Universidade de Aveiro Departamento de Biologia
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Avaliação ecotoxicológica integrada da área adjacente a uma mina de urânio abandonada

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tese apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Doutor em Biologia, realizada sob a orientação científica do Doutor Fernando José Mendes Gonçalves, Professor Associado com Agregação do Departamento de Biologia da Universidade de Aveiro

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…à minha “pequena” família: Maria João, Bruno e a todas as nossas vitórias...

o júri

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palavras-chave

minas de urânio, contaminação ambiental, compartimento aquático, compartimento terrestre, ensaios de toxicidade, análise de risco ecológico

resumo

A frequente e descoordenada acção antropogénica sobre o ambiente origina elevados graus de contaminação e produz alterações por vezes irreversíveis nos ecossistemas. A indústria e a actividade mineira são os exemplos mais frequentes desta questão. Reconhecido o problema ambiental, é urgente criar medidas de intervenção e de mitigação de modo a minimizar os impactos. A Análise de Risco Ecológico (ARE) é a metodologia recomendada para estes locais para avaliar os riscos e indicar medidas de intervenção. A mina de urânio da Cunha Baixa (Mangualde) foi identificada como uma das cerca de 60 áreas contaminadas requerendo intervenção prioritária, na medida em que apresenta riscos elevados para a vida humana e selvagem. A reduzida informação química e, sobretudo, biológica sobre estes locais torna difícil uma intervenção adequada. Deste modo, o principal objectivo desta tese foi gerar informação ecotoxicológica sobre a área adjacente à mina de urânio da Cunha Baixa. Na perspectiva da ARE esta informação enquadra-se nas etapas de 1 a 3 da análise de risco, visando reduzir incertezas para o processo de avaliação de riscos subsequente. O presente trabalho representa uma abordagem integrada aos compartimentos aquático (3 lagoas artificiais) e terrestre (solos adjacentes, aglomerados de escória e lamas de decantação), através da realização de baterias de bioensaios ecotoxicológicos (laboratoriais e *in situ*). Numa primeira fase, foi encetada a caracterização do compartimento aquático. Do ponto de vista físico-químico, o sistema aquático revelou altos teores em metais e baixo pH, na água e sedimentos. Todavia, a falta de concordância entre os limites legais estabelecidos para a maioria dos elementos (incluindo urânio) complica a avaliação dos riscos com base em dados físico-químicos. Os bioensaios agudos e crónicos, realizados com *Daphnia* spp. e *Pseudokirchneriella subcapitata* à coluna de água, revelaram efeitos agudos do efluente mineiro que aflora numa das lagoas (com pH≈4 e alto teor em metais). A reduzida toxicidade sedimentar, avaliada directamente no sedimento e indirectamente sob a forma de elutriado, sugere que os sedimentos funcionam sobretudo como barreira aos contaminantes. Numa segunda fase, foi efectuado o rastreio físico-químico e ecotoxicológico do compartimento terrestre, quer directamente no solo, quer indirectamente em elutriados produzidos a partir de amostras de solo. Também no solo foram registados valores preocupantes de metais, mais ou menos concordantes com os locais que revelaram toxicidade em ensaios de evitamento com *Eisenia andrei*. Aliás, estes últimos provaram ser ferramentas muito sensíveis à contaminação, tendo revelado toxicidade em alguns locais que a caracterização química classificaria como não perigosos. A última etapa do presente trabalho consistiu num ensaio *in situ* com *Eisenia andrei*, onde foram observados fenómenos de stress oxidativo e bioacumulação de metais, aquando da exposição a alguns dos locais. Em suma, este trabalho veio contribuir com informação biológica e química que minimiza incertezas na avaliação da contaminação da área adjacente à mina da Cunha Baixa.

