadors de contaminació produïda per l'abocador del Garraf (Barcelona, NE Espanya). Els exemplars de musaranya comuna, *Crocidura russula*, de l'abocador presentaren majors nivells de Pb, Cd, Mg, Zn, Cu i Cr als seus teixits que els exemplars de la zona control. Els valors de Hg van estar sota els nivells de detecció. Les concentracions de Pb, Cd i Cr variaren significativament amb l'edat, mentre que no es varen detectar diferències significatives entre mascles i femelles en l'acumulació de metalls. Mentre que els paràmetres morfològics no varen variar entre llocs de captura, els exemplars de l'abocador presentaren major freqüència de micronuclis en sang circulant que els animals control (1.786±0.272 *vs* 0.088±0.045 ‰; U=46.000, p<0.001). Els alts nivells de metalls potencialment tòxics, com el plom (fins a 59,71 µg/g) i el cadmi (fins a 56,57 µg/g), així com l'augment de micronuclis indiquen un efecte important sobre la salut d'aquests petits mamífers. Es considera que el biomonitoratge amb espècies autòctones és necessari en aquest abocador situat en una zona parcialment protegida.

Introducció

Els abocadors són la principal forma d'acumulació de residus sòlids en molts països, incloent els de la conca mediterrània. Si aquests dipòsits no estan adequadament controlats poden causar importants danys sobre el medi ambient ja que la descomposició dels residus acumulats genera gran quantitat i varietat de compostos gasosos i líquids. Els lixiviats, efluents líquids produïts per aquesta descomposició dels residus, són una font important de contaminació pels aqüífers i aigües superficials. Aquestes efluents són barreges complexes de substàncies potencialment tòxiques per la biota, incloent compostos orgànics i molts metalls pesants i altres elements com Pb, Cd, Mg, Fe, Zn, Cu, Mn, Mo i Cr. Encara que es disposa de poca informació sobre els efectes a poblacions naturals, s'ha demostrat que els lixiviats procedents d'abocadors produeixen un increment en les alteracions genètiques (increment de micronuclis, aberracions cromosòmiques i morfologies anormals d'espermatozoides) en rosegadors.

Els paràmetres morfològics s'han emprat sovint com a biomarcadors d'alteracions fisiològiques a poblacions naturals de petits mamífers exposats a diversos tipus de contaminació. A més, a causa de que alguns metalls produeixen dany al material genètic, el test de micronuclis, fàcil i ràpid, pot ser una eina per obtenir dades de clastogenicitat en poblacions naturals.

Com altres insectívors, la musaranya comuna, *Crocidura russula*, ha estat recentment emprada com a biodindicador de contaminació ambiental per metalls, encara que no hi ha dades sobre els efectes dels lixiviats d'un abocador sobre aquesta espècie.

Els objectius d'aquest estudi van ser: (i) quantificar les concentracions de metalls pesants a *C. russula* exposada als lixiviats de l'abocador del Garraf; (ii) avaluar els efectes fisiològics d'aquest tipus de contaminació bastant-se en diversos paràmetres morfològics i de genotoxicitat; (iii) identificar el pes que tenen dos factors biòtics (edat i sexe) en la variació dels paràmetres quantificats; i (iv) conèixer els efectes de l'abocador en les poblacions naturals, especialment a zones protegides.

Material i mètodes

L'abocador del Garraf es va començar a utilitzar al 1974 com a abocador il·legal. Fins al 2006 va rebre unes 850.000 tones de deixalles sòlides d'origen domèstic majoritàriament però també s'hi van abocar residus industrials i llots de depuradora. L'abocador es troba dins una zona que actualment té protecció ambiental (Parc del Garraf).

Del febrer a l'abril de 1998 es van capturar n=55 exemplars de musaranya comuna amb trampes de viu Sherman a una zona contaminada pels lixiviats de l'abocador (n=21) i a una zona control presumiblement no afectada per contaminació de cap tipus (n=34). L'esforç de captura total va ser de 1600 trampes/nit (TN). Els exemplars capturats van ser transportats al laboratori pel seu anàlisi seguint els procediments legals i ètics per animals d'experimentació. Tots els individus van ser sexats i establerta l'edat relativa mitjançant el desgast dentari, classificant-se en juvenils i adults. De cada exemplar es van mesurar la longitud corporal (BL, en mm) i la massa corporal (BM, en g) per calcular l'índex residual (RI). Aquest és un índex de condició corporal basat en els residuals originats a partir de la regressió de BM sobre BL i s'ha emprat per conèixer l'estat general de salut dels exemplars. També es va prendre el pes del fetge i ronyó de cada animal per obtenir dades de variació d'aquests òrgans diana.

Tot el material emprat per les anàlisis químiques va ser rentat prèviament per evitar contaminacions externes. Una part del fetge i el ronyó dret de cada exemplar van ser congelats per realitzar les anàlisis químiques. Per a aquestes anàlisis, entre 100 i 500 mg de cada òrgan van ser assecats fins a pes constant (60ºC, 48h) i digerits amb 5 ml d'àcid nítric i 2 ml d'àcid perclòric en tubs oberts a un microones Prolabo Microdigest A301 situat a una càmera blanca. Les mostres diluïdes 1:5 i amb rodi com a estàndard intern van ser analitzades mitjançant un espectròmetre òptic de plasma acoblat inductivament (ICP-OES) Perkin Elmer OPTIMA-3200RL que va quantificar Mg i Fe. El contingut de Pb, Hg, Cd, Zn, Cu, Mn, Mo i Cr es va quantificar amb un espectròmetre de masses de plasma acoblat inductivament (ICP-MS) Perkin Elmer ELAN-600. Es van emprar material de referència i blancs com a controls de la tècnica.

Mitjançant punció cardíaca es va extreure sang amb una xeringa heparinitzada i es van fer dos frotis de sang per espècimen que es van tenyir amb May-Grünwald Giemsa. Per cada individu es va observar la presència de micronuclis (MN) en 2000 eritròcits amb un objectiu d'immersió a un microscopi òptic.

Les dades vas ser transformades mitjançant transformació logarítmica i es va testar la normalitat (Shapiro-Wilk) i l'homogeneïtat de variàncies (Levene, *F*-test). Per cada teixit es va testar l'efecte de lloc de captura, sexe i edat en l'acumulació de metalls amb una anàlisis multivariant de la variància de tres factors (MANOVA). Les diferències intra i interpoblacionals de metalls i paràmetres morfomètrics van ser testades amb tests d'Student (*t*), mentre que les diferències de micronuclis van ser avaluades amb test de Mann-Whitney (*U*). Les correlacions entre metalls i paràmetres es van mesurar amb correlacions de Pearson o d'Spearman (*r*) segons si les dades tenien distribucions contínues o discretes. Pels anàlisis seqüencials es va utilitzar la correcció de Bonferroni. Totes les anàlisis es van realitzar amb SPSS versió 11.5.

Resultats

Es van capturar més exemplars a la zona de referència (34 musaranyes amb 550 TN) que a la contaminada (21 exemplars amb 1050 TN). No es van detectar diferències significatives entre llocs de captura en cap dels paràmetres morfològics quantificats.

Excepte el Hg, que no es va detectar en cap mostra, i el Cr que no es va detectar als ronyons dels exemplars de referència, la resta d'elements va ser detectat a totes les mostres. L'anàlisi MANOVA va evidenciar diferències significatives en funció del lloc de captura i de l'edat a fetge i ronyó. No es van detectar diferències significatives entre mascles i femelles als metalls quantificats, mentre que, per edat, va haver divergències significatives en Pb, Cd i Cr, mentre que Fe, Mo i Cu van tendir a augmentar als adults.

Els animals de la zona contaminada presentaren augments significatius en els nivells de Pb, Cd, Zn, Mg, Cu i Cr als seus teixits respecte a la zona control. La distribució de metalls per teixit va seguir patrons similars a les dues zones de captura: Fe, Cu, Mn i Mo es van acumular principalment al fetge i Pb i Cd al ronyó. La resta de metalls va presentar similars concentracions a ambdós teixits.

La freqüència de micronuclis va ser significativament major als exemplars de la zona contaminada. No es van detectar diferències per edat o sexe en la freqüència de MN. Els MN van mostrar correlacions amb Pb, Cd i Cr.

Discussió

Encara que els abocadors són freqüents a moltes regions, hi ha relativament pocs estudis de camp que valorin els efectes mediambientals de la contaminació produïda per aquestes estructures. Els nostres resultats de metalls són similars als obtinguts per altres autors, encara que les concentracions d'alguns metalls és més alta, probablement degut a les característiques físico-químiques de la zona d'estudi.

Les majors concentracions de Pb i Cd als exemplars de la zona impactada per l'abocador del Garraf són consistents amb resultats referits a la bibliografia i amb el fet que els insectívors solen ser bons bioacumuladors d'aquests dos metalls no essencials. Els baixos nivells de Hg als lixiviats del Garraf podrien explicar per què aquest metall va presentar concentracions tant baixes als teixits de musaranya comuna.

El Mg és un catió abundant als lixiviats, el que explicaria el seu augment als teixits dels animals exposats en relació als controls. Els augments de Zn i Cu als exemplars del Garraf podria ser degut a mecanismes protectors enfront l'exposició a substàncies tòxiques o a mecanismes de detoxificació, ja que la concentració d'aquests metalls als teixits de mamífers està molt ben regulat homeostàticament. Les infreqüentment altes concentracions de Cr en els teixits de les musaranyes de la zona contaminada de l'abocador del Garraf poden ser degudes a l'abundància d'aquest metall als lixiviats així com a la seva alta biodisponibilitat.

En els exemplars exposats als lixiviats, el Cd es va acumular amb l'edat als teixits, assolint fins a un factor de 6 al teixit renal. Aquests fenomen estaria relacionat amb la baixa taxa d'eliminació d'aquest metall i la formació de complexos estables Cd-metalotionina com a mecanisme per reduir la toxicitat als teixits. L'increment de Pb amb l'edat ha estat també reportada a altres poblacions de musaranya comuna. El decrement de Cr amb l'edat és un fenomen comú a varies espècies relacionat amb l'alta taxa d'absorció intestinal als juvenils i la menor absorció als adults. S'ha observat també una tendència a l'augment de Fe, Cu i Mo amb l'edat probablement relacionada amb diferències metabòliques entre juvenils i adults i/o, com en el cas d'altres metalls essencials, amb mecanismes protectors o de detoxificació.

Encara que no es van detectar diferències significatives en l'acumulació de metalls respecte al sexe, la major concentració de Mo a femelles, també observada a altres poblacions de *C. russula* i altres espècies d'insectívors, cal que sigui comprovada amb altres estudis degut al nombre asimètric d'animals de cada sexe.

La distribució de metalls per teixit correspon a l'observada a d'altres poblacions de *C. russula* i altres espècies de petits mamífers, ja que el patró d'acumulació per teixits sòl ser bastant constant. A l'igual que a altres espècies, només es van observar diferències en la distribució de Pb que podrien estar relacionades amb el temps i tipus d'exposició, com mecanisme per disminuir la toxicitat o al temps mig de vida dels metalls als teixits.

L'alt nombre de mascles capturats és degut a que es va fer l'experiment en època de reproducció en que els mascles estan més actius que les femelles. El menor nombre de musaranyes a la zona contaminada així com els menors índexs de condició física indiquen que la contaminació pels lixiviats de l'abocador del Garraf afecta significativament els individus i poblacions de musaranya comuna probablement degut a una sensibilitat major d'aquesta espècie i/o a fenòmens de competència amb rosegadors, de mida més gran.

Els valors de genotoxicitat van estar dins els rangs reportats per petits mamífers de poblacions naturals i indiquen que la barreja de substàncies que formen els lixiviats produeixen trencament del material genètic en la musaranya comuna. Aquest dany, produït en part per la formació de radicals lliures, també ha estat detectat a animals de laboratori exposats a lixiviats. A més, la significativa correlació MN-metalls indica el paper primordial d'aquests en la genotoxicitat observada.

Les altes concentracions d'alguns metalls no essencials a les musaranyes del Garraf fan pensar que els lixiviats de l'abocador produeixen un augment de disponibilitat d'aquests elements que s'acumulen fins a valors potencialment tòxics als teixits d'aquests petits mamífers. D'entre els elements analitzats, el Pb i el Cd van assolir concentracions per sobre el NOAEL ("No Observed Adverse Effects Level") als teixits de *C. russula*. La resta de metalls no semblen, per si sòls, representar cap risc de toxicitat, encara que els efectes acumulatius o les interaccions entre contaminants haurien també de ser avaluades.

En els últims anys s'ha fet un gran esforç en quant a protecció del medi per conservar ecosistemes i espècies i per millorar l'entorn en que es mou l'home. No obstant, algunes fonts de contaminació, com l'abocador del Garraf al Parc del Garraf, són anteriors a la protecció de l'espai. Per millorar el maneig d'aquesta àrea protegida, és necessari restaurar la zona alterada per l'abocador i avaluar els efectes d'aquesta font de contaminació al medi. Actualment hi ha poca informació sobre els efectes d'aquest abocador a les poblacions naturals que habiten a la zona afectada, pel que les dades presentades aquí poden ser importants a l'hora de plantejar estratègies de biomonitoratge de la contaminació a aquesta zona protegida. A més, queden molts aspectes sobre aquest focus de contaminació que caldria avaluar, com ara l'extensió de l'àrea afectada i la presència de compostos orgànics potencialment tòxics al medi procedents també dels lixiviats de l'abocador.

Conclusions

Els lixiviats de l'abocador són una font considerable de contaminació per metalls al medi ambient, incrementant els nivells de Pb, Cd, Zn, Cu i Cr als teixits de musaranya comuna exposada a aquesta font de contaminants. Alguns d'aquests metalls presenten un patró d'acumulació relacionat amb l'edat. A més, els lixiviats produeixen danys al material genètic en els individus d'aquesta espècie d'insectívor. Aquests resultats indiquen que l'espècie és sensible als canvis al medi i pot ser considerada una bona espècie bioindicadora en els programes de biomonitoratge de l'àrea protegida del Garraf.

Article 2: Bioaccumulation of metals and effects of a landfill in small mammals. Part III. Structural alterations.

Resum

Els lixiviats de l'abocador del Garraf contenen una barreja de substàncies potencialment tòxiques com ara els metalls pesants que poden afectar la biota de la zona protegida del Garraf. En aquest estudi s'ha realitzat un estudi histopatològic per analitzar qualitativament i quantitativament els efectes estructurals d'aquest tipus de contaminació en la musaranya comuna, *Crocidura russula*. El teixit hepàtic és el que va mostrar més alteracions degudes a l'exposició als lixiviats (apoptosi, necrosi, inflamació, vacuolització i microesteatosi). Els ronyons, menys alterats, van presentar necrosi i dilatació tubular, inflamació i cilindres, el que suggereix que les diferències metabòliques els fa més tolerants als efectes tòxics que el teixit hepàtic. No es van observar alteracions relacionades amb la contaminació a pulmó, melsa, pàncrees, gònades, esòfag, o intestí. Es conclou que l'espècie emprada pot ser considerada com a bon bioindicador de la contaminació produïda pels abocadors.

Introducció

Les poblacions naturals de llocs contaminats són sovint exposades a barreges de contaminants ingerits per la dieta. Aquesta exposició produeix estrés que afecta a molts nivells d'organització biològica, de molècules a ecosistemes. A més, el medi natural és altament dinàmic i hi influeixen multitud de paràmetres abiòtics i biòtics que compliquen l'avaluació dels efectes d'aquests contaminació. Aquest fet contrasta amb els experiments en laboratori en el que es pot seguir els efectes d'una substància tòxica en condicions controlades. Però les poblacions naturals, especialment les de zones protegides, proveeixen informació crucial per a conèixer l'estat del medi ambient i les espècies que poden ser bons bioindicadors. D'entre els petits mamífers, els rosegadors i els insectívors, que cobreixen diferents posicions tròfiques, han estat sovint emprat per a estudis ecotoxicològics, si bé les dades sobre espècies de crocidurins, com ara la musaranya comuna, *C. russula*, són limitats.

Els abocadors de deixalles domèstiques i industrials són freqüents a moltes zones, inclosa la conca mediterrània. Els lixiviats produïts per la descomposició de les deixalles són un bon exemple de barreja de contaminants orgànics i inorgànics que poden impactar greument la biota. Aquests efluents, carregats de substàncies orgàniques i inorgàniques, com molts metalls pesants, poden produir estrés oxidatiu i danys al material genètic que poden produir greus alteracions estructurals com necrosi o apoptosi. Tot i que el fetge i els ronyons són òrgans diana dels contaminants absorbits per l'intestí, altres òrgans poden veure's també afectats com ara el pàncrees, la melsa o les gònades. A l'abocador del

Garraf, el més gran de l'estat espanyol, s'ha constatat a *C. russula* l'acumulació de diversos metalls fins a concentracions potencialment tòxiques i diversos efectes tòxics a nivell morfològic i genètic que fan pensar en alteracions importants a les poblacions de musaranya comuna degut als lixiviats.

Els principals objectius d'aquest treball van ser: (i) fer una anàlisi qualitativa i quantitativa de les alteracions histològiques en òrgans diana d'exemplars de musaranya comuna exposats als lixiviats de l'abocador del Garraf; (ii) comparar la toxicitat dels lixiviats entre la musaranya comuna i una espècie simpàtrica de rosegador; (iii) identificar la influència del sexe i l'edat com a font de variabilitat intrapoblacional d'aquests efectes; (iv) correlacionar les alteracions histològiques amb l'acumulació de metalls i altres paràmetres; i (v) avaluar els efectes mediambientals dels lixiviats d'un abocador particularment a zones protegides.

Material i mètodes

Els exemplars emprats en aquest estudi són els mateixos que els del Capítol 4.1 (veure Material i mètodes). Durant la dissecció, es van fixar les gònades, el ronyó esquerre i una porció de fetge, pulmó, melsa, pàncrees, esòfag i intestí de cadascun dels exemplars en formaldehid al 10% tamponat. Els teixits que presentaven alteracions macroscòpiques també van ser fixats. Les mostres van ser processades seguint el protocol convencional per microscòpia òptica: deshidratació amb etanol, toluè com a líquid de transferència i inclusió en Paraplast. Les seccions de 5-10 µm van ser tenyides amb hematoxilina-eosina, muntades en DPX i observades amb un microscopi òptic Leica DMRB equipat amb una càmara Leica DC 500.

La incidència de les alteracions va ser avaluada qualitativament. Pel tractament estadístic de les dades, es van mesurar les alteracions amb una escala semiquantitativa per cada òrgan de cada exemplar. Segons la severitat de les lesions, es van classificar els teixits com: sense alteració (0), poc alterats (1), mitjanament alterats (2) i molt alterats (3).

Per cada teixit, es van avaluar els resultats per lloc de captura, edat i sexe mitjançant test de Mann-Whitney (*U*). Les relacions de les alteracions estructurals i dels altres paràmetres van ser calculades amb correlacions de Spearman (*r*). Les diferències van ser considerades significatives a $p<0.05$. Les anàlisis estadístiques van ser realitzades amb SPSS versió 15.0.

Resultats

En general, els òrgans dels exemplars de la zona control presentaven aspecte normal sense grans alteracions. En el teixit hepàtic, 9 dels 12 exemplars de la zona contaminada presentaven diverses alteracions relacionades amb la contaminació: apoptosi, necrosi, infiltrat periportal i perivascular de limfòcits i senyals de regeneració i vacuolització del citoplasma i microesteatosi. Dels 16 exemplars de la zona control, 13 no van mostrar cap alteració al fetge. Les lesions als ronyons van ser menys severes i extenses, tot i que es van detectar diversos casos de dilatació i necrosi tubular, cilindres hialins i processos inflamatoris. A la resta d'òrgans analitzats no es van observar alteracions relacionades amb la contaminació. Tampoc es van detectar diferències en les alteracions relacionades amb l'edat o el sexe. A més, les alteracions histopatològiques es van correlacionar significativament amb el pes relatiu del fetge i amb la bioacumulació de Mn.

Discussió

Degut a la importància cabdal de les conseqüències de la contaminació ambiental, s'han proposat diferents aproximacions per avaluar els efectes i predir el comportament dels contaminants en condicions tant dinàmiques com el medi natural. En aquest estudi s'ha avaluat per primer cop les alteracions estructurals en un insectívor, la musaranya comuna, exposat als lixiviats d'un abocador situat a una zona protegida. Les alteracions detectades són compatibles amb l'exposició a contaminants orgànics i inorgànics com els que estan presents als efluents de l'abocador. Quan la ingesta és la via principal d'entrada de tòxics, els teixits hepàtic i renal són els òrgans diana principals. En aquest estudi, el fetge va ser, degut a la seva funció principal en la detoxificació de contaminants, l'òrgan més afectat.

Els lixiviats indueixen estrés oxidatiu que pot danyar cèl·lules i teixits. D'entre els components d'aquests efluents, els metalls juguen un paper important en la toxicitat sobre els sistemes biològics, ja que la producció d'espècies reactives d'oxigen (ROS, "Reactive Oxygen Species") pot produir necrosi, apoptosi o processos carcinogènics com els observats als exemplars de la zona contaminada.

Les musaranyes de l'abocador presentaren també un augment de processos inflamatoris a fetge i ronyó, compatibles amb el que s'ha observat a petits mamífers d'altres zones contaminades i a animals de laboratori exposats a metalls. Cal remarcar que la inflamació crònica pot arribar a induir processos carcinogènics.

La vacuolització citoplasmàtica i la microesteatosi resulten de l'acumulació d'aigua, glicogen o lípids, i són indicatives de greus alteracions metabòliques com processos d'autofàgia. A més, els contaminants lipofílics poden acumular-se a les gotes lipídiques reduint així la seva toxicitat a les cèl·lules. Les zones amb aquestes alteracions estaven sovint rodejant zones necròtiques, formant amplies àrees de dany hepàtic.

Tot i que el ronyó és el principal acumulador de molts metalls, només s'han observat algunes alteracions en aquest teixit, compatibles amb l'exposició crònica a metalls que pot produir hiperfiltració als túbuls. Aquesta alteració pot produir necrosi tubular i cilindres hialins. L'òrgan, per compensar aquesta pèrdua de funcionalitat, pot desenvolupar una dilatació als túbuls. No s'observaren diferències per edat o sexe en les alteracions estructurals, el que indica que tota la població sofreix els efectes tòxics de l'exposició als lixiviats de l'abocador.

El projecte que es va dissenyar per avaluar els efectes tòxics d'aquests efluents incloïa la comparació de dues espècies simpàtriques de petits mamífers (el ratolí de bosc, *Apodemus sylvaticus*, i la musaranya comuna). Aquesta comparació d'espècies en diferent posició de la cadena tròfica pot donar important informació sobre tolerància interespecífica i comportament dels contaminants a les cadenes tròfiques terrestres. En general, les musaranyes presentaren altes taxes d'acumulació de metalls i menys alteracions que els ratolins. Els resultats obtinguts mostraren també una alta variabilitat d'efectes a nivell individual, des d'òrgans sense alteració a altres molt alterats, indicant l'exposició diferencial i la tolerància interespecífica en condicions reals. A més, diversos factors abiòtics i biòtics influeixen augmentant la variabilitat dels resultats. La gran quantitat de variables en joc fan que la comparació entre espècies sigui encara més interessant per obtenir una idea el més àmplia i exacta possible dels efectes dels contaminants.

Les alteracions observades a nivells morfològic i estructural poden ser indicadores de greus trastorns metabòlics que poden redundar en funcions bàsiques dels individus, com la reproducció o l'esperança de vida. L'avaluació dels efectes de la contaminació, per tant, es fa imprescindible en zones protegides com el Parc del Garraf, com a eina essencial per al maneig d'aquests àrees d'interès ecològic.

Conclusions

La histopatologia dels principals òrgans involucrats en el metabolisme i excreció de substàncies tòxiques és bona biomarcadora dels efectes de la contaminació produïda pels lixiviats procedents de l'abocador del Garraf. Les dades obtingudes amb musaranya comuna indiquen efectes tòxics deguts a l'acció directa dels contaminants, a la seva acumulació i a la seva excreció. S'observen també diferències entre una espècie omnívora, com el ratolí de bosc, i un insectívor, com la musaranya comuna. La comparació de diversos biomarcadors amb espècies simpàtriques a diferents nivells de l'escala tròfica proporciona informació interessant dels efectes del contaminants i per al maneig de zones protegides.

Article 3: Metal bioaccumulation in the greater white-toothed shrew, *Crocidura russula*, inhabiting an abandoned pyrite mine site.

Resum

Les concentracions de Mg, Zn, Pb, Cu, Mn, Hg, Cd, Mo, Cr i Ni van ser quantificades a fetge i ronyó de 54 musaranyes comunes *Crocidura russula* exposades als metalls d'una mina de pirita abandonada i a una zona control a l'Alentejo (Portugal). A més, es van quantificar diversos paràmetres morfològics com a mesura de dany fisiològics deguts a la contaminació. Els exemplars de la zona contaminada presentaren majors nivells de Fe, Pb, Hg, Cd, Mo i Ni que els espècimens de la zona control. A més, els adults presentaren augments de Cd i disminucions de Cr i Ni, mentre que, per sexe, es van detectar diferències significatives en els nivells de Mo i Ni. Els exemplars de la zona contaminada presentaren un augment del pes relatiu del fetge. Aquest està probablement correlacionat amb les elevades concentracions, potencialment tòxiques, de Pb, Hg, Cd i Ni detectades als teixits d'aquests exemplars. Després d'aquesta aproximació als efectes d'una mina de pirita abandonada a *C. russula*, caldria associar els paràmetres quantificats amb altres biomarcadors fisiològics, ecològics o genètics per conèixer més àmpliament els efectes d'aquest tipus de contaminació a les poblacions naturals.

Introducció

Molts metalls pesants són contaminants habituals que han incrementat la seva entrada al medi degut a activitats diverses com la mineria. Durant les últimes dècades, només aquesta indústria ha generat increments exponencials en l'entrada de metalls potencialment tòxics, com Pb, Cu, Zn, Hg o Cd. Quan deixen de ser econòmicament rentables, les mines es tanquen sense que s'acostumin a realitzar tasques de control de la contaminació que, en forma d'efluents àcids (AMD, "Acid Mine Drainage") poden impactar el medi durant llargs períodes. Aquests efluents, generats per la interacció aigua-roca estan carregats de metalls a pHs baixos, el que els fa altament disponibles per la biota, generant un important risc ambiental.

Diversos treballs han estudiat els efectes de les mines abandonades en rosegadors i insectívors (principalment als primers) degut a que són abundants, estan situats a diverses posicions tròfiques i tenen alta taxa metabòlica. No obstant, no es disposa de massa informació sobre l'ús d'espècies de crocidurins com a bioindicadors de contaminació ambiental, tot i que algunes espècies d'aquest grup, com la musaranya comuna, *Crocidura russula*, estan àmpliament distribuïdes i són relativament abundants. Concretament, aquest és el cas d'aquesta espècie, l'insectívor més estès i freqüent a la península Ibèrica.

A les poblacions naturals exposades a contaminants, com els metalls pesants, bona part de la variabilitat en les concentracions d'aquests elements pot ser explicada per factors abiòtics i biòtics com l'època anual, l'edat i el sexe. A més de l'acumulació de metalls als teixits, és important avaluar diversos paràmetres que indiquin l'estat fisiològic dels individus i poblacions. Dins d'aquest context, alguns paràmetres morfològics fàcilment mesurables, com els índexs de condició corporal o els pesos relatius d'òrgans diana dels contaminants, poden ser biomarcadors que donin informació valuosa sobre l'exposició i tolerància a metalls i altres contaminants de les poblacions naturals.

Els objectius d'aquest estudi van ser: (i) quantificar els principals metalls i altres elements pesants amb interès ecotoxicològic en teixits tous de musaranya comuna, *C. russula,* de la mina de pirita d'Aljustrel; (ii) examinar la influència estacional, d'edat i sexe sobre els patrons d'acumulació d'aquests elements: i (iii) avaluar alguns dels possibles efectes tòxics de les mines abandonades en petits mamífers insectívors.

Material i mètodes

A la zona del Baixo Alentejo (Portugal) existeixen moltes mines abandonades sense cap mesura de correcció mediambiental. Localitzada a l'anomenada Faixa de Pirita Ibèrica, la mina de pirita d'Aljustrel és una mina a cel obert explotada des de 1867 a 1996 i va ser emprada com a exemple de zona contaminada. La zona de Moura, a uns 70 kms de la zona contaminada, va ser emprada com a referència.

A la primavera i tardor de 2003 es van capturar 54 exemplars de musaranya comuna amb trampes Sherman a la zona contaminada (n=32) i a la zona control (n=22). L'esforç de captura total va ser de 1250 trampes/nit (TN). Els exemplars capturats van ser transportats al laboratori pel seu anàlisi seguint els procediments legals i ètics per animals d'experimentació. Tots els individus van ser sexats i establerta l'edat relativa mitjançant el desgast dentari, classificant-se en juvenils i adults. De cada exemplar es van mesurar diversos paràmetres morfològics, detallats al Capítol 4.1 (Material i mètodes).

El material emprat per les anàlisis químiques va ser rentat prèviament per evitar contaminacions. Una part del fetge i el ronyó dret de cada exemplar van ser congelats per realitzar les anàlisis químiques. Entre 50 i 100 mg de cada òrgan van ser assecats fins a pes constant (60ºC, 48h) i digerit amb 2 ml d'àcid nítric i 1 ml de peròxid d'hidrogen en bombes de Tefló. Un cop diluïdes 1:5 i emprant el rodi com a estàndard intern, es va quantificar Mg i Fe mitjançant un espectròmetre òptic de plasma acoblat inductivament (ICP-OES) Perkin Elmer OPTIMA-3200RL, mentre que amb un espectròmetre de masses de plasma acoblat inductivament (ICP-MS) Perkin Elmer ELAN-600, es va quantificar el contingut de Pb, Hg, Cd, Zn, Cu, Mn, Mo i Cr. Es van emprar material de referència i blancs com a controls de la tècnica. La freqüència de micronuclis es va avaluar segons el mètode explicat al Capítol 4.1 (veure Material i mètodes).

Les dades vas ser transformades mitjançant transformació logarítmica i es va testar la normalitat (Shapiro-Wilk) i l'homogeneïtat de variàncies (Levene, *F*-test). Per cada teixit es va testar l'efecte de l'estació de l'any, lloc de captura, sexe i edat en l'acumulació de metalls amb una MANOVA de quatre factors. La variació per estació, edat i sexe per cada lloc i teixit dels metalls va ser calculada amb proves *t* de Student, a l'igual que les diferències dels paràmetres morfomètrics per lloc de captura. Les correlacions entre metalls es van mesurar amb correlacions de Pearson (*r*). Pels anàlisis seqüencials es va utilitzar la correcció de Bonferroni. Totes les anàlisis es van realitzar amb SPSS 11.5.

Resultats

Es van capturar més juvenils a la primavera i més adults a la tardor, mentre que la relació de sexes va estar balancejada. Els exemplars de la zona contaminada mostraren un increment del pes relatiu del fetge. La MANOVA de totes les dades va indicar diferències significatives pels 4 factors testats a ambdós teixits. El patró d'acumulació de metalls per teixit va ser similar a les dues zones de captura: el fetge va ser el principal acumulador de Mg, Pb, Fe, Zn, Cu, Mn i Mo, mentre que els ronyons acumularen significativament més Hg, Cd i Ni. El Cr va tenir similars concentracions a ambdós teixits.

Els espècimens de la zona contaminada tenien majors concentracions de Pb, Hg i Ni a ambdós teixits. A més, al fetge presentaren també augments significatius de Fe, Cd i Mo i decrement de Cr.

L'estació de captura va influenciar l'acumulació de Mg, Cu, Pb, Hg, Cr i Ni. Els adults presentaren majors concentracions de Cd i menors de Cr i Ni. A més, els nivells de Mo i Ni van ser majors a femelles. Tots els metalls van presentar una o varies correlacions significatives entre ells a la zona d'Aljustrel.

Discussió

Diversos estudis han mostrat l'augment de metalls al medi degut a les mines abandonades a Portugal. Els efluents de la mina d'Aljustrel, situada a la mateixa faixa piritosa que la l'Aznalcóllar, han produït que les musaranyes que habiten a aquesta zona presentin grans increments de metalls no essencials i essencials als seus teixits. Aquests metalls no essencials acumulats estan associats amb la mena i poden ser transferits al medi, incrementant les seves concentracions als petits mamífers degut a la ingesta de preses contaminades. D'entre els essencials acumulats, el Fe es troba en altes concentracions als efluents de la mina mentre que l'augment de Mo pot ser degut a interaccions amb altres elements. Per altra banda, els nivells de metalls dels espècimens de la zona control van ser en general baixos, demostrant que aquesta zona pot ser una bona referència per estudis ecotoxicològics.

La gran variació en les concentracions de metalls a poblacions naturals pot ser explicada en part pels patrons d'acumulació deguts a factors abiòtics i biòtics. La variació estacional observada sembla relacionada amb el cicle vital (requeriments alimentaris, maduració gònades, reproducció) que indirectament pot produir un augment de la ingesta i la conseqüent acumulació de metalls.

L'acumulació de Cd relacionada amb l'edat pot ser deguda a mecanismes de detoxificació d'aquest metall que fan que s'acumuli associat a metalotionines per fer-lo menys tòxic als teixits. L'acumulació de Pb i Hg sembla relacionada amb la baixa taxa d'excreció d'aquests elements que s'acumulen al llarg de la vida dels individus en els casos d'exposició crònica. La disminució de Cr i Ni amb l'edat semblarien relacionades amb la baixa absorció intestinal a adults. Entre mascles i femelles va haver poques diferències en l'acumulació de metalls, només el Mo i Ni van presentar diferències per gènere degudes a requeriments nutricionals o a diferències en la ingesta o absorció de metalls entre sexes.

En general, els patrons d'acumulació de metalls per teixits van ser similars als observats a altres espècies. Les diferències en aquest patró al Pb entre els animals de ambdues zones podria estar relacionat amb mecanismes fisiològics per reduir la toxicitat i/o a l'exposició crònica i el període de vida mitja del metall a teixits tous.

Degut a la poca informació disponible en estudis ecotoxicològics sobre caràcters morfològics a mamífers insectívors, els resultats presentats aquí tenen una gran importància. A ambdós zones de captura, les musaranyes presentaren un estat físic semblant, però el fetge dels animals contaminats va ser superior als exemplars exposats a metalls, fet que pot indicar efectes tòxics a nivell hepàtic degut a l'exposició a metalls.

La contaminació ambiental sòl estressar els individus i poblacions degut a l'exposició crònica i els seus efectes no són sempre fàcils d'observar. Una aproximació als efectes tòxics dels contaminants bioacumulables, com els metalls pesants, és a partir dels seus nivells i dels òrgans on s'ha quantificat la seva acumulació. Tot i que els insectívors estan considerats com a més tolerants que altres grups, com els rosegadors, els nivells de Pb, Hg i Cd dels exemplars d'Aljustrel poden tenir efectes tòxics ja que són per sobre els 5-10 μg/g, 1.1 μg/g i 15.1 μg/g, respectivament, valors que es consideren com a indicadors d'exposició a aquests metalls. Aquests resultats indiquen que cal fer una atenció especial en l'acumulació dels 3 metalls no essencials en la biota que viu als voltants de les mines de pirita abandonades com la d'Aljustrel.

A més d'aquests efectes de cada metall, les musaranyes de la zona contaminada d'Aljustrel estan exposades a una barreja de metalls, el que pot incrementar les interaccions entre contaminants. Les correlacions observades en el present estudi entre metalls essencials i no essencials poden estar principalment relacionades amb mecanismes de competitivitat a l'absorció intestinal/unió amb enzims així com a mecanismes protectors per reduir la formació de ROS que podrien danyar els teixits.

Conclusions

Degut als alts requeriments alimentaris de les musaranyes, diversos metalls s'han acumulat fins a nivells potencialment tòxics als teixits dels espècimens propers a la zona de la mina de pirita d'Aljustrel, representant un important risc ambiental. Els paràmetres abiòtics i biòtics analitzats han estat importants per explicar la variabilitat observada en les concentracions de metalls. Les característiques fisiològiques i l'alta posició tròfica dels insectívors els fa *a priori* bons bioindicadors de contaminació de compostos bioacumulables com els metalls pesants.

Article 4: Haematology, genotoxicity, enzymatic activity and histopathology as biomarkers of metal pollution in the *shrew Crocidura russula*.

Resum

Es van quantificar diversos paràmetres hemàtics (WBC, RBC, Hgb, Hct) i de genotoxicitat (MNT), activitats enzimàtiques a fetge (GST, GPx, GR) i es va realitzar una avaluació histopatològica a fetge, ronyó i gònades com a biomarcadors de contaminació per metalls a exemplars silvestres de musaranya comuna, *Crocidura russula,* d'una zona minera abandonada. Els exemplars exposats als metalls presentaren alteracions significatives en comparació als exemplars d'una zona de referència: l'activitat de la GST va disminuir, la freqüència de micronuclis va augmentar i es van observar lesions hepàtiques relacionades amb aquesta exposició. Dels factors considerats, l'edat va ser el més important per explicar la variació observada, mentre que el sexe va ser el de menor importància. Alguns metalls van ésser significativament correlacionats amb els paràmetres avaluats, demostrant la gran influència d'aquests elements, potencialment tòxics, en les alteracions metabòliques. Aquestes dades constitueixen la primera mesura d'una bateria de biomarcadors en musaranyes d'una zona minera i una de les poques dades d'aquest tipus amb mamífers insectívors.

Introducció

Les mines abandonades constitueixen un risc mediambiental seriós a tot el món perquè poden impactar poblacions humanes i naturals per llargs períodes si no es prenen les mesures correctores necessàries. La mina d'Aljustrel, una de les més grans a Portugal, és un bon exemple d'aquesta problemàtica: aboca al medi grans quantitats d'efluents carregats de metalls pesants que poden entrar a les cadenes tròfiques degut a la seva alta disponibilitat. En contrast amb aquest risc ambiental evident, hi ha escassa informació ecotoxicològica sobre la influència de la contaminació per metalls a la salut d'espècies silvestres situades a una posició alta de la cadena tròfica, com les musaranyes.

L'avaluació dels efectes fisiològics de l'exposició crònica a contaminants ambientals emprant biomarcadors de toxicitat subletal és crucial per conèixer l'impacte de la contaminació sota condicions reals. Aquests efectes inclouen alteracions genètiques, enzimàtiques, hematològiques i histològiques que solen presentar-se quant hi ha un augment significatiu de metalls als teixits. Així, la correlació entre l'acumulació de metalls potencialment tòxics i els diferents biomarcadors pot donar informació valuosa sobre l'estat de salut i resposta dels exemplars de les poblacions silvestres. En aquest context, els petits mamífers són *a priori* bioindicadors ideals de pertorbacions del medi i per avaluar risc ambiental a poblacions humanes de zones contaminades.

Els objectius d'aquest estudi han estat: (i) quantificar diversos paràmetres hemàtics i de genotoxicitat, activitats enzimàtiques i una avaluació histopatològica a musaranyes d'una zona minera abandonada; (ii) establir la influència de l'edat i el sexe en la variació d'aquests paràmetres a poblacions silvestres: i (iii) discutir sobre el risc d'aquest tipus de contaminació així com sobre la idoneïtat dels insectívors com a bioindicadors.

Material i mètodes

En l'estudi es van emprar els 34 exemplars capturats vius en el treball referit al Capítol 4.3 (veure Material i Mètodes). Els paràmetres hemàtics van ser mesurats amb un "Coulter Counter Analizer" (Beckman Coulter, USA), la freqüència de micronuclis es va mesurar segons s'ha descrit en el Capítol 4.1 (veure Material i mètodes) i l'avaluació histològica es va efectuar seguint la descripció referida al Capítol 4.2 (veure Material i mètodes). En quant als paràmetres enzimàtics a fetge, es van realitzar seguint protocols prèviament descrits per mesurar GST, GPx i GR en homogenats de fetge.

Després de la transformació logarítmica de les dades i de testar la normalitat (prova de Kolmogorov-Smirnov) i l'homogeneïtat de les variàncies (prova de Levene), les variacions interpoblacionals es van avaluar emprant proves *t* de Student, en el cas de les dades contínues, i de *U*-Mann-Whitney, en el cas de les dades amb distribució discreta. Igualment, i depenent de la naturalesa de les distribucions, es van emprar els coeficients de correlació de Spearman i Pearson per detectar les relacions entre els paràmetres de dany i l'acumulació de metalls. Les anàlisis es van realitzar amb el programa SPSS 11.5 per Windows.

Resultats

No es van detectar diferències significatives segons el lloc de captura, l'estació, l'edat o el sexe en els paràmetres hemàtics, encara que es constata una tendència a incrementar els valors mitjos en els espècimens de la zona contaminada i entre juvenils i adults. Aquests paràmetres es van correlacionar amb diversos metalls en els animals de la zona contaminada.

Les musaranyes capturades a Aljustrel van presentar una freqüència de micronuclis significativament major que els de la zona control. No es van detectar diferències per edat o sexe en aquest paràmetre, mentre que es van detectar correlacions amb Fe, Mn, Cu, Pb, Cd i Cr.

L'activitat de la GST va disminuir als exemplars de la zona contaminada i va disminuir amb l'edat a les dues zones d'estudi. Les activitats de GPx i GR no van variar significativament per lloc de captura mentre que van incrementar-se als adults de les dues zones. El sexe no va ser una font significativa de variació en les activitats enzimàtiques quantificades. A l'igual que amb els paràmetres anteriors, es van detectar correlacions significatives entre les activitats enzimàtiques i diversos metalls (Pb, Cu, Mn i Cr).

Les musaranyes de la zona contaminada van presentar un augment de patologies a fetge (apoptosi, necrosi i vacuolització del citoplasma). Aquestes alteracions no van ser dependents de l'edat o el sexe ni van correlacionar-se amb l'acumulació de metalls. A més, no es van observar alteracions atribuïbles a la contaminació per metalls en els altres teixits analitzats.

Discussió

La problemàtica de les mines abandonades sense mesures per protegir el medi ambient no és un fet aïllat al sud de Portugal. Encara que aquestes mines produeixen alteracions severes a diversos nivells de les poblacions naturals, hi ha poca informació dels seus efectes tòxics.

Els valors dels diversos paràmetres hemàtics, de genotoxicitat i d'activitat enzimàtica obtinguts en el present estudi estan dins el rang obtingut per altres autors a petits mamífers de poblacions naturals. La tendència a augmentar d'alguns paràmetres hemàtics als exemplars de la zona contaminada del nostre estudi podria estar relacionada amb els efectes protectors de metalls essencials, com el ferro enfront l'exposició a contaminants a nivells tòxics, o amb la interacció de metalls tòxics amb el transport d'oxigen. En aquests paràmetres, els increments relacionats amb l'edat poden ser causats per la major quantitat de metalls als adults o a diferències en toxicitat o metabolisme entre adults i juvenils. L'augment de la freqüència de MN als animals de la zona contaminada mostra l'efecte clastogènic dels metalls de la mina de pirita sobre les musaranyes. La correlació entre MN i metalls essencials podria ser explicada per l'acció protectora o a efectes antagonístics en espècimens exposats a concentracions potencialment tòxiques de metalls.

Encara que hi ha abundant informació sobre l'alteració de les activitats enzimàtiques del sistema antioxidant a animals de laboratori, la informació referent a poblacions naturals és encara escassa. Les condicions metabòliques particulars de les musaranyes (mida petita, metabolisme alt, inclús més del predit per l'alometria) afecta diferents funcions com l'activitat enzimàtica dels sistemes de detoxificació. La disminució de l'activitat de la GST als animals d'Aljustrel és similar a la referenciada per altres autors a zones contaminades per metalls, mentre que la no divergència de les activitats dels altres enzims observada entre ambdues zones pot ser deguda a processos d'adaptació o tolerància enfront l'exposició crònica a metalls. Les variacions relacionades amb l'edat a les activitats enzimàtiques estudiades poden ser degudes a la toxicitat produïda per l'augment de metalls altament tòxics a adults junt amb el decrement de la resposta cel·lular a l'estrés oxidatiu a edats avançades. Tot i que no s'ha detectat diferències degut al sexe, però, caldrien estudis més extensos per dilucidar si aquest fet és característic de l'espècie estudiada.

Les dades d'histopatologia en mamífers insectívors són molt escasses i es limiten a lesions renals, tot i que l'avaluació de les alteracions estructurals d'òrgans diana pot ser una font important de dades qualitatives i quantitatives en ecotoxicologia. En aquest estudi es va detectar un increment d'alteracions hepàtiques relacionades amb la contaminació als exemplars de la zona de la mina d'Aljustrel, probablement relacionades amb l'alt contingut de metalls no essencials a concentracions per sobre el NOAEL. Aquestes concentracions potencialment tòxiques explicarien l'augment de necrosi, apoptosi i vacuolització del citoplasma, com l'observat en el present estudi.

Els petits mamífers han estat sovint emprats com a bioindicadors de contaminació ambiental, ja que juguen un paper fonamental en la transferència de contaminants al llarg de les cadenes tròfiques. Els insectívors, a més, acumulen altes concentracions de metalls i altres contaminants als seus teixits, el que pot servir per conèixer la dinàmica dels contaminants en condicions naturals.

Els danys produïts pels metalls poden afectar tots els nivells dels sistemes biològics, des de molècules a ecosistemes, el que implica que el seu estudi pot no ser sempre senzill. En el present estudi s'han relacionat l'acumulació de metalls amb els efectes a diversos nivells, detectant-se efectes tòxics a nivells estructural i de genotoxicitat, indicant que la espècie emprada pot ser un bon bioindicador de contaminació per metalls.

Conclusions

Les musaranyes de la zona contaminada d'Aljustrel van presentar alteracions atribuïbles a l'exposició a metalls a concentracions tòxiques. L'edat dels exemplars va ser un factor que explicava part de la variabilitat observada, mentre que el sexe no va ser una font significativa de variació. L'alt grau de correlació entre diversos paràmetres i l'acumulació de metalls va ser també un bon indicatiu de la importància d'aquests elements tòxics als resultats observats. Es constata que la utilització en conjunt de diversos paràmetres marcadors de dany a diferents nivells és una eina interessant per estudiar els efectes dels contaminants a poblacions naturals.

Article 5: Tissue, age, and sex distribution of thallium in shrews from Doñana, a protected area in SW Spain.

Resum

Al 1998, l'àrea protegida de Doñana, una de les principals reserves biològiques del sud-est europeu, va ser afectada per una gran quantitat de llots i aigües carregades de metalls, entre ells el tali (Tl), procedents del trencament de la bassa de residus de la mina de pirita d'Aznalcóllar. Des d'aquest desastre, s'han realitzat multitud d'estudis a la zona protegida per avaluar els efectes de la contaminació a flora i fauna. Tot i aquest esforç, hi ha poca informació sobre l'acumulació de Tl a Doñana, especialment a fauna terrestre. Aquest estudi mostra que hi ha un augment de fins a 10 cops de la concentració de Tl als teixits de musaranya comuna, *Crocidura russula*, de la zona afectada per l'abocament en relació als individus procedents de zones de referència. Els ronyons dels exemplars de la zona contaminada van presentar concentracions majors de Tl que els fetges, mentre que a la zona control els teixits hepàtic i renal van presentar concentracions similars. Es va observar una tendència a l'augment de Tl a adults respecte a juvenils, mentre que les femelles van presentar concentracions significativament majors que els mascles. El present estudi, a més de mostrar que la entrada de Tl a les cadenes tròfiques terrestres a Doñana és important, ofereix una de les poques referències existents sobre la concentració d'aquest metall a teixits de mamífers insectívors.

Introducció

El trencament de la bassa de residus de la mina de pirita d'Aznalcóllar, a l'abril de 1998, va provocar l'abocament de més de 5 Hm^3 de llots i aigües àcides carregades de metalls que van afectar la zona protegida de Doñana. Encara que des de l'accident s'han realitzat multitud d'estudis sobre els efectes d'aquest episodi a Doñana, hi ha escassa informació del que ha passat al medi terrestre, especialment en relació a metalls com el tal·li (Tl). En concret, aquest treball quantifica per primer cop l'esmentat metall en una espècie de petit mamífer de Doñana i constitueix un dels pocs estudis efectuats en poblacions naturals de petits mamífers.

El Tl és un metall no essencial altament tòxic, emprat a l'industria i que es troba als dipòsits de sulfit, com els de la mina d'Aznalcóllar. Recentment s'han publicat dades de la seva alta toxicitat, tant a experiments en laboratori com a poblacions humanes exposades, el que indicaria que l'augment de les seves concentracions a les cadenes tròfiques representaria un risc ambiental important.

El present estudi quantifica l'acumulació de Tl en teixits d'una espècie d'insectívor, la musaranya comuna, *Crocidura russula*, a una zona contaminada. A més, s'analitzen els efectes de dos factors biòtics (edat i sexe) en els patrons d'acumulació del metalls, així com la implicació dels resultats obtinguts en el maneig de l'àrea protegida de Doñana.

Material i mètodes

De novembre a desembre de 1999 es van capturar 29 exemplars de *C. russula*, de dues poblacions a l'àrea protegida de Doñana: una afectada per l'abocament de la mina d'Aznalcóllar (Entremuros; n=19) i altra no afectada i considerada com zona de referència (La Vera; n=10). Tal com a les altres zones d'estudi, de tots els exemplars es va determinar el sexe i va ser establerta l'edat relativa mitjançant el desgast dentari.

Per a les anàlisis químiques, el material emprat (fetges i ronyons de cadascun dels exemplars) va ser digerit seguint la metodologia exposada al Capítol 4.3 (veure Material i mètodes). El Tl es va quantificar mitjançant un espectròmetre de masses de plasma acoblat inductivament (ICP-MS) Perkin Elmer ELAN-600.

Les dades vas ser transformades mitjançant transformació logarítmica i es va testar la normalitat (Shapiro-Wilk) i l'homogeneïtat de variàncies (Levene, *F*-test). Inicialment es va testar l'efecte de lloc de captura, sexe i edat amb una ANOVA de tres factors. Les diferències en la concentració de metalls pesants per sexe a cada classe d'edat i les diferències interpoblacionals es van establir mitjançant proves *t* de Student, mentre que les diferències per cada classe d'edat es van avaluar mitjançant ANOVAs. Les diferències per lloc de captura de l'índex de condició corporal es van mesurar mitjançant *t*-Student. Per les anàlisis seqüencials es va utilitzar la correcció de Bonferroni. Totes les anàlisis estadístiques es van realitzar amb el programa SPSS 11.5 per Windows.

Resultats i Discussió

Els nivells de tali a totes les mostres va estar per sobre el límit de detecció. Comparats amb els espècimens de la zona control, les musaranyes de la zona contaminada presentaren augments significatius d'aquest metall a teixit hepàtic i renal, indicant una alta biodisponibilitat de Tl a la zona contaminada. Aquest metall és altament transferit dels sòls a plantes i animals, especialment als sòls amb molta humitat com és el cas de moltes zones a Doñana. De fet, s'ha observat l'increment d'aquest metall a altres espècies a la mateixa zona.

Els adults presentaren majors acumulacions d'aquest metall als seus teixits, però els increments no van ser significatius. Aquesta tendència a l'augment de Tl amb l'edat pot ser deguda a la menor excreció d'aquest metall en els adults, tal com s'ha observat en experiments controlats. A més, les femelles van tendir a acumular més Tl que els mascles, encara que s'esperaria un decrement de metalls a les femelles degut al pas de contaminants al fetus i als lactants. Aquestes majors concentracions podrien ser degudes a l'existència de diferències entre sexes en la exposició, absorció o excreció del metall, possiblement relacionades amb la reproducció o l'estat hormonal.

En quant als teixits analitzats, va haver majors concentracions de Tl als ronyons dels animals de la zona contaminada que als fetges, mentre que a la zona control ambdós teixits tenien concentracions semblants. Aquesta diferència es pot explicar degut a que a les zones contaminades el metall és transferit ràpidament de l'intestí al ronyó com a catió monovalent, mimetitzant el potassi, i acumulant-se a medul·la renal unit a proteïnes amb ponts sulfhidril.

Encara que no es coneix el mecanisme exacte de toxicitat del Tl, està considerat un fort oxidant amb activitat mutagènica, carcinogènica i teratogènica a mamífers. Com que s'ha descrit una clara dosi-resposta en la toxicitat d'aquest metall i els valors detectats a les musaranyes de

Doñana indiquen una alta disponibilitat i ingesta de Tl, caldria fer atenció als nivells d'aquest metall a aquesta àrea protegida. De fet, la gran quantitat d'estudis fets a Doñana després de l'accident de la mina de pirita són deguts principalment al gran interès ecològic d'aquest zona que conserva hàbitats, ecosistemes i espècies úniques o fortament amenaçades. Degut a que els petits mamífers com la musaranya comuna són font important a la dieta d'aus i mamífers carnívors, l'acumulació de metalls com el Tl en aquest sorícid pot ser que indiqui una alta disponibilitat d'aquest metall a posicions més altes de la cadena tròfica.

Conclusions

L'abocament de residus de la mina de pirita d'Aznalcóllar ha produït un augment significatiu en els nivells de Tl als teixits dels exemplars de musaranya comuna, que s'han acumulat seguint un patró d'edat, sexe i tipus de teixit. Donada la importància dels petits mamífers en la dieta d'aus i mamífers carnívors protegits i amb poblacions amenaçades, aquest augment de Tl a *C. russula* pot estar indicant un augment de la disponibilitat d'aquest metall als nivells alts de les cadenes tròfiques a Doñana.

Article 6: Metals in liver and kidneys and the effects of pollution in the shrew *Crocidura russula* inhabiting the protected wetland of Doñana.

Resum

Històricament impactada per diverses activitats humanes, la zona protegida de Doñana (SE Espanya) va patir els efectes del trencament de la bassa de residus de la mina de pirita d'Aznalcóllar a l'abril de 1998. Des d'aleshores, desenes d'estudis han avaluat concentracions i/o efectes dels metalls d'aquests residus a sòls, aigües, plantes i diverses espècies de vertebrats i invertebrats. No obstant, la informació ecotoxicològica a mamífers carnívors d'aquesta àrea protegida és molt limitada i per aquest motiu en aquest estudi s'ha avaluat l'acumulació de metalls i efectes (genotoxics, morfològics i estructurals) a 29 exemplars de musaranya comuna, *Crocidura russula,* capturats a una zona contaminada per metalls pesants i a una zona control. S'ha detectat un augment de metalls potencialment tòxics (Pb, Hg i Cd) i alteracions a diversos biomarcadors als exemplars de la zona contaminada per l'abocament de la mina de pirita. Degut a que els petits mamífers són part important de la dieta d'espècies carnívores d'aus i mamífers, moltes protegides i en perill d'extinció, que habiten a Doñana, aquest estudi és d'especial rellevància per conèixer els nivells de substàncies tòxiques a que estan exposades les poblacions naturals d'aquesta àrea protegida.

Introducció

La zona protegida de Doñana és un dels últims hàbitats de diverses espècies protegides. A l'abril de 1998 va ser afectada per l'abocament de la mina de pirita d'Aznalcóllar, que va abocar grans quantitats de llots i aigües amb metalls pesants com Pb, Hg, Cd, Sb i Tl. Des d'aleshores s'ha fet un gran esforç per monitoritzar aquests contaminants persistents a compartiments abiòtics (sòls i aigües) i biòtics (plantes i animals) d'aquesta àrea protegida. D'entre els animals, s'han avaluat diversos grups encara que les dades sobre mamífers terrestres són escasses.

Diversos metalls pesants poden causar trencament de DNA i poden induir la formació de ROS, que poden acabar alterant teixits i cèl·lules, induint mutacions i mort cel·lular, entre altres. Aquests efectes són sovint avaluats en experiments de laboratori però és imprescindible disposar de dades referents al què succeeix en condicions reals a poblacions naturals. En aquest sentit, els petits mamífers han estat sovint emprats com a bioindicadors de contaminació ambiental, incloent els metalls pesants, ja que presenten característiques òptimes per ser-ho.

En aquest estudi s'han avaluat l'acumulació de metalls i els seus efectes (a nivells morfològic, genotoxic i histològic) en poblacions naturals de musaranya comuna, *Crocidura russula*. A més, s'han estudiat dos factors biòtics (edat i sexe) com a fonts intraspecífiques de variació en aquestes poblacions.

Material i mètodes

En aquest estudi es van emprar els mateixos individus que els referits en el Capítol 4.5. De cada exemplar es van mesurar la longitud corporal (BL, en mm) i el pes (BM, en g) per calcular l'índex residual (RI) i diversos paràmetres morfològics seguint la metodologia detallada al Capítol 4.1 (Material i mètodes). De tots els exemplars es va determinar el sexe i es va establir l'edat relativa mitjançant el desgast dentari.

Per a les anàlisis químiques, el material emprat (fetges i ronyons de cadascun dels exemplars) va ser digerit seguint la metodologia exposada al Capítol 4.3 (veure Material i mètodes). Mitjançant un espectròmetre de masses de plasma acoblat inductivament (ICP-MS) Perkin Elmer ELAN-600, es va quantificar el contingut de Pb, Hg i Cd en fetge i ronyó dels 29 exemplars de musaranya comuna capturats a les zones de Entremuros (n=19) i de La Vera (n=10). Dels exemplars capturats vius (n=14) es van agafar mostres de fetge, ronyó, intestí i melsa per un anàlisis histopatològic (processades com es detalla al Material i mètodes al Capítol 4.2). A més, es va realitzar una anàlisi quantitativa capturant 4 imatges de cada mostra i processant-les amb el software "Image Tools" que va mesurar perímetre, elongació, rodonesa i compactació de 1588 cèl·lules. També es van processar mostres de sang per realitzar el Test de Micronuclis (TMN) seguint el protocol detallat a Material i mètodes del Capítol 4.1.

Les dades vas ser transformades mitjançant transformació logarítmica i es va testar la normalitat (Shapiro-Wilk) i l'homogeneïtat de variàncies (Levene, *F*-test). Inicialment es va testar l'efecte de lloc de captura, sexe i edat amb una MANOVA de tres factors. Les diferències en la concentració de metalls pesants i paràmetres morfomètrics per sexe a cada classe d'edat i les diferències interpoblacionals es van mesurar mitjançant tests d'Student (*t*). Les mateixes comparacions pel TMN i per la histopatologia es van realitzar mitjançant tests de Mann-Whitney (*U*). Per als anàlisis seqüencials es va utilitzar la correcció de Bonferroni. Totes les anàlisis estadístiques es van realitzar amb el programa SPSS 14.0 de Windows.

Resultats

El pes total i el pes relatiu del fetge van ser significativament majors als espècimens de la zona contaminada. Aquests exemplars van mostrar també una tendència a tenir majors pesos relatius dels ronyons i menor BW que els exemplars de la zona de referència.

La MANOVA per a fetge va mostrar diferències significatives per lloc de captura, edat i sexe. En relació al lloc de captura, es van detectar increments de Hg i Cd i un decrement de Cr als exemplars afectats pels metalls. Per edat, els adults de la zona contaminada presentaren més Hg i Cd que els juvenils, mentre que Pb, Mo i Fe tendiren a augmentar i Cu i Cr a disminuir als adults. A més, els mascles tenien majors concentracions de Pb i Co i les femelles més Mo als seus teixits.

Al teixit renal, la MANOVA va ser significativa només en relació al lloc de captura. Amb uns resultats molt semblants als obtinguts a fetge, els animals exposats als metalls de la mina tenien més Mg, Hg, Cd, Cu i Co que els control. L'edat va ser un factor que va influenciar les concentracions de Hg i Cd, mentre que es va observar la mateixa tendència a acumular metalls per gènere que a fetge.

La TMN va ser significativament major als exemplars de la zona pol·luïda. La histopatologia va mostrar que els animals contaminats per metalls presentaven focus de necrosi i apoptosi a teixit hepàtic. Aquestes lesions es van traduir en una tendència a l'augment del perímetre cel·lular als exemplars impactats pels metalls de la mina d'Aznalcóllar. A més, es van detectar correlacions significatives entre els paràmetres histològics analitzats i alguns metalls (Pb, Cr i Mn).

Discussió

Des de l'accident de la mina d'Aznalcóllar s'han realitzat multitud d'estudis sobre els seus efectes a la zona de Doñana, però la informació sobre biota de medi terrestre en general, i de mamífers carnívors en particular, és escassa o nul·la.

Divuit mesos després de la catàstrofe mediambiental, la disponibilitat de metalls potencialment tòxics presents als llots de la mina de pirita, com el Cd i Hg, segueix alta a la zona protegida afectada per l'abocament. Aquest metalls són bioacumulables i biodisponibles per la biota durant llarg temps en condicions com les de la reserva de Doñana. Les baixes concentracions de Pb pot ser deguda a la baixa disponibilitat a pHs bàsics. Tot i que molts metalls essencials eren abundants als llots de la mina, les seves concentracions als teixits de musaranya comuna no han variat significativament, degut principalment a la bona regulació de les seves concentracions als teixits de mamífers, encara que hi hagin alts increments en la seva ingestió. Les altes concentracions de Cr observades als exemplars de la zona control poden ser degudes a l'ús de fertilitzants que afecten les aigües i sòls de Doñana des de fa dècades.

Per edat s'observa un augment de Cd als adults, similar al referit anteriorment per a teixits tous en aquesta memòria, que sembla relacionat amb la formació de complexos Cd-metalotionina per tal de reduir la toxicitat d'aquest metall. Un mecanisme semblant podria explicar l'increment observat en l'acumulació de Hg i Pb als adults. La disminució de Cr als adults sembla relacionada amb una menor absorció intestinal d'aquest element amb l'edat.

A les femelles es va detectar una disminució de Hg i Pb relacionada amb la transferència de metalls al fetus via placenta i al lactant via llet materna durant la gestació i lactància. Les diferències entre mascles i femelles en les concentracions de metalls essencials pot ser deguda a diferències en la ingesta i concentració de metalls, atribuïbles a diferències hormonals o requeriments nutricionals, així com a les interaccions entre els diferents metalls.

L'augment dels efectes genotòxics als exemplars exposats a metalls ha estat anteriorment descrita a diverses espècies animals de la mateixa zona i indica un dels efectes tòxics de l'exposició crònica a metalls altament tòxics i amb activitat clastogènica.

L'augment del pes dels fetges dels exemplars de la zona contaminada és un indicatiu d'alteracions fisiològiques i histopatològiques, com s'ha mostrat a altres poblacions de petits mamífers exposats a metalls. La principal ruta d'exposició a aquests compostos és per la dieta i el fetge juga un paper metabòlic crucial en la detoxificació dels tòxics procedents de l'absorció intestinal.

La histopatologia pot proporcionar importants dades sobre els mecanismes de toxicitats i les alteracions estructurals a poblacions naturals exposades a diferents tipus de contaminació ambiental. Alguns metalls pesants, com Tl, Cd i Hg, amb altes concentracions als teixits de les musaranyes de l'àrea de Doñana, produeixen estrés oxidatiu que pot alterar les cèl·lules fins produir mort cel·lular o altres alteracions importants com processos mutagènics o carcinogènics. Les alteracions nuclears detectades al parènquima hepàtic a aquest treball poden ser indicatives de desorganització de la cromatina i poden tenir serioses conseqüències a l'expressió gènica.

En relació a la concentració de metalls a teixits tous, Cd i Hg han assolit valors indicatius de que els animals han estat exposats a aquests elements i que, per tant, poden veure's afectats pels efectes tòxics d'aquest elements (veure toxicitat a Capitol 1 i 3).

La zona protegida de Doñana és una de les més importants i menys alterades del sud-est europeu, i hàbitat d'importants poblacions de moltes espècies emblemàtiques i en perill d'extinció. Tot i que la musaranya comuna és un dels micromamífers més abundants a Doñana, no hi ha dades ecotoxicològiques d'aquest insectívor en llocs contaminats, per la qual cosa és essencial obtenir informació dels nivells i efectes dels contaminants a aquesta àrea protegida i comparar-la amb la d'altres espècies terrestres. En general, els insectívors acumulen més contaminants que altres micromamífers, però solen ser més tolerants. La comparació d'espècies simpàtriques pot donar informació important del comportament dels contaminants a les cadenes tròfiques i servir d'eina pel maneig de zones protegides.

Conclusions

Segons les dades obtingudes a musaranya comuna, Hg i Cd són els dos metalls que poden presentar problemes de toxicitat degut als alts nivells detectats als teixits tous als individus d'aquesta espècie afectats pels llots de la mina de pirita d'Aznalcóllar. A més, l'exposició a aquests contaminants ha produït diverses alteracions a nivell morfològic, histològic i de genotoxicitat, demostrant que la contaminació de la mina de pirita afecta també al normal desenvolupament de les funcions fisiològiques de les musaranyes afectades. Aquests resultats, junt amb l'alta posició tròfica de *C. russula* i la seva abundància, fa que aquesta espècie pugui ser tinguda en compte a l'hora d'avaluar la qualitat del medi ambient a zones protegides.

Article 7: Bioaccumulation of non-essential metals in the greater white-toothed shrew, *Crocidura russula*, in the Ebro Delta (NE Spain).

Resum

S'ha quantificat l'acumulació de Pb, Hg i Cd en ossos llargs de 105 exemplars de musaranya comuna, *Crocidura russula.* capturats al Delta de l'Ebre, una zona impactada per diverses activitats humanes, i a les Illes Medes, una zona control. Els nivells de Pb i Hg van variar significativament depenent de la localitat, edat i sexe, mentre el Cd no va mostrar variacions en funció d'aquests 3 factors. Les musaranyes capturades a la zona del Delta presentaren concentracions més elevades de Pb i Hg. L'increment de Pb amb l'edat va ésser especialment remarcable als animals del Delta de l'Ebre. Aquest augment s'atribueix a diverses activitats humanes, principalment la caça que incrementa la concentració i disponibilitat d'aquest element al medi natural. A diferència dels mascles, les femelles d'aquesta zona impactada presentaren nivells de Hg baixos a totes les edats, circumstància que pot estar associada amb la gestació i lactància. Aquest estudi mostra les primeres dades de metalls pesants en mamífers d'aquesta zona deltaica i indi-

ca que *C. russula* pot ser un bon indicador de contaminació. També es pot concloure que el sexe i l'edat representen una font important de variació en la bioacumulació d'aquests metalls en poblacions silvestres.

Introducció

Diverses activitats humanes poden generar contaminació per metalls a aire, aigües i sòls. A les últimes dècades, només la producció minera ha incrementat per 2, 2 i 15 la producció de plom, mercuri i cadmi, respectivament, amb la conseqüent entrada d'aquests metalls no essencials al medi natural. Els ecosistemes són un filtre efectiu per immobilitzar metalls als sòls, transferir-los als sistemes aquàtics i terrestres i, per tant, incrementant la biodisponibilitat i risc d'intoxicació a les poblacions naturals i a les humanes. Plom, mercuri i cadmi són tres metalls pesants no essencials àmpliament distribuïts als ecosistemes i reconeguts com a altament tòxics als sistemes biològics.

La zona protegida del Delta de L'Ebre ha estat històricament afectada per diverses activitats humanes, com la caça, l'agricultura o l'abocament de residus industrials i domèstics que, transportats pel riu Ebre, han produït un increment de diversos contaminants, inclosos certs metalls pesants, i episodis d'intoxicació massiva a poblacions silvestres d'aquest sistema deltaic. Per contra, la zona protegida de les Illes Medes no està afectada per activitats humanes contaminats. La informació ecotoxicològica de que es disposa en aquestes àrees protegides és escassa, especialment la referida a mamífers terrestres. Dintre d'aquest grup de vertebrats, els petits mamífers, i particularment els insectívors, s'han mostrat com a bons bioindicadors de contaminació degut, entre d'altres característiques, a la seva abundància, a la seva importància com a base de l'alimentació d'altres espècies, a que ocupen diferents nínxols tròfics i alts requeriments metabòlics que els fan òptims bioacumuladors de substàncies que biomagnifiquen al llarg de la cadena tròfica. Per avaluar aquests efectes de la contaminació a nivell individual o poblacional s'han emprat diversos biomarcadors genètics, morfològics, o bioquímics.

Els objectius d'aquest estudi van ser: (i) quantificar per primer cop la acumulació de metalls pesants no essencial a poblacions de *C. russula* del NE peninsular; (ii) analitzar els patrons de variació basats en el sexe i l'edat; (iii) avaluar l'impacte de la contaminació d'origen antròpic a dues zones protegides de clima mediterrani; i (iv) conèixer la idoneïtat del material osteològic de col·leccions biològiques com a font d'informació ecotoxicològica.

Material i mètodes

En aquest estudi es van quantificar els nivells de Pb, Hg i Cd en óssos llargs de 105 exemplars de musaranya comuna procedent de dues àrees costaneres protegides: El Delta de l'Ebre (n=73) i les Illes Medes (n=32). Els exemplars van ser capturats de 1976 a 1981 per estudis biològics i el material dipositat a la col·lecció del Dpt. de Biologia Animal de la Universitat de Barcelona.

De cada exemplar es va mesurar la longitud corporal (BL) i el pes (BM) per calcular l'índex residual (RI), índex de condició corporal que s'utilitza per conèixer l'estat general de salut dels animals. Tots els exemplars van ser sexats i establerta l'edat relativa mitjançant el desgast dentari.

Per a les anàlisis químiques, el material, emmagatzemat en borses de paper, va ser rentat amb aigua desionitzada, assecat fins pes constant (50ºC, 48h) i digerit amb 2 ml d'àcid nítric i 1ml de peròxid d'hidrogen en bombes de Tefló. Mitjançant un espectròmetre de masses de plasma acoblat inductivament (ICP-MS) Perkin Elmer ELAN-600, es va quantificar el contingut de Pb, Hg i Cd en óssos llargs. També es va mesurar la longitud cap-cos (BL, en mm) i pes total (BW, en g) de cadascun dels exemplars per calcular un índex de condició física basat en els residuals originats a partir de la regressió de BL sobre BW. Els animals es van sexar i classificar en tres categories d'edat relativa (1: juvenils; 2: adults; 3: senils) segons el desgast dentari.

Les dades vas ser transformades mitjançant transformació logarítmica i es va comprovar la normalitat (Kolmogorov-Smirnov) i l'homogeneïtat de variàncies (Levene, *F*-test). Inicialment es va avaluar l'efecte de lloc de captura, sexe i edat amb una MANOVA de tres factors. Les diferències en la concentració de metalls pesants per sexe a cada classe d'edat i les diferències interpoblacionals es van mesurar mitjançant proves *t* de Student, mentre que les diferències per cada classe d'edat es van mesurar mitjançant ANOVAs. Les diferències per lloc de captura de l'index de condició corporal es van mesurar mitjançant la *t* de Student. Per les anàlisis seqüencials es va utilitzar la correcció de Bonferroni. Totes les anàlisis estadístiques es van realitzar amb SPSS versió 11.5 per Windows.

Resultats

Les concentracions dels 3 elements van estar per sobre del límit de detecció en totes les mostres analitzades. La MANOVA va mostrar diferències significatives degut al sexe, edat i lloc de captura. Es van observar diferències entre sexes en l'acumulació de Pb i Hg, encara que els patrons d'acumulació van variar amb l'edat i el lloc de captura. Així, mentre que al Delta les diferències sexuals es varen detectar al Hg a adults, a les Medes aquestes diferències van ser significatives a juvenils i adults. A l'àrea control també el Pb va variar entre mascles i femelles adults i senils. El Cd no va variar significativament les seves concentracions per cap dels 3 factors estudiats.

Per edat, als exemplars dels dos llocs de captura les concentracions de Pb i Hg van ser significativament majors als senils. A l'analitzar les dades per lloc de captura, les musaranyes de la zona de l'Ebre presentaren valors mitjos de Pb i Hg significativament superiors als dels exemplars de les Medes. L'índex de condició física no va presentar variacions significatives entre àrees de captura.

Discussió

Els resultats obtinguts mostren que Pb i Hg són els dos metalls amb major importància, ja que a més d'assolir nivells potencialment tòxics (més de 10 i 1.1 µg/g, respectivament, a teixits tous), mostraren patrons d'acumulació en relació a sexe, edat i lloc de captura. L'augment de les concentracions d'aquests elements als exemplars de l'Ebre sembla degut a les dècades d'intensa activitat humana (industrial, domèstica, agricultura i caça) que ha incrementat tant les concentracions d'aquests metalls al medi com la seva biodisponibilitat. Grans quantitats de perdigons de plom provinents de la caça que s'acumulen als sòls del delta poden ser els principals causants de l'augment d'aquest metall a l'Ebre. En general, el mercuri i el cadmi s'acumulen principalment a teixits tous. Tot i això, s'han trobat alts nivells de Hg als ossos dels exemplars del Delta de l'Ebre. Aquest metall es biomagnifica al llarg de la cadena tròfica. Els alts nivells presentats als mascles poden ser indicatius d'un procés de transferència de Hg de les femelles gestants al fetus via placenta com també de les femelles lactants als nounats via llet. Encara que els sediments del riu Ebre tenen alts nivells de Cd, no s'ha observat un augment significatiu d'aquest metall, possiblement degut a que els ossos no són òrgans acumuladors de Cd.

Diversos estudis han mostrat que els petits mamífers exposats a metalls pesants poden patir una pèrdua de massa corporal o alterar el pes relatiu de diferents òrgans, el que quedaria reflectit en els índexs de condició física. En aquests estudi, encara que els exemplars del Delta de l'Ebre presentaren altes concentracions de Pb i Hg, el seu índex corporal no va ser inferior al dels exemplars de la zona control, indicant l'alta tolerància d'aquesta espècie a la contaminació ambiental per metalls. Tot i això, caldria examinar altres paràmetres a nivells genètic o bioquímic i emprar-los en conjunt amb els criteris morfològics per obtenir una millor mesura de la resposta de *C. russula* a l'impacte de la contaminació.

Les zones deltaiques són sovint diana de la contaminació d'origen antròpic i, malgrat la legislació mediambiental, han estat fortament alterades especialment als països desenvolupats. El Delta de l'Ebre s'ha vist impactat durant dècades per la caça, l'agricultura (especialment el cultiu d'arròs), i les indústries i poblacions situades al llarg del riu Ebre i la costa mediterrània propera. Els nostres resultats no només corroboren l'important contaminació per Pb i Hg sinó que també analitzen per primer cop aquests elements a mostres pertanyents a mamífers terrestres. A més, es constata que el material osteològic de col·leccions zoològiques pot ser una bona font per obtenir dades ecotoxicològiques sense haver de realitzar noves captures d'exemplars de poblacions naturals. Degut a que el material ossi emprat correspon a animals capturats fa 30 anys, seria interessant avaluar amb nous estudis com han variat els nivells de metalls a *C. russula* del Delta al llarg del temps i comparar aquestes noves dades sobre acumulació amb altres paràmetres morfològics, genètics, comportamentals o fisiològics per establir el grau d'afectació dels individus i poblacions a aquesta zona contaminada.

Conclusions

Les poblacions de musaranya comuna del Delta de l'Ebre estan exposades a nivells potencialment tòxics de Pb i Hg provinents d'activitats humanes diverses, com els perdigons emprats per caçar i diverses indústries que augmenten la entrada d'aquests elements i la seva acumulació a sediments, incrementant la seva biodisponibilitat per la biota. En contrast, els baixos nivells de Cd als exemplars de la zona contaminada suggereixen que aquest element no sembla representar un risc. El sexe i l'edat són dos paràmetres importants per explicar la variabilitat intrapoblacional en l'acumulació d'aquests metalls no essencials.

Article 8: Metal and metalloid accumulation in shrews (Soricomorpha, Mammalia) from two protected Mediterranean coastal sites.

Resum

Mentre que la informació ecotoxicològica disponible sobre alguns metalls molt tòxics és abundant, la referent a altres elements que poden presentar casos de deficiència o toxicitat és escassa. En aquest estudi s'ha quantificat les concentracions de zenc (Zn), coure (Cu), ferro (Fe), manganès (Mn), crom (Cr), molibdè (Mo), estronci (Sr), bari (Ba) i bor (B) en ossos de musaranya comuna, *Crocidura russula*, de dues zones protegides del litoral català, el Delta de l'Ebre, una zona humida impactada per diverses activitats humanes, i les Illes Medes, una zona control. L'augment de biodisponibilitat degut a fonts naturals i antropogèniques ha comportat l'increment significatiu de Fe, Mn, Mo, Sr, Ba i B als exemplars del Delta de l'Ebre, mentre que les musaranyes de les Illes Medes presentaren quantitats més elevades de Cu i Cr. L'anàlisi de components principals va separar completament els exemplars per lloc de captura, especialment degut als nivells de B, Sr i Cu. A més de proveir valors de referència de molts elements a ossos d'un mamífer insectívor, aquest estudi explora els factors de variabilitat i els patrons d'acumulació per sexe i edat i avalua el risc mediambiental degut a aquesta acumulació als teixits.

Introducció

Els metalls pesants no essencials, com Pb, Hg i Cd, i alguns d'essencials, com Zn i Cu, són contaminants àmpliament distribuïts als ecosistemes i ha estat extensivament estudiada tant la seva acumulació com els seus efectes a la biota. Per contra, d'altres elements (metàl·lics i no metàl·lics) que poden generar deficiències o toxicitat com Fe, Mn, Mo, Sr, Ba i B es disposa d'escassa informació. Aquests elements són constituents naturals dels sòls, però poden veure alterats la seva concentració i/o biodisponibilitat degut a diverses activitats humanes contaminants.

Els deltes són ecosistemes fràgils sovint afectats per activitats antropogèniques que els transformen o els fan desaparèixer especialment als països més industrialitzats. En les zones deltaiques impactades que encara sobreviuen, com el Delta de l'Ebre, és vital conèixer l'estat del medi i dels nivells d'elements potencialment tòxics que són disponibles per la biota. Aquest delta ha estat impactat durant dècades per diverses activitats humanes (indústries i residus domèstics abocats al riu Ebre, així com caça, pesca, ports i industries que afecten directament aquesta zona humida costanera). Tot i que existeix abundant informació sobre la contaminació al Delta, hi ha poques dades sobre metalls essencials i metal·loides a aquesta zona. En contraposició, les Illes Medes han estat menys alterades degut a activitats contaminants que puguin afectar el cicle geofísic dels metalls.

Els petits mamífers, especialment els insectívors com les musaranyes, són considerats bons bioindicadors de contaminació ambiental, tant per avaluar la bioacumulació de tòxics com per conèixer els seus efectes a les poblacions naturals, ja que tenen una alta taxa metabòlica basal i alts requeriments alimentaris. Malgrat això, hi ha poca informació sobre els nivells de molts elements a ossos de petits mamífers exposats a contaminació i del possible ús de material de col·leccions zoològiques per estudis ecotoxicològics.

Tenint totes aquestes circumstàncies presents, aquest estudi ha volgut: (i) determinar els nivells de diversos elements escassament quantificats en estudis ecotoxicològics; (ii) analitzar l'efecte del lloc de captura, edat i sexe en els patrons de bioacumulació d'aquests elements; (iii) proveir dades de referència d'aquests elements a ossos de poblacions naturals d'insectívors; i (iv) avaluar l'ús d'aquests paràmetres com a biomarcadors de contaminació en zones de clima mediterrani.

Material i mètodes

Per a aquest estudi es van emprar els 105 exemplars de musaranya comuna referits en l'estudi del Capítol 4.7. El Fe va ser quantificat amb un ICP-OES Perkin Elmer OPTIMA-3200RL i la resta d'elements (Zn, Cu, Mn, Cr, Mo, Sr, Ba, B, Ni i Co) amb un ICP-MS Perkin Elmer ELAN-6000. La normalitat de les variables i l'homogeneïtat de variàncies es va comprovar mitjançant les proves de Kolmogorov-Smirnov i Levene, respectivament. Inicialment, es va realitzar una anàlisi multivariant de la variància (MANOVA) de tres factors per avaluar l'efecte de la localitat, sexe i edat, i de les seves interaccions sobre els nivells dels metalls. En funció dels resultats obtinguts, les comparacions intra- i interpoblacionals es van avaluar amb proves *t* de Student o amb anàlisis univariants de la variància d'un factor (ANOVA). Per visualitzar les diferències interpoblacionals es va realitzar una anàlisi de component principals sobre les concentracions dels metalls aplicant una rotació Varimax. Les relacions entre els elements a la zona contaminada es van determinar amb correlacions de Pearson. Als tests seqüencials, els *p*-valors es van corregir per Bonferroni. Totes les anàlisis estadístiques van ser fetes utilitzant el programa SPSS 14.0 per Windows.

Resultats

Els nivells de Ni i Co van estar per sota els límits de detecció i no van ser inclosos a les anàlisis. La MANOVA va mostrat diferències significatives per lloc de captura, edat i la seva interacció. Com no es van detectar diferències per sexes, mascles i femelles van ser agrupats en les anàlisis estadístiques subseqüents. Per l'edat només es van detectar diferències significatives al Zn, mentre que per la localitat es van trobar divergències en tots els elements, excepte al Zn i al Cr. Tots els elements amb diferències per lloc de captura van presentar majors concentracions als animals de la zona contaminada, excepte el Cu. la zona del Delta de l'Ebre excepte el Cu que va assolir nivells més alts a la zona de les Medes.

Les dues primeres components principals van acumular el 61.46% de la variació total, essent el B i el Sr els elements amb més pes al primer eix factorial i el Cu al segon eix. La projecció va mostrar una completa separació entre els espècimens de les dues zones de captura. El nombre de correlacions significatives entre elements va ser particularment elevat en el cas del Cu (7) i del Fe (6). El Cr va ser l'únic metall no correlacionat amb cap altre element.

Discussió

Els nivells de metalls obtinguts a *C. russula* són similars als obtinguts per altres autors a ossos de petits mamífers. Les concentracions de Zn, similars en els dos llocs de captura, poden ser degudes a la bona regulació homeostàtica dels nivells d'aquest element essencial als teixits de mamífers. La disminució de Cu a la zona contaminada, també mostrada a altres tipus de mostres biològiques de la zona, es pot deure a una menor disponibilitat d'aquest element o a una possible disrupció degut als alts nivells de metalls no essencials, com Pb i Hg. L'increment dels nivells de Fe i Mn en els exemplars del Delta pot ser explicat pels efectes protectors enfront a l'exposició a diversos contaminants. Els nostres resultats indiquen una baixa biodisponibilitat de Cr al Delta de l'Ebre. L'augment de Mo a aquesta zona pot ser deguda a que els sòls rics en llim, com els deltaics, són paranys que capturen selectivament aquest element. Encara que aquest metall es considera un antagonista del Cu, en aquest estudi ambdós elements han presentat una correlació significativa, com ja s'ha descrit en alguns estudis precedents. L'estronci i el bari són dos elements no essencials àmpliament distribuïts que no magnifiquen al llarg de les cadenes tròfiques i que es presenten associats al calci al que mimetitzen als sistemes biològics. Els nivells de Sr als exemplars de l'Ebre van ser excepcionalment alts, principalment degut a les característiques geoquímiques dels sediments fluvials, a l'entrada d'aigua de mar a l'Encanyissada i a les aportacions d'origen humà. Per contra, els nivells de Ba van ser menors, tot i que sol captar-se millor que els seus associats, potser degut a que precipita com a $BaCO_3$ a sòls calcaris. A més, el Ba té un període mig curt als sistemes biològics i es excretat, reduint els nivells als teixits. El B es presenta sovint als sistemes naturals i pot incrementar la seva biodisponibilitat degut a diverses activitats humanes o a condicions ambientals, com llacunes amb poca circulació, com passa al Delta de l'Ebre. Aquest metal·loide afecta diferents teixits, entre ells els ossos. Els valors de B als exemplars del Delta són molt alts, indicant un increment en la biodisponibilitat d'aquest element que pot assolir concentracions potencialment tòxiques.

Els exemplars adults de musaranya comuna presentaren valors més alts de Zn que els juvenils. Aquest fet també succeeix a altres espècies de petits mamífers, però no s'havia detectat mai en teixits de *C. russula*. Però en el present estudi es detecta, probablement degut a que els teixits durs actuen com a magatzem de l'excés de Zn, els nivells del qual estan molt ben controlats als teixits de mamífer.

Les variacions de metalls relacionades amb el sexe van ser les que menys diferències van presentar a ossos de *C. russula*. Encara que no es van detectar diferències significatives, es va observar una tendència al increment de Mn, Mo i Fe a femelles, també documentat a altres poblacions de petits mamífers, probablement relacionat amb requeriments nutricionals, diferències en el metabolisme dels metalls degudes a l'acció d'hormones sexuals o a la interacció entre elements.

Una espècie es considera un bon bioindicador quan acumula altes concentracions de contaminants, com els metalls, i reacciona a la entrada d'aquests xenobiòtics al medi ambient. Els petits mamífers en general, i les musaranyes en particular, han estat sovint emprats per aquest propòsit a estudis ecotoxicològics ja que constitueixen un pas intermedi en la cadena tròfica. La dieta és la principal font de contaminants a mamífers i els insectívors s'alimenten de preses que solen ser grans acumuladors de contaminants com els metalls pesants. A més d'aquesta acumulació directa, les musaranyes solen ingerir grans quantitats de terra amb les preses, el que pot augmentar la ingesta de contaminants.

En la cerca de marcadors adequats per avaluar l'estat del medi, s'ha emprat sovint l'acumulació de metalls i metal·loides. Encara que l'element traçador adequat pot dependre del tipus de contaminació, l'anàlisi en conjunt de les dades dels diferents elements, com ara mitjançant una anàlisi de components principals, pot donar informació molt valuosa de l'estat del medi. En el nostre estudi, aquesta anàlisi ha permès separar perfectament les dues poblacions estudiades i a mostrat una major dispersió als exemplars de la zona afectada per activitats humanes, possiblement causat per la resposta individual i l'exposició diferencial en poblacions silvestres de zones contaminades. Els resultats obtinguts en aquest primer estudi amb ossos són semblants als referits per altres tipus de teixits, el que suggereix que la metodologia emprada es pot utilitzar a altres estudis semblants. Per últim, cal remarcar que l'ús de mostres òssies de col·leccions biològiques és un mètode no invasiu i pot ser una eina molt important a l'hora d'obtenir dades ecotoxicològiques. Els exemplars emprats en aquest estudi van ser capturats fa 30 anys a dues zones actualment protegides. Des d'aleshores, i especialment al Delta de l'Ebre, ha hagut una gran transformació del paisatge, a més de major control i protecció ambiental, el que fa més interessant realitzar noves prospeccions, quantificant tant metalls com altres paràmetres per conèixer els nivells actuals de contaminants, els seus efectes i les fonts emissores en zones protegides com aquestes.

Conclusions

Aquest estudi constitueix la primera quantificació de diversos metalls i metal·loides a ossos de musaranyes. Alguns d'aquests elements, que poden ocórrer naturalment o degut a activitats humanes, són tòxics per la biota i el seu monitoratge és imprescindible a les zones protegides.

La musaranya comuna, a l'igual que altres insectívors, sembla un bon bioindicador de metalls i bor ja que els acumula a altes concentracions, variant les concentracions als seus teixits en relació a les variacions al medi. La informació obtinguda en aquest estudi pot servir per conèixer la distribució i mobilitat dels elements en un medi ambient altament variable, com els deltes, i per predir el risc ambiental a zones protegides.

9. ANNEX 1

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Bioaccumulation of metals and effects of landfill pollution in small mammals. Part I. The greater white-toothed shrew, *Crocidura russula*

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Abstract

Here we quantified the bioaccumulation of metals (lead, mercury, cadmium, iron, magnesium, zinc, copper, manganese, molybdenum, and chromium) and assessed several morphological (RI, relative weights) and genotoxic parameters as biomarkers of pollution from the landfill of Garraf (Barcelona, NE Spain). Specimens of *Crocidura russula* (Insectivora, Mammalia) from the landfill site showed increased Pb, Cd, Mg, Zn, Cu, and Cr concentrations in their tissues. Levels of mercury were below detection limits. Concentrations of Cd, Pb, and Cr varied significantly with age and no differences were found between males and females. While no differences were found in morphological parameters between shrews from the two sites, those from the polluted one showed more micronuclei in blood than those from reference site (1.786 ± 0.272 vs. 0.088 ± 0.045‰; $U = 46.000$, $p < 0.001$). The considerable amounts of potentially toxic metals (Pb till 59.71 and Cd till 56.57 $\mu g\ g^{-1}$ DW in kidneys) and the genotoxic effects indicate the harmful effect on biota. We consider necessary biomonitoring this landfill sited in a partially protected area.

Keywords: *Crocidura russula*; Landfill; Heavy metals; Micronucleus test; Protected sites

1. Introduction

Landfills are the main form of solid waste accumulations in several regions, including Mediterranean countries (Loukidou and Zouboulis, 2001). However, if these sites are not adequately controlled they may have a severe environmental impact. Gaseous compounds, mainly methane and carbon dioxide, as well as volatile organic compounds (VOCs) are common pollutants of landfills (Christensen et al., 2001; Li et al., 2006; Wichmann et al, 2006). Also, liquid effluents named leachates are produced by the decomposition of wastes or by interaction between wastes and rain water, and are often a considerable source of contamination for groundwater aquifers as well as for adjacent soil and surface waters. Leachate composition, volume and toxicity vary depending on the nature and age of wastes, method of disposal, dump depth and climatic factors (De Rosa et al., 1996; Li et al., 2006). These effluents often contain a wide variety of organic and inorganic pollutants including metals such as Pb, Cd, Mg, Fe, Zn, Cu, Mn, Mo, and Cr (Ragle et al., 1995; Johnson et al., 1996; De Rosa et al., 1996; Christensen et al., 2001; Slack et al., 2005). These mixtures are highly toxic for biota (i.e. Cheung et al., 1993; Cabrera and Rodriguez, 1999; Sang and Li, 2004), however, information on the effects of landfill leachates on wildlife health is scarce. Genotoxic effects such as increases in micronuclei (MN), in chromosomal aberrations, and in abnormal sperm morphology frequencies, have been reported in laboratory rodents exposed to landfill leachates (Bakare et al., 2005; Li et al., 2006) and in wild mice from a hazardous waste site (i.e. Tull-Singleton et al., 1994).

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Morphological parameters are used as markers of physiological alterations in wild populations of small mammals exposed to some kinds of pollution (i.e. Ma, 1996; Ma and Talmage, 2001; Sánchez-Chardi et al., 2007a,b). In addition, some heavy metals have varied genotoxic effects because they may damage and induce mutations in DNA (Eisler, 1985, 1986; Seoane and Dulout, 2001; Palus et al., 2003). The micronucleus test (MNT) is an easy and quick method to obtain information about the clastogenic effect of xenobiotics and other environmental pollutants on wild mammals (i.e. Ieradi et al., 1996; Meier et al, 1999).

Like other insectivorous species (i.e. Talmage and Walton, 1991; Komarnicki, 2000), the greater white-toothed shrew, *Crocidura russula*, has been used as bioindicator of the bioaccumulation of metals and the effects of pollution (Sánchez-Chardi et al., 2007a,b). To our knowledge, the study presented here is the first to assess the accumulation of metals and effects of landfill pollution on insectivorous mammals.

The objectives of this study were: i) to quantify the concentrations of heavy metals in *C. russula* exposed to leachates from a landfill; ii) to evaluate the effects of this kind of pollution on the basis of several morphological and genotoxic parameters; iii) to identify the influence of sex and age as source of intrapopulation variation; and iv) to assess the environmental consequences of landfill pollution particularly in protected areas.

2. Material and methods

2.1. Study sites

Opened in 1974, each year the landfill of Garraf receives about 850000 t of solid wastes mainly of domestic origin but also significant amounts of industrial wastes and sewage sludge from sewage treatment plants. With a capacity of 17 M m^{-3}, the landfill is located on the Garraf massif, a karstic area characterized by Mediterranean climate and xerophytic vegetation. The chemical characterization of landfill leachates from the year 2001 was provided by the Metropolitan Environmental Authority and is shown in Table 1. Unfortunately, to our knowledge there are no data available for the same parameters corresponding to the year of captures. The reference site has similar vegetation and climate conditions to the polluted site, and is not affected by anthropogenic activities (Fig. 1). Moreover, since 1986 an area of 12376 ha, including both study sites, enjoys protected status as the "Parc Natural del Garraf".

From February to April 1998, $n = 55$ greater white-toothed shrews were collected with Sherman traps in the polluted site ($n = 21$), downstream of the leachate pool and affected by the landfill (Vall d'En Joan) and $n = 34$ from the reference site (Olesa de Bonesvalls). The total capture effort was 1600 traps $night^{-1}$ (TN). Specimens were transported to the laboratory and treated following legal and ethical procedures. The body weight (BW) to the nearest 0.01 g and body length (BL) to the nearest 0.01 mm of all specimens were measured. The shrews were lightly anaesthetized and killed by cervical dislocation. Liver and kidneys were immediately removed, weighted, and frozen at −20 °C prior to chemical analyses. Relative age and sex were determined for all individuals as described in Sánchez-Chardi et al. (2007a,b).

Table 1
Detection limit, mean ± standard deviation (M ± SD), and range of metals (in mg kg^{-1}), other compounds (in mg l^{-1}: cyanide, nitrites, nitrates, phenols; in mg kg^{-1}: Hydrocarbons), and pH in leachates from the Garraf landfill

	Detection limit	M ± SD	Range
Iron	0.1	6.38 ± 1.84	3.70–7.60
Magnesium	10	86.75 ± 16.34	77.00–111.00
Lead	0.5	n.d.	
Mercury	0.01	0.05 ± 0.01	n.d.–0.02
Cadmium	0.1	0.62 ± 0.10	n.d.–0.14
Zinc	0.1	1.15 ± 0.60	0.60–2.00
Copper	0.1	0.88 ± 0.21	0.70–1.10
Manganese	1	n.d.	n.d.
Chromium	0.1	0.62 ± 0.10	0.51–0.80
Nickel	0.1	0.36 ± 0.05	0.30–0.40
Cyanide	0.01	0.11 ± 0.09	n.d.–0.13
Nitrates	100	n.d.	
Nitrites	0.5	n.d.	
Phenols	0.2	3.47 ± 2.97	0.70–8.00
Hydrocarbons	5	29.05 ± 17.35	7.20–43.00
pH		8.41 ± 0.12	8.28–8.79

n.d.: non detected values.

2.2. Morphological parameters

The residual index (RI) was calculated following Jakob et al. (1996) as a regression of BL and BW. Specimens with positive RI values are considered to be in better condition than predicted for their weight and lenght. Consequently, negative values, down to the linear regression, are attributed to animals with lower body condition than expected. The relative hepatic and renal weight ratios were calculated as a percent ratio of somatic tissue (100× tissue weight/body weight). All morphologic parameters were calculated on a wet weight (WW) basis.

2.3. Chemical analyses

The material used for the digestion was thoroughly acid-rinsed. The tissues were dried at 60 °C till constant weight (48 h). From 50 to 100 mg of dry sample was digested by 5 ml of nitric acid (Instra, Baker-Analized) and 2 ml of perchloric acid (Instra, Baker-Analized), in open tubs in a Prolabo Microdigest A301 microwave placed in a clean room. Mg and Fe concentrations were determined by a Perkin–Elmer OPTIMA-3200RL Inductively Coupled Plasma Optical Spectrometer (ICP-OES), while Pb, Hg, Cd, Zn, Cu, Mn, Mo, and Cr were measured by a Perkin–Elmer ELAN-6000 Inductively Coupled Plasma Mass Spectrometer (ICP-MS) as described in Sánchez-Chardi et al. (2007a,b). For the purpose of statistical analyses, non-

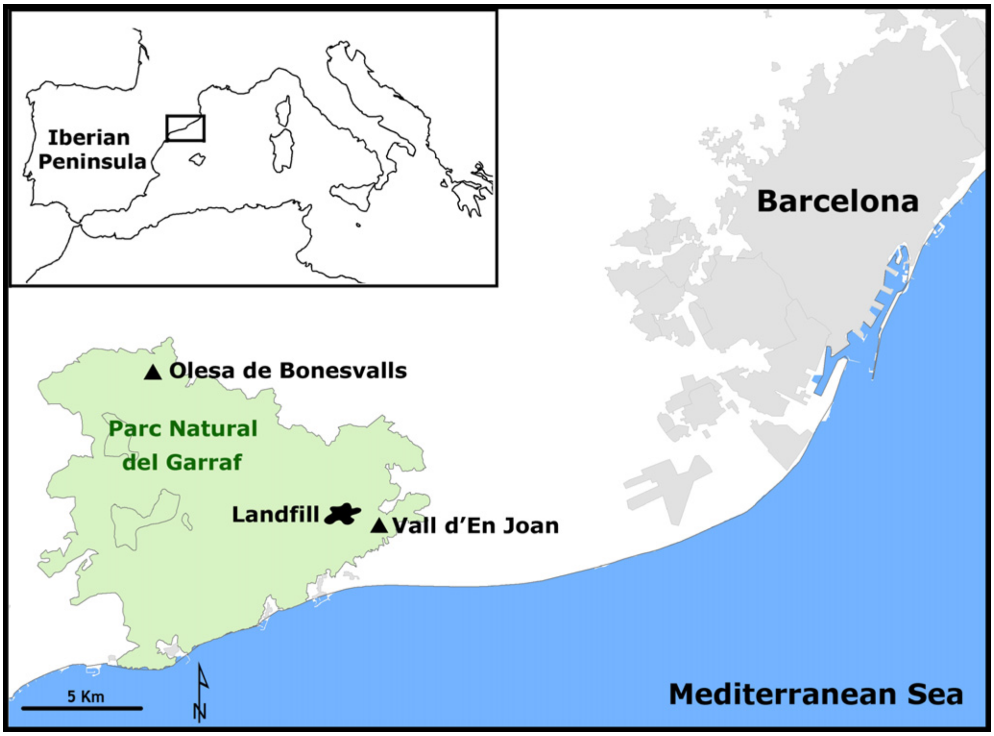

Fig. 1. Map showing the geographical location of the sites studied (▲).

detected values were replaced by the value equal to half the detection limit. The metal concentrations are presented as mean ± standard error (SEM) in $\mu g\, g^{-1}$ on dry weight (DW) basis.

2.4. Genotoxicity: the micronucleus test (MNT)

Peripheral blood was collected by cardiac punction into a heparinized syringe. Duplicate smears were made for each specimen on pre-cleaned microscope slides, they were then fixed with heat, and stained with conventional May-Grünwald Giemsa. For each individual, MN frequency was scored on 2000 blood erythrocytes through an oil immersion objective (×100) on a Leica Leitz DMRB microscope.

2.5. Statistical analyses

Data were log transformed and tested for normal distribution (Shapiro–Wilk test) and for homogeneity of variance (Levene test). From each tissue, the effect of sex, age, and site in metal concentration was obtained by three-way multivariate analysis of variance (MANOVA). Intra- and interpopulation comparisons of metals and divergences in RI and relative somatic ratios were evaluated by Student's tests (t), whereas differences in MN frequencies were assessed with Mann–Whitney test (U). To establish the relations between metal concentrations and MN frequency, Spearman correlation coefficients (r) were calculated in both liver and kidneys. Significant differences were accepted at $p < 0.05$. For all sequential tests, p-values were corrected by the Bonferroni adjustment (Rice, 1989). All statistical procedures were performed with SPSS (version 11.5 for Windows, SPSS Inc.).

3. Results

Captures of shrews were more abundant in the reference site (34 shrews in 550 TN) than in the landfill site (21 shrews in 1050 TN). In both sites, a large percentage of shrews were adults (30 out of 34 in the reference site and 14 out of 21 in the polluted site). A high number of males were captured in the reference site compared with the landfill site (Table 2). No differences in the morphological parameters measured were detected between sites (Table 3).