keywords Uranium mine, environmental contamination, aquatic compartment, terrestrial compartment, toxicity assays, ecological risk assessment

abstract

Frequent and uncoordinated anthropogenic actions produce high levels of environmental contamination and induce environmental change (sometimes irreversibly). Industrial and mining activities are the most frequent examples of man-induced change. After identifying the environmental problem, it is urgent to propose a mitigation plan for minimising impacts. Environmental Risk Assessment (ERA) is recommended as the most suitable tool to assess the environmental risks and to propose intervention measures. The Cunha Baixa uranium mine (Mangualde, central Portugal) has been identified as one of the ca. 60 contaminated areas requiring urgent intervention, as a consequence of high risks to humans and wildlife. The reduced background information of the area, particularly concerning local biota, impairs adequate intervention measures. Bearing this in mind, we proposed to generate ecotoxicological information for the area adjacent to the abandoned uranium mine. From the ERA perspective, this information can be incorporated in tiers 1-3, aiming at reducing uncertainty in the subsequent risk evaluation process. Thus, the present work represents an integrated approach to the aquatic and terrestrial (adjacent soils, mine tailings and sludge) compartments. This included both chemical and ecotoxicological characterisation, with the latter integrating biochemical, individual and population parameters, as well as laboratorial and *in situ* approaches. The first stage of the study was dedicated to the aquatic compartment of the Cunha Baixa mine, which consists of two artificial ponds that resulted from ore exploitation and a third one that works as a sedimentation basin. From the physical and chemical point of view, high levels of metals and low pH were found in the water column and sediments. However, the evaluation of risks solely based on chemical data is impaired by the lack of uniform (if any) threshold values for most elements (including uranium). Acute and chronic bioassays conducted with *Daphnia* spp. and *Pseudokirchneriella subcapitata* revealed acute effects of the aquatic effluent in one of the ponds. Sediment toxicity, which was evaluated in whole-sediment (direct) and elutriate (indirect) exposures, was found to be inexistent, suggesting that sediments mostly work as a barrier to contaminants. In the second stage of the study, the terrestrial compartment was targeted for chemical and ecotoxicological screening. Alarming concentrations of metals were also recorded in soils from some sites, more or less concordantly with those where avoidance behaviour of *Eisenia andrei* was observed. In fact, the latter proved to be sensible tools to environmental contamination, categorising some soil samples as toxic where chemical analysis did not. The last stage of this work consisted of an *in situ* assay with *Eisenia andrei*, which revealed oxidative stress and bioaccumulation of metals of earthworms exposed to contaminated media (soil and water), in some sites. Overall, the present study produced chemical and ecotoxicological information for the study area, giving a significant contribution towards the reduction of uncertainty for the ERA of the Cunha Baixa mining area.

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INTRODUÇÃO

Actualmente, é reconhecido que o Ambiente tem sido negligenciado por inúmeras actividades antropogénicas. A indústria, a exploração de minérios (minas) e a agricultura estão identificados como os principais focos de poluição, em consequência de todos os produtos resultantes das suas actividades. As alterações físicas, químicas e estéticas provocadas no ambiente levam a uma redução significativa da biodiversidade desses locais por vezes irreversível (Trontelj e Ponikvar-Zorko, 1998; Bengtsson et al., 2000; Gongalsky, 2003).