All the elements quantified in this study were detected in all samples of all specimens captured at either site, with two exceptions. Hg was not detected in any sample, and Cr was not detected in the kidneys of shrews from the reference site. MANOVA for all data showed that the importance of parameters in liver was: site ($F = 12.812$; $p < 0.001$) > age ($F = 3.010$; $p = 0.005$) > sex ($F = 1.365$; $p = 0.229$).

Table 2
Number of animals captured in the reference and landfill sites by sex and age

Site	Sex	Age		
		Juveniles	Adults	Total
Reference	Males	3	23	26
	Females	1	7	8
Landfill	Males	4	9	13
	Females	3	5	8

Table 3
Mean ± SEM of morphological parameters in shrews from the reference and landfill sites

	Reference site ($n = 34$)	Landfill site ($n = 21$)
BW (g)	7.64 ± 0.18	7.29 ± 0.34
BL (mm)	70.68 ± 0.61	69.02 ± 1.17
RI	0.357 ± 0.532	−0.578 ± 0.888
Liver (mg g^{-1} WW)	0.521 ± 0.016	0.492 ± 0.029
% Liver	6.83 ± 0.16	6.72 ± 0.17
Kidneys (mg g^{-1} WW)	0.116 ± 0.006	0.113 ± 0.005
% Kidneys	1.54 ± 0.08	1.61 ± 0.10

Metal bioaccumulation in kidneys showed a similar pattern, where site ($F = 7.526$; $p < 0.001$), age ($F = 3.052$; $p = 0.006$), and their interaction ($F = 2.546$; $p = 0.017$) were the main parameters influencing bioaccumulation, and sex ($F = 0.873$; $p = 0.581$) was the least important factor in variance.

No significant difference between males and females was found for any of the metals quantified. Adult shrews from both sites showed significantly higher Pb and Cd in liver (Landfill: $t = -2.121$, $p = 0.05$ and $t = -5.002$, $p < 0.001$, respectively; Reference: Cd: $t = -6.650$, $p < 0.001$) and kidneys (Landfill: $t = -2.245$, $p = 0.039$ and $t = -3133$, $p = 0.006$, respectively) and lower Cr concentrations in liver (Reference: $t = 3.679$, $p = 0.010$) and kidneys (Landfill: $t = 2.319$, $p = 0.035$). Moreover, levels of Fe, Mo, and Cu tended to increase with age in shrews from the two sites (Fig. 2).

Despite the age-dependent variation found in Pb, Cd and Cr, in order to increase sample size the age groups were pooled for comparisons of these elements by site. Specimens from the landfill site showed significantly more Pb, Cd, Zn, Mg, Cu, and Cr in liver and Pb, Cd, and Cr in kidneys compared with reference specimens (Table 4).

In both study sites, the liver was the main accumulator of Fe (Landfill: $t = 8.098$, $p < 0.001$; Reference: $t = 11.437$, $p < 0.001$), Mn (Landfill: $t = 14.851$, $p < 0.001$; Reference: $t = 15.845$, $p < 0.001$), and Mo (Landfill: $t = 4.680$, $p < 0.001$; Reference: $t = 7.283$, $p < 0.001$), whereas the kidneys bioaccumulated the highest concentrations of Pb (Landfill: $t = 7.382$, $p < 0.001$; Reference: $t = -6.482$, $p < 0.001$), and Cd (Landfill: $t = -3.756$, $p < 0.001$; Reference: $t = -2.614$, $p = 0.011$). Mg ($t = -2.702$, $p = 0.009$), Zn ($t = 2.803$, $p = 0.008$), and Cu ($t = 4.300$, $p < 0.001$) showed significantly high levels in livers of shrews from the polluted site. Moreover, in this polluted area Cr was significantly higher in kidneys compared with liver ($t = -2.658$, $p = 0.011$).

The shrews from the landfill site showed a significant increase in MN compared with those from the reference site (1.786 ± 0.272 vs. 0.088 ± 0.045‰; $U = 46.000$, $p < 0.001$). No difference was found by age or sex for this parameter at either site. Moreover, significant correlations were found between MN frequencies and Pb, Cd, and Cr for hepatic and renal tissues (Table 5).

4. Discussion

Although landfills are common in several countries, few ecotoxicological studies have addressed this kind of pollution. Leachate has been described as a complex mixture of organic compounds and metals (i.e. De Rosa et al., 1996; Wichmann et al, 2006). This chemical characterization is only the first step for a meaningful environmental impact (Pohland and Harber, 1986) and alone cannot generate sufficient information on impact because the absolute metal concentration alone does not reflect the degree to which these compounds affect the environment (Cheung et al., 1993). Data on the bioaccumulation and effects of leachates on wild populations are essential to assess the environmental impact of these disposal sites.

4.1. Metal bioaccumulation by site

Our results are consistent with those reported in liver and kidneys of insectivorous mammals (i.e. Talmage and Walton, 1991; Pankakoski et al., 1993, 1994; Komarnicki, 2000; Hamers et al., 2006). Nevertheless, concentrations of essential metals were slightly higher than data obtained for the same species (Sánchez-Chardi et al., 2007b), probably because of the particular conditions (alkaline pH, calcareous soil, dry climate) of the study sites.

When compared with reference specimens, the shrews from the landfill site showed more Pb, Cd, Mg, Zn, Cu, and Cr in their tissues. Our results on bioaccumulation of Pb and Cd are concordant but higher than those reported by Torres et al. (2006) in the wood mouse, *Apodemus sylvaticus*, from the same landfill site. In fact, insectivores are suitable bioindicators of these non-essential elements because they ingest and/or bioaccumulate more Pb and Cd than sympatric species of rodents (i.e. Talmage and Walton, 1991; Dodds-Smith et al., 1992; Ma and Talmage, 2001). In the shrews from the landfill site, Hg concentrations were under detection limits (0.20 μg kg^{-1} in the diluted solution, approximately 0.40 μg g^{-1} DW), which is in agreement with low levels in leachates of Garraf (Table 1) and other landfills (revision in Christensen et al., 2001).

Magnesium is an abundant cation and a main constituent of the colloidal mass in leachates (Gounaris et al., 1993) and it can reach high concentrations in soils near landfill sites. However, because of its low toxicity, few ecotoxicological studies have measured. An increase in this element was also reported in the Algerian mouse, *Mus spretus*, and the rat, *Rattus rattus*, from a pyrite mine site (Pereira et al., 2006).

The increase in Zn and Cu concentrations in liver of shrews from the landfill site may be related, at least partially, to protective and/or detoxification regulation. These elements have a strong physiological regulation in mammals and a high increase in concentrations in mammalian tissues has been reported only in cases of very high intake or disrupted metal metabolism (e.g. Goyer, 1997; Ma and

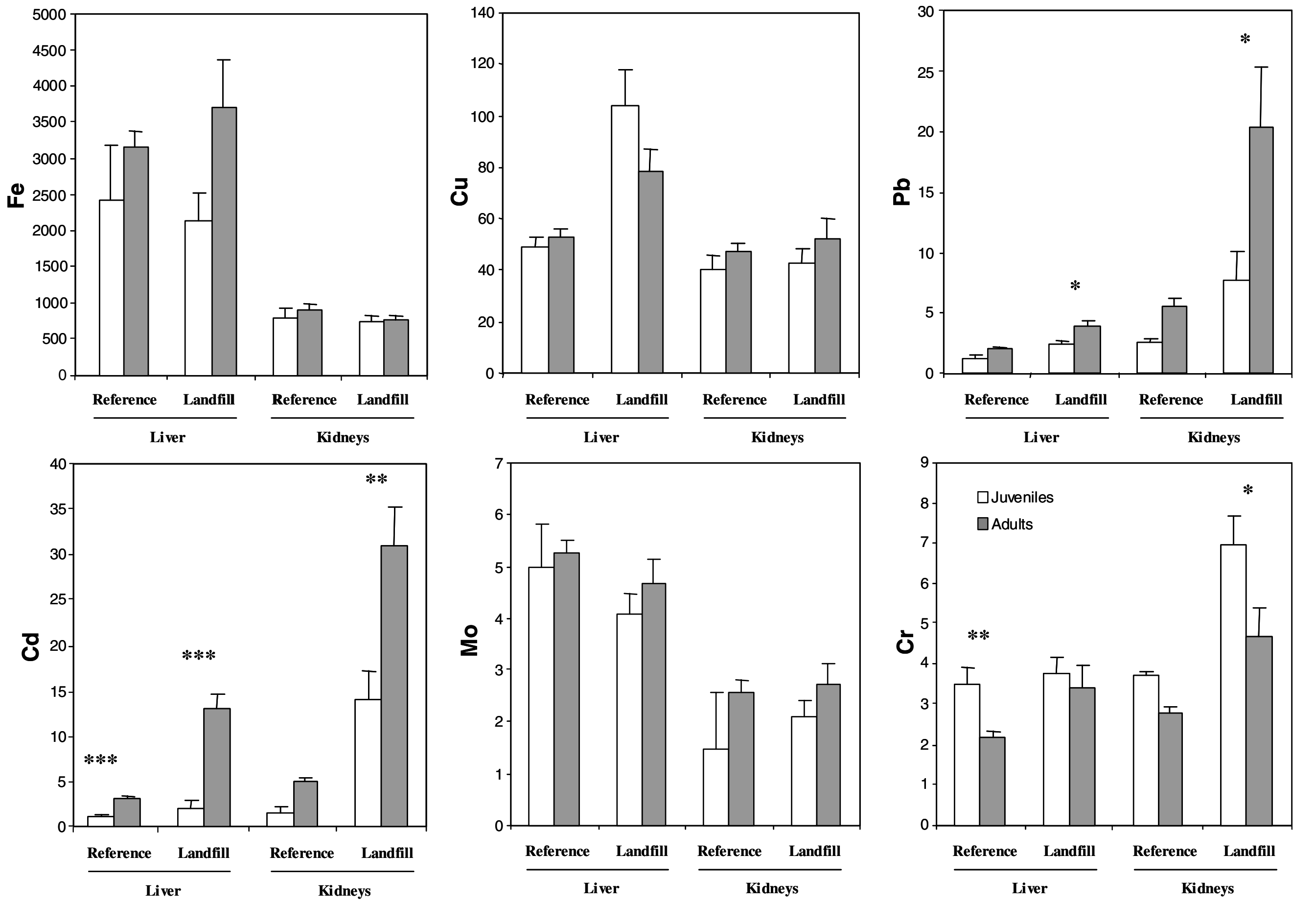

Fig. 2. Mean ± SEM values for metals (Fe, Pb, Cd, Cu, Mo, and Cr) in *C. russula* by tissue, site and age (in μg g^{-1} DW) (*$p \leqslant 0.05$; **$p \leqslant 0.01$; ***$p \leqslant 0.001$).

Table 4
Mean ± SEM values for metals in *C. russula* by tissue and site (in μg g^{-1} DW)

	Liver				Kidneys			
	Reference site ($n = 34$)	Landfill site ($n = 19$)	t	p	Reference site ($n = 31$)	Landfill site ($n = 19$)	t	p
Fe	3085.27 ± 230.29	3304.48 ± 502.00	–	–	895.76 ± 74.02	755.00 ± 39.33	–	–
Mg	1392.74 ± 182.09	1608.63 ± 76.49	3.145	0.003	1490.34 ± 20.93	1455.26 ± 37.42	–	–
Pb	1.93 ± 0.20	3.40 ± 0.49	3.263	0.002	5.37 ± 0.69	16.36 ± 3.78	3.213	0.003
Cd	3.03 ± 0.26	10.28 ± 1.63	3.202	0.004	4.65 ± 0.48	25.59 ± 3.58	9.129	<0.001
Zn	199.80 ± 6.80	232.26 ± 11.14	2.650	0.011	209.87 ± 6.23	194.65 ± 8.79	–	–
Cu	52.47 ± 3.03	84.90 ± 7.84	4.511	<0.001	46.97 ± 2.73	49.47 ± 5.42	–	–
Mn	37.42 ± 1.37	42.61 ± 2.20	–	–	16.52 ± 0.56	16.44 ± 0.69	–	–
Mo	5.24 ± 0.22	4.50 ± 0.38	–	–	2.49 ± 0.24	2.53 ± 0.29	–	–
Cr	2.31 ± 0.16	3.49 ± 0.45	2.864	0.006	n.d.	5.40 ± 0.59	3.611	0.001

Table 5
Spearman coefficients (r) and p-values between metals and MN frequencies in liver and kidneys of *C. russula*

	Liver		Kidneys	
	r	p	r	p
Pb	0.388	0.004	0.254	–
Cd	0.454	0.001	0.709	<0.001
Cr	0.104	–	0.317	0.025

Talmage, 2001). However, lower increases, as found in the present study, have been reported in small mammals exposed to heavy metals (i.e. Talmage and Walton, 1991; Pankakoski et al., 1993; Ieradi et al., 1996; Pereira et al., 2006). Zinc and copper interact with many chemicals and participate in detoxification processes, as part of the enzymes of the antioxidant systems, such as superoxide dismutase (SOD), and in metallothioneins (MT).

Chromium is also a common metal in leachates and landfill composts. This metal leaches rapidly and is often organically complexed in these effluents, thereby becoming more bioavailable and/or mobile through the soil (Gounaris et al., 1993; Outridge and Scheuhammer, 1993; De Rosa et al., 1996; Gagnon and Saulnier, 2003). Chromium concentrations in shrews from landfill site are among the highest found in liver and kidneys of small mammals (Eisler, 1986; Talmage and Walton, 1991; Pankakoski et al., 1993, 1994). This observation indicates the increased bioavailability of this metal in the polluted site.

Manganese and iron are redox-sensitive elements that generate hydroxides after oxygenation of leachates, thereby becoming important carriers of trace elements (i.e. Ragle et al., 1995). No significant difference between sites found in our study indicates a low bioavailability and/or a proper physiological regulation of these elements in shrews. Moreover, no differences in Mo concentrations between sites may be explained by low levels of this element derived from food consumption (Gagnon and Saulnier, 2003).

4.2. Metal bioaccumulation by age and sex

A clear bioaccumulation of Cd by age was observed in shrews from the two sites, in concordance with data reported in insectivores (i.e. Pankakoski et al., 1993, 1994; Sánchez-Chardi et al., 2007b). In particular, in the renal tissue the increase in concentration was up 6-fold between juveniles and adults from the landfill site. This bioaccumulation is related to the formation of stable Cd-MT complexes as a detoxification mechanism to reduce toxic effects (i.e. Ma and Talmage, 2001). The increase of Pb with age was also reported in *C. russula* bones from a polluted wetland (Sánchez-Chardi et al., 2007a), but was not demonstrated in soft tissues of this species inhabiting a pyrite mine site (Sánchez-Chardi et al., 2007b). In fact, adult shrews from this polluted mining site showed an increase of Pb in liver compared with juveniles (4.81 ± 0.97 vs. $5.81 \pm 1.20\ \mu g\ g^{-1}$, respectively), but this pattern was not observed in the reference site. This result may be attributed to the differences in exposure levels and/or chemical forms of Pb as well as to interpopulation variation in metal bioaccumulation. A decrease of Cr with age has also been reported for the same species and other insectivores (Pankakoski et al., 1993, 1994; Sánchez-Chardi et al., 2007b). This decrease was attributed to a high digestive absorption rate in juveniles and, therefore, a poor intestinal absorption in adults (Eisler, 1986; Outridge and Scheuhammer, 1993). Despite no statistically significant differences for Fe, Cu, and Mo, we observed a general tendency of increase concentrations of these metals in adults, as reported for *C. russula* (Sánchez-Chardi et al., 2007b). High concentrations of Cu in the liver of juveniles of several mammalian species in polluted sites have been reported, including *C. russula* inhabiting a pyrite mine (Sánchez-Chardi et al., 2007b). Because of the high metabolic rates during this growth period, young animals may are highly exposed to xenobiotics taken up mainly through diet. Cu has important roles in detoxifying function in Cu-containing metallothioneins, which may be found in high concentrations in livers of young mammals, and in protective systems against oxidative stress as part of enzymes such as CuZnSOD. These physiological functions of Cu may explain, at least in part, these increases found in juvenile shrews inhabiting metal-polluted sites.

In the present study, we did not detect any significant differences in metal concentrations between males and females. However, females showed more Mo than males, especially in the liver of shrews from the polluted site (5.24 ± 0.79 vs. $4.07 \pm 0.36\ \mu g\ g^{-1}$), in concordance with data obtained for the same species (Sánchez-Chardi et al., 2007b). On the whole, given the asymmetric number of captures by age and sex (see Table 2) as well as the high variation inherent in wild populations, as shown for the common shrew, *Sorex araneus*, and the mole, *Talpa europaea* (i.e. Talmage and Walton, 1991; Dodds-Smith et al., 1992; Komarnicki, 2000), we consider that more studies are required to confirm these results.

4.3. Metal bioaccumulation by tissue

Similar results on tissue distribution of Fe, Mg, Cd, Zn, Cu, Mn, and Mo were found in the same species inhabiting a pyrite mine site (Sánchez-Chardi et al., 2007b). In this previous article, Cr did not reach significantly high concentrations in livers, whereas in the present study significantly high concentrations were found in kidneys of shrews from the polluted site. Also, Pb distribution reached higher concentrations in kidneys when compared with liver in the shrews from the Garraf area. This distribution is not consistent with previous data reported for *Crocidura* species (Topashka-Ancheva and Metcheva, 1999; Sánchez-Chardi et al., 2007b) but agrees with main results for small mammals (i.e. Talmage and Walton, 1991; Pankakoski et al., 1994; Milton et al., 2003). Although tissue distribution of metals is usually constant within specific target organs, differences in bioaccumulation patterns have been reported for *S. araneus* and *T. europaea* (Talmage and Walton, 1991; Dodds-Smith et al, 1992; Komarnicki 2000; Swiergosz-Kowalewska et al., 2005). This phenomenon may be related to physiological mechanisms to decrease toxicity, type and time of exposure, and/or the half-life of metals in soft tissues.

4.4. Captures and morphological parameters

Distribution of captures by age and sex agrees with data reported by López-Fuster (1985) for this species. More males were captured because at the start of the breeding period (February–March) they are more active on the ground surface and wander in search of females (Churchfield, 1990). Moreover, specimens from the Garraf landfill had a lower body condition than reference shrews (see Table 3). The lower percentage of captures of *C. russula* in the former may indicate a fall in shrew population. In fact, an increase in bird and rodent populations and a decrease in captures of shrews in zones near landfill sites have been reported (Schroder and Hulse, 1979; Gabrey, 1997; Elliott et al., 2006). Although data are scarce, these observations indicate that landfill sites are not suitable habitats for these mammals. This apparent decrease in insectivorous populations may be a result of the specific toxicity of leachates in shrews and/or in their prey or of competition with rats and other rodents, which are abundant in waste disposal sites.

Our results for morphological parameters are similar to those reported for the same species (i.e. Bartels et al., 1979; Sánchez-Chardi et al., 2007a,b) and for other small mammals from polluted and reference sites (Ma, 1996; Milton et al., 2003; Pereira et al., 2006). No differences in these parameters were found between sites, indicating similar general health conditions in the two sites.

4.5. Genotoxicity

Our data are consistent with available literature reporting background mean MN frequencies of 2.43, 0.92, 0.1, and 0.00–1.74‰ in least shrew, *Cryptotis parva*, hedgedog, *Erinaceus europaeus*, bat *Artibeus jamaicencia*, and a few rodent species, respectively (Ieradi et al., 1996; Meier et al., 1999; Zúñiga-González et al., 2000). Leachates contain a large variety of compounds, and some are mutagens that may induce genotoxic effects in biota (Cabrera and Rodriguez, 1999; Sang and Li, 2004). In fact, the increase in MN frequencies and other genotoxic damage in laboratory mice exposed to landfill leachates may be induced by free radicals produced by oxidative stress in response to metal exposure (see Li et al., 2006). Moreover, Pb, Cr and Cd have clastogenic and/or genotoxic effects (i.e. Eisler, 1985, 1986; Tull-Singleton et al., 1994) and the MN frequencies increase after exposure to these elements (Ieradi et al., 1996; Seoane and Dulout, 2001; Palus et al., 2003). In agreement with these previous data, the shrews examined in our study showed high correlations between MN and Pb, Cr and Cd concentrations. However, wild populations from polluted sites are rarely exposed to a single compound and landfill sites are no an exception. Leachates are complex mixtures containing a wide range of organic and inorganic compounds that are potentially toxic. Despite the high metal-MN correlations detected, the influence of other potentially genotoxic compounds from leachates, such as several hydrocarbons and nitrogen-, chlorine- and phosphorous-containing compounds, on the increase in MN frequencies cannot be ruled out.

4.6. Toxicity effects of metals bioaccumulated

Landfill leachates form a widely variable and complex mixture of toxic compounds. A low pH is crucial to increase mobility and/or bioavailability of several metals, consequently a reduction of this transfer across the food-chain may be expected in the calcareous area of Garraf. In contrast, in these leachates Zn, Cu, and Cr, among other metals, are often associated with organic matter and colloids that increase their lability and enhance their dispersion in soils and waters (Gounaris et al., 1993; Yin et al., 2002; Slack et al., 2005). The high metal concentrations observed in shrews from the landfill site suggest that a high exchangeability of metals from leachates produces high bioavailability of metals at levels that are potentially toxic for biota (Cheung et al., 1993; Cabrera and Rodriguez, 1999; Li et al., 2006). Although insectivores show high tolerance to metal pollution (Talmage and Walton, 1991; Ma, 1996; Ma and Talmage, 2001), the Pb and Cd concentrations reported in the specimens from the landfill site may reach toxic levels. The mean Pb concentration in kidneys of shrews from this site ($16.36 \pm 3.78\ \mu g\ g^{-1}$ DW) was slightly higher than critical renal Pb level of $15\ \mu g\ g^{-1}$ DW (Ma, 1996) considered a chemical biomarker of toxic exposure to this metal in mammals. In our study, 7 out of 19 shrews in the Garraf landfill site showed more than $15\ \mu g\ g^{-1}$ of this metal in renal tissue (maximum of $59.71\ \mu g\ g^{-1}$ DW). Among small mammals, insectivores are more tolerant to Cd than rodents. Liver and kidney lesions in common shrews have been associated with high Cd concentrations of 577 and $253\ \mu g\ g^{-1}$ DW, respectively (revision in Ma and Talmage 2001), whereas concentrations of $110–260\ \mu g\ g^{-1}$ produce a nephrotoxic effect in mice (Pankakoski et al., 1993), and bank voles, *Clethrionomys glareolus*, show hepatic and renal alterations with Cd concentrations of 15.1 and $17.0\ \mu g\ g^{-1}$ DW, respectively (Wlostowski et al., 2003). The Cd concentrations observed in shrews from the landfill site were higher than the no-observable-adverse-effects-level (NOAEL) in rodents (maximum in liver and kidneys of 20.32 and $56.57\ \mu g\ g^{-1}$ DW, respectively) indicating a considerable environmental pollution. The concentrations for the remaining elements do not indicate a risk of poisoning or toxic effects on mammals. Furthermore, cumulative effects and/or interactions between potentially toxic metals and with other compounds cannot be disregarded.

4.7. Pollution in protected areas

Environmental legislation is abundant and in some cases very restrictive in developed countries. In fact, areas with partial or complete protection status are becoming increasingly more common, although in some cases protection status is established after a pollution event, as occurred in Garraf. The "Parc Natural del Garraf" reached a partial protection status 12 years after the opening of the largest landfill site in Spain. To improve the management of this natural interesting area, a plan was designed to close the landfill at the end of 2006 and restore the site. Disturbances that occur outside a protected area can affect biota. However, events within a protected area may alter the health status of animals by reducing life expectancy because specimens are more exposed to predators, by impairing reproduction, and/or by affecting population dynamics. Moreover, when natural communities are subjected to severe disturbances such as a landfill, an increase in the population density of opportunistic species may ocurr (i.e. Schroder and Hulse, 1979; Gabrey, 1997; Elliott et al., 2006) since these sites provide abundant nutrients (solid wastes and leachates with nitrogen and phosphorous). However, the landfill area may also become less suitable for wildlife due to these pollution sources often lead to increased environmental concentrations of toxic compounds, which stress wild populations, even if acute poisoning effects are not observed (Pankakoski et al, 1994). To our knowledge, the literature on ecotoxicological data in wild populations and terrestrial ecosystems exposed to landfill pollution is scarce. We therefore consider our data of particular relevance for the management of this protected area and for the biomonitoring of this source of pollution after the closure of the site.

Leachates contain compounds, such as metals, that may be long-term highly bioavailable for biota living some distance from the pollution source (i.e. Gagnon and Saulnier, 2003). In fact, the landfill of Garraf pollute groundwater, a very persistent pollution. Our results show that this site also has a considerable impact (bioaccumulation of metals at toxic levels and genotoxic damage) on wild populations of insectivorous mammals. However, the extent of the area affected by the dispersion of leachates and of the impact in individuals, communities and ecosystems is unknown.

5. Conclusions

The leachates from the Garraf landfill are a considerable source of metals. They produce an increase in Pb, Cd, Zn, Cu, and Cr in tissues of the shrew *C. russula*. Age-dependent was greater than sex-dependent variation on the intrapopulation variance of metal levels. Leachate pollution also produced genetic damage to shrews, which demonstrate the effects of this kind of pollution on health. This study also shows that the greater white-toothed shrew, *C. russula*, is a suitable species for biomonitoring this kind of pollution.

These findings are relevant for the management of wastes and for the evaluation of the hazardous effects of landfill leachates on biota. Moreover, to efficiently manage areas of ecological interest, we propose a continuous biomonitoring study to assess the effects of the Garraf landfill in terrestrial ecosystems.

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Bioaccumulation of metals and effects of a landfill in small mammals Part III: Structural alterations ☆

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ABSTRACT

The leachates from the Garraf landfill located in a protected site (NE Spain) contain several potentially toxic substances such as heavy metals. Here we report the histopathological alterations produced by this pollution in wild specimens of an omnivorous species, the wood mouse, *Apodemus sylvaticus*, and an insectivorous species, the greater white-toothed shrew, *Crocidura russula*. Hepatic tissue presented the most severe alterations in both the species, namely cell cycle arrest (apoptosis and necrosis), inflammation, preneoplasic nodules, vacuolation and microsteatosis. The kidneys were altered more in the mice (presenting tubular necrosis and dilatation, inflammation, and cylinders) than in the shrews, suggesting that different metabolic pathways render shrews more tolerant to renal toxicity induced by pollutants. No pollution-related alterations were observed in lung, spleen, pancreas, gonads, oesophagus, intestine, or adrenals. We conclude that the two species could be used in conjunction as bioindicators to assess the effects of environmental pollution at different trophic levels.

1. Introduction

Wild populations inhabiting polluted sites are often exposed to a mixture of chemical pollutants which are mainly taken in with the food. These pollutants result in multiple stresses and affect biological systems at virtually all levels, from molecules to ecosystems. The assessment of the risks of the diversity of pollutants to natural populations is difficult as this depends on a variety of abiotic (e.g. temperature, pluviosity) and biotic (e.g. age, gender, species) factors. However, studies under field conditions provide crucial ecotoxicological data on bioindicator species that can provide information on the quality of the environment (e.g. Pereira et al., 2006) and how to manage protected areas (e.g. Sánchez-Chardi and Nadal, 2007; Sánchez-Chardi et al., 2007, 2009). The species used for monitoring purposes should cover various levels of the food chain, like primary (herbivorous) and secondary (carnivorous) consumers. Thus, terrestrial small mammals (rodents and insectivores) have often been used as bioindicators of physical and chemical pollution (revisions in Ma and Talmage, 2001; Sheffield et al., 2001; Talmage and Walton, 1991). The wood mouse, *Apodemus sylvaticus* (Rodentia, Mammalia), is a widespread species often abundantly present in nature areas. Although occasionally showing carnivorous behaviour, this mouse is mainly a primary consumer and as such is often used as bioindicator of pollution (e.g. González et al., 2008; Rogival et al., 2007). The greater white-toothed shrew, *Crocidura russula* (Soricomorpha, Mammalia), is a relatively common secondary consumer in South Europe, predating on arthropods, molluscs and earthworms. It has recently been reported as a suitable species for ecotoxicological studies (González et al., 2008, 2009; Sánchez-Chardi and Nadal, 2007; Sánchez-Chardi et al., 2007, 2008, 2009; Wijnhoven et al., 2007). The use of wild specimens in ecotoxicological studies includes aspects of bioavailability, toxicity and detoxification mechanisms, and specific or individual exposure and susceptibility as determining factors for environmental risk under natural conditions. However, most information concerning the toxic effects of pollutants, such as heavy metals, on the structure and function of organs have derived from laboratory experiments on animals exposed to a single compound (Damek-Poprawa and Sawicka-Kapusta, 2004; Hoffmann et al., 1975; Koyu et al., 2006; Włostowski et al., 2000) and, less frequently, to multiple compounds (Jadhav et al., 2007; Silva de

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Assis et al., 2005) or complex mixtures such as landfill leachates (Li et al., 2006a, b; Świergosz-Kowalewska et al., 2006; Talorete et al., 2008). This controlled assessment of individual health status is the basis for further assessments of the true effects of pollutants on wild populations and dynamic natural systems.

Landfills are a considerable source of pollution in Mediterranean countries (Loukidou and Zouboulis, 2001), including Spain. The decomposition of disposed waste or the interaction between water and waste leads to the formation of liquid effluents named leachates. If landfills are not adequately controlled and sealed, leachates enter natural systems and cause toxic effects in plants and animals (Li et al., 2006a, b; Wilke et al., 2008). The quantity, composition and toxicity of leachates vary depending on the nature and age of wastes, the method of disposal, dump depth and climatic factors (see references in Sánchez-Chardi et al., 2007). Landfill effluents often contain a wide variety of organic and inorganic pollutants, including potentially toxic metals such as Pb, Cd, Fe, Zn, Cu, Mn, Mo, and Cr (see references in Sánchez-Chardi et al., 2007). These elements may be responsible for oxidative stress and a number of genotoxic effects reported in culture cells and laboratory rodents (Bakare et al., 2005; Li et al., 2006a, b; Talorete et al., 2008; Thomas et al., 2009) and wild mice from a waste site (Tull-Singleton et al., 1994). The induction of oxidative stress as a result of the production of reactive oxygen species (ROS) can damage lipids, thiol-proteins and nucleic acids (Jadhav et al., 2007 and references herein) thereby producing large alterations to tissues and ending in cell cycle arrest (Damek-Poprawa and Sawicka-Kapusta, 2003, 2004; Goyer, 1997; Sánchez-Chardi et al., 2008; Świergosz-Kowalewska et al., 2006). Although hepatic and renal tissues are the primary targets of pollutants ingested with the diet (e.g. Koyu et al., 2006; Włostowski et al., 2000), metals can also affect other organs and tissues, such as blood, gonads, spleen, lung, brain and bones (Damek-Poprawa and Sawicka-Kapusta, 2003; Jadhav et al., 2007; Li et al., 2006a, b), as reported in histopathological evaluations for wild rodents inhabiting near metallurgical industries (Damek-Poprawa and Sawicka-Kapusta, 2004) and abandoned mines (Pereira et al., 2006). To our knowledge, the present study is the first to compare the histopathological alterations caused by environmental pollution in two species of small mammals placed at different trophic position; a murid (wood mouse) and a crocidurine (greater white-toothed shrew). Despite the scarce information on structural alterations in wild mammals exposed to landfill leachates, previous studies in the Garraf landfill (NE Spain) reported the bioaccumulation of highly toxic metals such as Cd, Zn, Cu, and Cr and also morphological, biochemical and genotoxic alterations in *A. sylvaticus* and *C. russula* exposed to leachates (Sánchez-Chardi and Nadal, 2007; Sánchez-Chardi et al., 2007). From 1974 to 2006, the Garraf landfill served as a disposal site for domestic and industrial wastes and solid sludge from the metropole of Barcelona, accumulating about 25 million tonnes of waste. In 1986, the karstic area of Garraf granted protection status in recognition of its singular habitats and the endangered species found there. Therefore, the need to biomonitor of the landfill site increased.

The main objectives of this study are: (i) to qualify and quantify histological alterations in target tissues of two sympatric species of small mammals (a rodent and an insectivore) exposed to landfill leachates; (ii) to compare toxicity in these two species, which differ in their metabolism and trophic position; (iii) to identify the influence of sex and age as source of intra-species variation; (iv) to correlate histological alterations with metal bioaccumulation and other biomarkers; and (v) to assess the environmental consequences of landfill pollution particularly in protected areas.

2. Materials and methods

2.1. Study sites

The two study sites are located on the karstic massif of Garraf (NE Spain), a coastal system formed by hills of about 700 m, traversed by several valleys and covered by Mediterranean xerophytic vegetation. The area is located 30 km south of Barcelona city, and has granted partial protection as "Parc del Garraf". Using Sherman live traps, 49 specimens of the wood mouse, *A. sylvaticus*, and 28 specimens of the greater white-toothed shrew, *C. russula*, were trapped at two sites from February to May 1998. One polluted site called "Vall d'En Joan" is in the vicinity of the pool of the leachates, placed at the lower end of a valley. The reference site ("Olesa de Bonesvalls") is also a valley close to the landfill. No sources of pollution are known for this area.

Specimens were transported to the laboratory for dissection, following all ethical procedures for experimental animals. Sex was determined during dissection and relative age was determined on the basis of the tooth wear (see references in Sánchez-Chardi and Nadal, 2007; Sánchez-Chardi et al., 2007). The number of specimens by species, capture site, sex and age is described in Table 1. Concentrations of potentially toxic metals in liver and kidneys are reported in Table 2.

2.2. Histopathological evaluation

After dissection, the gonads, the adrenals and the left kidney and a small portion of lung, oesophagus, spleen, liver, intestine, and pancreas of all specimens were immediately fixed in 10% neutral-buffered formaldehyde. Samples of all lesions observed macroscopically were also taken and fixed. Tissues were individually dehydrated in ethanol series, cleared in toluene and embedded in Paraplast Histocomp (Vogel). Sections of 5–10 μm were stained with conventional hematoxylin and eosin and mounted in DPX. A Leitz DMRB light microscope with a camera Leica DC 500 was used for observations.

The incidence of alterations was reported in a qualitative evaluation. For statistical purposes, the alterations were measured on a semi-quantitative scale. A global score for each tissue was assigned to each specimen in a conventional scale on the basis of the severity and/or extent of lesions: without alterations (−), slightly altered (+), intermediately altered (++), and strongly altered (+++). A global score was given to each sample (0, 1, 2, 3). All results are expressed as mean and standard error of the mean (M $\pm$ SEM).

2.3. Statistical analyses

For each tissue and species, the results of the semi-quantitative assessment were compared by site, age and sex using Mann–Whitney tests (*U*). To detect relations between histopathology and other biomarkers, Spearman's correlation coefficients (*r*) were calculated between the histopathological evaluation and the bioaccumulation of metals and morphometric, plasmatic, and genotoxic parameters for each species. Significant differences were accepted at $p<0.05$. For all sequential tests, *p*-values were corrected using the Bonferroni adjustment. All statistical procedures were performed with the SPSS package (version 15.0 for Windows, SPSS Inc.).

3. Results

In general, the organs of specimens of *A. sylvaticus* and *C. russula* from the reference site showed healthy aspect. Livers had a compact structure and hepatocytes had a normal shape (Fig. 1A and F). Kidneys had a well developed cortex and medulla

Table 1
Number of animals captured in the reference and landfill site by species, site, age and sex (in brackets: males, females).

Species	Site	Age		Total
		Juveniles	Adults	
A. sylvaticus	**Reference**	5 (2, 3)	20 (12, 8)	25 (14, 11)
	Landfill	11 (6, 5)	13 (9, 4)	24 (15, 9)
C. russula	**Reference**	1 (1, 0)	15 (11, 4)	16 (12, 4)
	Landfill	3 (1, 2)	9 (9, 0)	12 (10, 2)

Table 2
Mean±SEM values for metals in liver and kidneys of wood mice, *A. sylvaticus*, and greater white-toothed shrews, *C. russula*, from the reference and the landfill sites (in $\mu g\,g^{-1}$ DW).

	Liver		Kidneys	
	Reference site ($n = 23$)	**Landfill site ($n = 27$)**	**Reference site ($n = 23$)**	**Landfill site ($n = 28$)**
A. sylvaticus				
Fe	619.23±41.50	874.74±66.64**	513.72±21.09	589.11±17.55**
Mg	1349.83±33.68	1428.49±38.35	1418.85±36.10	1525.62±36.06
Pb	0.41±0.05	0.68±0.11	0.73±0.08	1.10±0.18
Cd	0.30±0.08	0.44±0.15*	0.92±0.24	1.44±0.48
Zn	162.07±5.73	200.49±13.26*	142.76±4.99	135.88±3.13
Cu	20.68±1.36	39.16±10.36*	20.66±0.86	23.45±0.67*
Mn	7.95±0.95	10.23±1.80*	4.87±0.40	6.59±1.00
Mo	3.16±0.24	4.38±0.16***	1.65±0.11	1.92±0.20
Cr	0.71±0.05	1.32±0.10***	n.d.	3.61±0.32***
	Liver		**Kidneys**	
	Reference site ($n = 34$)	**Landfill site ($n = 19$)**	**Reference site ($n = 31$)**	**Landfill site ($n = 19$)**
C. russula				
Fe	3085.27±230.29	3304.48±502.00	895.76±74.02	755.00±39.33
Mg	1392.74±182.09	1608.63±76.49**	1490.34±20.93	1455.26±37.42
Pb	1.93±0.20	3.40±0.49**	5.37±0.69	16.36±3.78**
Cd	3.03±0.26	10.28±1.63**	4.65±0.48	25.59±3.58***
Zn	199.80±6.80	232.26±11.14*	209.87±6.23	194.65±8.79
Cu	52.47±3.03	84.90±7.84	46.97±2.73	49.47±5.42
Mn	37.42±1.37	42.61±2.20***	16.52±0.56	16.44±0.69
Mo	5.24±0.22	4.50±0.38	2.49±0.24	2.53±0.29
Cr	2.31±0.16	3.49±0.45*	n.d.	5.40±0.59***

*$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$ (after Sánchez-Chardi and Nadal, 2007; Sánchez-Chardi et al., 2007).
n.d.: Not detected.

(Fig. 2A, B, and J). The following alterations were observed by the following species:

- *A. sylvaticus*: A total of 21 out 24 specimens from the landfill site showed altered tissue structure. In contrast, only 11 out of 25 mice from the reference site showed histological alterations. Focal cell death, apparently produced by apoptosis (no surrounding inflammatory reaction was present), was observed in livers of a number of individuals from the two sites, although more frequent and severe in the specimens from the landfill site (Table 3). Extensive necrotic areas (with or without inflammation) often surrounded by vacuoled hepatocytes or with microsteatosis were simultaneously observed in several specimens irrespective of the zone; however, these alterations were also more frequent at the polluted site. One individual from the polluted site showed severe inflammation and another showed several preneoplasic foci in the liver (Fig. 1). There were no signs of morphological injury in the hepatic portal vein. The degree of hepatic damage was significantly higher in the animals from the polluted site than in controls (1.04±0.20 vs. 0.36±0.15, $U = 183{,}000$; $p = 0.005$). In general terms, the kidneys showed few pathological features, although some cases of interstitial nephritis, tubular dilatation, focal tubular necrosis and hyaline cylinders (formed by proteic casts and/or cellular debris) in tubular lumina were seen. Pathological features were almost exclusively found (Table 3) in animals from the landfill site (Fig. 2C–I). On the whole, the level of renal damage, although low at both sites, was significantly higher at the polluted site (0.32±0.12 vs. 0.04±0.04, $U = 249{,}000$; $p = 0.041$). No lesions related to pollution were observed in lung, spleen, gonads, oesophagus, intestine, adrenals or pancreas. It should be noted that no murine chronic respiratory disease (CRD) was detected. Only one case of a small alveolar carcinoma was found in a specimen from the control site. Inflammatory reactions related to parasitosis (helminths and candidiasis) in spleen, seminiferous tubules, and intestine in six specimens (three from each capture site) were found (data not shown). These alterations not related to pollution were not considered in the present histopathological evaluation.
- *C. russula*: Nine out of 12 shrews of the landfill site showed hepatic alterations related to pollution; apoptosis, necrosis, periportal and perivascular lymphocite infiltration and signs of regenerating activity, vacuolation, and microsteatosis (Fig. 1G–J). Thirteen out of 16 animals from the reference site showed healthy tissue structure. No severe pathologies were observed in kidneys (Fig. 2K–N). The mean scores of alterations in liver for polluted and reference site were 0.92±0.28 vs. 0.06±0.05 ($U = 48{,}000$; $p = 0.008$) and 0.15±0.10 vs. 0.05±0.04 ($U = 100{,}000$; $p = 0.680$), respectively. Like in wood mice, no lesions related to pollution were observed in the other tissues evaluated. However, severe acute and chronic inflammatory reactions related to the presence of helminths, with presence of plasma cells, neutrophils and a high number of eosinophils, were found in airways, spleen, and digestive tract of six shrews from the polluted site and three from the reference site (data not shown). Severity and frequency of histological pathologies related with pollution for both species is summarized in Table 3.

No significant relations were found between sex or age and structural alterations. Few significant correlations were detected between hepatic histopathology and other parameters previously reported. In *A. sylvaticus*, hepatic alterations were correlated with relative liver weight ($r = 0.451$, $p = 0.002$), kidney weight ($r = -0.315$, $p = 0.040$) and bioaccumulation of Cu in kidneys

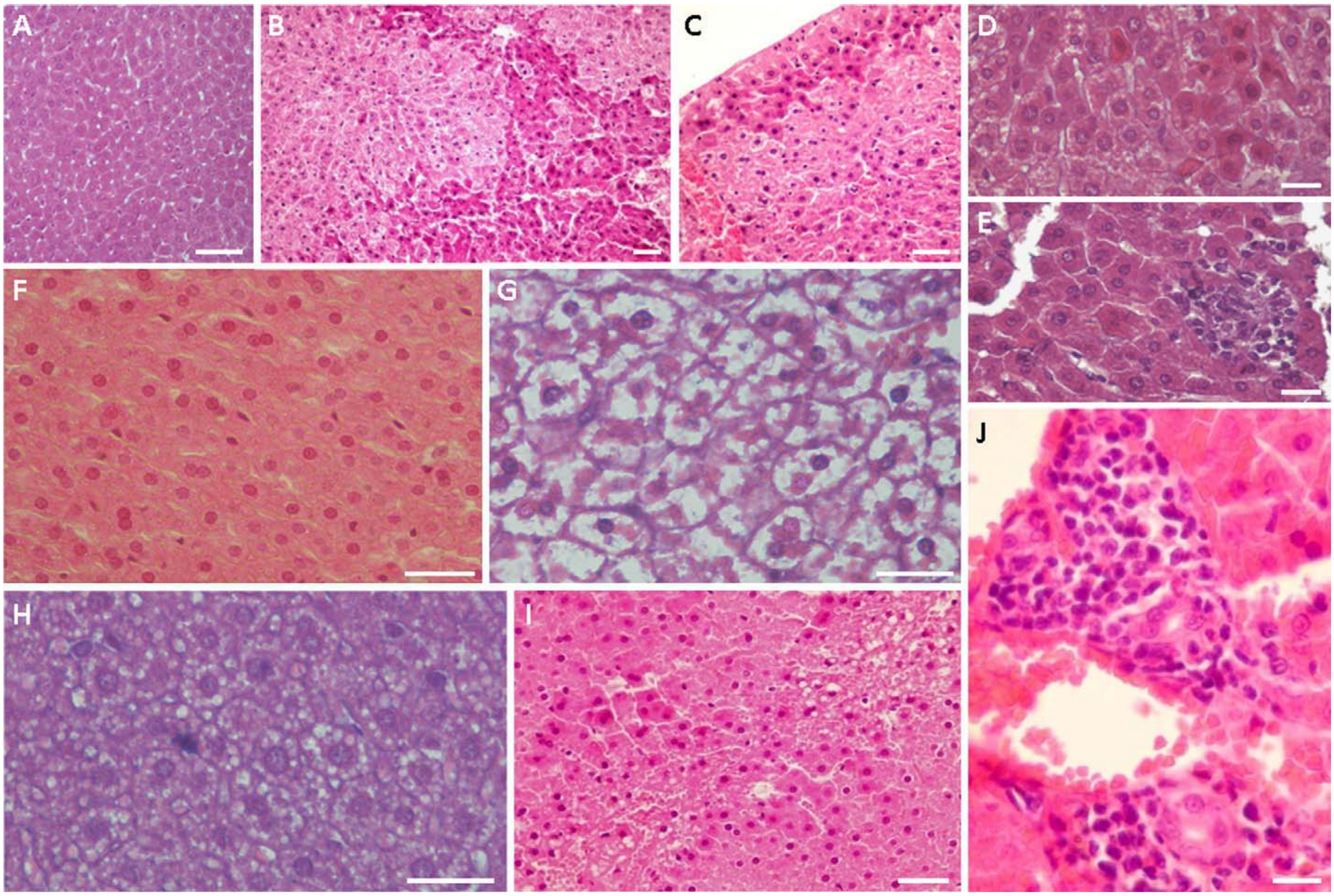

Fig. 1. Representative micrographs of hepatic sections from *A. sylvaticus* (A–E) and *C. russula* (F–J) showing: (A, F) non-altered tissue with hepatocytes with normal shape, (B) extensive necrosis, (C) supracapsular necrosis, (D) apoptotic cells, (E, J) inflammation, (G) highly vacuolated hepatocytes, (H) steatosis, and (I) extensive necrotic areas surrounding by vacuolated areas (bar size corresponding to 50 µm (A–C, I) or 25 µm (D–H)).

($r = 0.322$, $p = 0.040$). In *C. russula*, correlations were found between hepatic histopathology and relative liver weight ($r = 0.396$, $p = 0.041$) and bioaccumulation of Mn in kidneys ($r = 0.405$, $p = 0.045$).

4. Discussion

Given the concern about the environmental consequences of pollution, several approaches have been proposed for the assessment and prediction of pollutant behaviour and the effects of these substances on ecosystems. Laboratory studies under controlled conditions (Jadhav et al., 2007; Koyu et al., 2006; Zhang et al., 2009), models to predict toxicity or dispersion of pollutants (Kools et al., 2009) or the biomonitoring of abiotic or biotic components as well as several biomarkers in wild biota (Pereira et al., 2006; Scheirs et al., 2006; Tersago et al., 2004) attempt to address these issues. However, the exposure to mixtures of pollutants that interact among themselves or with abiotic and biotic components, the differential exposure or toxicity related to several biotic factors (nutritional status, age or gender among others) and other non-controlled variables of dynamic environments that alter bioavailability of pollutants are just some of the factors that hinder research to effects of mixtures in the field. Here we addressed the effects of landfill pollution under natural conditions by performing a comparative study of structural alterations in tissues of sympatric small mammals in a protected area. We propose that our approach can be a suitable as a biomarker to detect toxicity and pollution effects caused by chronic or acute exposure of wildlife more sensitive than other methods. The alterations observed in specimens from the polluted site are compatible with exposure to pollutants and, more specifically to landfill leachates. In general, hepatic and renal toxicity effects appear when primary or acquired tolerance of cells and tissues are overloaded. The main exposure route to metals for mammals is though the diet and hepatic and renal tissues are the primary targets (Clark et al., 1992; Koyu et al., 2006; Larregle et al., 2008; Pereira et al., 2006; Włostowski et al., 2008). In fact, in both species, liver was the organ in which most histological alterations caused by exposure to landfill pollution were observed and significant correlations between histopathological alterations in liver and liver weight reinforce this observation. The damage to this organ may be related to its metabolic functions in toxic transformation, bioaccumulation and excretion. In contrast to shrews, mice from the landfill site showed considerable alterations in kidneys, thereby indicating inter-specific differences in the response to toxicity. Kidney lesions concordant with our results have been reported in wild rodents from polluted areas (Stansley and Roscoe, 1996) and in laboratory shrews exposed to Pb (Pankakoski et al., 1994).

Leachates are inductors of oxidative stress that injure cells and tissues (e.g. Li et al., 2006a, b). Small mammals from the landfill site of Garraf accumulate heavy metals up to toxic concentrations in their tissues (Sánchez-Chardi and Nadal, 2007; Sánchez-Chardi

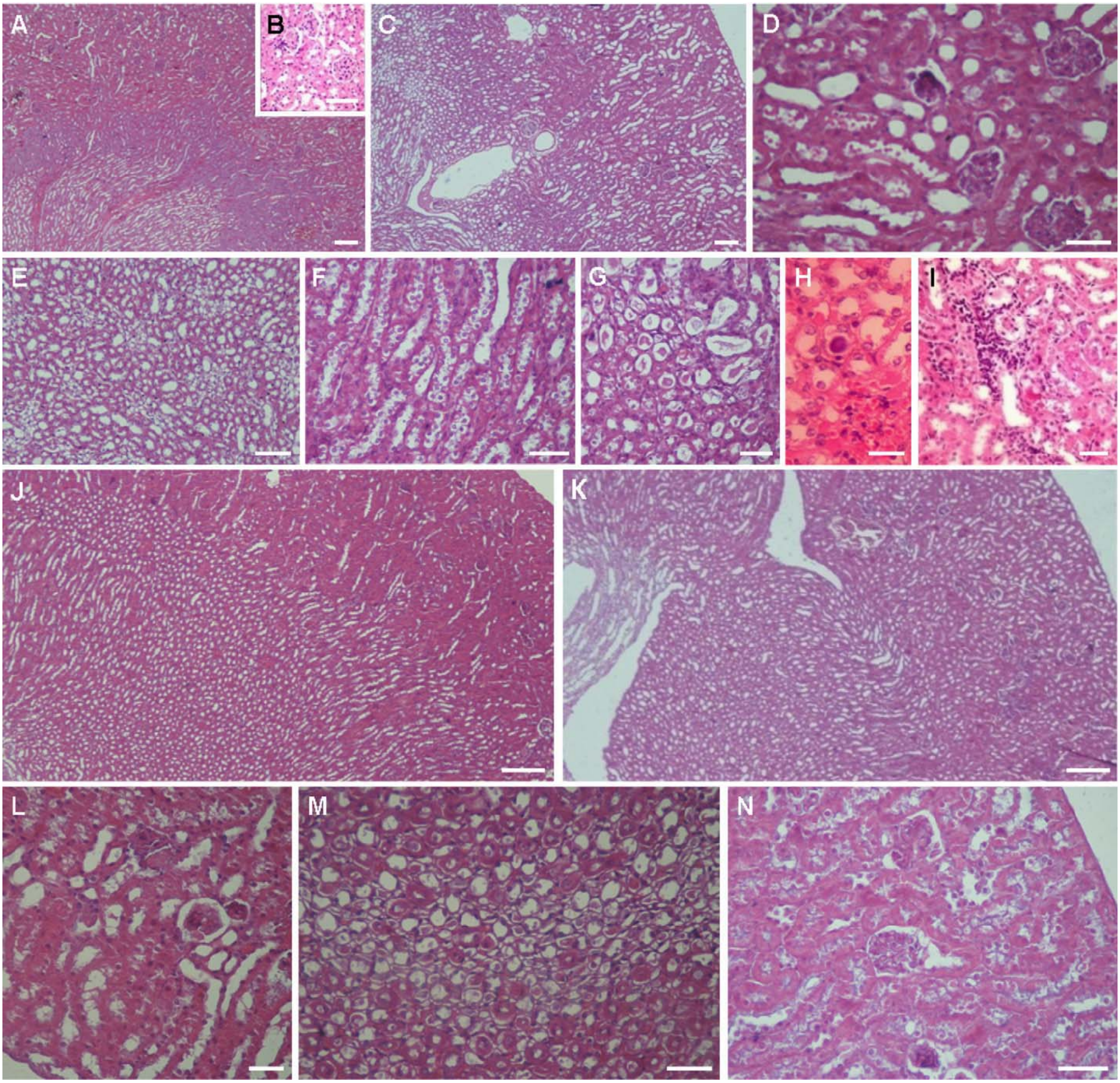

Fig. 2. Representative micrographs of renal sections from *A. sylvaticus* (A–I) and *C. russula* (J–N) showing: (A, B, J) normal structure of cortex and medulla, (C) tubular dilatation, (D, L) tubular dilatation in cortex, (E, M) tubular dilatation in medulla, (F, N) tubular necrosis, (G, H) cylinders, and (I) interstitial nephritis (bar size corresponding to 100 μm (A, C, J, K) or 50 μm (B, D–N)).

et al., 2007). This might indicates the important contribution of these elements to the effects reported. Although the precise molecular mechanism or mechanisms by which metals cause this damage remain unclear, the production of ROS is believed to be involved (Chwełatiuk et al., 2005; Włostowski et al., 2008). Depending on the nature of the injury, the generation of this oxidative stressor damages DNA, thereby producing apoptosis, necrosis or carcinogenic processes and altering membranes and lipid and protein metabolism (Jadhav et al., 2007; Larregle et al., 2008; Mena et al., 2009; Pereira et al., 2006). For example, the hepatotoxicity *in vivo* induced by metals such as Cd seems usually to be produced as ischemia caused by damage to endothelial cells and activation of Kupffer cells (Pagliara et al., 2003). A cascade of events involving inflammatory and cytotoxic mediators (Koyu et al., 2006) results in hepatocytes damage. In the wood mice, hepatocyte death (apoptosis) was maintained at a moderate level in controls suggesting that it is the expression of physiological cell turnover. However, *A. sylvaticus* from the polluted site showed apoptotic figures instead of the necrotic cells, surrounded by lymphocytes. This observation of cell cycle arrest is also commonly described in laboratory rodents and might indicate toxic effects caused by exposure to leachates. A similar response, although less severe and less frequently, was observed in shrews from the landfill site. Exposure to several pollutants, including

Table 3
Frequency and severity of histological alterations by site and tissue in wood mice (*A. sylvaticus*) and greater white-toothed shrews (*C. russula*) (without alterations (−), slightly altered (+), intermediately altered (++), strongly altered (+++)).

	Apodemus sylvaticus								*Crocidura russula*							
	Reference				Landfill				Reference				Landfill			
	−	+	++	+++	−	+	++	+++	−	+	++	+++	−	+	++	+++
Liver																
Apoptosis	15	8	1	1	7	10	4	3	16	–	–	–	8	2	2	–
Necrosis	24	1	–	–	19	1	1	3	15	1	–	–	6	3	1	2
Vacuolation/microsteatosis	22	2	1	–	20	1	1	2	15	1	–	–	7	1	3	1
Inflammation	25	–	–	–	22	1	1	–	15	1	–	–	9	3	–	–
Preneoplasic nodules	25	–	–	–	23	–	–	1	16	–	–	–	12	–	–	–
Kidneys																
Cylinders	22	–	–	–	17	2	4	–	14	1	–	–	9	1	–	–
Inflammation	21	1	–	–	20	–	2	1	15	–	–	–	9	–	1	–
Tubular necrosis	21	1	–	–	18	3	2	–	15	–	–	–	8	1	1	–
Tubular dilatation	22	–	–	–	19	1	2	1	15	–	–	–	7	2	1	–

heavy metals or mixtures as leachates has shown to produce apoptosis and necrosis in laboratory studies (Larregle et al., 2008; Talorete et al., 2008) and in wild small mammals (Clark et al., 1992; Damek-Poprawa and Sawicka-Kapusta, 2003; Sánchez-Chardi et al., 2008, 2009; Stansley and Roscoe, 1996; Świergosz-Kowalewska et al., 2006; Tersago et al., 2004).

Wood mice and shrews from the polluted site showed inflammatory processes similar to those reported for wild small mammals (Clark et al., 1992; Sánchez-Chardi et al., 2008) and laboratory rodents (Larregle et al., 2008; Włostowski et al., 2004) in other studies. Hepatotoxicity caused by metal exposure includes the involvement of proinflammatory cytokines and chemokines, Kupffer cell activation and neutrophil infiltration (Koyu et al., 2006 and references herein) producing inflammatory focuses such as observed in the present study. Nephritis was also reported in wild rodents exposed to environmental pollution (Clark et al., 1992). Chronic inflammation such as that produced by exposure to landfill leachates has been described as an inductor of carcinogenic processes (Talorete et al., 2008). Carcinogenesis was observed in one specimen of wood mouse from the landfill site. It is known that metals such as cadmium have carcinogenic activity (Damek-Poprawa and Sawicka-Kapusta, 2003).

Hepatocyte vacuolation and microsteatosis can be the result of lipids, glycogen or water accumulation, which are indicative of metabolic disturbances (Pereira et al., 2006) such as autophagic processes. Lipophilic pollutants can also be stored in these droplets (Włostowski et al., 2008), thereby being less available, which reduces the oxidative stress in cell components. We observed this alteration more frequently in livers of wood mice and shrews from the polluted site. This is consistent with previous studies on wild and laboratory small mammals exposed to metals and organic xenobiotics (Clark et al., 1992; Damek-Poprawa and Sawicka-Kapusta, 2004; Pereira et al., 2006; Sánchez-Chardi et al., 2008; Włostowski et al., 2008). In both small mammal species from Garraf, the vacuolated areas were often surrounded by necrotic zones, thereby indicating extensive damage in especially the liver such as occurs with steatohepatosis.

Renal tissue is the main target for bioaccumulation and effects of several pollutants, including metals such as Cd (Chmielnicka et al., 1989; Prozialeck et al., 2009). Tubular dilatation and proteic cylinders in kidneys were also reported in common shrews, *Sorex araneus*, experimentally exposed to Pb (Pankakoski et al., 1994) and in laboratory rodents exposed to essential and non-essential metals (Cerejeira Matos et al., 2009; Chmielnicka et al., 1989). Moreover, nephrotoxicity induced by metals includes cell death necrosis and apoptosis in tubules (references in Prozialeck et al., 2009). In general, these renal alterations may reflect a later phase of pollutant-induced hyperfiltration caused by chronic exposure to landfill leachates. Moreover, laboratory studies showed an increase in urinary output in rats exposed to metals (e.g. Chmielnicka et al., 1989; Prozialeck et al., 2009). In the present study, several renal alterations were not found in isolation: cylinders, tubular dilatation and necrosis were found together in those specimens altered. These results suggest that dilatation may be a compensatory mechanism after the loss of renal excretory function of nephrons by tubular necrosis and cylinders.

In addition to hepatic and renal tissues, pollutants can specifically affect other organs and tissues, such as blood, gonads, spleen, lung, brain and bones (e.g. Damek-Poprawa and Sawicka-Kapusta, 2003, 2004; Jadhav et al., 2007; Li et al., 2006a, b; Pereira et al., 2006). The spleen may be a suitable tissue to detect immune-depression effects as a response to pollution (Pereira et al., 2006; Tersago et al., 2004). In our case, the interference of parasitosis in spleen and intestine hindered the histopathological evaluation. The general health status may be decreased in animals heavily parasitized in digestive tract, spleen and lungs. Nevertheless, immuno-depression in stressed specimens by pollution exposure (Tersago et al., 2004) can result on an increase of parasites. These observations suggest further research focussing on quantitative studies and specific biomarkers for these tissues.

No sex- or gender-dependent variation was observed in the structural alterations detected in mice or shrews. Although gender and age-related toxicity rates have been found elsewhere (e.g. Gochfeld, 2007; Scheirs et al., 2006; Pereira et al., 2006), our result are consistent with previous data on *C. russula* (Sánchez-Chardi et al., 2008, 2009) and are indicative of similar toxic effects in all classes (juveniles, adults, males and females) of individuals.

The comparison of toxic effects between sympatric species in a distinct trophic compartment provides useful information on the effects of landfill pollution on terrestrial food chains. In general, our histopathological findings are compatible with morphological, haematological and biochemical results previously reported in the same specimens (Sánchez-Chardi and Nadal, 2007; Sánchez-Chardi et al., 2007). In agreement with our data, histological evaluations of wild mammals from polluted sites often describe different severity of a variety of lesions, from unaltered to highly damaged tissues, as well as alterations not related to pollution in both control specimens and in animals exposed to pollution. Although significant age- or sex-dependent variation was not observed in the present study, the differential exposure under natural conditions and/or the physiological response of each specimen to environmental disturbances is influenced by the genetic pool but differs depending on abiotic and biotic factors, including the photoperiod, temperature, and sex, age, or general health condition (e.g. Włostowski et al., 2008). This wide intraspecies variability implies that histopathological assessments in ecotoxicology should be quantitative or semi-quantitative in order to detect statistical significances or tendencies regarding histotoxic effects. Although mice and shrews showed histological alterations, the inter-specific differences in severity and frequency of the pathologies indicate the differences in sensibility of these animals to chronic pollution. In general, insectivores are considered more tolerant to toxicants than rodents as reported in non-observable-adverse-effects-level (NOAEL) for metals (Ma and Talmage, 2001; Sheffield et al., 2001; Talmage and Walton, 1991). However, the comparisons between small mammal species are usually made between rodents and soricine species (e.g. Hunter et al., 1987) and scarce comparative information is available on crocidurine species (González et al., 2008; Wijnhoven et al., 2007). Our results indicate similar toxic effects in liver in both mice and shrews but slightly lower toxicity rate in kidneys of the latter. Wood mice from the Garraf landfill showed several differences in morphological, plasmatic and genotoxic parameters while shrews showed differences in genotoxicity (Sánchez-Chardi et al., 2007). In contrast, the highest levels of toxic metals in tissues have been reported in *C. russula* (Sánchez-Chardi and Nadal, 2007). This observation is attributed to the fact that carnivores are usually more exposed to metals and accumulate more of these elements than omnivores and herbivores (Hunter et al., 1987; Ma and Talmage, 2001; Pankakoski et al., 1994; Talmage and Walton, 1991). Sympatric species of small mammals may differ dramatically in metal bioaccumulation in the absence of differences in external bioavailability mainly as a result of dietary uptake and ingestion of contaminated sediments. Thus environmental quality management programs should compare species with different trophic position.

Metabolic alterations caused by chronic exposure to pollutants can affect the basic functions of biota such as reproduction or life expectancy. Therefore, the assessment of environmental quality through the evaluation of structural alterations caused by pollution is of particular relevance in protected areas such as the Park of Garraf. Although the environmental quality of such zones is often high, long-term pollutant activities, such as landfilling, may disturb, modify or destroy ecosystems, thereby making them less suitable for wildlife. Therefore, continuous biomonitoring of pollutants by means of selected species is required. In this context, our results corroborate other studies that reported the wood mouse and the greater white-toothed shrew as effective bioindicators of non-essential metals and the effects of environmental pollution (González et al., 2008; Sánchez-Chardi and Nadal, 2007; Sánchez-Chardi et al., 2007; Wijnhoven et al., 2007). Moreover, further studies are necessary to elucidate more exactly the inter-specific differences in tolerance of environmental pollution between rodents and crocidurine species. These studies may include an ultrastructural evaluation or molecular studies to quantify genetic or metabolic alterations. With all this in mind and given the inter-specific differences between mice and shrews, we propose the use of both species in biomonitoring programs.

5. Conclusions

Histopathology of the major organs involved in metabolism and excretion of xenobiotics, as the liver and the kidney, are useful biomarkers of the effect of pollution in wild small mammals collected near the landfill of Garraf. Specimens of both species analysed showed toxic effects that could be attributed to direct action (cell cycle arrest, inflammation, preneoplasic nodules), storage (vacuolation, microsteatosis) and excretion (tubular dilatation, cylinders) of pollutants. The wood mice showed considerable alterations in liver and kidneys as a result of chronic exposure to leachates while the shrews showed main alterations in the liver. This result contrasts with higher bioaccumulation of metals in the shrews than in the mice. On the basis of our findings, we conclude that the comparison of several biomarkers in sympatric species of small mammals at different trophic levels provides suitable ecotoxicological data for a complete assessment of environmental pollution. Moreover, given the protected status of the Garraf area, the use of bioindicator species provides useful information for environmental quality management and to improve our understanding of the response capacity of natural populations to pollution in this area and in other areas with similar characteristics.

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Metal bioaccumulation in the greater white-toothed shrew, *Crocidura russula*, inhabiting an abandoned pyrite mine site

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Abstract

Hepatic and renal concentrations of iron, magnesium, zinc, lead, copper, manganese, mercury, cadmium, molybdenum, chromium, and nickel were quantified in shrews (*Crocidura russula*) inhabiting a pyrite mine site in Portugal. Several morphological parameters (body weight, residual index, and relative weights) were also examined to clarify the physiological effects of pollution. Shrews from the mine showed increased bioavailability of Fe, Pb, Hg, Cd, Mo, and Ni in comparison with reference specimens. Adult shrews had the highest Cd levels while Cr and Ni concentrations diminished. Intersexual differences were found for Mo and Ni. As a consequence of metal pollution, the relative hepatic weight was higher in shrews from the mine site when compared with reference specimens. These data indicate that *C. russula* is a good bioindicator of metal pollution. We also evaluated the toxic effects of Pb, Hg, Cd, and Ni, because several shrews from the polluted site showed high concentrations of these metals. To approximate at the real biological impact of abandoned mines, after this first step it is necessary to associate the bioaccumulation levels and morphological effects with other physiological, ecological and genetical biomarkers.

Keywords: *Crocidura russula*; Heavy metals; Liver; Kidneys; Pyrite mine; Portugal

1. Introduction

Heavy metals are common environmental pollutants that have increased their concentration and bioavailability by biota because of industrial activities such as mining. In fact, between 1930 and 1985 the mine production of Mg, Zn, Pb, Cu, Mn, Hg, Cd, Cr, and Ni alone increased 7-, 4-, 2-, 5-, 8-, 2-, 15-, 18-, and 35-fold respectively, with the subsequent release of these potentially toxic elements into the environment (Nriagu, 1988). Mines are closed down when they become economically non-viable and usually become a large and uncontrolled source of metal pollution. In the case of pyrite deposits, natural weathering interaction generates large amounts of effluents named "acid mine drainage" (AMD), characterized by the presence of toxic metals such as Zn, Pb, Cu, Mn, Cd, Mo, Cr, and Ni in low pH solution (Quevauviller et al., 1989; Santos Oliveira et al., 2002). These chemical conditions often increase the bioavailability of metals at potentially toxic levels (Scheuhammer, 1991; Lacal et al., 2003) and, consequently, the environmental risk of these deposits remain long after mines have been abandoned.

Several studies have reported the bioaccumulation of heavy metals in tissues of terrestrial small mammals inhabiting abandoned mines, mainly in rodent than in insectivorous species probably because mice and voles are, in general, more abundant and rapidly trapped than shrews. When comparing sympatric small mammal species,

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carnivores showed greater bioaccumulation than omnivores and herbivores, which is explained by their high metabolic rate, high food consumption rate, and position at the top of the food-chain (Andrews et al., 1984; Hunter et al., 1989; Shore, 1995; Ma and Talmage, 2001). In fact, a range of species of Talpidae and Soricine has been demonstrated as good bioindicators of metal pollution (e.g. Talmage and Walton, 1991; Pankakoski et al., 1994; Komarnicki, 2000). However, Crocidurine species have rarely been used as models in ecotoxicological studies on metal accumulation (Topashka-Ancheva and Metcheva, 1999; Sánchez-Chardi et al., 2007). The greater white-toothed shrew, *Crocidura russula* (*Mammalia, Insectivora*), inhabits the south-western Europe and is the most abundant and widely distributed shrew in the Iberian Peninsula.

Larger variation in the concentration of metals in the same population may be explained mostly by biotic and abiotic parameters. In fact, bioaccumulation patterns are dependent on age and sex, as well as physiological status and diet, which are subjected to seasonal differences (e.g. Cloutier et al., 1986; Lopes et al., 2002). However, few studies have addressed the variation in metal concentrations produced by these three parameters in insectivore populations (Smith and Rongstad, 1982; Komarnicki, 2000; Ma and Talmage, 2001).

In addition to the bioaccumulation levels, it is necessary to assess the physiological effects of chronic exposure to metals in order to evaluate the impact of pollution in "real world" conditions. Information on these effects can be partially attained by morphological parameters based on weight and/or length of animals. In fact, poisoning by some metals may result in a decrease in total body weight or an increase in relative organ weight, which may be indicative of histopathological alterations (e.g. Ma and Talmage, 2001). The wide range of responses shown by small mammals to heavy metal pollution suggests that natural populations are very tolerant, adapt to low quality environments and that only high metal exposure alter these morphological parameters (Ma, 1989; Stansley and Roscoe, 1996; Ma and Talmage, 2001).

Here we: (i) quantified the heavy metal concentrations in *C. russula* inhabiting an area near a pyrite mine, (ii) examined the seasonal, sexual, and age-bioaccumulation patterns in this species, and (iii) assessed the toxic effects of these abandoned mines on small mammals.

2. Materials and methods

In the Baixo Alentejo region (Southern Portugal) several mines have been closed down because of economic reasons and with no previous planning for environmental recovery. Located in the Iberian Pyrite Belt, the open pit and underground mine of Aljustrel was worked from 1867 to 1996. From 1900 to 1991 about 16 000 000 t of metals were mined, out of which 1 150 000 t from 1991 to 1996, including Fe = 1000 t, Cu = 6300 t, Zn = 27 500 t, and Pb = 4400 t (Instituto Geológico e Mineiro, SIORMINP, internal report). The polluted site of Aljustrel mine chosen for this study (37°53′08″N; 08°08′32″W) is affected by the AMD from the pyrite mine. A second area (Moura) where no sources of heavy metals have been reported was used as a reference. The Moura site is located 69 km northeast from Aljustrel mine (38°11′13″N; 07°24′34″W) (Fig. 1).

In spring and autumn 2003, a total of 54 greater white-toothed shrews were collected in the polluted ($n = 32$) and in the reference ($n = 22$) site. Shrews were live-trapped and transported to the laboratory, where they were lightly anaesthetized and killed by cervical dislocation. The body weight (BW) to the nearest 0.0001 g and body length (BL) to the nearest 0.01 mm of all shrews were measured. The liver, kidneys and spleen were removed and weighted. The residual index (RI) indicating body condition index was calculated following Jakob et al. (1996) as a regression of BL and BW. The liver:body, kidneys:body and spleen: body weight ratios were calculated as a percent ratio of somatic tissue (100× tissue wet weight/body wet weight). Sex was determined during dissection. For statistical analyses, animals were distributed into two relative age-classes (juveniles; adults) according to the degree of tooth wear (Vesmanis and Vesmanis, 1979).

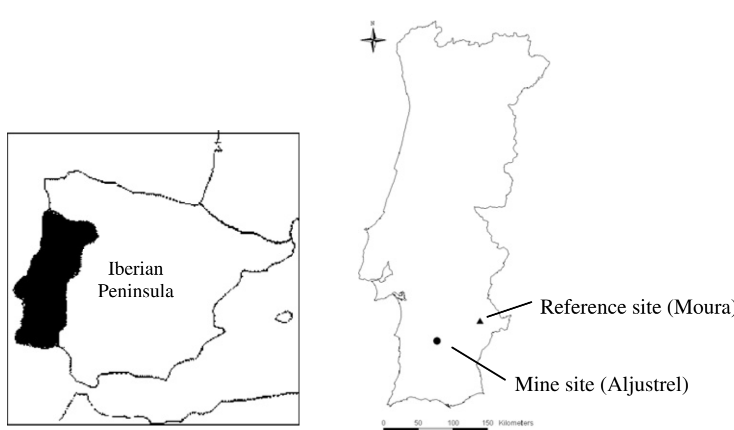

Fig. 1. Localization of the study areas in south of Portugal.

The material used for the digestion process was acid-rinsed. The liver and the right kidney from all shrews were acid digested in Teflon vessels following the methodology described in Sánchez-Chardi et al. (2007). Mg concentration was determined by a Perkin–Elmer OPTIMA-3200RL Inductively Coupled Plasma Optical Spectrometer (ICP-OES), while Pb, Hg, Cd, Cu, Zn, Mn, Mo, Cr and Ni were measured by a Perkin–Elmer ELAN-6000 Inductively Coupled Plasma Mass Spectrometer (ICP-MS). Two replicate subsamples and a standard reference material (Bovine Liver SRM-1577a) certified by the National Bureau of Standards (NBS) were included in the analyses. To obtain the concentration of elements, the mean values from 20 blanks were subtracted from each sample. The results of the metal analyses are presented as mean ± standard error (SEM) in $\mu g\ g^{-1}$ on dry weight (DW) basis.

Data were log transformed and tested for normal distribution (Shapiro–Wilk test) and for homogeneity of variance (Levene, *F*-test). From each tissue, an overall measure of the effect of season, sex, age, and site was obtained by four-way multivariate analysis of variance (MANOVA). Seasonal, sexual and age variation for each site and tissue were calculated by Student's *t*-tests (*t*). The divergences in RI, relative somatic ratios, site, and tissue were also calculated by Student's *t*-tests. Pearson's correlation coefficients (*r*) were calculated in liver and kidneys, in order to establish the relations between elements in the polluted site. Significant differences were accepted at $p < 0.05$. For all sequential tests, *p*-values were corrected by the Bonferroni adjustment (Rice, 1989). All statistical procedures were performed with SPSS (version 11.5 for Windows, SPSS Inc.).

3. Results

Metal concentrations in all the samples analysed were above the detection threshold for all individuals (in $\mu g\ kg^{-1}$, Fe: 10; Mg: 50; Zn: 0.50; Pb: 0.05; Cu: 0.10; Mn: 0.10; Hg: 0.20; Cd: 0.05; Mo: 0.05; Cr: 0.50; Ni: 0.20). In both sites, a large percentage of shrews caught in spring were juveniles, whereas adults were more abundant in autumn. A similar number of males and females were captured in the two sites (Table 1).

Table 1
Number of animals captured in the reference and mine site by season and age

Site	Season	Age		Total
		Juveniles	Adults	
Moura	Spring	14 (8,6)	6 (3,3)	20 (11,9)
	Autumn	3 (2,1)	9 (2,7)	12 (4,8)
Mine site	Spring	7 (2,5)	3 (2,1)	10 (4,6)
	Autumn	1 (0,1)	11 (6,5)	12 (6,6)

In brackets: males, females.

Table 2
Mean ± SEM of morphological parameters in shrews from the reference and mine site

	Reference site ($n = 17$)	Mine site ($n = 18$)
BW (g)	7.48 ± 0.99	7.20 ± 0.94
BL (mm)	70.60 ± 3.59	68.84 ± 5.49
RI	−0.215 ± 4.187	0.266 ± 0.899
Liver (mg g^{-1} WW)	0.418 ± 0.088	0.456 ± 0.107
% Liver	5.56 ± 0.16	6.27 ± 0.22*
Kidneys (mg g^{-1} WW)	0.125 ± 0.018	0.119 ± 0.016
% Kidneys	1.68 ± 0.03	1.66 ± 0.04
Spleen (mg g^{-1} WW)	0.030 ± 0.008	0.031 ± 0.014
% Spleen	0.41 ± 0.03	0.44 ± 0.43

* $p = 0.016$.

All morphologic parameters were calculated on wet weight (WW) basis. In comparison with specimens from the reference site, the shrews from the polluted site showed a significant increase in the hepatic ratio ($t = 2.529$; $p = 0.016$). No differences were detected between sites for the remaining morphological parameters (Table 2).

MANOVA for all data showed that the importance of parameters in liver was: site ($F = 8.600$; $p < 0.001$) > season ($F = 5.715$; $p < 0.001$) > age ($F = 3.614$; $p = 0.003$) > sex ($F = 2.451$; $p = 0.027$), and all factors were statistically significant. In kidneys, season ($F = 8.619$; $p < 0.001$), site ($F = 6.657$; $p < 0.001$) and age ($F = 3.449$; $p = 0.004$) were the main parameters influencing metal bioaccumulation, and sex ($F = 2.247$; $p = 0.041$) keeping as the less important in variance.

The accumulation pattern of metals by tissues was similar in the two sites. The liver was the main target organ for Fe, Zn, Cu, Mn, and Mo, whereas the kidneys bioaccumulate the highest concentrations of Hg, Cd and Ni. Cr has similar concentrations in these two organs analysed. Moreover, in the polluted area Mg and Pb were significantly higher in liver when compared with kidneys (Table 3). Copper and chromium showed no significant differences between tissues in either study sites.

The shrews from Aljustrel mine showed significantly high levels of Pb, Hg, and Ni in hepatic and renal tissues

Table 3
Results of the Student's *t*-tests for variation in metal concentrations according to tissue, for each site

	Reference site		Mine site	
	t	*p*	*t*	*p*
Fe	8.918	<0.001	15.391	<0.001
Mg	–	–	3.786	<0.001
Zn	4.183	<0.001	3.434	<0.001
Pb	–	–	7.382	<0.001
Mn	13.226	<0.001	9.401	<0.001
Hg	−6.009	<0.001	−5.541	<0.001
Cd	−4.386	<0.001	–	–
Mo	7.208	<0.001	9.851	<0.001
Ni	–	–	−8.210	<0.001

Table 4
Mean ± SEM values for metals in *C. russula* by tissue and site (in μg g^{-1} DW)

	Liver				Kidneys			
	Reference site ($n = 22$)	Mine site ($n = 31$)	t	p	Reference site ($n = 21$)	Mine site ($n = 32$)	t	p
Fe	1257.36 ± 160.03	2958.45 ± 395.21	5.054	<0.001	323.01 ± 22.99	381.02 ± 30.52	–	–
Mg	899.81 ± 27.51	987.32 ± 83.84	–	–	803.33 ± 45.56	746.87 ± 32.09	–	–
Zn	145.23 ± 7.27	167.26 ± 19.18	–	–	107.07 ± 7.97	109.46 ± 8.96	–	–
Pb	0.77 ± 0.09	5.26 ± 0.75	7.733	<0.001	0.70 ± 0.05	1.19 ± 0.21	–	–
Cu	23.83 ± 10.31	28.31 ± 16.17	–	–	20.82 ± 6.23	19.67 ± 2.69	–	–
Mn	17.56 ± 0.74	17.26 ± 1.42	–	–	5.84 ± 0.46	5.69 ± 0.52	–	–
Hg	0.26 ± 0.05	1.03 ± 0.26	5.004	<0.001	1.08 ± 0.16	2.70 ± 0.29	4.363	<0.001
Cd	2.27 ± 0.65	8.61 ± 1.47	4.428	<0.001	7.83 ± 2.06	18.17 ± 2.80	–	–
Mo	3.38 ± 0.24	5.30 ± 0.52	3.252	0.002	1.73 ± 0.08	1.79 ± 0.10	–	–
Cr	1.32 ± 0.11	1.12 ± 0.28	−3.360	0.002	0.97 ± 0.12	0.86 ± 0.08	–	–
Ni	0.67 ± 0.24	1.48 ± 0.17	4.114	<0.001	5.40 ± 1.80	15.28 ± 4.39	3.398	0.002

(see Table 4). Moreover, hepatic levels of Fe, Cd, and Mo were higher and Cr lower in shrews from the mine compared with reference specimens. The hepatic and renal values of the other elements (Mg, Zn, Cu, and Mn) remained statistically invariant between sites. These results indicate that the liver was the tissue with most significant differences in metal bioaccumulation between sites.

The bioaccumulation of several elements in *C. russula* tissues showed seasonal differences, and in some cases bioaccumulation patterns varied between tissues and sites of capture (see Table 5). In shrews from Aljustrel mine, liver showed higher concentrations of Mg and Cu in spring in comparison to autumn, whereas Pb and Ni increased in autumn. In shrews from the reference site, hepatic tissue showed significantly high levels of Mg and Pb in spring, whereas Hg decreased. Finally, chromium showed a significant decrease in autumn in the two sites and tissues studied. Similar pattern was revealed for manganese as a tendency without significant differences.

A clear pattern of age-dependent increase was detected in Cd levels in *C. russula*. In contrast, a decrease with age was observed for Cr and Ni. Moreover, Pb, Cu, and Hg also showed significant differences with age (Fig. 2). The female shrews from the two sites showed higher mean Mo and Ni concentrations in liver and kidneys than males. In the shrews from Aljustrel these sexual differences were significant in liver for Mo in autumn (2.76 ± 1.43 *vs* 6.04 ± 1.09 μg g^{-1}; $t = -2.876$, $p = 0.017$) and in renal tissue for Mo (1.34 ± 1.05 *vs* 1.90 ± 1.08 μg g^{-1}; $t = -3.439$, $p = 0.011$) and Ni (0.34 ± 3.67 *vs* 7.40 ± 1.39 μg g^{-1}; $t = -2.555$, $p = 0.038$) in autumn control shrews. No sex-dependent variation was detected in the concentrations of the other metals at the two sites.

Significant correlations between metal bioaccumulation in shrews caught in Aljustrel shown in Table 6.

4. Discussion

Studies on environmental pollution produced by mining activities in Portugal have reported metal increases in sediments, waters, plants, fish and mammals (Quevauviller et al., 1989; Nunes et al., 2001a,b; Lopes et al., 2001,

Table 5
Mean ± SEM values for metals in *C. russula* by site and season (in μg g^{-1} DW)

	Reference site				Mine site			
	Spring ($n = 10$)	Autumn ($n = 12$)	t	p	Spring ($n = 19$)	Autumn ($n = 12$)	t	p
Liver								
Mg	991.82 ± 39.48	823.14 ± 20.28	4.031	0.001	1118.18 ± 128.75	780.14 ± 14.63	3.359	0.002
Pb	1.07 ± 0.09	0.52 ± 0.10	3.466	0.002	4.24 ± 0.95	6.88 ± 1.12	–	–
Cu	25.51 ± 3.84	22.43 ± 2.54	–	–	33.66 ± 3.81	19.83 ± 3.35	2.290	0.007
Mn	19.34 ± 1.04	16.08 ± 0.86	–	–	19.31 ± 2.12	14.01 ± 1.05	–	–
Hg	0.05 ± 0.01	0.43 ± 0.05	−6.932	<0.001	1.32 ± 0.41	0.58 ± 0.78	–	–
Cr	1.47 ± 0.11	1.19 ± 0.17	–	–	1.65 ± 0.41	0.29 ± 0.09	3.189	0.003
Ni	0.72 ± 0.25	0.63 ± 0.40	–	–	0.95 ± 0.15	2.32 ± 0.19	−5.646	<0.001
Kidneys								
Mg	766.80 ± 15.70	830.72 ± 79.45	–	–	697.43 ± 12.94	829.26 ± 79.19	–	–
Pb	0.78 ± 0.04	0.64 ± 0.07	–	–	1.38 ± 0.33	0.88 ± 0.13	–	–
Cu	19.07 ± 0.27	22.13 ± 2.34	–	–	19.50 ± 0.38	19.96 ± 1.13	–	–
Mn	6.34 ± 0.33	5.46 ± 0.77	–	–	6.23 ± 0.61	4.78 ± 0.93	–	–
Hg	0.93 ± 0.05	1.19 ± 0.28	–	–	2.71 ± 0.39	2.68 ± 0.45	–	–
Cr	1.45 ± 0.12	0.60 ± 0.08	5.407	<0.001	1.16 ± 0.054	0.36 ± 0.08	8.959	<0.001
Ni	5.33 ± 1.79	5.45 ± 2.92	–	–	18.13 ± 6.96	10.55 ± 1.45	–	–

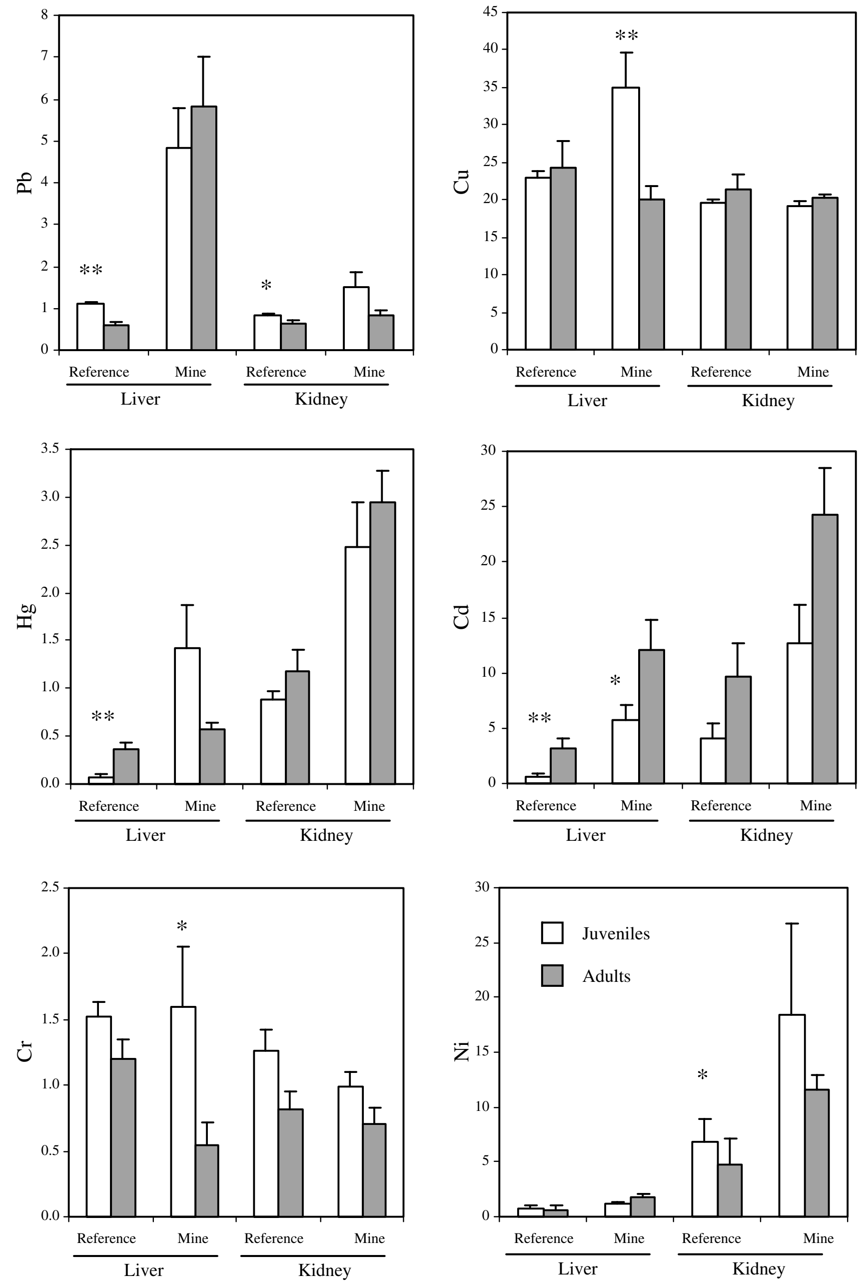

Fig. 2. Mean ± SEM values for metals (Pb, Cu, Hg, Cd, Cr, and Ni) in *C. russula* by tissue, site and age (in $\mu g\, g^{-1}$ DW) ($^{*}p \leqslant 0.05$; $^{**}p \leqslant 0.01$; $^{***}p \leqslant 0.001$).

2002; Viegas-Crespo et al., 2003; Pereira et al., 2006). These findings indicate that mines are an important source of metal pollution in this country. The pyrite mine of Aljustrel is located in the Iberian Pyrite Belt, a metalogenic province that crosses the Southeast of the Iberian Peninsula, which includes the mines of St. Domingos (Portugal) and Aznalcóllar (Spain), two sites known for environmental contaminants. The bioaccumulation and effects of toxic metals has been reported in rodents captured in St. Domingos (Pereira et al., 2006), while the Aznalcollar mine spill of 4.5 millions cubic meters of mine sludge in 1998 affecting the protected area of Doñana. This ecological disaster serves to highlight the great environmental risk of abandoned mines.

4.1. Metal bioaccumulation by site

The mine at Aljustrel releases large amounts of effluents loaded with potentially toxic metals into the environment (Quevauviller et al., 1989; Santos Oliveira et al., 2002). These metals may bioaccumulated and be biomagnified along the food-chain (Farag et al., 1998) long after mine closure. The main increases of metal concentrations in shrews from the mine site were observed for highly toxic

Table 6
Pearson coefficients and p-values between elements in liver and kidneys of the *C. russula* from the mine site

	Liver			Kidneys	
	r	p		r	p
Cu/Mg	0.578	<0.001	Cu/Fe	0.619	<0.001
Cu/Zn	0.653	<0.001	Cu/Mg	0.818	<0.001
Cu/Mn	0.411	0.002	Cu/Zn	0.752	<0.001
Pb/Fe	0.665	<0.001	Cu/Mn	0.591	<0.001
Pb/Hg	0.373	0.006	Cu/Mo	0.385	0.004
Pb/Cd	0.374	0.006	Fe/Mg	0.484	<0.001
Pb/Mo	0.556	<0.001	Fe/Zn	0.407	0.003
Pb/Cr	−0.498	<0.001	Fe/Mn	0.440	<0.001
Pb/Ni	0.523	<0.001	Mg/Zn	0.610	<0.001
Fe/Hg	0.500	<0.001	Mg/Mn	0.457	<0.001
Fe/Cd	0.429	<0.001	Mg/Mo	0.374	0.006
Fe/Mo	0.790	<0.001	Mn/Cd	0.470	<0.001
Fe/Cr	−0.381	0.005	Mn/Mo	0.570	<0.001
Mg/Zn	0.694	<0.001	Mn/Cr	0.510	<0.001
Mg/Mn	0.712	<0.001	Hg/Cd	0.431	<0.001
Mg/Mo	0.384	0.005	Cd/Mo	0.493	<0.001
Zn/Mg	0.694	<0.001	Mo/Cr	0.380	0.005
Zn/Mn	0.499	<0.001			
Mn/Mo	0.523	<0.001			
Mn/Cr	0.395	0.003			
Hg/Cd	0.525	<0.001			
Hg/Mo	0.507	<0.001			
Cd/Mo	0.590	<0.001			

non-essential elements (Pb, Hg, and Cd), as well as two essential elements (Fe and Mo). Nickel, an ubiquitous element that essentiality in mammals is discussed (e.g. Talmage and Walton, 1991; Eisler, 1998) and known to be carcinogen, teratogenic, genotoxic and hepatotoxic both to humans and animals (e.g. Kasprzak et al., 2003; Punshon et al., 2003), also increased in these shrews. Essential and non-essential metals displayed contrasting behaviour in their food-chain mobility within soil, plants, invertebrates, and shrews. Non-essential metals are associated with metal ores, pollute soils and waters, may be mobilized by several biological and chemical mechanisms and are highly transferred from soils and AMD across food-chains, and therefore bioaccumulated in small mammals (e.g. Johnson et al., 1978; Ma and Talmage, 2001; Lacal et al., 2003; Milton et al., 2004). Contaminated preys and soil ingest are the main routes of metal intake in mammals (Talmage and Walton, 1991; Stansley and Roscoe, 1996) and shrews ingest soil-residing organisms with relatively high bioaccumulation factors such as earthworms. The increase in Pb, Hg, Cd, and Ni in shrews from Aljustrel agrees with previous studies reporting significant amounts of these elements in AMD from pyrite mines in Portugal (Santos Oliveira et al., 2002), in soils from Aljustrel (Quevauviller et al., 1989), and in tissues of rodents from the St. Domingos pyrite mine (Pereira et al., 2006). Moreover, Pb, Cd, and Ni were accumulated in shrews inhabiting mine sites (Johnson et al., 1978; Smith and Rongstad, 1982; Świergosz-Kowalewska et al., 2005). To our knowledge, mercury is not reported as a common pollutant of pyrite mine tailings and data on the bioaccumulation in shrews from mine sites are lacking. Mercury concentrations in *C. russula* from mine site are higher than those recorded in other insectivores from polluted sites (Pankakoski et al., 1993, 1994). This observation supports the hypothesis of a high transfer of this element in the Aljustrel site probably because of the high mercury bioavailability in acidic conditions (Scheuhammer, 1991) such as AMD.

The high amounts of iron found in AMD and in mine soils (Quevauviller et al., 1989; Santos Oliveira et al., 2002) seem to be responsible for high dietary levels and consequent bioaccumulation in small mammals in pyrite mines (Pereira et al., 2006). Compared with highly toxic metals, environmental and ecotoxicological information on molybdenum is limited and levels of this metal in tissues of insectivorous are not commonly quantified. Pankakoski et al. (1993) reported lower liver Mo concentration in the mole, *Talpa europaea* ($M = 1.58–1.65\ \mu g\ g^{-1}$) that recorded in our study. The increased concentrations of this metal in shrews from Aljustrel might be related to physiological interaction between metals (Pankakoski et al., 1993). No significant differences between sites were found for the remaining essential elements quantified and their levels in shrew tissues did not appear to be related to increased environmental levels. Thus, in mammalian systems, it is likely that the uptake, bioaccumulation and excretion of these essential elements are effectively controlled physiologically and that they bioaccumulated only in cases of extremely high intake or disrupted metal metabolism (e.g. Goyer, 1997; Ma and Talmage, 2001).

Quantification of metals from reference sites is crucial to determine the current levels of clean sites and the loss of environmental quality in polluted zones. On a general trend, the metal concentrations in *C. russula* from our reference site were similar or lower than those reported in other insectivores from clean sites (Talmage and Walton, 1991; Pankakoski et al., 1993, 1994; Shore, 1995; Komarnicki, 2000; Ma and Talmage, 2001). Only Mo and Ni in kidneys and Mn in liver were among the highest reported in small mammal species. The characteristic species-dependent pattern of essential elements such Mn, the general high baseline metal values such Ni in insectivores, and the scarce information on levels of these three elements (Andrews et al., 1984; Hunter et al., 1989; Eisler, 1998; Ma and Talmage, 2001; Lopes et al., 2002) can explain these high concentrations.

4.2. Metal bioaccumulation by season, age, and sex

The wide range of variation observed in polluted areas may be partially explained by factors such as age and sex or by seasonal differences in diet and, therefore, by the individual response of uptake and bioaccumulation of metals. Few studies have addressed the seasonal changes in intake and bioaccumulation of metals in wild populations of mammals, but similar results were found for essential metals in the Algerian mouse, *Mus spretus*, and for non-essential metals in the Brown hare, *Lepus europaeus* (Massányi et al., 2003; Viegas-Crespo et al., 2003). In the case of variations in the concentration of essential metals, these may be related to life cycle (growth needs, gonad maturation, and reproduction). Moreover, seasonal differences may arise because of the indirect effects of pollution that affect food diversity and bioavailability and the consequent changes in exposure to essential and non-essential elements (e.g. Hunter and Johnson, 1982; Cloutier et al., 1986; Hunter et al., 1989; Lopes et al., 2002).

The adult shrews from the two study areas showed about a 2-fold in increase in Cd concentrations compared with juveniles. This metal bioaccumulates with age in insectivores (Pankakoski et al., 1993, 1994; Read and Martin, 1993) and rodents from mine sites (Smith and Rongstad, 1982; Milton et al., 2003). This age-bioaccumulation in renal tissue is associated with the formation of a stable cadmium-metallothionein complex as a detoxification mechanism to prevent toxic effects (Johnson et al., 1978; Ma and Talmage, 2001; Świergosz-Kowalewska et al., 2006). The increase in Hg with age also occurs in *C. russula* from a polluted wetland (Sánchez-Chardi et al., 2007). Although the livers from shrews caught in Aljustrel showed an increase in Pb with age, this metal did not show a clear bioaccumulation pattern with this factor. This observation contrasts with results from bones from the same species (Sánchez-Chardi et al., 2007). This discordant result could be attributed to the differences between hard and soft tissues bioaccumulation and/or by differences in exposure levels or chemical forms of Pb. The half-life of Pb in soft tissues is in the order of days or weeks and the level of this element indicates a relatively recent exposure, whereas the main Pb body burden in mammals is the bones, where it is stored in a lower toxic form. In contrast, Cr and Ni decrease with age in *C. russula*. Similar data for Cr was also reported in the common shrew, *Sorex araneus*, and *T. europaea* (Pankakoski et al., 1993, 1994) and for Ni in the meadow vole, *Microtus pennsylvanicus* (Smith and Rongstad, 1982). These observations may be explained by an analogous metabolic mechanism for Cr and Ni, and/or a higher digestive absorption rate in juveniles and, therefore, poor intestinal absorption of these elements in adults (Eisler, 1984; Outridge and Scheuhammer, 1993; Bonda et al., 2004; Pereira et al., 2006). While Fe, Mn, and Hg bioaccumulated with age in small mammals from polluted sites (e.g. Talmage and Walton, 1991; Stansley and Roscoe, 1996; Milton et al., 2003), we did not observe this trend in *C. russula* from the sites studied.

When compared with the other parameters, sex remains as less importance in bioaccumulation patterns. Metal bioaccumulation in mammals is related to reproduction and hormonal status. During pregnancy and/or lactation females may mobilized metals and transfer them across the placenta to the foetus or via milk to sucklings. Therefore, *a priori* a reduction in the concentration of toxic metals in females might be expected; however, contradictory results have been reported (e.g. Smith and Rongstad, 1982; Stansley and Roscoe, 1996; Lopes et al., 2002). The high concentrations of Mo and Ni in female shrews may be due to nutritional status as well as to differences in dietary intake and uptake of metals.

4.3. Metal bioaccumulation by tissue

The liver and kidneys are the main tissues involved in metabolic processes of toxic heavy metals such as biotransformation, bioaccumulation, detoxification and excretion. Our results on metal distribution in *C. russula* are consistent with those reported for other small mammal species (e.g. Talmage and Walton, 1991; Pankakoski et al., 1993, 1994; Eisler, 1998; Ma and Talmage, 2001; D'Havé et al., 2006). The liver was the main accumulator of Fe, Mg, Zn, Cu, Mn, and Mo, whereas Hg, Cd, and Ni were higher in kidneys and Cr showed similar mean values in both tissues and study sites. In contrast, lead exhibited distinct tissue pattern between sites: while Pb average values were similar between tissues in the reference site, the liver of shrews from Aljustrel showed higher concentrations. This latter distribution corroborates results for the bicoloured white-toothed shrew, *Crocidura leucodon*, and for the European hedgehog, *Erinaceus europaeus* (Topashka-Ancheva and Metcheva, 1999; D'Havé et al., 2006) and is discordant with data for other small mammal species that show higher renal than hepatic Pb concentrations (e.g. Talmage and Walton, 1991; Pankakoski et al., 1994). Although non-essential metals typically show accumulations within specific target organs, a change in tissue distribution has been reported in *S. araneus*, and *T. europaea* in relation with pollution (Andrews et al., 1984; Talmage and Walton, 1991; Komarnicki, 2000). This phenomenon seems related to physiological mechanisms to decrease toxicity, but the role of chronic exposure and half-life of Pb in soft tissues, as well as particular tissue distribution in *C. russula* must not be disregarded.

4.4. Morphological parameters

Given the scarce data on morphological parameters in insectivorous mammals, our results contribute to estimating the effects of pollution on wildlife. The results obtained for RI and relative weights in the two study sites were similar to those reported by other authors for small mammals inhabiting polluted and reference sites (Bartels et al., 1979; Ma, 1989; Milton et al., 2003; Pereira et al., 2006; Sánchez-Chardi et al., 2007). We found no differences in RI between the two sites, indicating similar fitness in the two areas; however, we did detect an increase in relative hepatic weight in shrews from mine site which may be indicative of hepatic edema (Milton et al., 2003). In fact, while body condition index is a measure of energy reserves and environmental quality, changes in the relative weight of somatic tissues may indicate metal exposure at toxic levels (Ma, 1989; Ma and Talmage, 2001). The increase in relative weight has been reported in kidneys of common shrews with extremely high Pb levels (Ma, 1989; Ma and Talmage, 2001) and in livers of bank voles, *Clethrionomys glareolus*, with similar Pb levels ($8.0 \pm 0.8\ \mu g\ g^{-1}$ DW; Milton et al., 2003) to those recorded in shrews from the Aljustrel mine site. These large differences between rodents and insectivores indicate that although these alterations are associated with Pb levels, they may also depends on other factors such as the interspecific tolerance of metals at toxic levels, exposure route and chemical form of the metal (Ma, 1989; Farag et al., 1998; Ma and Talmage, 2001).