A contaminação ambiental é uma preocupação global e que está amplamente identificada pelas entidades responsáveis e com competências de intervenção. No entanto, e apesar deste reconhecimento, é necessário sensibilizar as entidades com capacidade interventiva, para minimizar e/ou monitorizar este problema. Por outro lado, os métodos de avaliação ambiental para estes locais apresentam um elevado grau de incerteza, tornando-se numa tarefa árdua, na medida em que na maioria das vezes a contaminação apresenta-se como uma mistura complexa de agentes. Deste modo, e identificada a complexidade da contaminação, torna-se premente desenvolver medidas para proceder à avaliação dos riscos nesses locais. Assim, para locais com contaminação ambiental identificada a estratégia de acção, normalmente recomendada deve iniciar-se por uma análise de risco ecológico (ARE) e para a saúde humana, caso haja populações expostas (Hill et al., 2000; Stahl et al., 2000; Weeks et al , 2004; Weeks e Comber, 2005; O'Halloran, 2006). Esta metodologia é recomendada em diversos países, (e.g. Estados Unidos, Canadá, Holanda, Dinamarca, Alemanha) (USEPA, 1998; EC, 2003; Jensen e Mesman, 2006), diferindo apenas nalgumas estratégias de avaliação, mas tendo como base comum uma sequência de etapas com objectivos específicos para avaliação de riscos (Figura 1). Esta avaliação é precedida por uma recolha de dados, que deve ser o mais relevante para a área de estudo, visando diminuir as incertezas na avaliação de risco. A análise de risco assenta essencialmente sobre um princípio simples, na razão entre os valores ambientais registados e os critérios de qualidade legislados para os diferentes compartimentos. Segundo o modelo britânico, no qual este trabalho se baseiou (Weeks et al , 2004; Weeks e Comber, 2005), a Etapa 0 da análise de risco tem como objectivo a recolha de dados disponíveis sobre o local e integrá-los de modo a identificar os riscos e assim elaborar um modelo conceptual. No final desta etapa, devem estar identificados os contaminantes e os potenciais eco-receptores em risco

através da elaboração do modelo conceptual com a informação obtida. Na Etapa 1 pretende-se avaliar os riscos, com base somente na contaminação química dos compartimentos ambientais, através de uma abordagem rápida e simples. Após a identificação dos contaminantes e sua quantificação, procede-se à comparação com critérios de qualidade legalmente estabelecidos. Ainda nesta etapa é recomendada a realização de um rastreio ecotoxicológico baseado em ensaios de curta duração. Nomeadamente, ensaios agudos (avaliação de imobilização/morte dos organismos) integrando diferentes níveis tróficos e o teste de Microtox® (redução de bioluminiscência da bactéria *Vibrio fischeri*) para diferentes compartimentos (água, solo) de forma a reduzir as incertezas da avaliação química. Se depois desta etapa de avaliação, forem confirmados os riscos ou se subsistirem dúvidas então deve-se seguir para a etapa 2 da análise de risco ecológico (Figura 1). A Etapa 2 da ARE destina-se a confirmar quais os eco-receptores dos potenciais riscos e o modo de acção sobre eles. Nesta etapa, é realizada a caracterização ecotoxicológica do local recorrendo a ensaios de longa duração (crónicos e sub-letais) com amostras provenientes do local contaminado. Adicionalmente, e sempre que necessário, a quantificação química dessas amostras deve suplementar os dados biológicos. Se não forem observados efeitos biológicos, então a ARE pode terminar. Contudo, quando são evidentes efeitos biológicos e é registada a existência duma relação causa-efeito identificada para os contaminantes químicos, então avança-se de imediato para a gestão dos riscos. Por outro lado, se existem dúvidas ou incertezas quanto aos efeitos biológicos/químicos (Chapman, 2002) observados ou se no local a bioacumulação de compostos é identificada como um problema, então é necessário avançar para a Etapa 3 na ARE (Figura 1). Nesta última etapa de avaliação de riscos pretende-se avaliar a magnitude/amplitude dos riscos para eco-receptores no local. O avanço para esta etapa dá-se em função das prioridades e da minimização de incertezas, apenas para os locais registados como tóxicos ou que apresentaram resultados inconclusivos. Para tal, normalmente são desenvolvidos bioensaios *in situ* com espécies padrão, incorporando diferentes níveis tróficos. Este tipo de estratégia de avaliação (*in situ* e vários níveis tróficos) torna-se bastante vantajosa, uma vez que permite avaliar questões ao nível de efeitos de misturas complexas de contaminantes, biodisponibilidade/bioacessibilidade e bioacumulação dos contaminantes (Peijnenburg e Jager, 2003). No entanto, a Etapa 3 recorre essencialmente a modelos ecológicos e probabilísticos integrando toda a

informação gerada ao longo da ARE para assim prever os efeitos ao nível das populações. Deste modo, pode antever situações adversas e antecipar-se com medidas de intervenção e prevenção, ou, por outro lado, propor acções de recuperação e gestão para locais ambientalmente alterados.

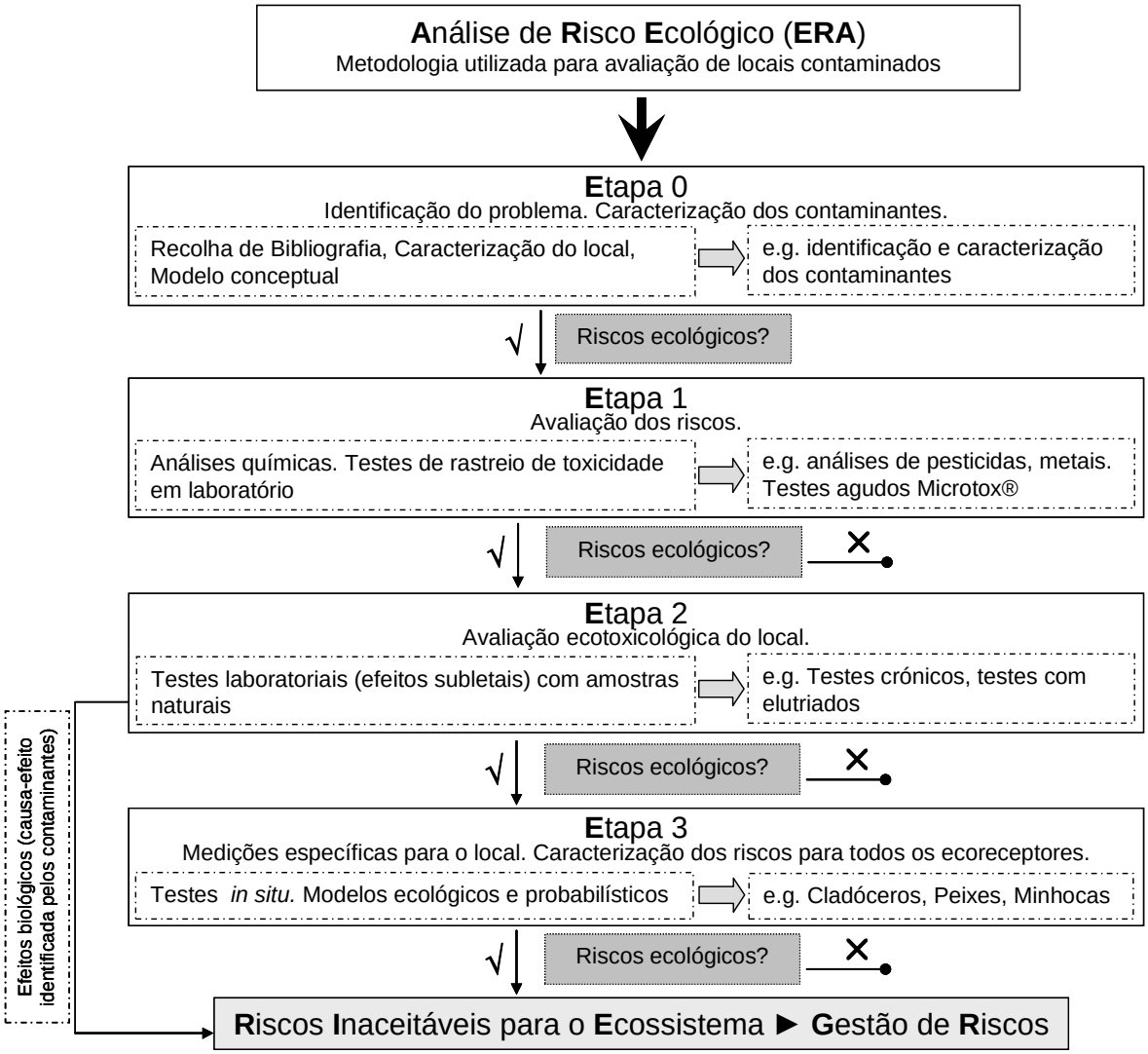

Figura 1 – Esquema simplificado da metodologia de análise de risco ecológico utilizada no Reino Unido (adaptado de Weeks, 2004).