4.5. Toxicity effects

Environmental pollution may stress animal populations, even if acute poisoning effects or levels are not observed (Pankakoski et al., 1994). From an ecotoxicological point of view, high metal exposure may produce toxic effects on wild specimens and animals may be more exposed to predators (Bonda et al., 2004). Although insectivores show high tolerance to metal pollution (Ma, 1989, 1996; Talmage and Walton, 1991; Shore, 1995; Ma and Talmage, 2001), the Pb, Hg, and Cd concentrations reported in specimens from Aljustrel may reach toxic levels. These non-essential elements have been associated with a wide range of toxicological responses in liver and kidneys of small mammals; these include oxidative stress, acute injury, apoptosis, and necrosis (e.g. Pankakoski et al., 1993; Stansley and Roscoe, 1996; Milton et al., 2003). In these organs, a wide range of critical Pb levels varying from $70\ \mu g\ g^{-1}$ DW to 5–$10\ \mu g\ g^{-1}$ DW (Scheuhammer, 1991; Ma, 1996) is considered a chemical biomarker of Pb toxic exposure in mammals. In our study, 11 out of 31 shrews in Aljustrel showed more than $5\ \mu g\ g^{-1}$ of Pb in liver (maximum of $15.99\ \mu g\ g^{-1}$ DW). Twenty-four out 32 specimens the mine site showed Hg concentrations above $1.1\ \mu g\ g^{-1}$ in kidneys (maximum of $5.65\ \mu g\ g^{-1}$DW) which is considered indicative of mercury pollution (Eisler, 1987). In contrast with rodents, insectivores are very tolerant to Cd as demonstrated in laboratory and field studies. While liver and kidney lesions in common shrews were associated with Cd concentrations of 577 and $253\ \mu g\ g^{-1}$ DW respectively, and $350\ \mu g\ g^{-1}$ DW is reported as indicative of harmful effects in insectivores (Hunter et al., 1989; Ma and Talmage, 2001), renal concentrations of about 110–$260\ \mu g\ g^{-1}$ produce nephrotoxic effect in mice (Pankakoski et al., 1993). Moreover, hepatic and renal alterations have been reported in bank voles with $15.1\ \mu g\ g^{-1}$ DW Cd in liver and 17.0 in kidneys (Wlostowski et al., 2003). The cadmium concentrations observed in shrews from the mine site did not reach toxic levels for these animals, but were higher than the no-observable-adverse-effects-level (NOAEL) in rodents and may indicate a considerable environmental pollution. Scarce information is available about food-chain transfer and uptake and tissue concentration of Ni (Andrews et al., 1984; Hunter et al., 1989; Eisler, 1998); however the high concentrations found in *C. russula* require more toxicological information that allows the assessment of their environmental consequences.

4.6. Correlation between metals

In spite of these toxic levels of single elements, the interaction and aggregative effect of metal mixture must be considered to determine subclinical environmental problems because these mixtures may increase toxic effects by synergism (Bellés et al., 2002). In fact, shrews from mining areas are, in general, exposed to a mixture of metals from mineral ores and associated inclusions (e.g. Johnson et al., 1978). Our data agree with relations reported for Fe, Zn and Cu with Pb, Cd, and Hg (e.g. Johnson et al., 1978; Wlostowski et al., 2003; Bonda et al., 2004; Pereira et al., 2006). On the whole, the role of the liver in homeostatic mechanisms and the kidneys in excretion processes may explain the correlations observed. Interaction between non-essential metals and essential metals is attributed mainly to competition in intestinal absorption, to competition mechanisms regarding metal enzymes complexes, and/or to the protective effects in front of oxidative processes produced by metal exposure. Despite of this, our results require confirmation due to the lack of information about these interactions.

5. Conclusion

The increase of metal in shrews due to their great feeding requirements of shrews may reveal low or current levels of pollution by bioaccumulation at detectable levels. Here we report that an abandoned pyrite mine produces a considerable increase in the bioavailability and bioaccumulation of potentially toxic metals (Pb, Hg, Cd, and Ni) in shrews. In fact, these elements might represent an environmental risk in these abandoned mines. Our results also indicate that season-, age- and sex-dependent variations must be considered when interpreting exposure, bioaccumulation and effects of metals in wild mammals. The trophic position and physiological characteristics of insectivores make them suitable bioindicators in the assessment of environmental quality and bioaccumulation in other species such as protected carnivorous mammals.

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Haematology, genotoxicity, enzymatic activity and histopathology as biomarkers of metal pollution in the shrew *Crocidura russula*

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Metals from an abandoned pyrite mine produce alterations in haematological parameters, GST, MNT, and histopathology in shrews.

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ABSTRACT

Haematological (WBC, RBC, Hgb and Hct) and genotoxicity (MNT) parameters, hepatic enzymatic activities (GST, GPx and GR), and a histopathological evaluation of liver, kidneys and gonads were assessed as general biomarkers of metal pollution in the shrew *Crocidura russula* inhabiting a pyrite mining area. Specimens exposed to metals presented a few significant alterations when compared with reference animals: GST activity decreased; micronuclei increased; and evident liver alterations related to metal exposure were observed. On the basis of all the parameters studied, age was an important factor that partly explained the observed variation, whereas sex was the least important factor. Significant correlations were also found between heavy metal concentrations and biomarkers evaluated, demonstrating the great influence of these metals in the metabolic alterations. To the best of our knowledge, these data constitute the first measurements of a battery of biomarkers in shrews from a mine site and are among the few available for insectivorous mammals.

1. Introduction

Abandoned mines constitute a serious environmental problem throughout the world. These sites represent a potential threat to human and ecosystems health and cause important impacts on wide areas over long periods if nothing is done toward pollution mitigation. The mine of Aljustrel is a good example of this situation. This mine site is one of the biggest deactivated pyrite mines in Portugal and spills out great amounts of liquid effluents, in what is known as acid mine drainage (AMD), characterized by high metal contents and low pH. In terrestrial food webs, toxic levels of some metals are easily reached in abandoned mining sites due to the high bioavailability of metals found in these areas. In fact, previous studies have reported the accumulation of several metals in soils, plants, and animals as well as their physiological effects in areas affected by deactivated mines in this country (e.g. Gerhardt et al., 2005; Pereira et al., 2006; Quevauviller et al., 1989; Sánchez-Chardi et al., 2007b). In spite of this high risk, scarce data are available concerning the influence of metal pollution on the health of species belonging to higher trophic levels, such as shrews. Almost no data were found on the effects of contamination in carnivorous small mammals inhabiting abandoned pyrite mining areas, Marques et al. (2007) quantify few haematological and enzymatic parameters in greater white-toothed shrews, *Crocidura russula*, from a Pb/Zn mine site. To our knowledge, the present study measures, for the first time, a battery of biomarkers in *C. russula*.

The assessment of physiological effects of chronic exposure to metals using biomarkers of sublethal toxicity is necessary in order to evaluate the impact of pollution under realistic conditions. These effects include genotoxic (Ieradi et al., 1996; Sánchez-Chardi and Nadal, 2007), enzymatic (Lopes et al., 2002; Świergosz-Kowalewska et al., 2006), haematological (Nunes et al., 2001; Reynolds et al., 2006; Rogival et al., 2006), and histological alterations (Clark et al., 1992; Damek-Poprawa and Sawicka-Kapusta, 2004; Pereira et al., 2006; Stansley and Roscoe, 1996) that in general only occur when substantial concentrations of metals are present in the tissues. In fact, the combined use of biomarkers with bioaccumulation data provides a suitable measure of health status,

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physiological condition, and response of terrestrial small mammal populations to pollution (e.g. Świergosz et al., 1998; Walker, 1998).

Environmental control should be multidirectional, but one of the most essential criteria of analysis is related to observations carried out on wild animals. Small mammals are ideal for monitoring environmental pollution as well as for evaluating the risk for human populations living in polluted areas such as abandoned metalliferous mining sites (e.g. Pereira et al., 2006; Sánchez-Chardi et al., 2007b; Talmage and Walton, 1991; Walker, 1998).

Here we quantify haematological and genotoxic parameters, enzymatic activities, and histopathological alterations in shrews inhabiting an abandoned pyrite mining site. Moreover, the effect of age and sex was also measured and correlations between metal bioaccumulation and biomarkers were determined in order to explain the variation found in these parameters. Finally, with all this information in mind, we discuss the risk involved with this kind of environmental pollution and the suitability of insectivorous mammals as bioindicators.

2. Material and methods

2.1. Study sites

This study was carried out in the vicinity of the deactivated pyrite mine of Aljustrel, located in the Baixo Alentejo province (southern Portugal). Deactivated about a decade ago, approximately 50,000,000 t of target metals were extracted from this mine during its 130 years of exploitation. The sampled area in Aljustrel (37°53′08″N; 08°08′32″W) is located along the "Água forte" riverside, where the stored acidic effluents are discharged during rainy periods. For comparative purposes, an area with similar relief, climate and vegetation, located 69 km northeast of the Aljustrel mine (38°11′13″N; 07°24′34″W) was selected as a reference, considering that no exogenous sources of heavy metals are known. The climate of the region where both sampling areas are located is characterized by hot dry summers and mild winters. The vegetation mainly consists of sparsely scattered specimens of *Quercus rotundifolia*, as well as several shrub and herbaceous species (*Rubus ulmifolius*, *Nerium oleander*, *Echium plantagineum*, *Bromus rigidus*, *Vulpia myuros*, and *Phleum phleoides*).

In 2003, a total of 34 specimens of the greater white-toothed shrew were live-trapped using Longworth® and Sherman® traps. Hepatic metal concentrations of these shrews were reported in a previous study (see Table 1). Distribution of captures by site, sex, and age is showed in Table 2. All captured animals appeared to be in good physical condition with no major signs of illness or deformity. Animals were sexed, weighed and transported to the laboratory. After a slight anaesthesia, specimens were sacrificed by cervical dislocation in strict accordance with ethical directives on the protection of animals.

2.2. Haematological and genotoxic parameters

Peripheral blood was collected by cardiac punction using heparinized syringes. White blood cells count (WBC, $\times 10^3\,mm^{-3}$), red blood cells count (RBC, $\times 10^6\,mm^{-3}$), haemoglobin concentration (Hgb, $g\,dl^{-1}$), and haematocrit (Hct, %) were quantified in a Coulter Counter Analyser (Beckman Coulter, USA).

For the micronucleus test, duplicate blood smears were made for each specimen on pre-cleaned microscope slides, fixed with heat, and stained with conventional May-Grünwald–Giemsa stain. For each individual, micronucleus (MN) frequency was scored on 2000 blood erythrocytes through an oil immersion objective (×100) on a Leica Leitz DMRB microscope.

Table 1
Mean ± SEM values for metals in liver of *C. russula* from the reference and the Aljustrel mine site (in $\mu g\,g^{-1}$ dry weight)

	Reference site ($n = 22$)	Mine site ($n = 31$)
Fe	1257.36 ± 160.03	2958.45 ± 395.21***
Mg	899.81 ± 27.51	987.32 ± 83.84
Zn	145.23 ± 7.27	167.26 ± 19.18
Pb	0.77 ± 0.09	5.26 ± 0.75***
Cu	23.83 ± 10.31	28.31 ± 16.17
Mn	17.56 ± 0.74	17.26 ± 1.42
Hg	0.26 ± 0.05	1.03 ± 0.26***
Cd	2.27 ± 0.65	8.61 ± 1.47***
Mo	3.38 ± 0.24	5.30 ± 0.52**
Cr	1.32 ± 0.11	1.12 ± 0.28**
Ni	0.67 ± 0.24	1.48 ± 0.17***

*$p \le 0.05$; **$p \le 0.01$; ***$p \le 0.001$.
After Sánchez-Chardi et al. (2007b).

Table 2
Distribution of specimens by site, sex and age

Site	Sex	Age		Total
		Juveniles	Adults	
Reference	Males	6	4	10
	Females	5	3	8
Mine	Males	4	4	8
	Females	4	4	8

2.3. Enzymatic parameters

A liver portion of about 300 mg was rinsed in an ice-cold 0.154 mM KCl solution immediately after dissection. Glutathione *S*-transferase (GST), glutathione peroxidase (GPx) and glutathione reductase (GR) activities were assessed according to Habig et al. (1974), Paglia and Valentine (1967) and Carlberg and Mannervik (1985), respectively. Total protein contents were determined according to the Biuret method (Gornall et al., 1949) using Bovine Serum Albumin (Sigma, Spain) as standard. Additional methodological descriptions may be found in Marques et al. (2007).

2.4. Histopathological evaluation

A small part of the liver, the right kidney and the gonads were fixed in 10% neutral-buffered formalin, dehydrated in ethanol, cleared in xylene and embedded in paraffin wax. Sections of 5 μm thick were stained with conventional hematoxylin and eosin and the slides were examined by light microscopy. The incidence of alterations was reported in a qualitative evaluation. Moreover, for statistical purposes the severity of alterations was measured on a semi-quantitative scale scored in four categories according to the intensity of alterations: without alteration (0), slightly altered (1), intermediately altered (2), and strongly altered (3).

2.5. Statistical analyses

All data were expressed as mean ± standard error ($M \pm$ SEM). When necessary, data were log transformed and then tested for normal distribution (Shapiro–Wilk test) and homogeneity of variance (Levene, *F*-test). Comparisons of haematological and enzymatic parameters by age, sex, and site were performed with Student's tests (t). Significant genotoxic and histopathological divergences for these three factors were assessed using Mann–Whitney tests (U). Moreover, for each of these factors, data were pooled in order to obtain an appropriate sample size for statistical analyses. To investigate associations between bioaccumulation and its effects, Spearman or Pearson correlation coefficients (r) were calculated between hepatic metal concentrations and enzymatic, haematological, histopathological, and genotoxic parameters for both study sites. All statistical analyses were performed with SPSS 11.5 software for Windows.

3. Results

3.1. Haematological and genotoxic parameters

Descriptive statistics for haematological parameters are shown in Table 3. Though no significant differences were found by study site, season, age, or sex in these parameters, a tendency toward increase was detected in specimens from the polluted site. An age-dependent increase in both studied sites was also observed (Table 3). Moreover, at the polluted site, significant correlations were observed between RBC and Pb ($r = -0.609$, $p = 0.009$), RBC and Cr ($r = 0.618$, $p = 0.008$), Hct and Pb ($r = -0.510$, $p = 0.036$), and Hgb and Cu ($r = 0.525$, $p = 0.031$).

Shrews collected on the polluted site exhibited significantly higher micronuclei mean frequencies than specimens from the reference site (1.00 ± 0.15 *vs* 0.14 ± 0.09‰, respectively; $U = 4.195$, $p = 0.001$). No relationships were found in MN frequencies with age or sex; whereas significant correlations were detected between MN and several metals, namely Fe ($r = 0.722$, $p = 0.002$), Mn ($r = -0.592$, $p = 0.020$), Cu ($r = -0.565$, $p = 0.028$), Pb ($r = 0.524$, $p = 0.045$), Cd ($r = 0.537$, $p = 0.022$), and Cr ($r = -0.744$, $p = 0.001$).

Table 3
Haematological parameters in *C. russula* by age and site

	Reference site			Mine site		
	Juveniles ($n = 8$)	Adults ($n = 8$)	Total ($n = 16$)	Juveniles ($n = 5$)	Adults ($n = 10$)	Total ($n = 15$)
WBC ($\times 10^3$ mm^{-3})	3.150 ± 0.327	4.625 ± 0.740	3.887 ± 0.435	3.840 ± 0.819	4.060 ± 0.749	3.987 ± 0.553
RBC ($\times 10^6$ mm^{-3})	5.997 ± 0.917	8.147 ± 0.664	7.072 ± 0.613	7.507 ± 0.468	8.544 ± 0.526	7.853 ± 0.371
Hgb (g dl^{-1})	12.212 ± 0.861	13.887 ± 0.487	13.050 ± 0.524	13.300 ± 0.595	14.130 ± 0.427	13.853 ± 0.350
Hct (%)	25.012 ± 3.816	34.700 ± 2.887	29.856 ± 2.628	34.920 ± 1.970	33.330 ± 2.226	33.860 ± 1.592

3.2. *Enzymatic parameters*

Specimens from the abandoned mine site showed a significant decrease in GST activity ($t = -3.422$; $p = 0.002$). No site variation was found in the activity of the other quantified enzymes (Table 4). With age, a general tendency toward a decrease in GST activity was detected, while GPx (Reference site: $t = -2.336$, $p = 0.034$) and GR tended to increase (Table 4). No sex-dependent variation was observed in enzymatic activities of *C. russula* from both study sites. Moreover, in the specimens from the polluted area, significant positive correlations were identified between GPx activity and hepatic concentrations of Pb ($r = -0.587$, $p = 0.013$), Cu ($r = 0.791$, $p < 0.001$), Mn ($r = 0.651$, $p = 0.005$), and Cr ($r = 0.536$, $p = 0.027$).

3.3. *Histopathological evaluation*

Specimens exposed to metal pollution in the mining area showed a significant increase in number and severity of pathological alterations, namely foci of cell necrosis, apoptosis, and cytoplasmic vacuolization in the hepatic tissue, when compared with reference shrews ($U = 17.500$; $p < 0.001$). In fact, 86% of the analysed specimens from the mine site showed evidences of necrosis and 79% of vacuolization. On the other hand, 8% of the specimens from the reference site showed evidences of necrosis and 58% presented signs of vacuolization. Moreover, a specimen from the polluted site showed evidences of apoptosis in the hepatic tissue with nuclei fragmentation and pyknotic nuclei. This was confirmed with an immunohistochemistry analysis, namely the detection of cysteine protease Caspase 3 (data not shown). Table 5 reports a semi-quantitative severity scale of alterations in the liver and Fig. 1 shows examples of control and altered tissues. No significant age- or sex-dependent variations were detected in these pathologies for either of the studied sites. There were also no significant relationships between pathologies and hepatic metal concentrations. Moreover, no alterations related with metal pollution were observed in the kidneys, testes or ovaries of any of the analysed shrews.

4. Discussion

Several pyrite mines have been closed in southern Portugal in the past few decades, mainly due to economic reasons. Most of them were abandoned without any previous environmental recovery plan, and thus have continued to be an environmental threat long after mining activities have ceased. Harmful effects to biota include deep changes in cells, tissues, individuals, populations, and ecosystems. However, scarce information is available on effects on higher trophic level species such as insectivorous mammals.

4.1. *Haematological and genotoxic parameters*

Registered values for haematological parameters are in agreement with available literature on small mammal species (see revision in Table 6). No significant differences were found between study sites in spite of the higher mean values detected in the polluted area. Such a result could, at least partially, be due to the great dispersion of data that may mask any significant difference. In fact, homeostasis of mammalian systems under chronic exposure to environmental pollution may decrease and/or attenuate differences in wild populations, in contrast with laboratory animals that often showed significant differences when acutely exposed to metals (e.g. Jadhav et al., 2007; Medina et al., 2007; Włostowski et al., 2003). Several studies have reported alterations of haematological parameters in Algerian mice, *Mus spretus*, house mice, *Mus musculus*, wood mice, *Apodemus sylvaticus*, and northern pocket gophers, *Thomomys talpoides*, inhabiting polluted sites (see Table 6). In our study, this tendency toward an increase of haematological values might be related with protective effects of essential metals such as iron or with interactions of toxic metals with oxygen transport, as suggested by the low level of efficiency of their mitochondria in the elimination of reactive oxygen species (ROS) and/or immunotoxic effects of pollutants (e.g. Tersago et al., 2004; Stewart et al., 2005). Moreover, the higher levels of toxic metals in adults as compared with juveniles and the alterations of metabolism in old animals could explain the tendency toward an increase of some haematological parameters with age. In accordance with our results, no sex-dependent variation was showed for the same analysed parameters in *M. spretus* and *A. sylvaticus* (Nunes et al., 2001; Rogival et al., 2006).

The micronuclei frequencies obtained for the reference area are in agreement with previously published data of background levels in *C. russula* and other small mammal species collected from different reference sites (see references in Sánchez-Chardi and Nadal, 2007). The significant positive correlation found between MN frequencies and non-essential metal concentrations in shrews from the pyrite mining site may be indicative of clastogenic effects of these elements in wild specimens (e.g. Ieradi et al., 1996; Sánchez-Chardi and Nadal, 2007; Topashka-Ancheva et al., 2003; Tull-Singleton et al., 1994). Correlations between MN and essential elements could be partly explained by the protective and/or antagonistic effects between elements in specimens exposed to high concentrations of toxic metals.

Table 4
Enzymes activities (μmol/min/g protein) in the liver of *C. russula* by age and site

	Reference site			Mine site		
	Juveniles ($n = 8$)	Adults ($n = 9$)	Total ($n = 17$)	Juveniles ($n = 6$)	Adults ($n = 11$)	Total ($n = 17$)
GST	1094.01 ± 96.99	891.44 ± 67.65	986.76 ± 61.54	791.57 ± 74.71	628.36 ± 89.37	685.96 ± 65.01
GPx	113.20 ± 19.93	161.57 ± 11.34	139.24 ± 12.63	128.55 ± 10.45	143.25 ± 16.60	133.14 ± 8.73
GR	66.25 ± 3.61	68.11 ± 3.12	67.24 ± 2.31	64.67 ± 4.24	76.00 ± 5.67	71.75 ± 4.04

Table 5
Frequency and intensity of histological alterations in livers of *C. russula* (without alteration (−), slightly altered (+), intermediately altered (++), strongly altered (+++))

	Reference site ($n = 12$)				Mine site ($n = 14$)			
	−	+	++	+++	−	+	++	+++
Necrosis and degeneration	11	1	0	0	2	8	4	0
Apoptosis	12	0	0	0	13	0	0	1
Vacuolization	5	4	2	1	3	1	4	6

4.2. Enzymatic parameters

It is well documented that heavy metal exposure alters the normal activity of antioxidant enzymes, as reported in laboratory and wild mammals (e.g. Li et al., 2006; Lopes et al., 2002; Pinheiro et al., 2001; Reynolds et al., 2006; Viegas-Crespo et al., 2003). However, data with measurements on these parameters are quite scarce for shrews (Hamers et al., 2006; Marques et al., 2007). Some shrew species are among the smallest mammals and in order to maintain their body temperature, they have extremely high food requirements due to their high metabolic rate, which is higher than predicted by allometric scaling (e.g. Stewart et al., 2005). These atypical physiological conditions also affect enzyme activities in detoxification systems, as reported for the short-tailed shrew, *Blarina brevicauda* (Stewart et al., 2005). In the present study, though no significant differences were found for GPx and GR between study areas, a decrease of GST activity was registered in shrews from Aljustrel. GST catalyzes the conjugation of various substances with glutathione, playing an important role against oxidative stress. This result agrees with data provided by Świergosz-Kowalewska et al. (2006) for bank voles, *Clethrionomys glareolus*, exposed to metals from a zinc–lead smelter. Similar levels of Cd and Pb bioaccumulation in renal tissue (maximum 47 $\mu g\ g^{-1}$ and 20 $\mu g\ g^{-1}$, respectively) were reported in shrews from the Aljustrel mining site (Sánchez-Chardi et al., 2007b). In general, the non-significant differences detected in GPx and GR activities between

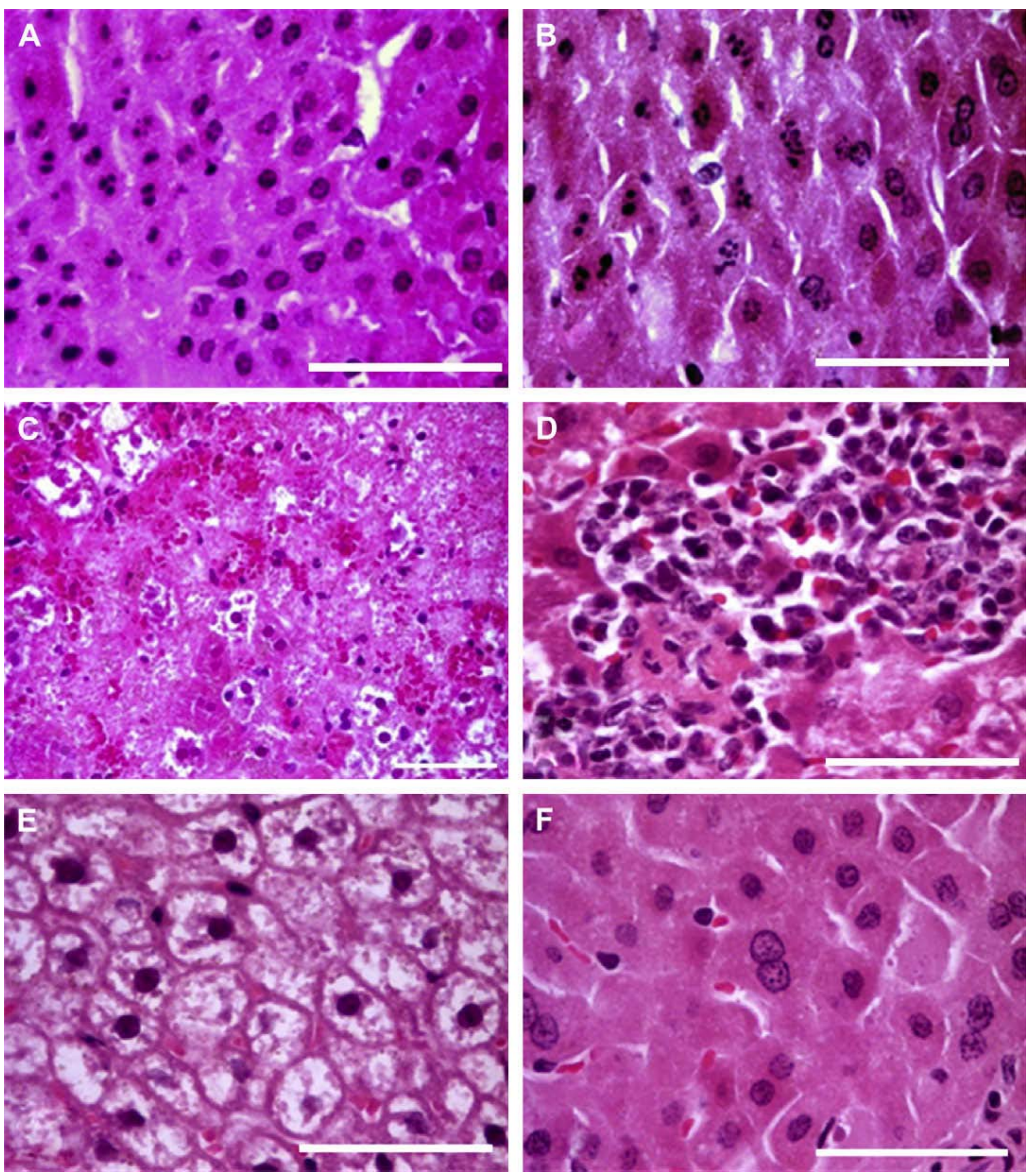

Fig. 1. Sections of hepatic tissue of *C. russula* showing: (A, B) apoptosis with nuclei fragmentation; (C, D) degeneration and necrosis with lymphoid elements; (E) more rounded cells, swollen parenchyma with vacuolization; and (F) normal hepatic tissue (bar size corresponding to 50 μm).

Table 6

Haematological parameters and hepatic enzymes activities (mean, mean ± SD, mean ± SEM or mean and range) reported for wild small mammals from polluted (P) and reference (R) sites

Species		*n*	Haematological parameters				Enzymes activities				Descriptive statistics	References
			WBC ($\times 10^3$ mm^{-3})	RBC ($\times 10^6$ mm^{-3})	Hgb (g l^{-1})	Hct (%)	GST	GPx	GR	Units		
Crocidura russula	P	16	4.80	8.13	14.0	35.7	1.18	0.128	0.067	μM/mg prot/min	Median (range)	Marques et al. (2007)
	R	17	3.30	6.64	13.2	29.3	0.933	0.168	0.067			
	R	6		10.8 ± 0.5	15.6 ± 1.7	44.3 ± 3.0					*M* ± SD	Bartels et al. (1979)
Suncus etruscus	R	3		18.3 ± 0.3	17.4 ± 0.8	50.3 ± 1.5						
Apodemus sylvaticus	P	25	1.42 ± 2.25	8.20 ± 1.66	16.00 ± 1.53	45.83 ± 3.24					*M* ± SD	Gorriz et al. (1996)
	P	30	1.21 ± 1.43	8.43 ± 1.42	16.59 ± 1.59	47.40 ± 4.43						
	R	27	1.51 ± 1.36	8.57 ± 1.78	16.69 ± 1.61	46.77 ± 4.06						
	R	10–5					96.8 ± 31.1–89.2 ± 32.7			μM/g WW liver/min	*M* ± SD	Lopes et al. (2002)
	P	6	1.34 ± 0.15	7.10 ± 0.05							*M* ± SEM	Tersago et al. (2004)
	P	7–8	1.56 ± 0.23	8.92 ± 0.03								
	R	7–9	2.03 ± 0.24	8.94 ± 0.04								
Apodemus flavicollis	P	8	3.68 ± 1.26	8.16 ± 0.88	18.44 ± 0.79	54.4 ± 4.08					*M* ± SEM	Topashka-Ancheva et al. (2003)
	P	12	3.17 ± 1.33	7.8 ± 3.2	18.40 ± 1.8	56.2 ± 2.7						
Clethrionomys glareolus	P	6					0.921 ± 0.292	21.622 ± 6.950	0.053 ± 0.009	μM/mg prot/min	*M* ± SEM	Świergosz-Kowalewska et al. (2006)
	P	5					0.334 ± 0.057	5.642 ± 1.017	0.134 ± 0.016			
	R	10					0.558 ± 0.113	27.134 ± 9.124	0.196 ± 0.042			
	P	9	3.73 ± 8.58	8.25 ± 1.00	17.82 ± 1.8	53.92 ± 3.39					*M* ± SEM	Topashka-Ancheva et al. (2003)
	P	8	3.15 ± 1.25		17.80 ± 1.0	54 ± 0.8						
	R	11	2.83 ± 0.75	9.97 ± 1.56	20.30 ± 1.3	55.31 ± 4.75						
Chionomys nivalis	P	5	3.62 ± 1.16	8.92 ± 1.81	14.8 ± 2.34	49.95 ± 4.87					*M* ± SEM	Topashka-Ancheva et al. (2003)
	R	4		10.96 ± 1.36	16.85 ± 2.1	46.15 ± 3.43						
Microtus pennsylvanicus	P	23	4.4 ± 0.5								*M* ± SEM	Knopper and Mineau (2004)
	P	27	5.2 ± 0.6									
	P	5	4.6 ± 1.1									
	P	11	4.5 ± 0.5									
	R	10	5.2 ± 0.6									
Mus spretus	P	60–80	7.92 ± 5.81	8.23 ± 1.57	15.6 ± 2.6	37.7 ± 7.9					*M* ± SD	Nunes et al. (2001)
	R	36–46	6.83 ± 4.35	8.49 ± 0.84	15.1 ± 1.7	39.1 ± 4.6						
	R	16–14					162.8 ± 93.8–129.5 ± 37.1			μM/g WW liver/min	*M* ± SD	Lopes et al. (2002)
	P	49					187.8 ± 56.9			μM/g WW liver/min	*M* ± SD	Viegas-Crespo et al. (2003)
	R	30					147.3 ± 73.9					
Peromyscus leucopus	P	11			14.0 (11.5–16.0)	47 (37–55)					Mean (range)	Stansley and Roscoe (1996)
	R	12			15.0 (12.5–17.0)	49 (42–56)						
Peromyscus maniculatus	P	19			14.6	44.3					*M*	Kucera (1988)
	P	34			15.2	48.5						
	R	44			12.4	40.7						
	R	44			12.7	41.4						
Thomomys talpoides	P	10	2.71 ± 0.44								*M* ± SEM	Reynolds et al. (2006)
	P	9	5.29 ± 1.17									
	R	10	4.81 ± 0.78									

study sites can possibly be related to an adaptation or increasing tolerance of specimens to chronic metal exposure.

The free radical theory proposed by Harman (1992) postulates an increase of ROS with age and, therefore, a consequent increase of antioxidant defences. Moreover, age-dependent variations were reported in antioxidant activity of rats (e.g. Gupta et al., 1991) and in the activity of hepatic cytochrome P450 in common shrews, *Sorex araneus* (Hamers et al., 2006). Since toxic metals such as Cd, Pb, and Hg reached significantly higher levels in adult shrews as compared with juveniles collected in Aljustrel (Sánchez-Chardi et al., 2007b), the correlation found between enzyme activities and age might be indicative of metal toxicity when a certain level of bioaccumulation is reached, as well as a decline in cellular response to oxidative stress with increasing age (e.g. Holbrook and Ikeyama, 2002). In fact, several studies have showed that metal exposure can cause alterations of GPx, GST, and GR activities, preventing some adverse effects of oxidative stress induced by the production of ROS species (e.g. Cnubben et al., 2001; Jadhav et al., 2007; Li et al., 2006; Viegas-Crespo et al., 2003).

In rodents (Fouchécourt and Rivière, 1995; Li et al., 2006) and insectivores (Hamers et al., 2006) sex-dependent variation has been previously reported in enzymes from detoxification systems. Nonetheless, no differences were found in *C. russula* in the present study, in agreement with other surveys using wild populations of small mammals (Lopes et al., 2002; Viegas-Crespo et al., 2003). These divergent results may be indicative of inter-species and/or inter-populations differences. Due to the scarce information available on biotic parameters obtained from wild populations of small mammals exposed to metal contamination, it is essential that more studies dealing with these aspects be carried out.

4.3. Histopathological evaluation

One of the goals of this study was to assess, for the first time, histological alterations related to metal exposure in livers of wild shrews. As far as we know, available data on the histopathological effects of metal pollution in wild insectivorous mammals are very scarce and limited to renal lesions (e.g. Stansley and Roscoe, 1996). Histopathological evaluation of target tissues is a suitable biomarker that provides important qualitative and quantitative information about acute or chronic effects of toxic compounds, sometimes not so finely predicted by other parameters (e.g. Reynolds et al., 2006; Jadhav et al., 2007; Thijssen et al., 2007). This approach is commonly used on laboratory animals but seldom on wild populations of insectivorous mammals. Several other studies have used the liver as the main target organ for the assessment of histopathological alterations, examining various rodent species exposed to non-essential metals on polluted sites (Clark et al., 1992; Damek-Poprawa and Sawicka-Kapusta, 2004; Pereira et al., 2006).

In the present study, shrews from the mining area presented an increase in the number and severity of pathological alterations in the liver attributed to the effects of bioaccumulation of toxic compounds in this organ. In fact, the relative liver weight increases and the hepatic levels of Pb, Hg, and Cd in these specimens were above the no-observed-adverse-effects-level (NOAEL) and, therefore, susceptible to toxic effects (Sánchez-Chardi et al., 2007a,b). Among tissues and cellular alterations, chronic exposure to these elements induces hepatic cell necrosis and apoptosis, and produces cytoplasmic vacuolization in hepatocytes, showing metal toxicity and carcinogenicity (e.g. Świergosz et al., 1998; Damek-Poprawa and Sawicka-Kapusta, 2004; Jadhav et al., 2007). However, it was impossible to determine which metal caused which specific lesion due to wild populations are often exposed to a mixture of toxics. These signs of hepatic damage may be due to oxygen deficiency and/or the presence of ROS induced by metal exposure. This can often occur when detoxifying systems such as metallothioneins are not responding efficiently enough to bind all metal ions (Świergosz et al., 1998; Włostowski et al., 2003; Jadhav et al., 2007).

Despite the abundance of data relating toxic effects with age and gender, no dependent variation was found in *C. russula* for the two parameters. The absence of histological changes in the kidneys, testes or ovaries of any of the analysed shrews may reflect these tissues higher tolerance to heavy metals. This physiological adaptation to chronic exposure is probably connected with efficient detoxification systems acting in these tissues or low exposure to toxic species of metals due to the important role of the liver in detoxification of xenobiotics uptaken in food.

4.4. Small mammals as bioindicators

Small mammals are often considered to represent an intermediate stage between low and high trophic levels, since they constitute important items in the diet of carnivorous birds and mammals. However, shrews are insectivorous and can be considered predators with a high position in the food-chain. In fact, they usually accumulate larger amounts of toxic pollutants than rodents, which makes these insectivores suitable bioindicators of environmental contamination (e.g. Talmage and Walton, 1991).

Wild mammals from polluted environments can uptake metals until toxic levels are reached. The assessment of this potential risk can be measured by daily intake, metal bioaccumulation in tissues, or through biomarkers that report toxic effects at different levels. Daily metal intake in shrews from the pyrite mine of Aljustrel remains unknown, but considering the high concentrations of non-essential metals found in tissues of the analysed specimens (Sánchez-Chardi et al., 2007b) it is obvious that it exceeds the threshold of toxicity. Even though biomarkers can be influenced by a multiplicity of factors under wild conditions, they provide suitable information on animals' health status when exposed to some sort of pollution in their natural habitat. In fact, the assessment of environmental pollution effects in wild biota has been a vital challenge for ecotoxicologists in spite of evident difficulties caused by constantly changing environments and high intra-specific variability.

Organelles, cells, tissues, organs, individuals, populations, communities, or ecosystems can suffer the deleterious consequences of toxic substances. An important and complicated issue is to understand precisely how pollutants affect each of these organizational levels. Here we have quantified several parameters in order to better understand possible deleterious effects of non-essential metal exposure from the sub-cellular to the individual and population levels. As a result, we reported significant genotoxic, enzymatic and histological alterations, as well as a tendency toward an increase in haematological parameters in shrews chronically exposed to metals in an abandoned mining area.

Several authors have suggested that wild small mammals inhabiting polluted sites are more sensitive than animals that are the subjects of laboratory experiments (e.g. Reynolds et al., 2006). Laboratory animals are usually under strict, controlled conditions, with minimal variation in abiotic (temperature, humidity, photoperiod, etc.) and biotic factors (such as gender or age). In addition, controlled contamination protocols usually involve animals uniformly exposed to a single toxic compound at a known and constant concentration. Conversely, specimens from natural populations live under numerous (and often uncontrolled) circumstances, such as parasitosis or reduced food availability, that can contribute to a decline in their health status. Thus the amount of energy available for essential activities and defence against toxic effects of metal exposure is considerably diminished. Moreover, in the wild, metal exposure frequently includes a combination of potentially toxic compounds that are not distributed constantly in time and space. These factors can lead to intra-specific variation, differential exposure and response (Talmage and Walton, 1991), contributing to the great variability observed in some parameters in polluted areas (e.g. Sánchez-Chardi et al., 2007a; Marques et al., 2007). Pollutants may also be transformed before exposure, producing cumulative effects and/or undergoing interactions between them. So wild populations chronically exposed to pollution apparently make use of adaptive processes to better tolerate toxicants in a changing environment (e.g. Marques et al., 2007; Medina et al., 2007). All these factors can complicate the interpretation of ecotoxicological data as some parameters often do not show statistical significance, but only increasing/decreasing tendencies. A multidirectional approach such as the one described in the present study is extremely important for a wider and more accurate view of the effects of environmental pollution.

5. Conclusions

Shrews from the abandoned pyrite mine of Aljustrel not only showed significant heavy metals accumulation in their tissues (above toxic levels) but also presented physiological alterations. Age was a factor that particularly contributed to the observed variability, whereas sex was the one that contributed the least to the variation of the quantified parameters. Statistically significant correlations were reported between biomarkers and heavy metal contents. In spite of these relationships, none of the analysed metals can be pointed to as being chiefly responsible for the variation of the quantified biomarkers. A number of other metals, as well as other xenobiotics often present in wild conditions, may have also contributed to these results.

The combination of multiple biomarkers at different levels of organization can contribute to an integrative view of overall effects of environmental pollution at realistic conditions in wild specimens and populations. Here we have showed that non-destructive biomarkers such as MNT and haematological parameters provide suitable information, but histopathological evaluation of target tissues has revealed itself to be an important, specific, and sensitive tool for ecotoxicological assessment.

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Short communication

Tissue, age, and sex distribution of thallium in shrews from Doñana, a protected area in SW Spain

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Abstract

In 1998, the protected area of Doñana, an important natural region in SW Europe, was affected with great amount of acidic waters and sludge from a pyrite mine loaded with toxic metals such as thallium (Tl). Since this ecological catastrophe, several studies have addressed the effects of this pollution on the flora and fauna in this protected area. However, in contrast to other non-essential metals, scarce information on Tl was available after this disaster, especially in terrestrial environments. This study reported a 3- and 10-fold increase in Tl in liver and kidneys, respectively, of the greater white-toothed shrew, *Crocidura russula*, in the polluted site in comparison with reference animals. Kidneys showed the highest concentrations of this metal in the polluted site, whereas both organs analysed have similar concentrations in the reference site. Although no significant age-dependent variation was found, adults had higher concentrations than juveniles. Moreover, females showed higher concentrations than males. These results demonstrate the high entrance and transfer of Tl in terrestrial food-chains. To the best of my knowledge, these data constitute the first measurements of Tl in mammals from the protected area of Doñana and are among the few available for insectivorous mammals.

Keywords: *Crocidura russula*; Thallium; Doñana; Protected areas; Pyrite mine

1. Introduction

When the tailing dam at the Los Frailes Mine in Aznalcóllar (SW Spain) broke in April 1998, a total of $5Hm^3$ of acidic waters and sludge from mining activities was released into the Guadiamar river affecting the protected area of Doñana. This spill included high amounts of toxic metals such as lead (Pb), mercury (Hg), cadmium (Cd), zinc (Zn), copper (Cu), and thallium (Tl). Since this accident, several studies have measured these elements and their effects in Doñana. However, while abundant information is available for Pb, Hg, Cd, Zn and/or Cu in biotic and abiotic compartments, little attention has been devoted to Tl (e.g. Prat et al., 1999; Simón et al., 1999; Madejón et al., 2003, 2006, 2007; Solà and Prat, 2006). To my knowledge, no data are available on the concentrations this metal in terrestrial small mammals in Doñana. In fact, although wild rodents and insectivorous mammals are often used as bioindicator of pollution (Talmage and Walton, 1991; Ma and Talmage, 2001), data on the concentrations of Tl in this group are scarce. The only report available was made by Dmowski

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et al. (1998), who described extremely high Tl bioaccumulation in three rodent species from a polluted site and low concentrations in the common shrew, *Sorex araneus*, from a reference site.

Tl is a non-essential heavy metal used in several industrial activities. It is found in sulfite deposits such as those in the Aznalcóllar mine, worked since Roman times (e.g. Grimalt et al., 1999; Kazantzis, 2000). Although this metal is not as widely distributed as other heavy metals, it has recently been examined in ecotoxicological studies as it is more toxic for organisms than other elements (Nriagu, 1998; Lin et al., 2001; Ma and Talmage, 2001; Rocha et al., 2004; Wierzbicka et al., 2004; Gao et al., 2007). In fact, chronic exposure to and poisoning by thallium has been reported in humans (Léonard and Gerber, 1997; John Peter and Viraraghavan, 2005) and the increase in levels and bioavailability of this element in the environment presents a risk for human and environment.

This study provides the first data on thallium bioaccumulation in an insectivorous mammal, the greater white-toothed shrew, *Crocidura russula*, from a polluted site and analyse the tissue distribution and age- and sex-dependent variation of this non-essential element in this species. I also discuss the implications of thallium pollution for environmental risk and management of protected areas.

2. Materials and methods

A total of 29 shrews were captured during November and December 1999 in two sites in the protected area of Doñana. The first is an area affected by the spill from the Aznalcóllar mine ("Entremuros, Parque Natural", 37° 01′12″N, 6° 16′38″W) and the second a reference site not affected by this pollution ("La Vera, Reserva Biológica", 36° 59′25″N, 6° 26′41″W). Specimens were transported to the laboratory and killed by cervical dislocation following ethical procedures. Sex was determined during dissection. Shrews were classified as juveniles or adults on the basis of the degree of tooth wear.

Liver and right kidneys were dissected, dried and digested in Teflon vessels with 2 ml of nitric acid and 1 ml of hydrogen peroxide (Instra, Baker Analized), as described in Sánchez-Chardi et al. (2007). Tl concentrations were measured in diluted samples using rhodium as internal standard in a Perkin-Elmer ELAN-6000 Inductively Coupled Plasma Mass Spectrometer (ICP-MS). Two replicate subsamples were analysed and a reference material (Bovine Liver SRM-1577a) certificated by the National Bureau of Standards was included in the analysis. To calculate the concentration of elements, the mean values from 10 blanks were subtracted from each sample. Results are expressed in $\mu g\ kg^{-1}$ on a dry weight basis (DW). Chemical analyses were performed at the Elemental Analysis Facility of the Scientific-Technical Services at the University of Barcelona.

After log-transformation and test for normal distribution (Shapiro–Wilk test) and for homogeneity of variance (Levene, F-test), differences in Tl concentrations by site, sex and age were analysed by Student tests (t) using SPSS (version 11.5 for Windows, SPSS Inc.). Significant differences were assumed at $p<0.05$. Details of the procedures can be found in Sánchez-Chardi et al. (2007).

3. Results and discussion

Tl concentrations in all the samples were above the detection limit (0.01 $\mu g\ kg^{-1}$). Compared with reference specimens, the shrews from the polluted site showed a significant increase in Tl concentrations in liver (147.69±44.47 *vs.* 52.68±7.76 $\mu g\ kg^{-1}$; $t=2.717$, $p=0.012$) and kidneys (325.27±33.59 *vs.* 33.32±7.63 $\mu g\ kg^{-1}$; $t=8.477$, $p<0.001$). These results indicate high bioavailability of Tl in shrews collected near the Guadiamar river, in an area with tidal fluctuation. In fact, when present in the environment, this metal is often dissolved and may be adsorbed onto clay minerals and, particularly in wet soils, may be highly transferred to plants and animals (Dmowski et al., 1998; Gao et al., 2007; Madejón et al., 2007). This great availability of Tl has also been reported in plants, periphyton, plankton, and macroinvertebrates collected in the Guadiamar river (Prat et al., 1999; Madejón et al., 2003, 2006, 2007; Solà and Prat, 2006). In contrast, low concentrations of this element have been reported in blood from birds captured in Doñana (Benito et al., 1999). These low levels could be explained by the short half-life of Tl in blood or to differences in metal intake or turnover between birds and mammals.

Compared with previous studies in wild mammals, the Tl concentrations registered in *C. russula* from the reference site were lower or similar to those reported in liver and kidneys of the Japan mongoose, *Herpestes javanicus*, the common shrew and three rodent species, and in the liver of Amami rabbit, *Pentalagus furnessi* (Dmowski et al., 1998; Horai et al., 2006).

Although mean values of Tl were high in the liver (95.97±47.37 *vs.* 168.04±52.65 $\mu g\ kg^{-1}$) and kidneys (310.59±35.77 *vs.* 353.03±35.77 $\mu g\ kg^{-1}$) of adults from the polluted site, no significant age-dependent variation was found in specimens from this site. No age comparisons were possible in shrews from the reference site because all the specimens captured were adults. Laboratory data indicate an increase in Tl with age as a result of decreased excretion by kidneys in adult rats was observed in relation to juveniles (Fleck and Appenroth, 1996). Moreover, although field studies show the same increase with age in polluted areas (e.g. Lin et al., 2001), the high variability in wild specimens reduces statistical significance, as occurs in this study. I consider that more studies are required to elucidate this question.

Females of *C. russula* tended to accumulate more Tl than males (Fig. 1). This increase was significant in the reference site (Liver: $t=2.554$; $p=0.038$; Kidneys: $t=-3.705, p=0.014$). Although a decrease in Tl is expected in females because this metal is transferred across the placenta to the foetus (Dmowski et al., 1998; Nriagu, 1998), similar results have been reported for several metals in small mammals (see references in Sánchez-Chardi et al., 2007). These findings may be explained, at least in part, by differences in metal intake, uptake and/or turnover because metal bioaccumulation in mammals is related to reproduction and hormonal status. However, specific studies are necessary to identify the mechanisms that might explain these gender differences in Tl accumulation.

The tissue distribution pattern of Tl differed between specimens from the polluted and reference sites. While significantly high Tl concentrations were found in kidneys when compared with liver in animals from the polluted site ($t=-3.002$, $p<0.001$), no statistical differences were detected between tissues from specimens in the reference site. Background concentrations of this metal with low toxic interference could explain the data in the former. High bioaccumulation of Tl in renal tissue could be attributed to considerable exposure to this metal in polluted sites. This element is rapidly absorbed through the gastrointestinal

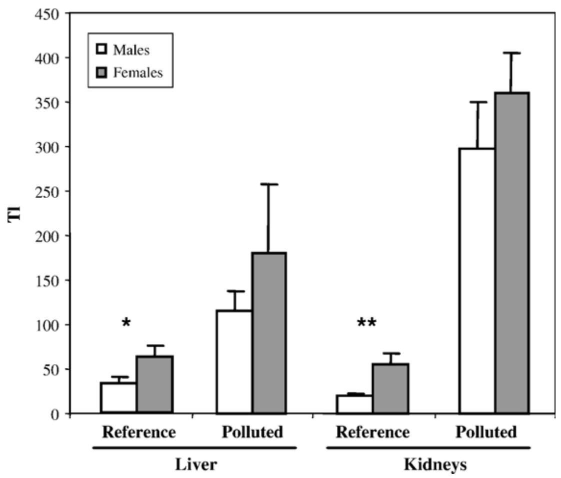

Fig. 1. Mean±SEM values for thallium in *C. russula*, by tissue, sex and site (in μg kg^{-1} DW) (* $p \leq 0.038$; ** $p \leq 0.014$).

tract and through skin. It circulates in intra-and extra-cellular fluid as a monovalent cation, mimics potassium cations, and binds with sulfhydryl groups of proteins at the mitochondrial membrane, as its toxicity seems related with K-dependent processes, i.e. substituting this element in Na^+/K^+-ATPase (Nriagu, 1998; Rocha et al., 2004; Wierzbicka et al., 2004). These observations may explain why the highest concentrations of Tl in mammals have been reported in renal medulla, where it interferes with filtration and excretion processes and produces serious toxic effects (Fleck and Appenroth, 1996).