Ao longo da ARE é bem evidente o recurso à ciência que avalia os efeitos de factores externos (físicos, químicos e biológicos) sobre os organismos vivos e ecossistemas – ecotoxicologia. Esta ciência é bastante vantajosa e útil na ARE uma vez que possui estratégias e metodologias padronizadas de avaliação para diferentes organismos que possibilitam a integração de vários níveis de organização biológica (*ver* ASTM, 1997,

2000; Environment Canada, 1992; ISO, 1996, 2000, 2005; OECD, 1984, 2000a,b; USEPA, 1994). A avaliação de parâmetros desde o nível molecular (e.g. biomarcadores, Hyne et al., 2003; Ribera et al., 2001) ao ecossistema (e.g. comunidades) seria o cenário ideal para quantificar os riscos na ARE. Todavia, o leque de metodologias exigido tornaria a sua execução uma tarefa morosa e com elevados custos, levando os cientistas, na maior parte das vezes, a recorrer ao melhor compromisso para a obtenção de informação. Assim, não surpreende que vários autores (e.g. Costan et al., 1993; Castillo et al., 2000; Robidoux et al., 2004) defendam a utilização de baterias de ensaios com diferentes espécies (diferentes sensibilidades) para avaliar a contaminação de um compartimento do ecossistema como sendo uma mais valia. Relativamente aos organismos padrão usados nos ensaios de avaliação, na perspectiva da ARE, estes devem ser preteridos em função de organismos nativos da área na medida em que torna os resultados ecologicamente mais relevantes. Becker et al. (1998) e Fernandéz et al. (2006) apresentam mesmo metodologias de selecção de espécies para análise de risco ecológico tendo em consideração a utilização do ecossistema. A redução de incertezas acrescida com dados ecologicamente relevantes aumenta a possibilidade de uma intervenção mais correcta e de aplicação de medidas interventivas mais direccionadas.

A aplicação das metodologias da ARE está amplamente documentada (e.g. Stahl et al., 2000; Pereira et al., 2004c, Mattson e Angermeier, 2007). A actividade mineira é um dos exemplos comummente utilizados na descrição e aplicação da análise de risco ecológico ou das diferentes etapas (Lozano et al., 2000; Laurence, 2001; Pereira et al., 2004c). Os principais riscos que provêm da generalidade da actividade mineira estão identificados e prendem-se sobretudo com questões de natureza química (e.g. elevadas concentrações de metais, Ribeiro et al., 2000). Nomeadamente, a ocorrência de baixos valores de pH nas águas (Lopes et al., 1999) e solos, circundantes aos complexos mineiros, em consequência de processos de lixiviação (Kelly, 1988). Por outro lado, a acidez contribui activamente para o incremento da dissolução e transporte de vários elementos químicos tóxicos (nomeadamente metais), arrastando o problema por vezes até distâncias consideráveis do foco poluente (e.g. Brown et al., 1998). Uma outra questão que ocorre neste locais, apresentando-se como uma preocupação adicional, prende-se com a acumulação em escombreiras de produtos "estéreis" da extracção e de rejeitados dos processos de concentração dos minérios, portadores de metais pesados e de diversos