Tl is a strong oxidant with high bioavailability. It is soluble in physiological conditions and has mutagenic, carcinogenic, and teratogenic effects in mammals (Léonard and Gerber, 1997; Nriagu, 1998; Rocha et al., 2004; Wierzbicka et al., 2004). The exact mechanism of toxicity is unclear (John Peter and Viraraghavan, 2005) and critical tissue levels in mammals are unknown. However, there is a clear dose–response relationship between low mean Tl concentrations (5.2±8.3 μg l^{-1}) in urine and adverse effects in humans (Kazantzis, 2000). Moreover, its average dietary intake in natural unpolluted environments is usually less than 5 μg day^{-1} and soils with concentrations ranging from 1 to 500 mg kg^{-1} produce toxic effects on biota (Heim et al., 2002; Wierzbicka et al., 2004). Concentrations found in the present study indicate a higher daily Tl intake and therefore reason of concern.

The protected area of Doñana is of considerable ecological importance. This area is one of the largest and most conserved areas in SW Europe. However, during centuries, pyrite mines have continuously released heavy metals into the Guadiamar river, thereby producing chronic exposure that may affect individuals, communities and ecosystems (Grimalt et al., 1999; Simón et al., 1999; Prat et al., 1999). A mining accident, such the one in Aznalcóllar, intensifies these chronic effects and can increase the morbidity and mortality of specimens, reduce population viability, and deeply alter or even destroy entire habitats. Once deposited, Tl persists in soils for long periods (Kazantzis, 2000; Rocha et al., 2004). Because of this extended bioavailability, it is taken up by plants and biota. Soil bioavailability and persistence increase in clay-rich and non-acidic soils such as in the polluted site examined in the present study (Madejón et al., 2006). Moreover, small mammals are common prey for endangered carnivorous birds and mammals found in Doñana. Given that the maximum admissible daily dose of Tl for humans is 15.4 μg kg^{-1} DW (see references in Kazantzis, 2000), these results indicate a greater increase in Tl intake for carnivores that feed on these small mammals and, therefore, bioaccumulation to toxic concentrations at high trophic levels.

4. Conclusion

The spillage from the Aznalcóllar mine leads to the bioaccumulation of harmful concentrations of Tl in kidneys of shrews in the protected area of Doñana. Moreover, biotic factors (age and sex) determine Tl bioaccumulation patterns.

Given the high concentrations of Tl found in shrew tissues as well as the serious long-term toxic effects and persistence of this element in the environment, a biomonitoring programme is required to assess levels and sublethal effects in individuals, populations, and ecosystems in this protected area.

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Metals in liver and kidneys and the effects of chronic exposure to pyrite mine pollution in the shrew *Crocidura russula* inhabiting the protected wetland of Doñana

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ABSTRACT

Historically impacted by anthropogenic activities, the nature reserve of Doñana (SW Spain) was affected by an unprecedented spillage of mud and acidic water from the Aznalcóllar pyrite mine in April 1998. Although several studies have addressed the influence of this spill on soils, water, and biota, there is little information on mammals, especially carnivorous species. We measured the concentrations of Fe, Mg, Pb, Hg, Cd, Zn, Cu, Mn, Mo, Co, and Cr in specimens of the greater white-toothed shrew, *Crocidura russula*, inhabiting the protected area affected by the mine spillage. We also examined other parameters to approach at the physiological effects of pollution. We found an increase in non-essential metals (Pb, Cd, and Hg), and morphometric, histological and genotoxic alterations. Age and gender were two significant factors explaining metal bioaccumulation: adults had higher Hg and Cd levels than juveniles, whereas males bioaccumulated more Pb and Co and less Mo than females. The micronucleus frequencies in blood erythrocytes were significantly higher in specimens from the polluted site than animals from the control site. Shrews from the impacted area also had hepatic alterations, namely increased liver-body ratio, focal necrosis, and signs of apoptosis in hepatocytes. Due to the relevance of small mammals in the diet of endangered species such as carnivorous birds and mammals, the findings of our study are of practical use for the management of the Doñana wildlife reserve and other protected Mediterranean wetlands.

1. Introduction

Doñana wildlife reserve is inhabited by several protected and endangered species, especially birds and mammals. On 25 April 1998, an incident at the pyrite mine "Los Frailes" in Aznalcóllar discharged about 5 Hm^3 of acidic waters and toxic mud carrying heavy metals such as Pb, Cd, Hg, Zn, Sb, and Tl into the Agrio river, thereby affecting this protected wetland. Since this event, several studies have provided abundant information on metal pollution in sediments and waters as well as on bioaccumulation and effects on plants, invertebrates, fish, reptiles, birds, rodents, carnivorous mammals, and human populations (e.g. Bonilla-Valverde et al., 2004; Fletcher et al., 2006; Gil et al., 2006; Madejón et al., 2007; Smits et al., 2007; Millán et al., 2008; Turner et al., 2008; Márquez-Ferrando et al., 2009). However, information on metal concentrations and their effects on insectivorous mammals is scarce, with only one study reporting a great increase in Tl in shrew tissues (Sánchez-Chardi, 2007).

Several heavy metals lead to clastogenic effects as a result of DNA breakage and can induce the generation of reactive oxygen species (ROS), which can lead to cell damage or death. Depending on the nature of the injury, the damage to the DNA may be repaired, or it may induce mutation or lead to apoptosis (Bragadin et al., 2003; Leonard et al., 2004), necrosis (Pereira et al., 2006; Jadhav et al., 2007) or other important cell alterations in somatic and germinative tissues (e.g. Damek-Poprawa and Sawicka-Kapusta, 2004; Nordberg et al., 2007). Effects are generally assessed or modelled in laboratory animals or culture cells in controlled conditions for a single compound or a few metals. However, biota are often exposed to a mixture of several chemical pollutants (Bellés et al., 2002). Field studies on ecotoxicological effects are useful to identify bioindicator species and assess the quality of the environment. Insectivorous mammals (shrews, hedgehogs, and moles) have been widely used as site-specific bioindicators of anthropogenic pollution, including heavy metals (e.g. Talmage and Walton, 1991; Ma and Talmage, 2001; D'Have et al., 2006). When non-degradable pollutants are released into the environment, they can be taken

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up and transferred through food chains and may accumulate in predators such as the greater white-toothed shrew *Crocidura russula* (Alleva et al., 2006; Wijnhoven et al., 2007). In particular, this species has small home ranges, and shows high food requirements and a high metabolic rate (e.g. Talmage and Walton, 1991; Ma and Talmage, 2001; Stewart et al., 2005).

Here we examine the effect of a recent large increase in the environmental concentration of heavy metals to *C. russula* from the protected reserve of Doñana. We measure the bioaccumulation of Fe, Mg, Pb, Cd, Hg, Zn, Cu, Mn, Mo, Co, and Cr, and assessed several morphometric, genotoxic, and histopathological parameters as biomarkers of environmental metal pollution in shrews. Moreover, the relation between age and sex and the bioaccumulation patterns was evaluated as inherent sources of variability.

2. Materials and methods

2.1. Study areas

During November and December 1999, 29 specimens of the greater white-toothed shrew, *C. russula* were trapped. Of these, 19 were caught in an area affected by the spill from the Aznalcóllar mine (''Entremuros, Parque Nacional'', 37°01′12″N, 6°16′38″W) and 10 were collected at a reference site not affected by this pollution (''La Vera, Reserva Biológica'', 36°59′25″N, 6°26′41″W). Traps were placed 5–8 m apart for about 400 m in a transect running along the intertidal zone. Both sites are situated in the protected reserve of Doñana (Fig. 1), an extensive ecological coastal wetland in SW Spain inhabited by several protected species. The total capture effort was 1950 traps per night. Shrews were transported to the laboratory and killed by cervical dislocation. The study was conducted in accordance with the European and Spanish legislation for laboratory animals (Guideline of the comission of the European Communities 86/609/EEC of 24/11/1986 and Real Ordinance, 1201/2005 of 10/10/2005). Sex was determined during dissection. Specimens were classified into two relative age classes (juveniles and adults) on the basis of the degree of tooth wear (Vesmanis and Vesmanis, 1979). In the polluted area, three females were pregnant and another two more lactating, whereas only three males showed signs of sexual activity. In the reference site, one male presented sexual activity and one female was lactating.

2.2. Morphometric parameters

The body weight (BW) to the nearest 0.01 g and head and body length (BL) to the nearest 0.01 mm of all specimens were measured during dissection. The residual index (RI) is calculated by the regression of BW and BL (Jakob et al., 1996). Positive values are an indication for specimens with relative higher body condition than expected for their weight and length. Animals with negative values have a relative lower body condition than predicted by the BW to BL ratio. Hepatic and renal weight to the nearest 0.001 g was measured on a wet weight basis (WW). The relative weights were calculated as a percent ratio of somatic tissue (100× tissue weight/body weight).

2.3. Chemical analyses

About 300–500 mg of the liver and right kidney of each specimen were dissected, dried to constant weight (48 h, 60 °C), weighed, and then digested in Teflon vessels with 2 mL of nitric acid and 1 mL of hydrogen peroxide (Instra, Baker Analized). Duplicate subsamples diluted (1:5), with rhodium as internal standard, were measured for Pb, Hg, Cd, Zn, Cu, Mn, Mo, Co, and Cr using a Perkin–Elmer ELAN-6000 Inductively Coupled Plasma Mass Spectrometer (ICP-MS) and for Fe and Mg by a Perkin–Elmer OPTIMA-3200RL Inductively Coupled Plasma Optical Spectrometer (ICP-OES). Moreover, standard reference material (Bovine Liver SRM-1577a) and 6 blanks were included in the analyses. Metal concentrations were expressed in $\mu g\, g^{-1}$ on a dry weight basis (DW). All chemical analyses were performed at the Elemental Analysis Facility of the Scientific-Technical Services at the University of Barcelona.

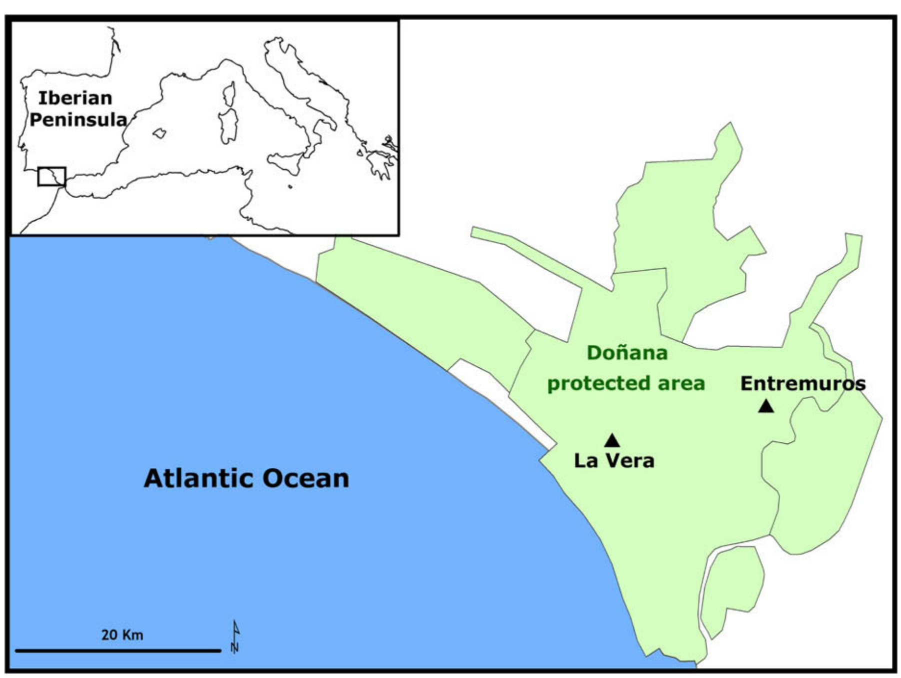

Fig. 1. Map showing the geographical location of the sites studied in Doñana protected area (▲): ''Entremuros'' (polluted site) and ''La Vera'' (reference site).

Table 1
Mean ± SEM values for morphometric parameters (BW, BL, RI, weights and relative weight ratios) of *C. russula* by site.

	Reference site ($n = 10$)	Polluted site ($n = 19$)
BW (g)	6.57 ± 0.18	6.14 ± 0.20
BL (mm)	66.80 ± 0.47	64.88 ± 0.73
RI	−0.559 ± 0.246	0.265 ± 0.226
Liver (g)	0.263 ± 0.016	0.324 ± 0.019*
% Liver	4.02 ± 0.28	5.47 ± 0.27**
Kidneys (g)	0.102 ± 0.005	0.115 ± 0.009
% Kidneys	1.58 ± 0.09	1.87 ± 0.15

* $p \leqslant 0.05$.
** $p \leqslant 0.01$.

2.4. Micronucleus test (MNT)

Blood samples are obtained by cardiac punction with a heparinized syringe. For each specimen, duplicate smears were made on pre-cleaned microscope slides, fixed with heat and stained with conventional May Grünwald-Giemsa staining. Micronuclei (MN) frequencies were scored by two observers on 2,000 blood erythrocytes for each specimen through an oil immersion objective (100×) on a Leica Leitz DMRB microscope. The frequencies were expressed as arithmetic mean and standard error of the mean ($M \pm$ SEM).

2.5. Histopathology

A fraction of liver and the left kidney of six adult shrews from the polluted site and eight from the reference site were fixed by 10% neutral-buffered formaldehyde and prepared for histological studies following conventional procedures, as described in Sánchez-Chardi et al. (2008). For the qualitative analysis, the stained sections were analysed under a Leica Leitz DMRB light microscope using a Sony Cyber-Shot, 7.2 mega pixels, to capture the images. Four images from each slide were captured under a 40× objective. These images were then processed by Image Tools software for quantitative analysis. The endpoints used were the presence of necrosis areas or apoptosis, inflammatory response, fibrosis, neoplasia, pre-neoplasic focuses and cytoplasmic vacuolization. For the quantitative analyses, we measured the perimeter, elongation, roundness, and compactness of a total of 1588 cells.

2.6. Statistical analyses

After log-transformation and test for normal distribution (Shapiro–Wilk test) and for homogeneity of variance (Levene, F-test), a three-way multivariate analysis of variance (MANOVA) was performed to obtain an overall estimation of the effects of on the parameters evaluated of site, age, and sex, together with their interactions. In order to increase sample size, the data were pooled and the effects of age and sex on metal bioaccumulation were analysed for each site. The differences in metal concentrations and morphometric parameters were analysed for site, age and gender classes with Student's tests (t). The same comparisons of MNT and histopathology were performed with Mann–Whitney tests (U). In order to increase sample size, sexual activity was not considered as factor since significant differences had not been found in previous analyses. Significant differences were assumed at $p < 0.05$. For all statistical analyses, SPSS 14.0 (2005) was used.

3. Results

Among the morphometric parameters, liver weight and relative liver weight were significantly higher in specimens from the polluted area than reference specimens ($t = 2.544$, $p = 0.017$; $t = 3.233$, $p = 0.003$, respectively). Moreover, the former tend to have greater relative renal weights and lower body weight than reference shrews (Table 1).

In liver, significant differences in metal levels between sites ($F = 33.421$, $p < 0.001$), ages ($F = 48.191$, $p < 0.001$), and sexes ($F = 52.930$, $p < 0.001$) were found. Hepatic mean concentrations of Hg and Cd were significantly higher in shrews from the polluted area, while more Cr was found in high concentrations in specimens from the reference site (Table 2). Adults from the polluted site had more Hg ($t = 3.240$; $p = 0.005$) and Cd ($t = 3.252$; $p = 0.005$) than the juveniles (Fig. 2A). Moreover, levels of Pb, Mo and Fe tend to increase and Cu and Cr decrease in adults. Among gender differences, males had significantly high mean values of Pb and Co at the polluted site ($t = 2.520$; $p = 0.023$, $t = 2.358$; $p = 0.031$, respectively), whereas females bioaccumulated more Mo ($t = -3.119$; $p = 0.017$) at the reference site (Table 3).

In renal tissue, the importance of the factors regarding metal differences were more site related ($F = 3.624$, $p = 0.024$), than sex related ($F = 1.315$, $p = 0.329$) and to a lesser extent age related ($F = 1.133$, $p = 0.415$). In a similar pattern to that of hepatic tissue, the shrews from the polluted site had elevated Mg, Hg, Cd, Cu, and Co concentrations (Table 2). Moreover, adults from the polluted site had higher Hg ($t = 3.068$; $p = 0.018$) and Cd ($t = 3.886$; $p = 0.001$) concentrations than juveniles (Fig. 2B). No significant sex-dependent variation was detected in kidneys; however, males and females showed a similar pattern of metal bioaccumulation as that detected in livers (Table 3).

The specimens from the polluted site showed a significant increase in micronuclei frequencies in peripheral erythrocytes compared with those from the reference site (1.222 ± 0.169 *vs.* 0.250 ± 0.119‰; $U = 1.500$, $p < 0.001$).

The histopathological findings did not reveal the lesions that are commonly described in animals exposed to toxic metals or other

Table 2
Mean ± SEM values for several metals (Fe, Mg, Pb, Hg, Cd, Zn, Cu, Mn, Mo, Co, and Cr) in tissues of *C. russula* compared by site (in μg g^{-1} DW).

	Liver				Kidneys			
	Reference site ($n = 10$)	Polluted site ($n = 19$)	t	p	Reference site ($n = 9$)	Polluted site ($n = 19$)	t	p
Fe	732.16 ± 230.29	952.37 ± 170.16	–	–	351.36 ± 15.08	362.49 ± 12.99	–	–
Mg	390.17 ± 20.92	394.14 ± 46.08	–	–	239.52 ± 21.19	313.83 ± 9.83	3.635	0.001
Pb	2.16 ± 0.39	2.79 ± 0.37	–	–	2.09 ± 0.19	2.43 ± 0.19	–	–
Hg	0.53 ± 0.12	1.28 ± 0.13	4.167	<0.001	1.08 ± 0.54	3.04 ± 0.24	3.976	<0.001
Cd	1.57 ± 0.21	5.56 ± 1.04	4.008	0.001	4.66 ± 1.01	16.74 ± 2.76	2.670	0.014
Zn	52.18 ± 3.52	49.34 ± 5.92	–	–	160.10 ± 11.34	177.12 ± 6.44	–	–
Cu	51.97 ± 10.30	56.16 ± 7.87	–	–	37.63 ± 4.98	48.95 ± 1.78	2.720	0.012
Mn	36.78 ± 1.83	38.27 ± 7.31	–	–	17.81 ± 2.23	20.02 ± 0.89	–	–
Mo	4.27 ± 1.22	6.81 ± 0.91	–	–	3.32 ± 0.58	3.75 ± 0.24	–	–
Co	0.64 ± 0.11	0.54 ± 0.10	–	–	0.62 ± 0.12	1.09 ± 0.10	2.589	0.016
Cr	3.00 ± 0.48	1.74 ± 0.60	2.955	0.006	2.07 ± 0.27	1.91 ± 0.19	–	–

pollutants; lesions such as fibrosis, vacuolization of the cytoplasm, inflammatory response, or neoplasic focus. However, our data showed some hepatic alterations in specimens from the polluted area, namely the occurrence of necrotic areas in four out of six individuals (Fig. 3) and high incidence of apoptotic figures (Fig. 3). When alterations were observed in hepatocyte nuclei, 1036 cells from specimens from the polluted site were compared by image analysis with 552 cells from specimens from the reference site. There was no significant difference in these parameters. However, shrews exposed to metals showed a tendency to increase the cellular perimeter when compared with reference shrews (7.531 ± 0.356 *vs.* 6.914 ± 0.222 μm), whereas the elongation, roundness, and compactness remained invariant between sites (M ± SEM: 1.190 ± 0.020 *vs.* 1.193 ± 0.021 μm; 0.724 ± 0.021 *vs.* 0.755 ± 0.010 μm; 0.912 ± 0.009 *vs.* 0.917 ± 0.007 μm, respectively). These parameters were significantly correlated with Pb, Mn and Cr contents in liver. The perimeter was correlated with Pb and Cr ($r = -0.665$, $p = 0.018$; $r = -0.597$, $p = 0.041$, respectively), the roundness with Pb ($r = -0.597$, $p = 0.041$), Mn ($r = -0.597$, $p = 0.041$), and Cr ($r = -0.597$, $p = 0.041$), and compactness with Mn ($r = -0.597$, $p = 0.041$). Moreover, no alterations related to toxic metals were detected in renal tissue. Given the small number of animals examined, micronucleus and histopathological data were not analysed to assess the effect of age or gender or correlations with metal bioaccumulation.

4. Discussion

4.1. Bioaccumulation of metals

Since the Aznalcóllar mine spillage, several studies have reported an increase in heavy metal concentrations in soil and water (Tovar-Sanchez et al., 2006) which lead to toxic effects for wildlife of Doñana (e.g. Blasco et al., 1999; Bonilla-Valverde et al., 2004; Sánchez-Chardi, 2007). After this ecological disaster, most of the sludge from the floodplains was removed to minimize pollution effects. However, even after this remediation measure, later studies reported the continued presence of metals in the environment (e.g. Fletcher et al., 2006; Tovar-Sanchez et al., 2006; ; Smits et al., 2007; Millán et al., 2008; Márquez-Ferrando et al., 2009).

Eighteen months after this environmental disaster, the availability of potentially toxic metals such as Cd and Hg in the impacted site was still higher than in the reference site and they were accumulated in the tissues of shrews. In general, these non-essential elements are available to biota over a long time and are usually strongly accumulated in soft tissues of mammals recently exposed to contaminated air, food or water. Cd is a highly bioavailable metal and is mobile in less acidic soils such as those found in the Doñana area. It is retained with Fe oxides (Kraus and Wiegand, 2006) and is one of the main pollutants of mine residues. Mercury was also present in considerable amounts in the residues (e.g. Bonilla-Valverde et al., 2004). The low increase in Pb concentrations detected in shrews from the polluted area could be explained by low bioavailability several months after the spillage, probably because this metal is immobile in less acidic soils (Kraus and Wiegand, 2006). Zn, Cu, Co, Mn, and Mo were abundant in waters and slurry spilled from the mine and remained in high concentrations in the water and soil of the polluted area several years after the incident (Tovar-Sanchez et al., 2006).

Essential metals had similar concentrations in tissues from shrews from the polluted and reference sites, as previously reported in the Algerian mouse *Mus spretus* (Bonilla-Valverde et al., 2004). This is a result of metabolic regulation that prevents disruption of metabolic turnover and high bioaccumulation in mamma-

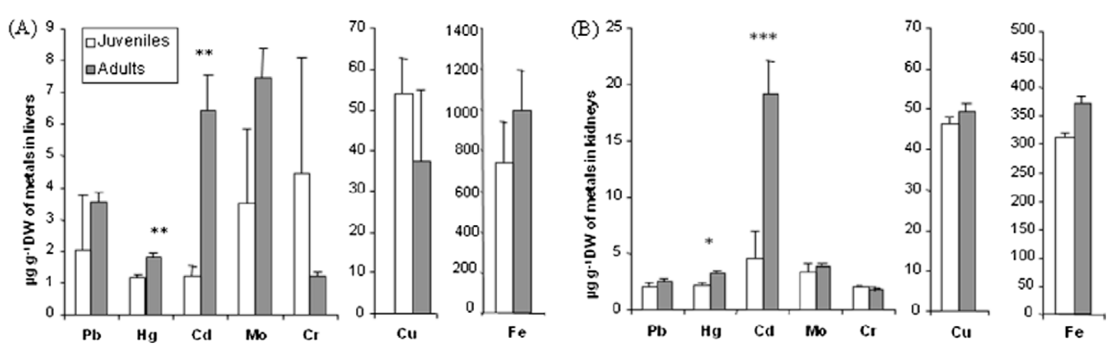

Fig. 2. Mean ± SEM values for Pb, Hg, Cd, Mo, Cr, Cu, and Fe in *C. russula* collected at the polluted site, by age in liver (A) and kidneys (B) (in μg g^{-1} DW), ($^{*}p \leqslant 0.05$; $^{**}p \leqslant 0.01$; $^{***}p \leqslant 0.001$).

Table 3
Mean ± SEM values for metals (Fe, Pb, Hg, Zn, Mo, and Co) in *C. russula* by tissue, site and sex (in μg g^{-1} DW).

		Reference site		Polluted site	
		Males (n = 6)	Females (n = 3)	Males (n = 9)	Females (n = 10)
Liver	Fe	803.27 ± 223.87	589.92 ± 248.45	1086.3 ± 361.3	845.2 ± 119.8
	Pb	2.88 ± 0.53	2.52 ± 0.56	3.20 ± 0.69	1.56 ± 0.18*
	Hg	0.83 ± 0.07	0.38 ± 0.14	1.49 ± 0.15	1.11 ± 0.20
	Zn	24.54 ± 2.41	63.94 ± 22.96	67.93 ± 22.37	77.85 ± 15.71
	Mo	2.41 ± 0.22	8.00 ± 2.69*	6.07 ± 1.30	7.74 ± 1.25
	Co	0.63 ± 0.08	0.67 ± 0.25	0.63 ± 0.19	0.29 ± 0.05*
Kidneys	Fe	162.30 ± 19.67	136.77 ± 19.67	161.41 ± 14.92	163.36 ± 20.86
	Pb	2.40 ± 0.33	2.14 ± 0.70	2.36 ± 0.21	2.52 ± 0.27
	Hg	2.57 ± 0.56	1.00 ± 0.51	3.09 ± 0.29	3.03 ± 0.37
	Zn	157.31 ± 7.89	163.81 ± 0.51	175.78 ± 11.81	178.19 ± 7.34
	Mo	3.44 ± 0.62	3.27 ± 1.26	3.37 ± 0.38	4.05 ± 0.27
	Co	0.64 ± 0.09	0.59 ± 0.27	0.86 ± 0.09	1.26 ± 0.14

* $p \leqslant 0.05$.

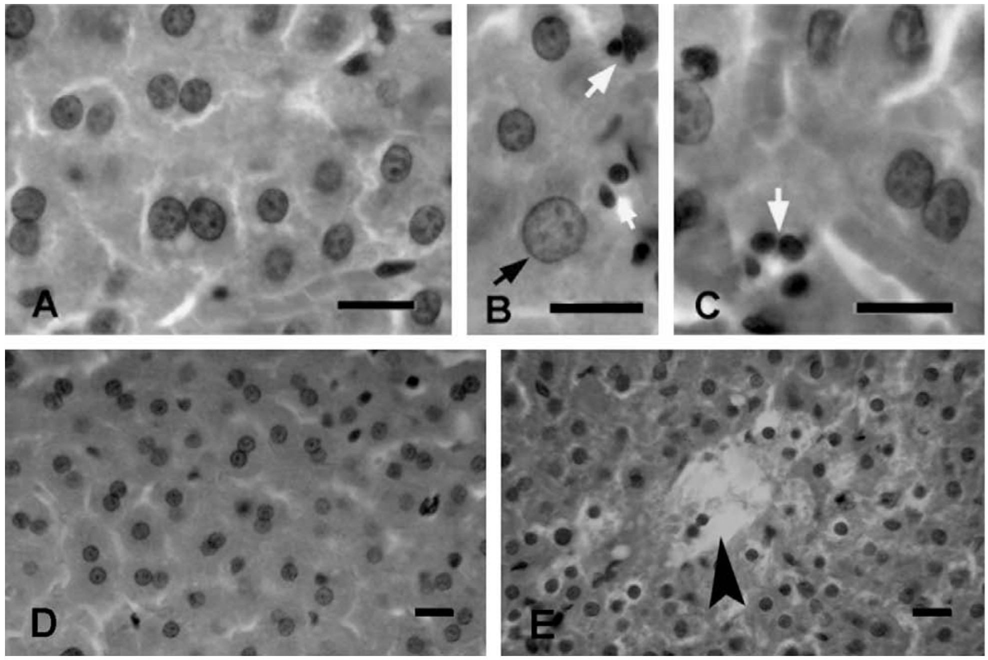

Fig. 3. Hepatic sections showing normal and altered tissue in *C. russula*. (A, D) Healthy livers from shrews collected at the reference site; (B, C, E) livers from shrews collected at the polluted site: observe the enlarged nuclei (black arrow) (B), the apoptotic figure (white arrow) (B, C), and the foci of cell necrosis (black arrow) (E). Haematoxilin and eosin stains. (Scale bars: A, D, E = 50 μm; B, C = 20 μm).

lian tissues despite substantially elevated dietary intake (e.g. Talmage and Walton, 1991; Goyer, 1997; Ma and Talmage, 2001). In contrast, the higher mean values of Cr, another essential element, in specimens from the reference site, could be partially explained by the effect of fertilizers, which load the waters and soils of the reference area with metals (Tovar-Sanchez et al., 2006). The Doñana area has been exposed to agricultural practices and mining extraction for centuries, which contributed metals to the environment before the spillage of 1998 (Arambarri et al., 1996; Fletcher et al., 2006).

The adult shrews from Doñana had higher Cd accumulation than juveniles, which is consistent with previous reports in soft tissues of *C. russula* (Sánchez-Chardi and Nadal, 2007; Sánchez-Chardi et al., 2007b) and other small mammals species exposed to mine pollution (Bonilla-Valverde et al., 2004; Smith and Rongstad, 1982). This age-dependent bioaccumulation is associated with detoxification mechanisms, namely the formation of cadmium-metallothionein in liver, which is transported by blood and then stored mainly in the renal cortex (e.g. Bonilla-Valverde et al., 2004; Sánchez-Chardi et al., 2007b). A similar mechanism could explain the age-dependent accumulation of Hg and Pb, as also reported for other small mammals species (e.g. Pankakoski et al., 1993, 1994; Stansley and Roscoe, 1996; Damek-Poprawa and Sawicka-Kapusta, 2004). The tendency of essential elements that are physiologically well regulated in mammals to increase with age (Fig. 2) could be related to the metabolic requirements of adult specimens as well as to interferences with non-essential elements (Pankakoski et al., 1994; Goyer, 1997; Bellés et al., 2002; López Alonso et al., 2004). The decrease in Cr detected is concordant with a lower intestinal absorption of this essential metal in adults (Outridge and Scheuhammer, 1993; Sánchez-Chardi et al., 2007a).

Gender differences in the bioaccumulation pattern of non-essential metals have been reported in several species of mammals including human (Smith and Rongstad, 1982; Komarnicki, 2000; Clark et al., 1992; Vahter et al., 2007). Low Hg and Pb levels in females could be related mainly to the reduction of metal burden mobilized and transferred during gestation to the foetus and/or during lactation to the young (see references in Sánchez-Chardi et al., 2007b). No sex-dependent variation was reported for Mo in *Talpa europaea* (Pankakoski et al., 1993). In contrast, the females of *C. russula* collected in a pyrite mine site and in a landfill site had higher Mo concentrations than males (Sánchez-Chardi and Nadal, 2007; Sánchez-Chardi et al., 2007a). More Fe was detected in males of different species including human (Khan et al., 1995; Clark et al., 1992). Zn also tends to increase in soft tissues in the females of some mammals such as the mole (Komarnicki, 2000). More specifically, steroid hormone receptors are Zn-finger proteins and Zn is also part of several enzymes, including some related to antioxidant systems such as cytosolic superoxide dismutase (Lopes et al., 2002). Generally speaking, the gender differences of these essential elements (Co, Fe, Zn, and Mo) may be associated with differences in the metabolic profile of metals involved in the activity of sexual hormones, the intake or uptake of metals, nutritional requirements or interactions between elements (Goyer, 1997; Chmielnicka and Sowa, 2000; Bellés et al., 2002; Lopes et al., 2002; López Alonso et al., 2004; Vahter et al., 2007).

4.2. Genotoxicity

The micronucleus numbers found in shrews from the central part of the Doñana reserve ("La Vera") could be considered references for genotoxicity rates and are in concordance with data previously reported (Tanzanella et al., 2001; Festa et al., 2003; Mateos et al., 2008). The MN frequencies were higher in shrews exposed to metals from the pyrite mine, thereby indicating the clastogenic effects of this kind of pollution, which is bioavailability to biota over a long period of time. Similar results were reported by Festa et al. (2003) and Tanzanella et al. (2001) in a survey of the Algerian mouse, *M. spretus*, collected at the same sites during the same period. These authors speculate on the contribution of the chronic pollution that occurred before the mine accident to genotoxicity. In fact, the Guadiamar River was severely affected by anthropogenic activities before this accident (some mines have been operating since Roman times), thereby causing increased levels of toxic substances in the area (e.g. Arambarri et al., 1996; Fletcher et al., 2006). However, the significant effect of the spillage in 1998 on the bioavailability of toxic metals and on the genotoxic damage to the Doñana reserve is evident, as shown by the increase in metal concentrations in soil, water, plants and animals (e.g. Blasco et al., 1999; Bonilla-Valverde et al., 2004; Fletcher et al., 2006; Gil et al., 2006; Kraus and Wiegand, 2006; Tovar-Sanchez et al., 2006; Madejón et al., 2007; Smits et al., 2007).

4.3. Morphometric parameters

In shrews from the polluted site, the increase in liver weight and the tendency to show increased renal weight and decreased body weight may be indicative of considerable physiological and histological alterations (Shore and Douben, 1994; Sánchez-Chardi et al., 2007b, 2008). The presence we detected of both histological alterations in the livers and genotoxic effects in the blood of shrews from the polluted area are consistent with these morphological findings and could be explained by exposure to toxic levels of non-essential metals. Similar results of morphometric parameters were previously obtained for small mammals exposed to metals (Ma and Talmage, 2001; Pereira et al., 2006; Sánchez-Chardi et al., 2007a,b). In mammals, the main exposure route to pollutants is through diet. The liver is the most important detoxifying tissue. This organ plays a crucial role in food conversion, biotransformation of xenobiotics, and vitellogenesis for reproduction purposes. Consequently, the impairments of hepatic function have a number of negative consequences on growth, health, life expectancy and reproductive success of individuals and may therefore adversely affect whole populations.

4.4. Histopathology

Histopathological endpoints could also contribute to the sensitivity of organs to heavy metal pollution and could provide information on the mechanism of action of pollutants and on target organs (Wester et al., 2002). Cellular biomarkers act as early warning signals of stress by organisms exposed to contamination. They may provide information on the level of the developing stress, ranging from initial biological effects to the impact on cell physiology. There are wide descriptions that exposure to heavy metals generates ROS via Fenton-type or Haber-Weiss-type reactions. Heavy metals such as Tl, Cd, Co, Fe, Pb, and Cr also react directly with cell molecules within the cytosol and cause a wide range of cellular responses such as apoptosis and finally necrosis (e.g. Bragadin et al., 2003; Leonard et al., 2004; Chia et al., 2005; Oliveira Ribeiro et al., 2005), depending on the level of oxidative stress generated. The occurrence of cell cycle arrest (necrosis and apoptotic figures) in liver of *C. russula* chronically exposed to high concentrations of Tl, Pb, Cd, and Hg corroborates the above effects reported in the laboratory. In addition, the nuclear alterations described here are also additional evidence that the uptake and high bioaccumulation of toxic metals in livers of *C. russula* are related to these findings. Theses alterations may represent chromatin disorganization and have serious consequences on gene expression by altering the transmission of information from the nucleus to the cytoplasm and outside the cell. In fact, the presence of distinct nuclear forms reported here provides evidence that the toxic mechanism of metals also disturbs the DNA and protein array within the nucleus.

A few studies have described renal alterations (see references in Sánchez-Chardi et al., 2008) in Soricine species of shrews. However, the response of *C. russula*, a Crocidurine species, to metal pollution is more similar to that shown by rodents (Damek-Poprawa and Sawicka-Kapusta, 2004; Pereira et al., 2006). Given their particular metabolic characteristics (e.g. Stewart et al., 2005) and differences in phylogenic origin and ecological strategy, further studies including biochemical, physiological or morphological parameters, are required to explain the differences observed between groups of shrews in response to exposure to heavy metals.

4.5. Toxicity effects

Several studies with laboratory specimens have reported toxic effects of a single metal under controlled conditions. However, data on wild populations of species of higher trophic levels are scarce. The effects of heavy metals considered alone as well as cumulative toxicity rates or interactions such as synergism could explain the increase in genotoxicity and histological alterations observed in shrews from the Doñana reserve. No complete guideline on the toxic levels of the heavy metals addressed in this study is currently available for shrews. However, considered alone, the levels found for essential metals and Pb do not appear to be of toxicological hazard in the specimens collected in Doñana. In contrast, Cd and Hg levels in renal and hepatic tissues of shrews from the polluted area merit concern. The mean values of Hg higher than 1.1 $\mu g\ g^{-1}$ should be regarded as presumptive evidence of an environmental mercury problem in wild mammals (Eisler, 1987). Wild specimens with high loads of non-essential metals may suffer toxic effects such as impairment of reproduction or a decrease in life expectancy. When assessing the status of wild animals, the toxic effects of environmental pollution could be biased because of the limited number of animals studied as well as by the intra-specific variation and selection in natural populations, which may reduce the metal load or eliminate impacted animals that are easier preys to predators. These features may remove extreme values that can be measured in laboratory studies. In fact, laboratory rodents showed alterations such as necrosis, apoptosis, vacuolization and fibrosis when chronically exposed to realistic concentrations of metals (e.g. Jadhav et al., 2007).

4.6. Environmental quality assessment in protected sites

Inhabited by several endangered vertebrate species, including carnivorous birds and mammals, the area of Doñana is one of largest protected coastal sites and one of the last great wildernesses in Europe. These protected species feed on small mammals and one of the most abundant in Doñana is the greater white-toothed shrew (Cagnin et al., 1998). Could *C. russula* serve as a bioindicator to assess environmental quality? Given that this species has a high metabolic rate, food requirements and tolerance to toxins, it reacts to pollution by bioaccumulating higher concentrations of heavy metals than sympatric rodents (Alleva et al., 2006; Sánchez-Chardi and Nadal, 2007; Wijnhoven et al., 2007), as occurs with other insectivores (Talmage and Walton, 1991; Ma and Talmage, 2001). These metabolic characteristics as well as the ecological interest of abundant species of non-migratory and easily available carnivores make shrews as suitable biomonitoring species to detect very finely an increase of xenobiotics (Talmage and Walton, 1991; Shore and Douben, 1994; Komarnicki, 2000; Ma and Talmage, 2001). In contrast, the shrews appear to be more tolerant to pollutants than rodents as shown by less alteration of physiological parameters such as biochemical biomarkers (e.g. Ma and Talmage, 2001; Sánchez-Chardi et al., 2008). Comparative studies of sympatric species are required for correct assessment of environmental quality and risk in order to determine inter-specific sensitivity to pollutants and detect the most sensitive species. Thus, it may be possible to assess the critical loads, namely highest levels of pollutants without toxic effects, for wild populations and ecosystems. Moreover, a database for heavy metal concentrations in autochthonous biota with diverse trophic strategies is required to allow studies on the bioavailability, transfer and behaviour of chemicals through a variety of protected ecosystems. The study of temporal variation of metals throughout the ecosystems could also be important for these protected areas. In fact, our samples were collected a few months after the spillage from the pyrite mine. Later studies in Doñana showed differences in bioavailability of metals in soils, waters and plants. However, to our knowledge, terrestrial mammals at high trophic position have not been biomonitored. We consider that *C. russula* is a suitable species to assess environmental quality especially for bioaccumulable pollutants and recommend that it be included as part of management programmes for protected sites.

5. Conclusions

Here we have identified Hg and Cd as toxic elements that are highly bioaccumulated in terrestrial mammals in Doñana. We have also reported age and sex as two relevant factors to explain variability in bioaccumulation patterns in wild populations recently exposed to extremely high amounts of metals. Moreover, we have found morphometric, genotoxic, and histological effects of metal pollution in shrews from the area affected by the mine spillage. These effects and the considerable concentrations of non-essential metals in tissues of shrews from this protected area also indicate the need for frequent sampling to evaluate the food chain transfer of these long-term persistent pollutants. The high trophic position of shrews as well as their abundance makes them a suitable species for biomonitoring programmes especially in areas of ecologic value such as Doñana.

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Bioaccumulation of lead, mercury, and cadmium in the greater white-toothed shrew, *Crocidura russula*, from the Ebro Delta (NE Spain): Sex- and age-dependent variation

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Bioaccumulation patterns of Pb and Hg reveal sex and age-related differences in the large bones of the greater white-toothed shrew from a polluted Mediterranean wetland.

Abstract

We quantified bioaccumulation of lead, mercury, and cadmium in bones from 105 greater white-toothed shrews (*Crocidura russula*) collected at the Ebro Delta, a polluted area, and the Medas Islands, a control site. Lead and mercury levels varied with site, age, and sex, although statistical significances depended on each factor. Globally, shrews from the polluted area exhibited significantly higher concentrations of Pb and Hg. Increment of Pb with age was particularly remarkable in wetland animals and was interpreted in relation to human activities, namely hunting. Unlike males, females from the Ebro Delta maintained low Hg levels, which were associated with gestation and lactation. Cadmium levels did not differ between sites, sexes, or ages. This study provides the first data on heavy metals in mammals from this wetland and suggests that *C. russula* is a good bioindicator of metal pollution. We concluded that sex and age may represent an important source of variation in the bioaccumulation of these metals in wild populations.

Keywords: *Crocidura russula*; Heavy metals; Ebro Delta; Sex; Age

1. Introduction

Anthropogenic activities may generate metal pollution in air, soil, and water. Over the last decades, the production of lead (Pb), mercury (Hg), and cadmium (Cd) from mining increased 2-, 2- and 15-fold, respectively, and the subsequent release of these metals into the environment is of some concern (Nriagu, 1988). The ecosystem seems to offer an effective filter, retaining contaminants in soil profiles, transferring them into aquatic (Négrel and Roy, 2002) and/or terrestrial systems, and thereby increasing the bioavailability and poisoning risk both to humans and the environment. Lead, Hg, and Cd, three non-essential elements widely distributed, are well known for their highly toxic effects on biological systems (e.g. Lewis et al., 2001; Wolfe et al., 1998). In countries where lead additive is prohibited, the use of lead shot pellets, which are composed by 98% Pb and by traces of other metals such as Cd (Mozafar et al., 2002), may become the primary source of environmental lead pollution (Ma, 1989). Although mercury is not an abundant element in the environment, it is widely distributed due to various industrial and agricultural practices. The source of cadmium may be natural or anthropogenic resulting among other activities from the use of superphosphate fertilizers or the spill from industrial and domestic sewage.

The pollution status at the Ebro Delta, a partially protected area in NE Spain, is well documented, particularly for certain heavy metals present in freshwater and marine habitats. In this wetland, fertilizers, mainly employed in rice farming, and

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especially the lead shot pellets from hunting activities remain the primary sources of ''in situ'' metal pollution (Mañosa et al., 2001). Up to 4–9 t of these pellets are spread across rice fields and lagoons each year and even 2.5×10^6 pellets/ha were found in sediments (Mateo et al., 1997). Direct lead poisoning by shot is very common among granivorous waterbirds, but it has also been described in several species of birds of prey, which may feed on game birds and mammals (Mateo et al., 1997, 2003; Mateo and Guitart, 2003). Moreover, industrial and domestic effluents transported by the Ebro River are also responsible for ''ex situ'' pollution, spreading large amounts of Pb, Hg, and Cd in this protected area (Lacorte et al., 2006; Lavado et al., 2006; Mañosa et al., 2001; Schuhmacher et al., 1994). As a consequence, considerable amounts of Pb and Hg have been detected in the river water, sediments, earthworms, fish, and marine organisms close to this deltaic area (Lavado et al., 2006; Morera et al., 1997; Ramos et al., 1999; Schuhmacher et al., 1990, 1993). In the Ebro Delta, Cd concentration was high in sediments, but low in soil, water, rice, and seabird eggs (Mañosa et al., 2001; Morera et al., 1997; Ramos et al., 1999), suggesting that terrestrial environments may be less affected by Cd pollution.

Although the intake and bioaccumulation of pollutants by mammals is known to occur (Komarnicki, 2000; Shore, 1995; revision in Talmage and Walton, 1991), to our knowledge, metal concentrations in mammals from the Ebro Delta have never been examined. Several studies have shown that insectivores are mammals suitable for toxicological research, especially because of their high trophic chain position and metabolic rate, as well as their demanding feeding requirements (see revision in Ma and Talmage, 2001). They accumulate more potentially harmful metals than other small mammal species, and their exposure to pollutants tends to exceed other mammals with lower metabolic rates (Ma, 1989; Ma and Talmage, 2001; Talmage and Walton, 1991). Moreover, consumers at higher trophic levels in terrestrial ecosystems may be useful in predicting risks to human health (Komarnicki, 2000). For the present work, we used the greater white-toothed shrew, *Crocidura russula* (Hermann, 1780), an insectivore species widely distributed throughout south-western Europe (Ramalhinho et al., 1999) and very common to the Mediterranean region under study.

In general, the biological effects of pollutants on individuals and populations are assessed by means of a variety of factors, including several genetic, morphological, and/or biochemical parameters (Ma and Talmage, 2001; Talmage and Walton, 1991; Topashka-Ancheva and Metcheva, 1999). Some criteria regarding physiology, such as a body condition index, can be used as rapid and easy measures of adaptability in wild mammals in polluted environments (Ma, 1989; Jakob et al., 1996; Nunes et al., 2001a).

With all this in mind, the main goals of this study were: (i) to quantify for the first time the bioaccumulation of non-essential metals in the greater white-toothed shrew, *C. russula*, from NE Spain; (ii) to analyse patterns of variation based on sex and age; (iii) to assess the impact of metal pollution from human activities on a protected Mediterranean area; and (iv) to evaluate the use of osteological material drawn from biological collections as a source of ecotoxicological information on mammalian populations.

2. Materials and methods

We used large bones from 105 greater white-toothed shrews, *C. russula*, originally collected during the period 1976–1981 from two partially protected coastal areas in north-eastern Spain (Fig. 1): the Ebro Delta (40°43′N, 00°40E) and the Medas Islands (42°20′N, 03°13′E). The Ebro Delta, the second most important wetland in the Iberian Peninsula, has an area of 32,000 ha, of which 7802 ha corresponds to a Natural Park. In this site, specimens ($n = 73$) were captured at L'Encanyissada lagoon, characterized by helophytic vegetation (*Phragmitetea*). This zone is polluted by Pb shot pellets into the soil, as well as by Hg and Cd from industrial, domestic, and agricultural activities (mainly rice farming). With an area of 21.5 ha and about 1 mile off the nearest mainland (L'Estartit), the Medas Islands constitute a small archipelago formed by seven isles. In this clean and uninhabited area, which is not affected by human activities, animals ($n = 32$) were caught on the Meda Gran Island in halophilous vegetation (*Carpobrotetosum*).

For each animal, body mass (BM, in g) and body length (BL, in mm) were measured; in pregnant females the weight of the embryos were subtracted. To evaluate the general health status of animals, we used the residual index (RI), as a body condition index, in which BM is regressed on BL after the data were appropriately transformed to meet the assumptions of regression. The residual distances of individual points from this regression line were then used as the estimator of condition (see e.g. Jakob et al., 1996). For statistical analyses, animals were distributed into three relative age-classes (1: juveniles; 2: adults; 3: seniles), based on the degree of toothwear (Vesmanis and Vesmanis, 1979) and reproductive status (López-Fuster et al., 1985). The bones of each animal were kept in single metal-free paperbacks and stored in the zoological collection of the Animal Biology Department (University of Barcelona) until the chemical analyses were performed.

Materials used in the digestion process were thoroughly acid-rinsed. The bones were then washed with high purity grade water (Milli-Q© system, 18.2 MΩ cm^2) and dried until constant weight was attained (50 °C, 48 h). Tissue (80–100 μg) from each animal was placed on Teflon vessels and digested (120 °C, 12 h) with 2 ml of nitric acid 70% (Instra, Baker-Analized) and 1 ml oxygen peroxide 30% (Instra, Baker-Analized). Samples were diluted to 1:5 in Milli-Q© water with 1% HNO_3 and rhodium as an internal standard. Concentrations of Pb, Hg, and Cd were determined by Inductively Coupled Plasma Mass Spectrometer Perkin Elmer ELAN-6000. Two replicate subsamples were analysed and a standard reference material (Bovine Liver SRM-1577a) certificated by the National Bureau of Standards was included in the analysis. The results regarding heavy metals were expressed as microgram/gram (μg/g) on a dry weight basis.

Data were log transformed and tested for both normal distribution (Kolmogorov–Smirnov) and homogeneity of variance (Levene, *F*-test). Initially,

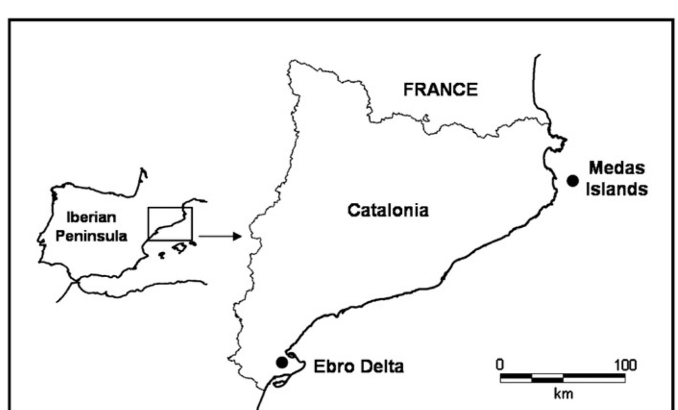

Fig. 1. Map showing the geographical location of the samples analysed.

an overall measure of the effects of sex, age group, and site was obtained by a three-way multivariate analysis of variance (MANOVA). Intersexual differences for each age-class and site were calculated by Student's *t*-tests. For each site and sex, divergences in the concentration of elements by age were performed using one-way analyses of variance (ANOVAs), and pairwise comparisons of sample means were conducted by Scheffe's method. Interpopulation differences were assessed by the Student's *t*-test for each sex and age group. To minimize seasonal, reproductive, and growth variations, only non-reproductive adults from winter were used to calculate the RI; comparison between sites was evaluated by a Student's *t*-test. For all sequential tests, *p*-values were corrected by the Bonferroni adjustment (Rice, 1989). All statistical procedures were performed using SPSS 11.5 software (SPSS Inc., Chicago, IL).