produtos de reacção. Actualmente, esta situação observa-se em áreas mineiras abandonadas aumentando o perigo de dispersão de contaminantes e os riscos para a saúde pública. Assim, e com o intuito de salvaguardar a vida humana e selvagem, é necessário identificar os riscos ambientais associados às actividades mineiras e a toda a área abandonada nas imediações das explorações. Numa primeira instância, e logo após o encerramento e abandono da exploração, é necessário impor medidas de segurança de modo a minimizar acidentes. A falta de vedações em ruínas abandonadas (antigas infra-estruturas mineiras), escavações, poços, galerias, aglomerados de escórias, eventuais abatimentos/desmoronamentos de trabalhos subterrâneos, são alguns dos exemplos mais observados em antigos locais de exploração mineira (Santos Oliveira et al., 2002). Nas fases seguintes, concernentes à identificação e avaliação dos potenciais riscos ecológicos para o local, recomenda-se a implementação da ARE.

A exploração de minérios radioactivos apresenta os perigos e questões ambientais anteriormente abordados. No entanto, acresce nestes locais (sobretudo em minas de urânio e volfrâmio) o perigo da radioactividade. A radioactividade é um fenómeno natural ou artificial, em que algumas substâncias ou elementos químicos (radioactivos) são capazes de emitir radiações. A radioactividade resulta da instabilidade de alguns desses elementos químicos (e.g. urânio, tório, rádio) cujo decaimento liberta constantemente partículas alfa, beta e gama (raios-X). O urânio, em particular, é um elemento tóxico (ATSDR, 1999) que existe normalmente no ambiente (Elless e Lee, 1998). Apresenta-se como bastante instável e decai rapidamente noutros elementos igualmente tóxicos (radão, rádio, chumbo, polónio e bismuto) (Vera Tomé et al. 2002). Quando foi descoberto, o urânio tinha uma aplicação limitada e era utilizado apenas na fotografia, indústrias de cabedal e de madeira. No entanto, com o crescimento da energia nuclear, o urânio tornou-se um elemento de extrema importância na obtenção de energia através de reacções de fissão. Mais recentemente, uma outra forma de urânio (urânio empobrecido) tem sido alvo de estudos dado que, devido ao seu carácter de utilização (militar), expõe os soldados a elevados valores de radiação (Durante e Pugliese, 2002, 2003; Bleise et al., 2003). Os perigos específicos associados à exploração deste minério são a radioactividade, os altos teores de metais, baixos valores de pH e dureza (água e solos) (Charles et al., 2002; Gongalsky, 2003; Özmen et al., 2004; Pyle et al., 2002).

EXPLORAÇÃO DE URÂNIO EM PORTUGAL

A exploração de urânio em Portugal decorreu ao longo de quase um século (1907-2000) desta resultaram cerca de 62 minas de exploração de urânio confinada maioritariamente à faixa Centro/Norte do país, mais particularmente aos distritos de Viseu, Guarda, Portalegre e Évora (Figura 2). Durante a exploração do urânio, foram identificadas reservas com volume de recursos razoavelmente ricos em U_3O_8 (teor de corte 0,05%), nas duas maiores regiões de exploração, na ordem de 4370 toneladas na Região das Beiras e de cerca de 3200 toneladas no Alto Alentejo (Dias e da Costa, 1980; Nero et al, 2003).