3. Results

Lead, mercury, and cadmium concentrations in the bones analysed were above the detection threshold for all animals (in μg/kg, Pb: 0.05; Hg: 0.20; Cd: 0.05). MANOVA revealed significant differences by sex ($F = 3.799$, $p = 0.013$), age ($F = 9.611$, $p = 0.001$) and site ($F = 24.502$, $p = 0.001$). Therefore, basic descriptive statistics (sample size, arithmetic mean, standard error, and range) of heavy metal concentrations are shown for each factor (Table 1).

Intersexual differences were observed for Pb and Hg, although bioaccumulation patterns varied according to the age and capture site. Thus, while in the Ebro Delta significant sexual differences in the concentration of Hg were detected for adults ($p = 0.017$) and seniles ($p = 0.001$), in the control area these differences corresponded to juveniles ($p = 0.008$) and adults ($p = 0.024$). Moreover, in the latter area Pb accumulation also differed significantly between the sexes for both adults ($p = 0.013$) and seniles ($p = 0.021$). The bioaccumulation of Cd did not exhibit significant sex-dependent variation in any of the studied sites.

Age-dependent statistical signification in the metal concentrations for each sex and site is shown in Table 2. Pairwise comparisons between sample means revealed that in the Ebro Delta, Pb concentration was significantly higher in seniles than in juveniles, both in males ($p = 0.002$) and females ($p = 0.001$). As for Hg, concentration increased significantly in senile males with respect to juveniles ($p = 0.001$) and adults ($p = 0.014$), whereas it did not vary in females. In the Medas Islands, increase in Pb was significant in senile males with respect to juveniles ($p = 0.004$) and adults ($p = 0.012$), while for Hg significantly greater values were found only between senile and juvenile females ($p = 0.02$). No variation of Cd concentration with age was observed either in the polluted or in the control site.

Globally, shrews from the polluted site showed a significant increase in Pb (8.91 ± 0.72 μg/g *vs* 1.34 ± 0.13 μg/g) and Hg (2.24 ± 0.29 μg/g *vs* 0.93 ± 0.66 μg/g) than individuals from the control site. In males, significantly higher values were found in the Ebro Delta compared with the Medas Islands for Pb (juveniles, $p = 0.015$; seniles, $p = 0.001$) and Hg (seniles, $p = 0.001$). In females, concentrations of Pb and Hg were significantly higher in seniles ($p = 0.001$) and juveniles ($p = 0.005$) from the Ebro Delta. No difference in the concentration of Cd was observed between the two sites (0.18 ± 0.02 μg/g *vs* 0.13 ± 0.01 μg/g). Likewise, a comparison of residual indexes between the sites (Ebro: 0.242 ± 0.134, $n = 23$; Medas: -0.253 ± 0.260, $n = 22$) did not show any statistical difference ($t = 1.713$, $p = 0.094$).

Table 1
Descriptive statistics of metal concentrations (μg/g, in dry weight) in the samples analysed according to sex and age

	Age	Males					Females				
		n	$\bar{X}$	SEM	Min	Max	n	$\bar{X}$	SEM	Min	Max
Ebro Delta											
Pb	1	14	4.88	1.05	0.13	12.47	13	2.56	0.79	0.11	9.30
	2	6	6.14	1.97	0.30	12.98	6	6.47	2.20	0.30	15.18
	3	16	13.03	1.04	6.09	19.60	18	14.69	0.80	10.09	23.00
Hg	1	14	1.79	0.27	0.27	3.73	13	1.21	0.16	0.56	2.30
	2	6	1.93	0.59	0.55	3.99	6	0.55	0.12	0.19	1.00
	3	16	5.53	0.82	0.61	12.63	18	1.09	0.19	0.12	3.09
Cd	1	14	0.19	0.03	0.08	0.47	13	0.16	0.02	0.06	0.34
	2	6	0.09	0.01	0.06	0.13	6	0.13	0.02	0.08	0.22
	3	16	0.20	0.05	0.04	0.77	18	0.22	0.04	0.07	0.85
Medas Islands											
Pb	1	3	0.49	0.09	0.35	0.67	6	0.97	0.25	0.07	1.68
	2	5	0.73	0.15	0.32	1.21	7	1.46	0.20	1.01	2.46
	3	7	1.70	0.24	1.04	2.80	4	2.50	0.10	2.30	2.76
Hg	1	3	1.04	0.03	0.97	1.08	6	0.66	0.06	0.47	0.88
	2	5	0.68	0.08	0.38	0.85	7	1.01	0.08	0.64	1.34
	3	7	0.95	0.18	0.47	1.61	4	1.42	0.22	0.79	1.74
Cd	1	3	0.09	0.01	0.07	0.11	6	0.10	0.02	0.05	0.18
	2	5	0.15	0.03	0.10	0.23	7	0.15	0.02	0.06	0.25
	3	7	0.14	0.01	0.11	0.17	4	0.14	0.02	0.10	0.20

Table 2
Results of the one-way ANOVA for variation in metal concentrations according to age, for each site and sex

Site	Sex	d.f.	Pb		Hg		Cd	
			F	*p*	*F*	*p*	*F*	*p*
Ebro Delta	Males	2, 33	8.64	0.001	10.22	0.001	1.27	–
	Females	2, 34	24.80	0.001	3.33	–	1.34	–
Medas Islands	Males	2, 12	11.60	0.002	1.40	–	4.24	–
	Females	2, 14	3.75	–	10.12	0.002	1.45	–

p-Values corrected by the Bonferroni adjustment.

4. Discussion

Our results revealed that in the shrews analysed Pb and Hg bioaccumulation varied according to site, age, and sex, arranged in ascending order of importance, whereas Cd remained statistically invariable for all three factors. The high Pb and Hg levels found in the shrews from the Ebro Delta can be easily explained as a result of many decades of hunting and of intense industrial and agricultural activities carried out in this area (Schuhmacher et al., 1990; Mateo and Guitart, 2003; Mateo et al., 1997, 2003; Mañosa et al., 2001). Such anthropogenic actions have led to an increase in the bioavailability of non-essential heavy metals by biota, and consequently to chronic exposure for wild mammals inhabiting the Ebro Delta. Because of Pb exposure, shrews from the polluted site experienced a 6-fold increase in their Pb levels compared with control animals. Values obtained in the shrews from the Ebro Delta are comparable to those reported for the bi-coloured white-toothed shrew, *Crocidura leucodon* (Topashka-Ancheva and Metcheva, 1999). In contrast, Pb concentrations in the femur of the common shrew, *Sorex araneus*, were considerably higher, ranging from 134 to 1469 µg/g (Ma, 1989; see Table 3). The difference in Pb concentration between Crocidurinae and Soricinae may be particularly linked to their metabolic rates (*C. russula*: 2.45 ml O_2/g h; *S. araneus*: 7.74 ml O_2/g h; Churchfield, 1990). In fact, Ma and Talmage (2001) noted that the crocidurine species exhibited bioaccumulation levels similar to rodents and lower than those of the soricine species. Additionally, these authors stated that the exposure of insectivores depended strongly on the soil conditions governing the bioavailability of Pb intake by preys, especially earthworms. For instance, it has been pointed out that a higher shot pellet corrosion rate occurs in acidic soils, demonstrating that soil pH can affect Pb bioavailability (Ma, 1989, 1996; Shore, 1995). Thus, the alkaline character of the Ebro sediments (8.03 ± 0.32; Ramos et al., 1999) may also partially explain the lower Pb values observed in *C. russula* with respect to *S. araneus* collected in acidic soils (Ma, 1989). Other conditional factors, such as interspecific diet variation and/or intraspecific seasonal differences in prey items, cannot be disregarded.

Bones are considered a useful indicator of cumulative lead exposure, since most of the Pb intake via ingestion (90%) is transferred to them, where it has a low excretion rate and is stored in a relatively stable chemical form (Ma, 1989; Scheuhammer, 1991; Shore and Douben, 1994). This fact may explain the increase in lead burden that occurs with aging and which was observed in *C. russula* from the Ebro Delta, a finding similarly reported for other insectivores such as the common shrew, the pygmy shrew, *Sorex minutus*, and the common mole, *Talpa europaea* (Komarnicki, 2000; Pankakoski et al., 1993, 1994; Read and Martin, 1993; Stansley and Roscoe, 1996; Talmage and Walton, 1991).

Although mercury and cadmium accumulate mainly in soft tissues, it is well known that they directly affect the physiology of bone cells. These heavy metals interfere with calcium metabolism and bone remodelling, suppress bone cell activities, inhibit osteoclastic activities, and generate hypocalcemia and histopathological changes such as osteopenia and osteomalacia (Gdula-Argasinska et al., 2004; Goyer, 1997; Suzuki et al., 2004). Mercury pollution is of crucial concern in aquatic ecosystems, where inorganic mercury is highly transformed into methylmercury and bioaccumulates in biota (Schuhmacher et al., 1993). In fact, fish and seafood are not only the main culprits in mercury poisoning incidents, but also one of the main dietary exposure routes for humans (López Alonso et al., 2003). The available information on mercury concentrations in terrestrial biota is scarce, the Ebro Delta being no exception. Indeed regarding this region, evaluations of the effect this metal exerts have mainly focused on freshwater and marine ecosystems (Lacorte et al., 2006; Lavado et al., 2006; Mañosa et al., 2001; Schuhmacher et al., 1993). Our study provides the first data on Hg accumulation in a terrestrial mammal from this wetland and shows that in this area a significant increase of about 2.5-fold in mercury levels was produced compared with the control site. The relatively high values recorded in the shrews from the Ebro Delta indicate that there is some transfer of this metal from aquatic to terrestrial environments. Similar high Hg levels have also been reported in the bones of other terrestrial mammals (see Talmage and Walton, 1991 for a revision) and humans (e.g. Lindh et al., 1980) inhabiting polluted sites. Furthermore, different patterns of mercury bioaccumulation were observed between sexes in *C. russula* from the Ebro Delta: males exhibited a significant increase as they aged, whereas Hg levels remained unvarying throughout the life of females. This finding suggests that the mercury burden in females is reduced by transfer to the foetus via placenta and to offspring via milk, as widely described for other mammals (see e.g. Frodello et al., 2000; Yoshida et al., 1994). In the control area no significant sexual variation was observed, consistent with observations made at other sites with low mercury levels,

Table 3
Lead and cadmium concentrations (mean, mean ± SD, mean ± SEM or mean and range) reported for bones of several insectivore species from polluted (P) and control (C) sites

Species	Site	N	Pb	Cd	Reference
Blarina brevicauda	P	49	67.1 ± 53.0 SD		Getz et al. (1977)
	P	67	19.9 ± 21.0 SD		
	P	32	12.2 ± 8.3 SD		
Blarina brevicauda	P	1	437		Stansley and Roscoe (1996)
	C	4	12.3 (6.27–16.4)		
Crocidura leucodon	P	3	89.6 ± 25 SD	0.95 ± 0 SD	Topashka-Ancheva and Metcheva (1999)
Myosorex varius	P	3	372.4 ± 171.6 SEM		Reinecke et al. (2000)
Sorex araneus	P	20	193.0		Chmiel and Harrison (1981)
	C	6	41.0		
Sorex araneus	P	7		3.00 ± 0.65 SEM	Hunter and Johnson (1982)
	P	8		3.30 ± 0.28 SEM	
	C	8		3.65 ± 0.16 SEM	
Sorex araneus	P	17		1.89 ± 0.19 SEM	Andrews et al. (1984)
	C	13		1.00 ± 0.10 SEM	
Sorex araneus	P	23	610 ± 53.06 SEM		Andrews et al. (1989)
	C	18	55.3 ± 11.89 SEM		
Sorex araneus	P	16		1.83 ± 0.23 SEM	Hunter et al. (1989)
	P	20		2.84 ± 0.51 SEM	
	C	21		2.04 ± 0.22 SEM	
Sorex araneus	P	12	550 (134–1469)		Ma (1989)
	C	10	53.7 (20.0–102)		
Talpa europaea	P	7	42.4 ± 33.3 SD	0.032 ± 0.017 SD	Komarnicki (2000)
	P	5	24.8 ± 15.6 SD	0.038 ± 0.018 SD	
	P	9	22.0 ± 11.1 SD	0.039 ± 0.019 SD	

Values in dry weight, except for *Myosorex.*

in which the normal excretion rate is high enough to prevent bioaccumulation (López Alonso et al., 2003; Talmage and Walton, 1993).

Although sediments carried by the river and fertilizers from agriculture activities are sources of cadmium in the Ebro Delta (Lavado et al., 2006; Ramos et al., 1999; Schuhmacher et al., 1990), several studies have shown that levels of this metal remain low or moderate (revision in Mañosa et al., 2001). This agrees with our own findings, which demonstrate that Cd concentrations in shrews from this delta did not differ from those obtained in Medas' animals. In fact, the values achieved from both sites were similar or lower to those recorded in the bones of other insectivore species from unpolluted sites (Table 3). Nevertheless, it must be taken into account that since cadmium is excreted via osteoclasts (Suzuki et al., 2004), it does not appear to bioaccumulate in hard tissues in polluted areas. Thus, for example, when comparing *S. araneus* from control and polluted sites, levels of this metal in liver and kidney increased until 82- and 33-fold, respectively, whereas in femur it hardly varied (Andrews et al., 1984; Hunter and Johnson, 1982). Therefore, considering that insectivores are effective bioindicators of this metal in polluted areas (e.g. Komarnicki, 2000; Ma and Talmage, 2001; Talmage and Walton, 1991), we conclude that an additional examination of the soft tissues of *C. russula* is needed to further elucidate the entry of Cd into the biota of this wetland.

Our results showed that, in comparison with the control site, *C. russula* from the Ebro Delta bioaccumulated lead and mercury in levels that "a priori" were expected to have adverse effects. Ma (1996) found concentrations of Pb above 10 μg/g in the liver or 25 μg/g in the kidney, to be harmful to the common shrew. Assuming Pb ratios of 1:2 to 1:6 for kidney:femur and 1:30 for liver:femur from Ma's data on *S. araneus* (1989), values obtained in the present study seem far below the risk threshold for acute poisoning postulated in *C. russula*. Nonetheless, neither subclinical exposure nor direct mortality can be assessed by our data, and therefore cannot be disregarded. In the case of mercury, levels at which it may prove toxic vary greatly in wildlife. Based on the available literature, Eisler (1987) deduced that Hg concentrations greater than 1.1 μg/g for liver and kidney, should be regarded as presumptive evidence of an environmental mercury problem in wild mammals. Moreover, 30 μg/g for hepatic and renal tissues is considered the intoxication threshold for mammals, with levels of 13–69 μg/g reported in the kidneys of wild and laboratory mammals whose deaths had been attributed to mercury poisoning (Wren, 1986; Lord et al., 2002). As all of these Hg levels were expressed in terms of wet weight, a ratio of 2:1 must be applied to obtain comparable dry weight concentration since the water contained in bones constitutes approximately 50% of total weight. While Hg ratio between bone and soft tissues is not generally provided, using the data published by Jefferies and French (1976) for the wood mouse, *Apodemus sylvaticus*, we have considered an approximate Hg ratio of 1:4 for bone:kidney and bone:liver. The mercury concentrations detected in shrews from the Ebro Delta (up to 12.44 μg/g; i.e. approximately 24.88 μg/g wet weight in soft tissues) would indicate that mercury pollution affects this protected area.

Several studies have shown that small mammals exposed to heavy metals may lose body mass and/or may experience relative weight alterations in some tissues (e.g. Ma, 1989, 1996;

Nunes et al., 2001a). Body weight variation is reflected in the body condition index, a parameter that has been used to correlate individual responses and physiological alterations in animals inhabiting environments of diminished quality, for example by metal exposure. Although shrews from the Ebro Delta present high levels of lead and mercury, their fitness did not differ from that of control animals as can be deduced from their body condition index. This result is in agreement with those observed in seabirds and waterfowls from the Ebro Delta, where metal pollution was not associated with impairment of reproduction or body condition (Mateo et al., 1997; Morera et al., 1997). As previously reported (Ma, 1989; Milton et al., 2003; Nunes et al., 2001a), our results indicate that *C. russula* exhibits a high tolerance to metal pollution, similar to other small mammals. Body condition among shrews varied greatly in the two study sites suggesting that it is not driven by metal pollution but rather by particular conditions, such as habitat characteristics, food availability, and predation, among others. Thus, as has already been suggested in other studies (e.g. Nunes et al., 2001a,b), genetic, haematological, biochemical and developmental parameters should be used in conjunction with morphological criteria, to obtain a more suitable measure for assessing the response of terrestrial small mammals inhabiting polluted areas.

As can be deduced from published data, the range of variation in metal concentration is generally wider in polluted areas than in clean sites (Table 3). This observation was noted in the Ebro Delta, not only in *C. russula* but also in several bird (Mateo et al., 1997, 2003; Sanpera et al., 2000) and invertebrate species (Schuhmacher et al., 1990, 1994). This circumstance may indicate an individual response due to a different metal exposure and/or to particular ecological, genetic, and physiological factors in chronically exposed animals (Talmage and Walton, 1991). Additionally, the tissular turnover that occurs in bones causes changes in metal concentration, particularly in lead, over the life of an animal, which may also partially explain the great variation observed at the population level. In contrast, in non-polluted sites, animals do not need detoxicant mechanisms to control the intake of metals and to prevent toxic effects to their organs.

Because anthropogenic contamination frequently often ends up in wetlands, the degradation of deltaic areas is a common phenomenon in most developed countries, despite increased environmental legislation and protection. Specifically in the Ebro Delta, hunting, land use, and other anthropogenic activities have considerably increased non-essential heavy metal exposure, diminishing the environmental quality. Our results not only corroborate previous and recent studies reporting increased environmental levels of lead and mercury in this protected wetland (e.g. Lacorte et al., 2006; Lavado et al., 2006; Mañosa et al., 2001), but also analyse mammal data, which allows improving the assessment of environmental risk in this particular habitat. Furthermore, this study demonstrates that osteological material from mammal collections offers an effective tool for ecotoxicological studies, constituting a non-invasive method since it does not involve unnecessary animal captures. Moreover, it provides specific reference values to better assess the time variation of pollutants in wild populations in future research, particularly when relevant toxicological episodes occur. In fact, our samples were collected over 25 years ago, when environmental legislation in Spain was scarce. Nowadays, the use of lead shot pellets is forbidden and industrial and domestic wastewaters are more controlled and/or are treated in order to reduce river pollution. Consequently, a decrease in metal input in the Ebro Delta may be expected. Nevertheless, given the long environmental persistence of Pb and Hg, further studies are required to evaluate temporal variation in the levels of these metals and the efficacy of the recent legislation to improve the environmental quality of this wetland.

We conclude that terrestrial wildlife is exposed to non-essential heavy metals (Pb and Hg) in the protected area of the Ebro Delta. The lead from shot pellets and the mercury from anthropogenic activities deposited in the sediments from terrestrial environments and transported by water may become highly bioavailable to biota in this region. In contrast, cadmium does not appear to bioaccumulate in shrews. Sex and age are two important factors to be considered when conducting ecotoxicological studies, since they may represent a source of variability in wild populations. Apparently, the exposure to environmental pollutants does not affect the fitness of the shrews from the Ebro Delta, suggesting a high tolerance to toxic heavy metals. The greater white-toothed shrew may be regarded as a good indicator of Pb and Hg accumulation in the Mediterranean climate. In addition, bones from collections are a suitable source for assessing contamination levels in mammals. Further studies combining several criteria (including morphological, genetic, behavioural, physiological or developmental parameters) are needed in order to obtain additional information on the exposure, dietary uptake, pollutant bioavailability, and environmental quality of this protected area.

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Metal and metalloid accumulation in shrews (Soricomorpha, Mammalia) from two protected Mediterranean coastal sites

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Sr, Ba, Fe, Mn, Mo and B were bioaccumulated in bones of shrews from the Ebro Delta area and Cu in Medas Islands, whereas Cr and Zn showed similar levels at both sites.

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Although ecotoxicological data on heavy metals are abundant, information on other potentially toxic elements with attributed deficiency and/or toxic disturbances is scarce. Here we quantify zinc, copper, iron, manganese, chromium, molybdenum, strontium, barium, and boron in bones of greater white-toothed shrews, *Crocidura russula*, inhabiting two protected Mediterranean coastal sites: the Ebro Delta, a wetland impacted by human activities, and the Medas Islands, a reference site. Natural and anthropogenic inputs significantly increase Fe, Mn, Mo, Sr, Ba, and B in specimens from the Ebro Delta, whereas Cu and Cr were higher in Medas' shrews. Principal component analysis allowed complete separation between sites along the first two axes in particular due to B, Sr, and Cu. This study provides metal reference values in bones of insectivores, explores their variability and bioaccumulation patterns in depth, and assesses the potential environmental risk and toxicity for biota exposed to the above elements.

1. Introduction

Non-essential metals, such as lead (Pb), mercury (Hg), and cadmium (Cd), and essential elements, such as zinc (Zn) and copper (Cu), are common pollutants widely distributed throughout the ecosystems. They have been extensively quantified and their toxic effects for biota are well-known (e.g. Goyer, 1997; Mañosa et al., 2001; Nriagu, 1988; Sánchez-Chardi et al., 2007a). In contrast, environmental information concerning other metals and metalloids, like iron (Fe), manganese (Mn), molybdenum (Mo), strontium (Sr), barium (Ba), and boron (B), is less readily available. All these elements are natural constituents of soils and sediments (Fernández-Turiel et al., 2003; He et al., 2005; Nriagu, 1988; West et al., 2001) and their levels in mammalian tissues may depend on season, age and/or sex, as well as physiological, pathological, and ecological conditions (Komarnicki, 2000; Lopes et al., 2002; Pankakoski et al., 1993, 1994; Sánchez-Chardi and Nadal, 2007; Sánchez-Chardi et al., 2007a,b). Moreover, their background levels and/or bioavailability may increase due to industrial, agricultural, and domestic pollution, and other human activities such as hunting. Thus, lead shot pellets may contain traces of Zn and Cu (Mozafar et al., 2002), fertilizers are a source of Zn, Cu, Fe, Mn, Cr, Mo, and B (He et al., 2005; Otero et al., 2005), and plaguicides may have traces of Zn, Cu, Fe, Mn, Ba, and B (He et al., 2005; Mañosa et al., 2001). Additionally, effluents and atmospheric deposition from industries may contain virtually all elements, and domestic effluents may be an important input of Zn, Cu, Fe, Mn, Cr, and B (e.g. Lucho-Constantino et al., 2005; Outridge and Scheuhammer, 1993). Generally, data on some of these metals and metalloid in wild populations are scarce or absent, probably due to their low toxicity compared with those of widely distributed non-essential elements and the complexity of behaviour of metal mixtures at real conditions. This lack of data hinders our understanding of metal and metalloid migration through food chains.

Deltaic environments are fragile ecosystems often affected by heavy metals and other pollutants, especially in developed countries (e.g. Oliveira Ribeiro et al., 2005; Sánchez-Chardi et al., 2007a) and one of the main goals when analysing heavy metal concentrations is to distinguish between natural background levels and those originating from anthropogenic contaminant sources (Rodríguez Martín et al., 2006). For many decades, the Ebro Delta

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has been impacted by several human activities such as lead shot pellets from hunting, fertilizers and pesticides from agricultural processes, industrial poles, and domestic sewage (Lacorte et al., 2006; Lavado et al., 2006; Mañosa et al., 2001; Ocampo-Duque et al., 2008; Schuhmacher et al., 1993). However, most studies focusing on the Ebro area have reported fragmentary, not always consistent, information about elements at both biotic and abiotic levels (e.g. Grimalt and Albaigés, 1990; Lavado et al., 2006; Mañosa et al., 2001; Navas and Machín, 2002; Ocampo-Duque et al., 2008). The Medas Islands are a nature reserve that is barely impacted by pollution sources and considered a pristine site with background levels of toxic metals and other pollutants (Pastor et al., 1995; Sánchez-Chardi et al., 2007a).

Small mammals, especially shrews, are reliable bioindicators of environmental pollution (bioaccumulation of metals and physiological effects) because they show high food requirements and their metabolic rate is high (e.g. Komarnicki, 2000; Pankakoski et al., 1993, 1994; Sánchez-Chardi et al., 2007a,b; Talmage and Walton, 1991). Likewise, bones accumulate several metals, especially bivalent elements that mimic calcium (e.g. EPA, 1998; Nielsen, 2004). In addition, bones from zoological collections have been used successfully to study contamination levels in small mammals (e.g. Sánchez-Chardi et al., 2007a). However, there is little information on the accumulation of several elements in the hard tissues in wild mammals.

With the above considerations, the aims of this study were: (i) to determine levels of those elements scarcely quantified in previous ecotoxicological studies; (ii) to analyse site, age-, and sex-dependent variation in the bioaccumulation patterns; (iii) to provide reference values for metal and metalloid contents in hard tissues of shrews; and (iv) to assess the use of these parameters as biomarkers in Mediterranean sites.

2. Materials and methods

We analysed the element content in large bones of 105 greater white-toothed shrews, *C. russula*. Animals were collected from1976 to 1981 in the Ebro Delta ($n = 73$) and the Medas Islands ($n = 32$), two partially protected coastal areas in north-eastern Spain. Specimens were sexed and aged: (1. Juveniles: immature shrews in their first year of life; 2. Adults: mature shrews in their first year of life; 3. Seniles: shrews in their second year of life) according to toothwear (Vesmanis and Vesmanis, 1979) and reproductive condition (López-Fuster et al., 1985). Bones were cleaned by exposure to dermestid larvae (Dermestidae, Coleoptera), kept in single metal-free paperbags and stored in the zoological collection of the Animal Biology Department (University of Barcelona). Initially, this material constituted the basis of several investigations on morphometric and biological aspects of the species (López-Fuster, 1985; López-Fuster and Ventura, 1992; López-Fuster et al., 1985, 1986).

For chemical quantification, dried samples were placed in Teflon vessels and digested by nitric acid and hydrogen peroxide (Instra, Baker Analyzed). Samples were diluted 1:5 in deionized (Milli-Q©) water with 1% nitric acid. Rhodium was used as internal standard and bovine liver (SRM-1577a), certified by the National Bureau of Standards, was included as reference material in the analysis. Concentrations of Fe were quantified by a Perkin-Elmer OPTIMA-3200RL inductively coupled plasma optical spectrometer (ICP-OES) whereas the rest of the metals (Zn, Cu, Mn, Cr, Mo, Sr, Ba, Ni, and Co) and a metalloid (B) were determined by a Perkin-Elmer ELAN-6000 inductively coupled plasma mass spectrometer (ICP-MS). The results of element concentrations were expressed as mean ± standard error of the mean ($M \pm SEM$) in micrograms/gram (μg/g) on a dry weight basis. Detailed methodological information is described by Sánchez-Chardi et al. (2007a,b).

Normal distribution and homogeneity of variance of the log transformed data were assessed by the Kolmogorov–Smirnov test and the Levene F-test, respectively. Initially, a three-way multivariate analysis of variance (MANOVA) was performed to obtain an overall estimation of the effects of site, age, and sex and their interactions on the element levels. When element concentrations did not differ according to age and/or sex, data were combined to increase the sample size of each site. Depending on this previous procedure, intra- and interpopulational comparisons were evaluated either by Student's t-test or by one-way analysis of variance (ANOVA). When ANOVA was applied, pair-wise comparisons of sample means were conducted by Scheffe's method. In an attempt to visualize the degree of divergence between both populations considering all elements analysed, a principal component analysis was performed. This statistical technique reduces multidimensional data sets to lower dimensions while retaining those characteristics of the data set that contribute most to its variance. After factor extraction, a Varimax rotation was employed to aid interpretability of the low-variance principal components. Pearson's correlation coefficients were calculated to evaluate the relationship between the elements analysed in shrews from the polluted site. For all sequential tests, p-values were corrected by the Bonferroni adjustment (Rice, 1989), as modified by Chandler (1995). For all statistical analyses, SPSS 14.0 (2005) was used.

3. Results

Nickel and cobalt concentrations were under threshold limit of ICP-MS (in μg/kg in diluted acidic solution: Ni 0.20, Co 0.05) and therefore were not included in the analyses. The rest of elements were above the detection threshold in all specimens (in μg/kg in diluted acidic solution: Fe 10, Zn 0.50, Cu 0.10, Mn 0.10, Cr 0.50, Mo 0.05, Sr 0.10, Ba 0.05, B 0.05).

MANOVA showed significant differences in the element concentrations by site ($F = 103.487$, $p = 0.001$), age ($F = 2.528$, $p = 0.001$), and their interaction ($F = 2.434$, $p = 0.002$). Since sexual divergence was not observed, sexes were pooled in subsequent analyses. Descriptive statistics of the elements quantified according to site and age are shown in Table 1.

Significant age-dependent variation was only observed in Zn levels in the Medas Islands ($F = 8.226$, $p < 0.001$), with statistical divergences between juveniles and both adults ($p = 0.009$) and seniles ($p = 0.003$). Mean values of this metal increased with age in the two capture sites although in the Ebro Delta differences were not significant.

Element concentrations varied significantly between sites (in all cases $p < 0.001$), except for Zn (differences evaluated for each age-class) and Cr (Table 1). Globally, shrews from the polluted area showed the highest mean values, with the exception of Cu.

Principal component analysis was performed on the log transformed data excluded Zn due to the age-dependent variation observed in the Medas' animals and the absence of interpopulational differences. Two rotated principal components with eigenvalues greater than 1 were extracted, which accounted for 61.46% of the total variance. Variables with higher positive loadings were B and Sr on the first factor (PC I) and Cu on the second factor (PC II; Table 2). Projection of the specimens onto the first two axes showed a complete separation along PC I, due to the lower scores exhibited by the Medas' shrews (Fig. 1).

Significant Pearson's correlation coefficients and the corresponding p-values evaluated in shrews from the Ebro Delta are shown in Table 3. In general, the highest number of correlations appeared between Cu and Fe and the rest of elements (7 and 6 cases, respectively), whereas Cr correlated with none of the elements analysed.

4. Discussion

4.1. Bioaccumulation by site, age, and sex

Levels of Zn found in *C. russula* agreed with those reported in the bones of other Soricomorpha (Andrews et al., 1989; Komarnicki, 2000; Topashka-Ancheva and Metcheva, 1999). Similar concentrations observed between the sites studied might be mainly due to the good homeostatic mechanisms of this metal in mammalian tissues (Goyer, 1997).

Copper levels found in bones of *C. russula* from Medas Islands were in agreement with data reported for the common shrew, *Sorex araneus*, from reference sites and lower than those observed in *S. araneus* and *C. leucodon* from polluted sites (Hunter and Johnson, 1982; Hunter et al., 1989; Topashka-Ancheva and Metcheva, 1999). In the Delta shrews, Cu levels were significantly low, a circumstance that has also been reported in fish and eggs of seabirds from the same area (Lavado et al., 2006; Morera et al., 1997; Sanpera et al.,

Table 1
Descriptive statistics (sample size (*n*), arithmetic mean (*M*), standard error of the mean (SEM), minimum (Min.) and maximum (Max.)) of element concentrations (μg/g, in dry weight) in the samples analysed according to site and age (1, juveniles; 2, adults; 3, seniles).

	Age	Ebro Delta					Medas Islands				
		n	*M*	SEM	Min.	Max.	*n*	*M*	SEM	Min.	Max.
Zn	1	27	213.09	13.63	111.21	358.44	9	203.52	3.52	191.95	217.85
	2	12	227.73	16.39	126.78	345.61	12	228.15	4.54	202.00	252.11
	3	34	238.09	8.68	81.12	360.31	11	232.94	6.78	187.99	265.96
	Total		227.14	7.03				222.87	3.69		
Cu	1	27	4.67	0.50	2.31	15.73	9	5.68	0.63	3.70	9.65
	2	12	3.51	0.29	2.12	5.17	12	5.09	0.42	2.63	8.14
	3	34	4.78	0.48	1.02	13.93	11	6.11	0.41	4.33	8.39
	Total		4.53	0.29				5.61	0.28		
Fe	1	27	258.93	18.55	145.85	501.96	9	154.03	11.69	112.11	209.52
	2	12	220.41	13.78	153.43	324.34	12	158.35	8.32	122.45	206.77
	3	34	211.86	18.34	117.55	602.00	11	176.57	4.80	152.23	199.88
	Total		230.67	11.36				163.39	4.97		
Mn	1	27	7.45	0.69	1.94	16.01	9	4.06	0.20	2.83	4.79
	2	12	10.81	1.32	5.24	18.82	12	4.89	0.25	3.78	6.77
	3	34	7.02	0.57	3.65	15.47	11	4.66	0.26	3.64	6.35
	Total		7.80	0.45				4.58	0.15		
Cr	1	27	2.28	0.16	1.06	5.47	9	1.84	0.12	1.44	2.67
	2	12	1.81	0.17	1.18	3.02	12	2.07	0.17	1.31	3.61
	3	34	1.78	0.17	0.75	5.97	11	1.91	0.11	1.36	2.72
	Total		1.97	0.11				1.95	0.08		
Mo	1	27	0.18	0.02	0.08	0.57	9	0.11	0.01	0.09	0.14
	2	12	0.13	0.01	0.08	0.24	12	0.11	0.01	0.09	0.16
	3	34	0.20	0.06	0.07	2.16	11	0.12	0.01	0.09	0.15
	Total		0.18	0.25				0.11	0.02		
Sr	1	27	250.65	16.61	126.69	474.88	9	68.41	1.81	59.46	74.93
	2	12	228.79	17.32	100.32	321.30	12	73.27	2.58	61.89	91.03
	3	34	246.93	20.80	55.87	680.58	11	78.28	2.82	58.38	91.57
	Total		245.32	11.73				73.63	1.58		
Ba	1	27	17.64	2.06	9.48	53.17	9	8.96	1.08	5.14	14.41
	2	12	13.70	0.98	8.65	18.29	12	10.05	0.67	7.15	15.48
	3	34	18.19	1.87	2.73	64.77	11	12.63	1.33	9.29	24.26
	Total		17.25	1.17				10.63	0.64		
B	1	27	143.99	6.49	75.58	209.22	9	28.44	8.54	7.73	86.97
	2	12	136.05	7.59	102.34	174.68	12	11.93	1.81	5.87	22.48
	3	34	169.01	18.38	39.42	445.11	11	26.92	3.92	7.46	52.54
	Total		154.34	9.05				21.73	3.05		

1997). Although high percentage of sand in Ebro Delta soils reduces Cu availability for biota (Grimalt and Albaigés, 1990), low values of essential elements in polluted areas are often related to the interaction between essential and non-essential metals. In mammalian tissues, this metal is effectively controlled by homeostatic mechanisms (Goyer, 1997; Hunter et al., 1989; Johnson et al., 1978; López Alonso et al., 2004) that may be disrupted by high exposure to Pb and Hg (Blanuša et al., 1989; Lavado et al., 2006; Pankakoski et al., 1994), heavy metals that appeared in high concentrations in the specimens from the Ebro Delta (Sánchez-Chardi et al., 2007a).

Iron concentrations found in *C. russula* did not differ from those previously obtained in the bones of bank voles, *Myodes glareolus* (Damek-Poprawa and Sawicka-Kapusta, 2004; Sawicka-Kapusta et al., 1990). The increase in shrews from the Ebro Delta may be explained by the protective effects of iron, as previously observed in this species (Sánchez-Chardi et al., 2007b) and in several tissues of other species, for example bone medule (see e.g. López Alonso et al., 2004; Pereira et al., 2006; Włostowski et al., 2003). There are several cellular detoxification mechanisms involving iron, such as

Table 2
Component correlations obtained after applying a PCA with Varimax rotation to the metals and metalloids in bones of shrews.

	Components	
	PC I	PC II
B	0.854	−0.026
Fe	0.555	0.612
Ba	0.579	0.351
Sr	0.898	0.180
Cu	−0.382	0.735
Mn	0.552	0.446
Mo	0.345	0.649
Cr	−0.265	−0.639

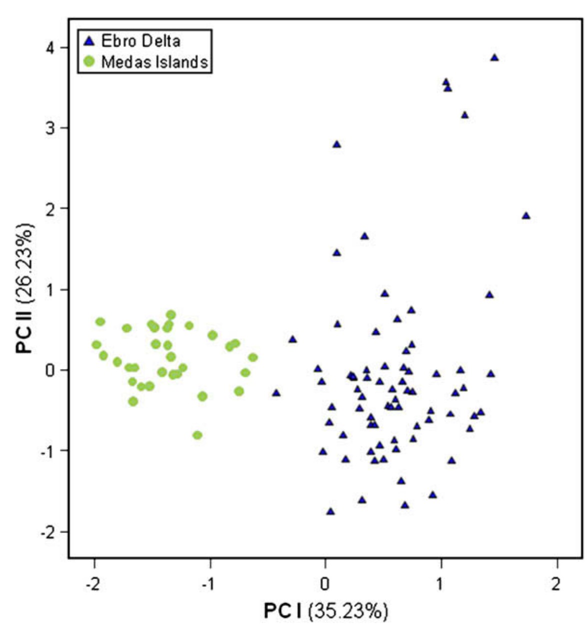

Fig. 1. Biplot of the individual scores onto the first two component axes according to site.

Table 3
Significant Pearson correlation coefficients (r) and p-values (p) between metals analysed in shrews from the polluted site.

	r	p		r	p
Zn/Cu	0.263	0.025	Cu/B	0.266	0.023
Zn/Fe	0.520	<0.001	Fe/Mn	0.600	<0.001
Zn/Mn	0.409	<0.001	Fe/Mo	0.322	0.005
Zn/Mo	0.246	0.036	Fe/Sr	0.492	<0.001
Zn/Sr	0.424	<0.001	Fe/Ba	0.302	0.009
Zn/Ba	0.211	0.073	Mn/Sr	0.333	0.004
Cu/Fe	0.329	0.004	Mn/Ba	0.235	0.045
Cu/Mn	0.332	0.004	Mo/Ba	0.096	<0.001
Cu/Mo	0.405	<0.001	Mo/B	0.439	<0.001
Cu/Sr	0.449	<0.001	Sr/Ba	0.721	<0.001
Cu/Ba	0.382	0.001			

the induction of synthesis of proteins like cytochromes and ferritin, that protect mammalian cells from the toxic effects of both essential and non-essential metals (Lopes et al., 2002; Pereira et al., 2006), which might explain our findings.

Manganese levels were low and similar to those reported for the bank vole, the field vole, *Microtus agrestis*, and the wood mouse, *Apodemus sylvaticus* (Beardsley et al., 1978; Gdula-Argasinska et al., 2004; Gorriz, 1996). The levels of this metal are physiologically regulated in mammals and environmental increases are not generally related to high levels in tissues (Beardsley et al., 1978). As with iron, the higher levels observed in the Ebro Delta shrews in relation to the Medas' specimens might be related to detoxification mechanisms and/or interaction between elements (see references in Lopes et al., 2002; Sánchez-Chardi et al., 2008).

Previous studies on the Ebro Delta indicate high variability in the concentration of chromium (Ocampo-Duque et al., 2008) with no clear pattern of bioaccumulation (Schuhmacher et al., 1993, 1994, 1995). As occurs in other small mammals (Pereira et al., 2006; Talmage and Walton, 1991), we found the highest chromium concentrations in shrews from the reference site. Chromium levels are mainly influenced by natural content of parent rocks on a regional scale (Rodríguez Martín et al., 2006). Consequently, and considering that Cr was not significantly correlated with the rest of the elements analysed, our results might indicate a low bioavailability of this metal in the Ebro Delta rather than competitive mechanisms between essential and non-essential elements as suggested in previous studies (e.g. Pereira et al., 2006).

The molybdenum levels found in specimens from the Ebro Delta may be related to the high redox potential of deltaic soils that act as a net to catch this element as well as these alkaline lime-rich environments also facilitate Mo solubilization and mobilization (Fernández-Turiel, personal communication; Frank, 2004). Although molybdenum is known to be a copper antagonist (Pankakoski et al., 1993) due to the formation of a sulphur–molybdenum complex that binds Cu, we found significant correlation between both elements, as has also been reported in other mammals (Blanco-Penedo et al., 2006; López Alonso et al., 2004).

Strontium and barium are widely distributed non-essential elements that do not biomagnify throughout the trophic chain, and calcium mimics in biological systems (EPA, 1998; Malina, 2004; Nielsen, 2004; Purdey, 2004). Whereas Sr levels found in *C. russula* from Medas Islands did not differ from those found in other natural environments, levels in the Ebro Delta specimens were significantly higher than those reported in the whole body, soft and hard tissues, of other small mammals inhabiting polluted and reference sites (Appleton et al., 2000; Cloutier et al., 1986; Seifert et al., 1999). Strontium is considered toxic only at high concentrations (Nielsen, 2004), producing hypocalcaemia in exposed animals . Nevertheless, in the present study neither calcium decrease (M ± SEM: Ebro Delta 224.01 ± 3.02 mg/ g; Medas Islands 229.65 ± 1.98 mg/ g) nor body weight loss (Sánchez-Chardi et al., 2007a) were observed in shrews from the Ebro Delta. The input of Sr in this area may be partially explained by the geochemistry of river sediments and soils in the Ebro Basin (with high evaporitic rock content) and the seawater entrance of the Encanyissada lagoon that are rich in this element (Fernández-Turiel et al., 2003), although, the importance of anthropogenic sources of Sr cannot be disregarded. Conversely, although the uptake and accumulation of Ba in bones of mammals is higher than their associates (EPA, 1998; Nielsen, 2004), the low Ba concentrations found in shrews may be due, at least partially, to its tendency to precipitate as $BaCO_3$ when there is a high $CaCO_3$ content. Additionally, Ba has a short half-time in biological systems, as it is excreted in faeces and urine, reducing body burden in mammals (EPA, 1998; Malina, 2004).

Boron is a metalloid naturally occurring throughout the environment (Malina, 2004) and its essentiality for animals is discussed (Chapin et al., 1997). Low levels found in shrews from the reference site agreed with data obtained in bones of laboratory mammals (Chapin et al., 1997) and could be considered as background concentrations. The high B levels observed in shrews from the Ebro Delta may be due to anthropogenic activities, as occurs in other areas (Lucho-Constantino et al., 2005; Powell et al., 1997). Moreover, limited water circulation in the lagoons and soils with high clay content in the Ebro Delta (Lacorte et al., 2006) might increase boron bioavailability (e.g. Lucho-Constantino et al., 2005). Boron affects weight and size of bones, as well as their essential elements and fat content (Chapin et al., 1997). Considering the data reported by these authors for laboratory rodents, a very high B intake, up to levels related to bone alteration, would be expected in shrews from the Ebro Delta. However, this metalloid showed a wide range of non observable adverse effects levels (NOAEL) depending on the species and parameters tested (review in Eisler, 1990).

As for age is concerned, bones of adult shrews tended to have higher Zn concentrations than those of juveniles, as has also been reported for *S. araneus*, the meadow vole, *Microtus pennsylvanicus*, and the wood mouse (Cloutier et al., 1986; Pankakoski et al., 1993, 1994; Read and Martin, 1993; Tersago et al., 2004). However, this pattern has not been observed in soft tissues of *C. russula* (Sánchez-Chardi et al., 2007b; Sánchez-Chardi and Nadal, 2007), probably because bone acts as a storage tissue for excess zinc (where it accumulates as metabolically inactive) and/or due to interaction with other elements (Johnson et al., 1978; Pankakoski et al., 1993, 1994; Talmage and Walton, 1991).

Among parameters analysed in the present study, gender remained the least important factor, as reported for the common shrew and the lesser shrew, *S. minutus* (Pankakoski et al., 1994). In *C. russula*, this circumstance also applies to heavy metal bioaccumulation (Sánchez-Chardi et al., 2007a,b). Although sexual differences were not significant, females showed higher levels of Mn, Mo, and Fe than males (data not shown). A similar trend has also been reported for the species and other wild mammals (Lopes et al., 2002; Pankakoski et al., 1993; Sánchez-Chardi et al., 2007a,b), although gestation and lactation are expected to reduce metal body burden. Nutritional requirements, different metabolic profiles of metals related to sexual hormones, and/or interaction between elements (see references in Goyer, 1997; Lopes et al., 2002; Pankakoski et al., 1993) may be responsible for the results obtained concerning these essential metals.

4.2. Biomarkers and bioindicators of environmental quality

Usually a species is considered a suitable bioindicator when it accumulates high concentrations of toxic heavy metals and reacts to the entrance of xenobiotics into the environment. For this purpose, small mammals in general and shrews in particular have proved as suitable bioindicators of pollution because they constitute an intermediate step between top predators and invertebrates. Earthworms, snails, slugs, and arthropods are common shrew preys, some of them well-known as metal accumulators (Hunter and Johnson, 1982; West et al., 2001), and diet is the main source of metals in mammals (e.g. Johnson et al., 1978; Appleton et al., 2000; Torres and Johnson, 2001). Apart from the direct accumulation of metal content through the preys, the shrews also intake xenobiotics indirectly through soil ingestion (Stansley and Roscoe, 1996). These feeding characteristics, as well as high metabolic rate and tolerance to toxic compounds, make shrews high accumulators of several elements (e.g. Komarnicki, 2000; Pankakoski et al., 1993, 1994; Talmage and Walton, 1991).

In the search for suitable parameters (i.e. biomarkers) to assess environmental quality, metal and metalloid concentrations may be used. In general, the adequate tracer element may depend on the kind of pollution, the physicochemical conditions of the soil and water, or the species studied, among other factors. Moreover, the combined data of several elements and their interactions may provide useful information for ecotoxicologists. Nevertheless, the interaction of elements, as well as the information derived from analysis of essential metals and other elements, is often forgotten in ecotoxicological studies. We propose the use of the whole data set provided by several elements as a valuable information source on environmental quality. Principal component analysis performed on these data allowed us to obtain a good separation of the specimens according to the study sites. Thus, whereas specimens from the reference area appeared as a homogeneous group, shrews from the polluted zone exhibited more dispersed scores. This increase of variability in the accumulation of pollutants has also been reported in disturbed areas as a consequence of intraspecific variation and individual exposure and response to pollutants (e.g. Sánchez-Chardi et al., 2007a and references therein). Similar methodology has been previously used with several kinds of samples from reference and polluted areas but not, to our knowledge, with bones of shrews. Moreover, our results with PCA must be checked in other polluted areas and with other species in order to use hard tissues as a suitable source of ecotoxicological data.

As has been demonstrated in the case of highly toxic metals (Sánchez-Chardi et al., 2007a), examination of bones from zoological collections is a reliable and non-invasive method of studying elements with ecotoxicological interest. The samples used in this study were collected 30 years ago and since then environmental conditions have been strongly modified. On the one hand, environmental laws have protected places of ecological significance, such as the Ebro Delta, reducing and controlling human impact. Since a reduction in industrial and domestic untreated wastewaters is expected by this restrictive environmental legislation, metal entrance into the Ebro River should have diminished throughout this time. On the other hand, the increase in human population and its pollutant activities (traffic, industries and domestic wastes) may increase metal pollution in the Ebro Delta. Therefore, having demonstrated the bioaccumulation of toxic metals in biota of the Ebro Delta, we consider that it would be interesting to undertake further analysis based on recent material to assess current levels, effects, and sources of potentially toxic elements in protected areas.

5. Conclusions

This study constitutes the first quantification of several metals and metalloids in the bones of shrews. A few of these elements may be toxic for biota and may be of special concern in protected areas. Main entrances of these elements seem to occur naturally, but further analyses are needed to quantify anthropogenic inputs.

Like other insectivorous species, the greater white-toothed shrew, *Crocidura russula*, seems to be a suitable bioindicator of metals and boron because it is a strong bioaccumulator and the element concentrations in their tissues vary in relation to environmental changes. Moreover, the information obtained may be useful to know the distribution and mobility of metals and metalloids within ecosystems and to predict the risk of environmental pollution in protected Mediterranean sites.

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10. ANNEX 2

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How does the greater white-toothed shrew, *Crocidura russula*, responds to long-term heavy metal contamination? — A case study

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Abstract

Heavy metals accumulation in parallel with the evaluation of physiological and biochemical effects resulting from continued metal exposure were considered here using for the first time the great white-toothed shrew *Crocidura russula* as an *in vivo* model. Shrews were originated from an abandoned lead/zinc mining area and from a reference area, both in Alentejo, southern Portugal. Hepatic contents of nickel, copper, zinc, cadmium, mercury and lead were quantified by Inductively Coupled Plasma Mass Spectrometer (ICP-MS). Haematological parameters (white blood cells, red blood cells, haemoglobin and haematocrit) were obtained in a Coulter Counter Analyser and biochemical markers of the redox balance (glutathione *S*-transferase, glutathione peroxidase, and glutathione reductase) activities were measured spectrophotometrically using a Duo-50 spectrophotometer. Compared with control animals, significantly higher concentration of hepatic cadmium (9.29 *vs.* 1.18 μg/g dry weight) and nickel (1.56 *vs.* 0.343 μg/g dry weight) were detected in the shrews collected in the mining area. However, no significant changes were observed on haematological or enzymatic parameters in animals exposed to metal pollution. The obtained results show that shrews are good bioaccumulators of toxic heavy metals, but very tolerant to their effects, revealing an interesting long-term adaptation to polluted environments. In addition, this study provides reference values for haematological parameters and antioxidant enzymes levels in *C. russula*, which may be relevant for comparative purposes in further studies.

Keywords: *Crocidura russula*; Abandoned mining area; Heavy metals; Haematological parameters; Antioxidant enzymes

1. Introduction

Mining wastes remaining after the extraction of target metals are referred as important sources of environmental contamination, reaching in some cases levels that might become toxic to wildlife and an environmental risk to human health. Portugal has a legacy of about 85 old mines deactivated for economical reasons. Most of these mines were deactivated without any previous environmental recovery plan. As a consequence, tones of metal residues are still circulating in their surroundings (Santos Oliveira et al., 2002). Preguiça, a lead/zinc mine located in Alentejo, southern Portugal was deactivated 40 years ago, being a fine illustration of this reality. Over the last decades, the area once occupied by the mine has been covered by vegetation, hiding all the tailings and scoria produced and accumulated in the soil.