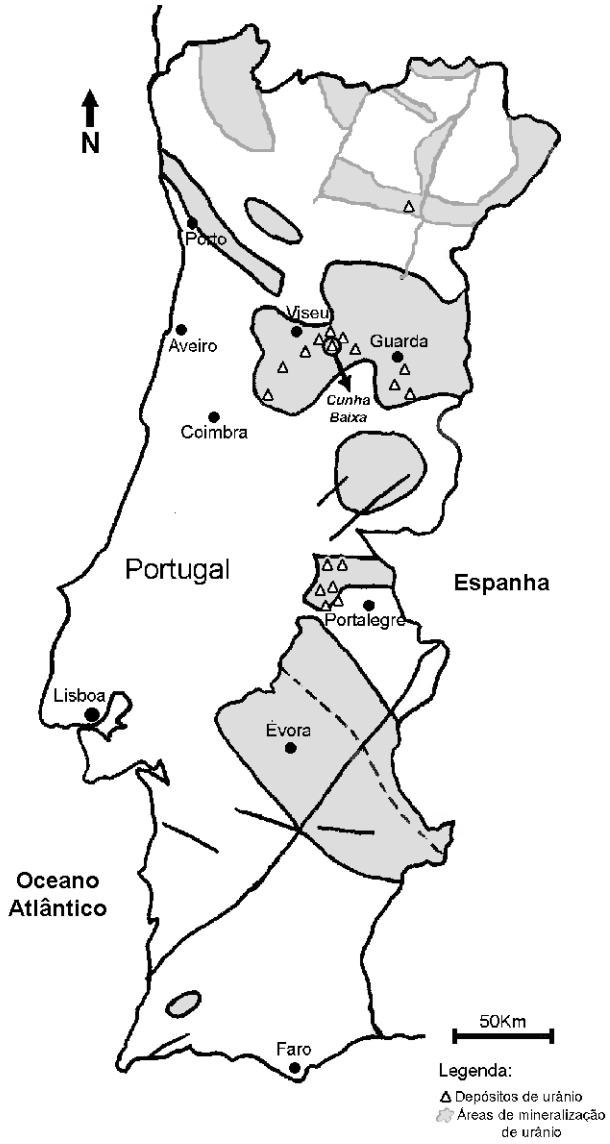

Figura 2 – Mapa representativo das zonas de mineralização de urânio em Portugal (a cinzento) e localização dos principais depósitos de urânio onde se realizou exploração de minério.

No entanto, a exploração com maior significado deu-se na Região das Beiras, onde o urânio foi explorado em diversas minas, das quais se salientam as minas Urgeiriça, Bica, Castelejo, Cunha Baixa, Quinta do Bispo e Pinhal do Souto. O urânio foi um dos minérios com maior viabilidade em Portugal, com rápida expansão em termos de exploração e de descoberta de novos jazigos minerais, uma vez que o país se encontra numa área de elevada favorabilidade uranífera. Quando o auge da exploração de urânio ocorreu tornou-se necessário acelerar a extracção e industrialização do minério, de modo a rentabilizar economicamente as minas. Tal situação levou à deposição descoordenada de diversos tipos de resíduos, na sua maioria radioactivos, nas áreas envolventes a estas explorações (Nero et al, 2003). De modo a minimizar os impactos da exploração mineira após o seu encerramento, o governo, na década de 90, legislou a obrigatoriedade de implementação de processos de recuperação ambiental para explorações com área superior a 5ha e ou com uma produção anual superior a 150000t (M.I.A., D.L. nº88/90, 16 de Março). Assim, as empresas exploradoras do minério são obrigadas a criar e a implementar medidas de remediação para o local de modo a minimizar os riscos/impactos ambientais, antes de poderem encerrar definitivamente a actividade.

As diferentes jazidas uraníferas que foram identificadas como economicamente rentáveis deram origem à exploração intensa de minério. As suas características morfológicas distintas levaram a métodos de exploração diferenciados: desmonte subterrâneo, desmonte a céu aberto e desmonte conjunto subterrâneo/céu aberto (Nero et al., 2003). Após o encerramento da exploração convencional, e aproveitando a elevada solubilidade do urânio, foi utilizada lixiviação "*in situ*" com soluções ácidas, como um método adicional de desmonte. Esta metodologia era usada essencialmente para recuperar o urânio residual em minas já exploradas por outros métodos, através da recuperação dos licores da lixiviação (Pereira et al., 2004b; Cordeiro Santo e Pereira Freire, 1983).