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Several studies have confirmed that insectivores may accumulate superior amounts of heavy metals in their tissues, suggesting their major role as biomonitors (*e.g.* Talmage and Walton, 1991; Ma et al., 1991; Ma, 1996; Pankakoski et al., 1993, 1994; Komarnicki, 2000; Ma and Talmage, 2001). The potential interest of ground-living insectivores as *in situ* models, such as shrews, is explained by their widespread occurrence; limited home range; small body size; high metabolic rate; and invertebrate-based diet, especially because invertebrates are known to accumulate high levels of metals (Hunter and Johnson, 1982; Scanlon, 1987; Ma et al., 1991; Shore, 1995). In the present study, the greater white-toothed shrew, *Crocidura russula*, a widely distributed species in western and southern Europe, including Portugal (ICN/CBA, 1999), is tested as a model of environmental long-term mining contamination.

Ecotoxicological surveys usually focus on bioaccumulation but rarely determine biological effects of long-term exposure to mining residues. So, only a few studies report morphological, biochemical, haematological, or genetic alterations for metal-exposed insectivores (*e.g.* Ma, 1989; Dodds-Smith et al., 1992; Hendricks et al., 1995) or other small mammals (Nunes et al., 2001; Viegas-Crespo et al., 2003). In this study, in addition to hepatic heavy metal accumulation, haematological and biochemical effects are investigated in *C. russula* inhabiting an abandoned mine area. Haematological values (white and red blood cells, haemoglobin and haematocrit) can be indicative of the physiological status of wild animals (Marques et al., 2006). Biochemical parameters, such as antioxidant enzyme activities (glutathione *S*-transferase, glutathione peroxidase and glutathione reductase) are often used as markers of oxidative stress, considering the active role they play in the detoxification of deleterious compounds produced by several metals and other environmental pollutants (Cnubben et al., 2001).

The obtained results will also allow i) to assess the role of the white-toothed shrew as a bioindicator of heavy metal pollution, ii) to determine haematological and biochemical reference values for this species and at last iii) to confirm the potential environmental risk of abandoned mines.

2. Materials and methods

2.1. Study areas

This study was carried out in a riparian area in the surroundings of an old lead/zinc mine (Preguiça mine), located in Alentejo, southern Portugal (38°02′15″N; 07°17′01″W). This mining area is included in the Iberian Magnetitic–Zinciferous Belt, characterized by the presence of lead and zinc oxides in soils, as well as several other metals, present in trace amounts (Vairinho and Fonseca, 1989) (Table 1). The climate in this region is characterized by hot dry summers and mild winters. The average annual temperature and precipitation are approximately 17 °C and 600 mm, respectively. The vegetation of this riparian area is dominated by trees and shrub species (*Quercus rotundifolia*, *Cistus ladanifer*, *Rubus ulmifolius* and *Nerium oleander*). Herbaceous species (*Echium plantagineum*, *Bromus rigidus*, *Vulpia myunos* and *Phleum phleoides*) were also present.

For comparative purposes, an area without known exogenous sources of heavy metals and located 30 Km northwest from Preguiça mine (38°11′18″N; 07°24′34″ W) was chosen as reference. Both sites have similar climate, vegetation and relief.

2.2. Animal and tissue collections

A total of 33 adult *C. russula* (reference area: 9 males, 8 females; Preguiça area: 7 males, 9 females) were live-trapped using Longworth® and Sherman® traps baited with a mixture of sardine, oil and wheat flour. The captures were performed in 3-night trapping sessions, using 150 traps along 800 m transects.

Animals were collected, sexed, weighed (to the nearest ±0.1 g) and transported to the laboratory. Blood samples were collected by cardiac punction using heparinized syringes. Liver was promptly removed, weighed and separated in two fractions, one for the immediate determination of antioxidant enzyme activities, while the other was stored at −20 °C for later quantification of heavy metal contents. All methodologies were conducted in strict accordance with the directive 86/609/EEC on the protection of laboratory animals.

2.3. Metal analyses

Liver fractions were dried (60 °C) till constant weight (dry weight: dw). For each specimen, 80 to 100 µg of

Table 1
Soil elemental composition in the Preguiça area [Adapted from the Vairinho and Fonseca (1989)]

Elements	Preguiça area
Ni (mg/kg)	27
Cu (mg/kg)	194
Zn (%)	0.74
Cd (mg/kg)	40
Pb (%)	0.19

Table 2
Hepatic concentrations (μg/g dw) of heavy metals in *C. russula* in study areas

Metals	Reference area ($n=17$)		Preguiça area ($n=16$)	
	Median	Range	Median	Range
Ni	0.34	0.02–4.95	1.30*	0.05–3.76
Cu	20.3	15.0–46.6	17.7	11.0–33.6
Zn	145	100–243	130	92–222
Cd	1.18	0.04–12.50	9.28**	0.22–49.60
Hg	0.100	n.d.–0.420	0.240	n.d.–0.490
Pb	0.93	0.07–1.63	1.17	0.13–3.01

(*) (**) Significantly different from the reference area (Mann–Whitney's *U*-test).
* $p<0.05$, ** $p<0.01$.
n: number of animals.
n.d.: not detectable.

liver tissue was digested in Teflon vessels (120 °C, 12 h) with 2.0 ml 70% nitric acid (Baker Instra Analysed) and 1.0 ml 30% hydrogen peroxide (Baker Instra Analysed). Nickel (Ni), copper (Cu), zinc (Zn), cadmium (Cd), mercury (Hg) and lead (Pb) were quantified by a Perkin Elmer ELAN-6000 Inductively Coupled Plasma Mass Spectrometer (ICP-MS). Two replicate subsamples and a standard reference material (Bovine Liver SRM-1577a) certified by the National Bureau of Standards (NBS) were included in the analyses. The mean values from twenty-six blanks were subtracted from each sample. Method detection limits (in ppb) were 0.20 for nickel, 0.10 for copper, 0.50 for zinc, 0.05 for cadmium, 0.20 for mercury, and 0.05 for lead.

2.4. Haematological and enzyme analyses

A Coulter Counter Analyser (Beckman Coulter, USA) was used to determine blood parameters: white blood cell count (WBC, $\times 10^3$ mm^{-3}), red blood cell count (RBC, $\times 10^6$ mm^{-3}), haemoglobin concentration (HGB, g dl^{-1}) and haematocrit (HCT, %).

For enzymatic determinations, liver was rinsed in an ice-cold 0.154 mm KCl solution, for immediate determination of glutathione *S*-transferase (GST), selenium dependent glutathione peroxidase (Se-GPx) and glutathione reductase (GR) activities. Liver homogenates (1:10 w/v) were prepared with approximately 0.3 g of fresh liver and ice-cold 0.25 M potassium phosphate buffer (pH = 7.0), using a Potter–Helvehjem homogenizer (B. Braun Melsungen, Germany), and immediately centrifuged at 1000 *g* for 15 min at 4 °C. Then the supernatant was collected and further diluted with 0.25 M potassium phosphate buffer, according to each enzyme's specific protocol. Enzyme activities were measured spectrophotometrically using a Duo-50 spectrophotometer (Beckman Instruments, USA). Assays were performed in triplicate and average values were calculated.

GST activity was determined according to the method of Habig et al. (1974), by following the change in the absorbance of the substrate 1-cloro-2,4-dinitrobenzene (CDNB), conjugated with GSH, at 340 nm and expressed as μmol of product/min/mg protein. Se-GPx activity was determined according to the method developed by Paglia and Valentine (1967) and expressed as μmol NADPH oxidized/min/mg protein. GR activity was determined using the method described by Carlberg and Mannervik (1985) and expressed as μmol NADPH oxidized/min/mg protein. Total protein contents were determined in triplicate according to the biuret method (Gornall et al., 1949) in liver homogenates using Bovine Serum Albumin (Sigma, Spain) as standard.

2.5. Statistical analyses

Statistical analyses were performed by SPSS 11.0 for Windows (SPSS Inc., 1999). Most variables did not exhibit normal distribution and/or variances homogeneity, so all variables were compared using Mann–Whitney's *U*-test. Results are shown as median and range values (Minimum–Maximum). Differences were considered statistically significant at $p<0.05$.

3. Results

3.1. Metal concentrations

Significantly higher concentrations of cadmium and nickel were detected in shrews collected in the mining area comparing with the reference area ($p<0.05$ and

Table 3
Haematological parameters in *C. russula* in study areas

Haematology	Reference area ($n=17$)		Preguiça area ($n=16$)	
	Median	Range	Median	Range
WBC ($\times 10^3$ mm^{-3})	3.30[a]	0.60–9.10	4.80[b]	1.20–6.80
RBC ($\times 10^6$ mm^{-3})	6.64	2.20–10.30	8.13	4.85–10.60
HGB (g/dl)	13.2[a]	9.5–15.6	14.0	10.1–16.8
HCT (%)	29.3	8.7–42.3	35.7	21.7–46.5

WBC, white blood cells; RBC, red blood cells; HGB, haemoglobin; HCT, haematocrit.
n: number of animals.
[a] $n=16$.
[b] $n=15$.

Table 4
Enzymes activities (μmol/min/mg protein) in the liver of *C. russula* in study areas

Enzymes	Reference area		Preguiça area	
	(n=17)		(n=16)	
	Median	Range	Median	Range
GST	0.93	0.53–1.49	1.18	0.62–1.98
Se-GPx	0.168 [a]	0.095–0.184	0.128 [b]	0.095–0.186
GR	0.067	0.052–0.082	0.067 [c]	0.041–0.098

GST — Glutathione *S*-transferase; Se-GPx — Selenium-dependent glutathione peroxidase; GR — Glutathione reductase.
n: number of animals.
[a] n=10.
[b] n=11.
[c] n=15.

p<0.01, respectively) (Table 2). Concerning the hepatic values of other metals (copper, zinc, mercury and lead), no statistically differences were observed between study areas. Additionally, for most metals, hepatic levels revealed a wider range in Preguiça when compared with the unpolluted area (Table 2).

3.2. Haematological parameters and enzyme activities

No statistical differences between study areas were reported for any haematological parameter (Table 3), although median values were always higher in shrews from Preguiça.

Hepatic activities of GST, Se-GPx and GR were unaltered in *C. russula* from the mining area, although GST activity revealed a higher median and larger dispersion of values, when compared with reference area (Table 4).

4. Discussion

This study was designed to evaluate, for the first time, the role of *C. russula* as a bioindicator of environmental pollution in an abandoned mining area.

Results showed that in reference shrews, metal levels were similar or lower than those obtained in other insectivores species from unpolluted sites (revision in Talmage and Walton, 1991, Pankakoski et al., 1993, 1994; Mertens et al., 2001). On the contrary, shrews from Preguiça mine, revealed significantly high concentrations of hepatic cadmium and nickel. Cadmium is a non-essential metal that usually accompanies most ores of lead and zinc. Mainly due to anthropogenic activities, this metal has been widely distributed throughout the food chains (Hunter and Johnson, 1982; Andrews et al., 1984; Torres and Johnson, 2001). Considered highly toxic to mammals (*e.g.* Talmage and Walton, 1991), laboratory studies have shown that cadmium exposure induces hepatic necrosis, and also oxidative stress, which might contribute to its hazardous toxicity and carcinogenicity (Karmakar and Chatterjee, 1998). Shrews inhabiting the mining area showed, on average, a 7-fold cadmium increased compared with the low accumulation detected in shrews collected in the reference area (9.29 and 1.18 μg/g dw, respectively). This result points towards a contamination through direct ingestion of soil particles and/or via transfer through the food web (Torres and Johnson, 2001). In liver, ingested cadmium forms very stable complexes with sulfhydryl rich protein, metallothionein, which have a biological half-life of several years (Scheuhammer, 1991; Wlostowski et al., 2000, 2003). These complexes protect host tissues from cadmium damage (Goering and Klaassen, 1983; Klaassen et al., 1999). Generally, in mammals, the critical hepatic cadmium concentration is considered to be 20.0–30.0 μg/g dw (Nogawa et al., 1986), whereas hepatic and renal lesions have been reported in common shrew *Sorex araneus* with cadmium concentrations over 253 μg/g dw (Ma and Talmage, 2001). In Preguiça mine 31% of the individuals of *C. russula* showed cadmium concentrations in liver above the critical level reported by Nogawa et al. (1986).

In contrast to cadmium, information about nickel's hepatic concentration in biota is scarce and inconclusive, particularly for shrews. Previous nickel levels reported in polluted areas by Pankakoski et al. (1993, 1994) in common shrew (0.00–0.64 μg/g dw), pygmy shrew *Sorex minutus* (3.39 μg/g dw), and mole *Talpa europaea* (0.13–0.25 μg/g dw) are consistent with our data. Nickel is a ubiquitous element easily transferred throughout the food chain (Torres and Johnson, 2001; Punshon et al., 2003), although some studies reported a low increase or even a decrease of this metal in small mammals inhabiting nickel contaminated areas (Cloutier et al., 1986; Fendick et al., 1989; Talmage and Walton, 1991; Punshon et al., 2003). Nickel is known to be carcinogen, teratogenic, genotoxic and hepatotoxic (Pandey and Srivastava, 2000; Punshon et al., 2003), and concern should be taken on the accumulation of this element in the body (Kasprzak et al., 2003). Although, data on critical nickel residues in whole body and target organs associated with acute or chronic effects for terrestrial wildlife are lacking (Torres and Johnson, 2001).

No significant differences were observed for the other elements between study areas. However, shrews from Preguiça showed hepatic levels of mercury and lead slightly higher when compared with the reference shrews. This tendency might indicate either present-day reduced levels of these elements in the environment and/or its non-homogeneous distribution (Torres and Johnson, 2001). Lead and mercury levels detected in *C. russula* from Preguiça mine are far below those associate with liver pathologies or metal pollution in wild mammals, considered to be 5–10 μg/g dw for lead (Ma, 1996) and 1.1 μg/g wet weight for mercury (Eisler, 1987).

It is well known that soils from lead/zinc mines contain high amounts of copper and zinc (Andrews et al., 1984; Santos Oliveira et al., 2002), but the bioaccumulation of these two elements was not verified in *C. russula*. As previously reported, the uptake of copper and zinc is correlated with their amount in the gastrointestinal tract (Torres and Johnson, 2001). Nevertheless, their absorption is generally regulated by an effective homeostatic mechanism (*e.g.* induction of metallothionein) and does not correlate with soil contents (Talmage and Walton, 1991; Mertens et al., 2001; Milton et al., 2003).

In this study, the physiological and biochemical responses to long-term metal contamination appeared to be negligible, suggesting that the potential deleterious effects of metal contamination are compensated by additional detoxification pathways. In vertebrates, metal binding metallothioneins are thought to be one of the major routes for metal detoxification. Besides, exposure to increased levels of metals over many generations may impose a selective pressure on *C. russula* populations, resulting in a selection of more metal-tolerant individuals that adapt to low quality environments (Holloman et al., 2000; Viegas-Crespo et al., 2003).

Despite no significant differences were found in the contaminated area, the collected shrews show a greater data dispersion both on haematological values and biochemical parameters. These results suggest an individual response of shrews inhabiting a low quality environment, as earlier reported in Algerian mice (Nunes et al., 2001; Viegas-Crespo et al., 2003). Furthermore, it is assumed that studied antioxidant enzymes, GST, Se-GPx and GR, play an important role in the detoxification of oxidant compounds, including lipoperoxides, which can be partly produced by metal pollutants. However, according to Wlostowski et al. (2000), high concentrations of cadmium in bank vole's diet, produced histophatological changes in the liver and metallothioneins induction, but paradoxically reduced the hepatic levels of lipoperoxides. The authors explain this result based on the observed decreased of hepatic levels of iron and copper in voles. In fact, these elements are essential components of several proteins and enzymes involved in the mithocondrial respiratory chain, possibly leading to some disturbances in the ATP production (the main endogenous source of free radicals) and consequently to lipid peroxidation. So, a similar response may explain the non-significant changes in antioxidant enzyme activities in cadmium contaminated shrews from the Preguiça mining area.

5. Conclusions

The results of the present study have illustrated the relevance of *C. russula* as a bioindicator species in environmental quality assessment. Besides, abandoned mines, such as Preguiça mine, may constitute unpredictable long-term sources of heavy metal contamination. Considering the position of shrews in food webs, we can speculate about the accumulation of heavy metals in higher trophic levels and assume an important biomagnification scenario of potentially toxic elements. So, it cannot be disregarded the potential health risk of old mines, even for humans, and the need of controlling their negative environmental impact.

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Bioaccumulation of metals and effects of a landfill in small mammals. Part II. The wood mouse, *Apodemus sylvaticus*

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Abstract

We assess the bioaccumulation of metals (Pb, Hg, Cd, Fe, Mg, Zn, Cu, Mn, Mo, Cr) and effects of landfill leachates on morphological (RI, relative weights), plasma (GPT, GOT, creatinine), and genotoxic (MNT) parameters in wood mice, *Apodemus sylvaticus*, inhabiting close the Garraf landfill site (NE Spain). Due to the high age- and sex-dependent variation in wild populations, we also studied the effect of these biotic factors on the parameters studied. Wood mice from the landfill site, sited in a partially protected area, showed more Cd, Fe, Zn, Cu, Mn, Mo, and Cr than specimens from the reference site. Moreover, mice near the landfill registered low RI and high relative renal weight, GPT, and MN frequency, which indicate that the landfill affects the health of wild mice. In contrast to sympatric shrews from a previous study, wood mice showed lower bioaccumulation of metals and lower variation caused by biotic factors. Moreover, the morphological and physiological alterations demonstrated that they were also more sensitive at environmental pollution. Given the contribution of small mammals to ecosystem function and the scarce ecotoxicological data on the effects of landfill pollution on wild terrestrial mammals, we consider that our study can be used to improve the management of this protected area.

Keywords: *Apodemus sylvaticus*; Landfill; Heavy metals; Micronucleus test; Plasma parameters

1. Introduction

The wood mouse, *Apodemus sylvaticus* (Linnaeus, 1758) is widespread in Europe, Asia minor and north Africa and is used as bioindicator of bioaccumulation and/or effects of heavy metals (see references in Talmage and Walton, 1991; Gorriz, 1996; Sheffield et al., 2001; Rogival et al., 2007). However, information on the suitability of this species as monitor of a number of biomarkers and of trace element bioaccumulation is fragmentary or lacking.

Landfills accumulate great amounts of waste and are common in some regions including Mediterranean countries (Loukidou and Zouboulis, 2001). These sites are also an important source of liquid effluents, named leachates, which, because of their high toxicity for biota, can have an adverse impact on the environment when released in an uncontrolled manner (Cheung et al., 1993; Bakare et al., 2005; Li et al., 2006). Indeed, highly stable contaminants, such as heavy metals, are common constituents of landfill effluents. However, in spite of this evident risk posed by these sites, only a few studies have examined the bioaccumulation of metals and the toxic effects of hazardous waste and landfill leachates in small mammals (Sheffield et al., 2001; Torres et al., 2006; Sánchez-Chardi and Nadal, 2007).

A current challenge for ecotoxicologists is to identify biomarkers for the best possible diagnostic. Over the last

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decade, the value of morphological findings for the field assessment of environmental pollution has been recognized (e.g. Liro, 1985; Tersago et al., 2004; Pereira et al., 2006; Rogival et al., 2007; Sánchez-Chardi and Nadal, 2007). Although morphological biomarkers are not usually indicative of a specific contaminant, they are sensitive for monitoring the impact of anthropogenic activities and can provide useful data for the identification of the deleterious consequences on populations inhabiting polluted environments (see references in Sheffield et al., 2001). In addition, several plasma parameters are indicative of tissue and cell alterations and are routinely used in the diagnosis of mammal pathologies (Gorriz et al., 1996; Silva et al., 1999; Świergosz-Kowalewska et al., 2006). The micronucleus test (MNT) is also an easy and non-lethal method to assess the genotoxic effects of environmental pollutants on natural populations (Ieradi et al., 1996; Meier et al., 1999; Zúñiga-González et al., 2000; Seoane and Dulout, 2001). The background levels of these morphological, plasma, and genotoxicity parameters can be altered by exposure to metals (Eisler, 1985, 1986; Meier et al., 1999; Palus et al., 2003).

The present study aims (i) to determine the bioaccumulation of heavy metals in *A. sylvaticus* as a result of exposure to leachates from the Garraf landfill; (ii) to assess the effects of these effluents on morphological, plasma, and genotoxic parameters; (iii) to identify the influence of sex and age on intra-population variation of these biomarkers; (iv) to evaluate the use of this species as bioindicator in a region of Mediterranean climate, particularly in protected areas; and (v) to compare results on this rodent with those from sympatric shrews.

2. Material and methods

2.1. Study sites

Initially an illegal waste disposal site, the Garraf landfill was more or less adapted to receive large amounts of domestic and industrial wastes from the metropolitan area of Barcelona. When it closed in 2006, this landfill was largest of its kind in Spain, having accumulated a total of 25 millions tonnes of waste. Located in a karstic area that was granted partial protection status in 1986 and inhabited by several endangered and protected species of mammals, birds, and amphibians, this landfill is a pollutant of ground and surface waters.

From February to April 1998, we collected 52 wood mice by means of Sherman traps in two areas. The "Vall d'En Joan" capture site (41°17′30″N, 1°57′0″E), herein referred to as the polluted site, is downstream of the landfill and affected by leachates while the "Olesa de Bonesvalls" site (41°20′29″N, 1°51′5″E) was considered a reference site. The total capture effort was 1550 traps night^{-1} (TN). Specimens were killed by cervical dislocation and liver and kidneys were immediately removed, weighed, and frozen at −20 °C prior to chemical analyses. Sex was determined during the dissection and relative age of mice was established on the basis of the degree of tooth wear (Felten, 1952).

2.2. Chemical analyses

A piece of liver and right kidney of all specimens were dried till constant weight and digested by nitric and perchloric acids (Instra, Baker-Analized) in open tubes in a Prolabo Microdigest A301 microwave placed in a clean room (see Sánchez-Chardi and Nadal, 2007). Mg and Fe concentration were determined by a Perkin-Elmer OPTIMA-3200RL Inductively Coupled Plasma Optical Spectrometer (ICP-OES), and Pb, Hg, Cd, Zn, Cu, Mn, Mo, and Cr were measured by a Perkin-Elmer ELAN-6000 Inductively Coupled Plasma Mass Spectrometer (ICP-MS) as described in Sánchez-Chardi and Nadal (2007) and Sánchez-Chardi et al. (2007). Metal concentrations were calculated as mean ± standard error (M ± SEM) in μg g^{-1} on a dry weight (DW) basis. Moreover, for statistical purposes, the values below the detection limit were assigned as half of this limit.

2.3. Morphological parameters

For all specimens, we measured body weight (BW) to the nearest 0.01 g and body length (BL) to the nearest 0.01 mm. Body condition indices, such as the residual index (RI), are a measure of energy reserves. The residual index (RI) is a linear regression of BL and BW (Jakob et al., 1996; Sánchez-Chardi and Nadal, 2007). Specimens with positive RI values are considered to be in better condition than predicted for their weight and length. Consequently, negative values, down to the linear regression, are attributed to animals with lower body condition than expected. Liver and kidneys were weighed to the nearest 0.001 g on a wet weight basis. The relative hepatic and renal weight ratios were calculated as a percent ratio of somatic tissue (100 × tissue wet weight/body wet weight).

2.4. Plasma parameters and genotoxicity test (MNT)

Peripheral blood was collected from the periorbital eye sinus into a heparinized microhaematocrit tube and placed on Eppendorf tubes. An aliquot was centrifuged at 4000 rpm for 5 min and the supernatant was collected to measure plasma parameters. Commercial kits (Menarini) for glutamic oxalacetic transaminase (GOT), glutamic pyruvic transaminase (GPT), and creatinine quantification were used to determine these parameters in a Spotchem automatic analyzer (Menarini). These compounds may be indicative of organ-specific physiological disorders. Transaminases are intracellular enzymes whose large presence in blood indicates liver damage, whereas an increase in plasma creatinine is a biomarker of renal injury. GOT and GPT were expressed as M ± SEM in U l^{-1}, while creatinine was expressed as M ± SEM in mg dl^{-1}.

With another blood aliquot, duplicate smears were made for each specimen on pre-cleaned microscope slides. These were fixed with heat and stained with conventional MayGrünwald-Giemsa stain. For each individual, MN frequency was scored on 2000 blood erythrocytes through an oil immersion objective (×100) on a Leica Leitz DMRB microscope. Plasma parameters and MN frequencies were expressed as M ± SEM.

2.5. Statistical analyses

Parameters with continuous distribution were log-transformed and tested for normal distribution (Shapiro–Wilk test) and for homogeneity of variance (Levene, F-test). From each tissue, an overall measure of the effects of site, age, and sex on metal concentration was obtained by three-way multivariate analysis of variance (MANOVA). Intra- and inter-population comparisons of metal bioaccumulation were evaluated by Student's tests (t). The inter-site divergences in RI, relative somatic ratios, and plasma parameters were also calculated by this test. RI was calculated for the whole population. Since we did not find significant differences by sex, results for males and females were pooled in order to increase number of data. Therefore, morphological parameters were compared by site and age, owing to the asymmetric percentage of juveniles and adults in the two capture sites. Variation in MN frequency was assessed by the Mann–Whitney test (U). Pearson's correlation coefficients (r) were calculated in liver and kidneys separately, to establish the relations between metal concentrations and plasma parameters, whereas the correlation between metal concentration and MN frequency was evaluated by Spearman's correlation coefficients (r). Significant differences were accepted at $p < 0.05$. For all sequential tests, p-values were corrected by the Bonferroni adjustment (Rice, 1989). All statistical procedures were performed with SPSS package (version 11.5 for Windows, SPSS Inc.).

3. Results

The results of captures by site, sex and age were reported in Table 1. Capture effort at the polluted site was 350 TN for 28 mice (Capture efficiency: 8%), while for the reference site it was 1200 TN for 24 specimens (Capture efficiency: 2%). Although no significant differences were found in RI by site (Reference site: 0.701 ± 0.558; Landfill site: −0.601 ± 0.679), a tendency to decrease in BW and BL and increase in relative liver weight as well as an increase in relative kidneys weight, significant in adults ($t = 3.015$, $p = 0.005$), was detected in mice from the polluted site (Table 2).

Table 1
Number of animals captured in the reference and landfill site by sex and age

Site	Sex	Age		Total
		Juveniles	Adults	
Reference	Males	2	12	14
	Females	2	8	10
Landfill	Males	7	11	18
	Females	6	4	10

MANOVA of metal variation in liver showed the importance of parameters to be site ($F = 7.809$, $p < 0.001$) > age ($F = 1.487$, $p = 0.188$) > sex ($F = 1.150$; $p = 0.357$), while kidneys showed a similar pattern: site ($F = 4.458$, $p < 0.001$) > age ($F = 0.962$, $p = 0.492$) > sex ($F = 0.626$; $p = 0.781$). Significant increases in Fe, Cd, Zn, Cu, Mn, Mo and Cr were found in mice from the polluted site when compared with reference specimens. Moreover, Pb and Mg also tended to be greater in the former (Table 3).

Pb, Mg, Fe, Cu, Mn, and Cr concentrations varied with age (Fig. 1). In adult mice from the polluted site, Pb and Mg decreased in livers ($t = 0.022$, $p = 0.022$; $t = 2.389$, $p = 0.025$, respectively), whereas Fe and Cu decreased in kidneys ($t = 2.256$, $p = 0.033$; $t = 2.504$, $p = 0.019$, respectively). Although wood mice from the reference site showed similar patterns of age-dependent variation for Pb, Mg, Fe and Cu, significant differences were found only for Pb levels in liver ($t = 3.476$, $p = 0.002$). Moreover, Mn and Cr tended to decrease in the livers and kidneys of mice from both sites. No sex-dependent variation was detected for any metal in either organ.

Wood mice showed similar tissue distribution of metals in the two study sites. Liver showed significantly higher bioaccumulation of Fe (Landfill: $t = 4.131$, $p < 0.001$), Zn (Landfill: $t = 5.841$, $p < 0.001$; Reference: $t = 2.414$, $p = 0.020$), Cu (Landfill: $t = 2.197$, $p = 0.032$) Mn

Table 2
Mean ± SEM of morphological parameters in juvenile and adult wood mice from the reference and landfill site

	Juveniles		Adults	
	Reference site ($n = 4$)	Landfill site ($n = 13$)	Reference site ($n = 20$)	Landfill site ($n = 15$)
BW (g)	17.95 ± 0.36	14.86 ± 0.83	23.53 ± 0.75	22.55 ± 0.95
BL (mm)	86.41 ± 0.82	80.05 ± 1.62	93.23 ± 1.30	92.47 ± 2.05
Liver (g)	0.890 ± 0.023	0.862 ± 0.058	1.222 ± 0.043	1.137 ± 0.156
% Liver	4.96 ± 0.99	5.97 ± 0.48	5.21 ± 0.13	6.20 ± 0.78
Kidneys (g)	0.218 ± 0.006	0.189 ± 0.018	0.216 ± 0.010	0.258 ± 0.016
% Kidneys	1.21 ± 0.02	1.38 ± 0.22	0.93 ± 0.04	1.15 ± 0.07**

*$p \leqslant 0.05$; **$p \leqslant 0.01$; ***$p \leqslant 0.001$.

Table 3
Mean ± SEM values for metals in *A. sylvaticus* by tissue and site (in μg g^{-1} DW)

	Liver				Kidneys			
	Reference site ($n = 23$)	Landfill site ($n = 27$)	t	p	Reference site ($n = 23$)	Landfill site ($n = 28$)	t	p
Fe	619.23 ± 41.50	874.74 ± 66.64	3.136	0.003	513.72 ± 21.09	589.11 ± 17.55	2.922	0.005
Mg	1349.83 ± 33.68	1428.49 ± 38.35	–	–	1418.85 ± 36.10	1525.62 ± 36.06	–	–
Pb	0.41 ± 0.05	0.68 ± 0.11	–	–	0.73 ± 0.08	1.10 ± 0.18	–	–
Cd	0.30 ± 0.08	0.44 ± 0.15	2.308	0.025	0.92 ± 0.24	1.44 ± 0.48	–	–
Zn	162.07 ± 5.73	200.49 ± 13.26	2.709	0.009	142.76 ± 4.99	135.88 ± 3.13	–	–
Cu	20.68 ± 1.36	39.16 ± 10.36	2.841	0.007	20.66 ± 0.86	23.45 ± 0.67	2.536	0.014
Mn	7.95 ± 0.95	10.23 ± 1.80	2.495	0.016	4.87 ± 0.40	6.59 ± 1.00	–	–
Mo	3.16 ± 0.24	4.38 ± 0.16	3.987	<0.001	1.65 ± 0.11	1.92 ± 0.20	–	–
Cr	0.71 ± 0.05	1.32 ± 0.10	6.163	<0.001	n.d.	3.61 ± 0.32	7.570	< 0.001

n.d.: non detected values.

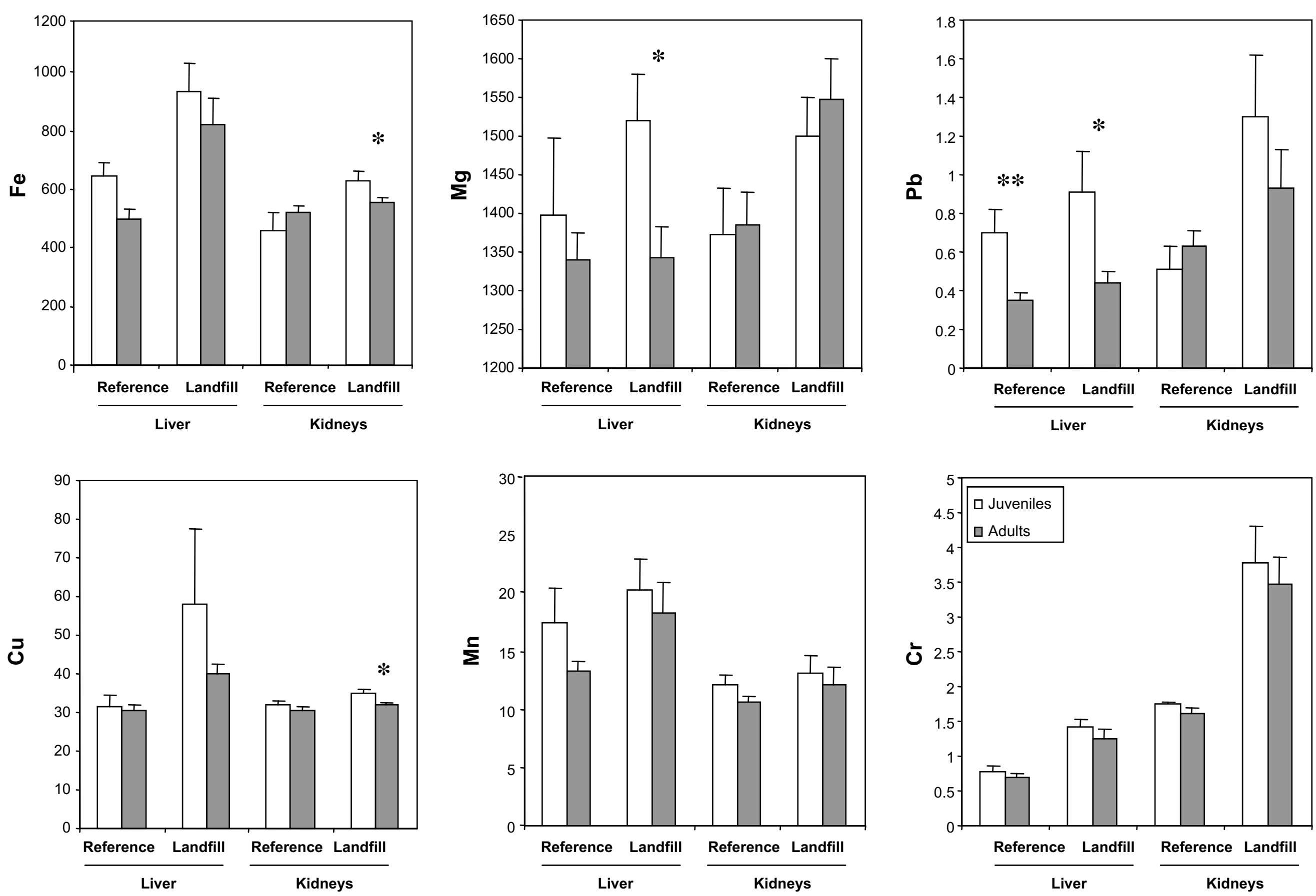

Fig. 1. Age-related differences of Fe, Mg, Pb, Cu, Mn, and Cr in *A. sylvaticus* by tissue and site. Metal concentrations in M ± SEM in μg g^{-1} DW. ($^{*}p \leqslant 0.05$; $^{**}p \leqslant 0.01$; $^{***}p \leqslant 0.001$.)

(Landfill: $t = 3.713$, $p < 0.001$; Reference: $t = 2.817$, $p = 0.007$), and Mo (Landfill: $t = 9.591$, $p < 0.001$; Reference: $t = 2.817$, $p < 0.001$) than kidneys. In contrast, renal tissue had significantly higher levels of Pb (Landfill: $t = -2.763$, $p = 0.008$; Reference: $t = -3.074$, $p = 0.004$), Cd (Landfill: $t = -2.740$, $p = 0.008$; Reference: $t = -3.035$, $p = 0.004$), and Cr (Landfill: $t = -8.897$, $p < 0.001$; Reference: $t = -9.757$, $p < 0.001$).

The levels of GPT in plasma were significantly higher in specimens from the polluted site compared with reference specimens ($t = 2.068$, $p = 0.047$). Moreover, GOT values tended to be increased in mice from the polluted site (Table 4). No inter-site variation was observed for creatinine. In addition, significantly higher mean MN frequencies were observed in mice from landfill site than those from the reference site ($U = 21.000$, $p < 0.001$). Neither age- or sex-dependent variation was observed in plasma parameters or in MN frequency. While no correlations between metal concentrations and plasma parameters were found, significant correlations were detected between MN frequency and Fe, Cd, Mo and Cr concentrations in liver and kidneys (Table 5).

Table 4
Mean ± SEM values for plasma parameters (GPT, GOT and Creatinine) and micronucleus frequency in *A. sylvaticus* by site

	Reference site	Landfill site
	($n = 16$)	($n = 19$)
GPT ($U\ l^{-1}$)	99.25 ± 11.61	147.37 ± 20.21**
GOT ($U\ l^{-1}$)	63.56 ± 9.12	93.58 ± 24.33
Creatinine ($mg\ dl^{-1}$)	1.62 ± 0.18	1.96 ± 0.28
MN frequency (‰)	0.281 ± 0.079	1.182 ± 0.110*

* $p < 0.001$.
** $p = 0.047$.

Table 5
Spearman coefficients and *p*-values between elements and MNT in liver and kidneys of *A. sylvaticus*

	Liver		Kidneys	
	r	*p*	*r*	*p*
Fe	0.359	0.034	0.309	0.027
Cd	0.266	–	0.388	0.021
Mo	0.478	0.004	−0.152	–
Cr	0.482	0.003	0.568	<0.001

4. Discussion

Landfill sites for the disposal of solid waste continue to be common in many countries. The accumulated waste produces large amounts of leachates, complex mixtures that comprise several compounds at potentially toxic levels. Landfill management practices include the analyses of these effluents; however, this chemical characterization alone does not provide sufficient information on their environmental risk (Cheung et al., 1993) because absolute metal concentrations do not reflect the degree to which they affect biota. Here we examined the bioaccumulation of metals and the effects of landfill pollution in individuals and populations of wood mice.

4.1. Metal bioaccumulation by site

The concentrations of metals in wood mice from the reference site were consistent with those reported for the same species and other rodents inhabiting non-polluted sites (revisions in Talmage and Walton, 1991; Gorriz, 1996; Sheffield et al., 2001; Beernaert et al., 2007; Rogival et al., 2007). Pb and Cd are non-essential elements that are widely distributed throughout ecosystems and they affect a wide range of physiological functions including nervous, excretory, reproductive, and haematopoietic systems. The bioaccumulation of Pb and Cd in the liver and kidneys of small mammals in polluted areas, including landfill sites, has been reported (Torres et al., 2006; Sánchez-Chardi and Nadal, 2007). These observations indicate that the food chain transfer of these metals is of some concern. However, despite the increases detected in wood mice, these animals showed higher levels of the latter, which could attributed to the bioavailability of this labile element to small mammals even in slightly alkaline soils (Mertens et al., 2001) such as the Garraf site. Mercury accumulation was insignificant, which is consistent with the low mercury levels in leachates and in tissues of sympatric greater white-toothed shrew, *Crocidura russula* (Sánchez-Chardi and Nadal, 2007).

Essential elements (Fe, Zn, Cu, Cr, Mn, and Mo) are usually present in leachates and are often organically complexed, becoming highly available through food chains (e.g. De Rosa et al., 1996; Kaschl et al., 2002; Sánchez-Chardi and Nadal, 2007). However, changes in metal bioavailability and turnover may increase the concentrations of these elements in tissue and this increase may be related, at least in part, to detoxification functions. In fact, increases in the accumulation of Fe, Zn, Cu, and Mn in mice from the landfill site were moderate and did not appear to indicate a disruption of homeostatic control of these metals. High Fe concentrations reduce the toxic effects of pollutants such as Cd, by preventing lipid peroxidation or by participating in detoxification systems such as transferrin/ferritin. Zn and Cu have critical functions in antioxidant systems as these metals block the formation of reactive oxygen species as well as some metallothioneins (Włostowski et al., 2003; Pereira et al., 2006). Mn is also involved in antioxidant functions such as a cofactor of superoxide dismutase. Like *C. russula* in the Garraf landfill (Sánchez-Chardi and Nadal, 2007), *A. sylvaticus* showed high concentrations of Cr in tissues, demonstrating that this metal is highly transferred from leachates to mammals. In contrast, the shrews captured at the same landfill site did not show significant increases in Fe, Mn, or Mo (Sánchez-Chardi and Nadal, 2007). The differences between wood mice and shrews can be attributed to interspecies differences in exposure, uptake, and/or excretion of these elements.

4.2. Metal bioaccumulation by age, sex and tissue

Data on the effect of biotic factors, such as age and sex, on metal bioaccumulation in the wood mouse are scarce. Those available do not show clear patterns as the effects of these factors vary greatly between populations. We observed a general decrease in concentrations of essential (Fe, Mg, Mn, Cu, and Cr) and non-essential elements (Pb) with age. This finding is consistent with results for several metals in the same species (Lopes et al., 2002; Beernaert et al., 2007) and in other mammals (Scheirs et al., 2006; Outridge and Scheuhammer, 1993; Sánchez-Chardi and Nadal, 2007). Concentrations of Fe, Mg, and Cu in juvenile mice may be related to high intake and incorporation during this growing period and also to a decrease in intestinal absorption of certain metals such as Cr in adults. Moreover, greater energy requirements of juveniles, implying high uptake of food, may explain increased Pb values observed in the tissues of these specimens at the polluted site.

Although several authors have reported sex-dependent variation of metal concentrations in wood mice (e.g. Lopes et al., 2002; Scheirs et al., 2006; Beernaert et al., 2007), we did not detect these differences. This discordant result may be attributed mainly to inter-population variation caused by differences in exposure and uptake of elements.

In spite of the discordance of information on metal bioaccumulation in the wood mice, the distribution of Cd, Fe, Pb, Hg, Cd, Zn, Cu, Mn, and Cr observed in our study is consistent with previous data on this species (Talmage and Walton, 1991; Gorriz, 1996; Bargagli et al., 1997; Sheffield et al., 2001; González et al., 2006; Torres et al., 2006; Beernaert et al., 2007; Rogival et al., 2007; Scheirs et al., 2006; Wijnhoven et al., 2007). Although both Mg and Mo are important in ecotoxicology (they may reach toxic concentrations and their deficiency can be critical) no data are available on these metals in wood mice. Our data also corroborate findings on Mg and Mo in other small mammals (e.g. Pankakoski et al., 1993; Pereira et al., 2006; Sánchez-Chardi et al., 2007; Sánchez-Chardi and Nadal, 2007).

4.3. Captures and morphological parameters

The distribution of captures of wood mice by age and sex is consistent with data reported for sympatric *C. russula* (Sánchez-Chardi and Nadal, 2007). At the end of the winter, adults are the most abundant age group in populations of wood mice. Like shrews, more males were captured in this study because at the start of the breeding period (February–March) they are more active on the surface.

More wood mice were captured at the polluted site than at the reference. Our observation is consistent with reports on other rodent species and contrasts with the decrease in captures of shrews (e.g. Schroder and Hulse, 1979; Gabrey, 1997; Sánchez-Chardi and Nadal, 2007). The results indicate deep changes in the structure of the small mammal community in response to intense disturbance such as this extensive waste disposal site. These community changes may arise as a result of the competitive selection of the most tolerant species. In fact, several rodent species are opportunistic and waste provides protection from predators. In addiction, the organic wastes increase availability of food and leachates load soils with nitrogen and phosphorus compounds, thereby increasing vegetation and nutrients for herbivores at these sites.

Our results on morphological parameters are consistent with those reported for the same species (Liro, 1985; Tersago et al., 2004). Specimens from the reference site showed a better health status than mice from the polluted site, reflected by high BW and BL and positive RI values, up to the linear regression. This tendency is concordant with results obtained in relative renal weight, which was greater in specimens from the latter site. This increase may be indicative of renal edema (Simmons et al., 1994; Ma and Talmage, 2001) caused by exposure to elements or compounds at toxic levels.

4.4. Plasma parameters and MN frequency

The values of plasma parameters and MN frequency in specimens from the reference site are consistent with data on *A. sylvaticus* and also on other wild and laboratory small mammals (Ieradi et al., 1996; Silva et al., 1999; Sánchez-Chardi and Nadal, 2007). In liver and kidneys, cell damage followed by a release of cytoplasmic enzymes into blood and by an increase in these compounds in plasma provides the basis for clinical diagnosis (Silva et al., 1999). The low creatinine values in mice from the polluted site indicate that renal function is not greatly affected by landfill pollutants. However, the increase observed in transaminases may be indicative of hepatic damage caused by the inflammatory response to pollutants (Peakall, 1992; Simmons et al., 1994; Gorriz et al., 1996). However, given that other factors, such as parasitosis, can also increase transaminases in wild mammals, the degree of hepatic injury in response to environmental pollutants can be established only through a histopathological study of specimens (Sánchez-Chardi, unpublished data). Taken together, our data show that exposure of wood mice to landfill pollution leads to liver injury. In contrast, kidneys reach a degree of tolerance or adaptation at exposure of landfill pollution by excretion and/or detoxification mechanisms and, therefore, the deleterious effects of xenobiotics are prevented. The absence of correlation between metal concentrations and plasma parameters point to the influence of other xenobiotics from leachates. In fact, wild populations of mammals are often exposed to a wide range of potentially toxic compounds and landfill pollution is not an exception. Several hydrocarbons and N-, Cl- and P-containing compounds such as CN-, pesticides, phenols, phthalates or polyvinyl aromatic hydrocarbons, all of which are usually present in leachates may reach toxic levels.

Wild populations of rodents are suitable bioindicators of genotoxic damage (e.g. Sheffield et al., 2001; Hamers et al., 2006; Scheirs et al., 2006). In agreement with our results, Ieradi et al. (1996) and Sánchez-Chardi and Nadal (2007) report strong correlations between several heavy metals and MN frequency in erythrocytes of house mice, *Mus domesticus*, and *C. russula*. These correlations seem corroborate that these elements contribute to clastogenic effects in wild mammals (revision in Eisler, 1985, 1986; Seoane and Dulout, 2001; Palus et al., 2003).

4.5. Toxicity effects of metals bioaccumulated

When heavy metals are released into the environment they can produce toxic effects in biota. Information about these effects in wild mammals is generally very limited but may be relevant to predict environmental risk (Hamers et al., 2006). Moreover, the degree of extrapolation of biochemical biomarkers to harm at the individual or population level varies greatly among parameters and species. Consequently, it is difficult to give an absolute definition of a body concentration range of metals that reflects "normal" conditions. In the present study, the concentrations of the most toxic metals were below the LOAEL, lowest-observed-adverse-effect-level (Ma and Talmage, 2001; Sheffield et al., 2001). Moreover, upon capture, none of mice showed any external or internal sign of alteration caused by metal toxicity. However, sublethal effects at "real world" conditions of environmental pollution such as those observed in morphological, plasma, and genotoxic parameters in the present study, can be considered. These effects of chronic exposure to pollution may produce very important alterations in population dynamics such as a decrease of life expectancy, greater vulnerability to predators and/or dysfunctions in reproduction.

Moreover, leachates often contain a wide variety of organic compounds (Simmons et al., 1994) that may also contribute to altering physiology as observed in wood mice from the Garraf landfill. These potentially toxic compounds and synergic metal–metal and/or metal–organic compounds interactions cannot be overlooked as they may constitute an environmental risk and explain, at least partially, the toxic effects observed.

4.6. Pollution in protected areas

The environmental quality of zones that have partial or complete protection status is often high. These areas are usually subjected to low disturbance regimes and are inhabited by protected and endangered species. However, long-term pollutant activities such as landfilling, may disturb or destroy ecosystems, thereby making them less suitable for wildlife. In fact, these waste disposal sites may produce an increase in the levels and bioavailability of toxic compounds, such as heavy metals, which stress populations during multiple generations as a result of extended bioavailability. Therefore, continuous biomonitoring of pollutants by means of selected species is required. In this context, our results corroborate other studies that reported the wood mouse to an effective bioindicator of non-essential metals and the effects of environmental pollution (Talmage and Walton, 1991; Bargagli et al., 1997; Sheffield et al., 2001; González et al., 2006; Scheirs et al., 2006; Wijnhoven et al., 2007; Rogival et al., 2007).

Due to the deep environmental impact of the Garraf landfill, a comparison between two species, the insectivorous *C. russula* and the rodent *A. sylvaticus*, two abundant and widely distributed small mammal species, provides useful information about the effects of the landfill on terrestrial food chains. In the case of metals, inter-specific differences may provide important ecotoxicological information about exposure, bioaccumulation, excretion and tolerance pathways between low (omnivorous mice) and high (insectivorous shrew) trophic levels. Small mammals may differ dramatically in tissues and body contents of a heavy metal in the absence of differences in external bioavailability mainly by dietary uptake and ingestion of contaminated sediments. Carnivores are usually more exposed to metals and accumulate more of these elements than omnivores and herbivores (revision in Talmage and Walton, 1991; Ma and Talmage, 2001; Hamers et al., 2006). This observation is significant for ecotoxicological studies because the metals transferred from lower to higher trophic levels may enter the human food chain. Moreover, rodents are usually less tolerant to metal pollution and show toxic effects at lower metal concentrations than insectivorous species (Talmage and Walton, 1991; Ma and Talmage, 2001; Sheffield et al., 2001). However, these comparisons were usually made between rodents and Soricine species of shrews and scarce comparative information is available on Crocidurine species. Our results showed a similar pattern of metals bioaccumulated in *Apodemus* (present study) and *Crocidura* (Sánchez-Chardi and Nadal, 2007) to that reported recently by Wijnhoven et al. (2007). However, the Crocidurine shrews accumulate higher concentrations of more toxic elements, especially non-essential metals, while wood mice are less tolerant to pollution. This finding is consistent with previous data obtained between rodents and insectivores.

5. Conclusions

A. sylvaticus is suitable species to use as bioindicator of landfill pollution, demonstrating variation of metal levels in their tissues and morphological, plasma and genotoxic effects. The alterations in these parameters demonstrate the impact of the Garraf landfill on an omnivorous species inhabiting this area. In comparison with a sympatric species of insectivorous mammal, wood mice show lower concentrations of potentially toxic metals and less tolerance to the toxic effects of leachate exposure.

Biomonitoring pollution through wild animals is crucial for the assessment of environmental quality and to improve our understanding of the response capacity of natural populations to pollution. The requirement for this systematic control is greater in protected areas inhabited by endangered species.

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