A partir de 1991, a produção de minério de urânio diminuiu de importância, contribuindo, este facto, para a diminuição relativa deste subsector no país. Os custos demasiado elevados para a extracção de minério rentável sobre o minério pobre e/ou estéril levaram à diminuição significativa da exploração de urânio. Não obstante este facto, a exploração de urânio prolongou-se até 2000, apenas na mina da Quinta do Bispo, envolvendo somente do tratamento de minérios pobres pelo processo de lixiviação. Após a cessação e abandono destas minas, o foco de poluição foi identificado pelas entidades

responsáveis e, na maior parte das vezes, negligenciado por falta de monitorização pelas empresas responsáveis e controlo dos minérios pobres e escombreiras. Além da elevada contaminação química (metais pesados) registada nestes locais, a exploração de urânio acresce com outro tipo de contaminação – a radioactividade (Durante e Pugliese, 2003). Dentre os elementos de decaimento do urânio, o radão apresenta-se como ligeiramente mais estável e tem sido alvo de vários estudos, essencialmente num grupo de freguesias na Região da Beiras. Estes estudos consistiram em medir a concentração de radão no interior de habitações, nos solos e no ar exterior, onde têm sido registados valores bastante elevados (habitações ≈ 2396 Bqm^{-3}, solo ≈ 5681 $Bqm^{-2}s^{-1}$, ar ≈ 5266 Bqm^{-3}) (Marinho Falcão et al., 2005).

O EXEMPLO DA MINA DA CUNHA BAIXA

As minas da Cunha Baixa e Quinta do Bispo situam-se na "região uranífera das Beiras", no concelho de Mangualde, pertencendo ao mesmo complexo mineiro, da Urgeiriça e foram as duas explorações de urânio mais importantes em Portugal. A mina da Cunha Baixa apresenta a particularidade de se situar na área adjacente a um aglomerado populacional, aldeia com o mesmo nome (concelho de Mangualde). A mina, é estruturalmente constituída por dois sistemas de falhas conjugadas, 40ºN± 5ºE subvertical e 70ºN±5ºW com inclinação 75ºN. O jazígo uranífero, do ponto de vista geomorfológico é considerado uma peneplanície caracterizada pelo rejuvenescimento do relevo com a formação de um "horst" tectónico que inclui a Serra da Estrela (Santos Oliveira e Ávila, 2001). É uma zona que se caracteriza (tipologia – F_2D_2, filoniano com quartzo defumado e leitoso, disseminação metassedimentos) por granitos metassedimentares metamorfizados que datam da idade Hercínica (Dias e da Costa, 1980). A mina esteve em laboração desde 1967 a 1993, sob a tutela da Junta de Energia Nuclear (JEN) e já no final da exploração pela Empresa Nacional de Urânio (ENU). A concessão foi depois transferida para a Exmin - Companhia de Indústria e Serviços Mineiros e Ambientais (recentemente, fundida na "holding" do Estado para o sector mineiro EDM –Empresa de Desenvolvimento Mineiro) que assumiu a responsabilidade da requalificação ambiental após o encerramento dos trabalhos de exploração.

Durante a actividade de exploração, esta mina recorreu à lavra subterrânea (1967 a 1987), atingindo uma profundidade máxima de 150m. Já no final da exploração (1984 a

1991), recorreu-se à lavra de céu aberto (Santos Oliveira e Ávila, 2001). Durante o recurso a este método de exploração, e de modo a recuperar uma maior quantidade de minério viável de minérios de baixos teores, recorreu-se a processos de lixiviação estática *in situ* com ácido sulfúrico (H_2SO_4) (Santos Oliveira e Ávila, 2001; Pedrosa e Martins, 1999). Através deste procedimento eram recolhidos os licores da lixiviação e posteriormente levados para o complexo principal (Urgeiriça) para se proceder à sua industrialização (Cordeiro Santo e Pereira Freire, 1983). Como resultado da exploração da mina da Cunha Baixa foram extraídas cerca de 500 000 toneladas de minério e 76 000 kg de óxidos de urânio (U_3O_8) provenientes das operações de lixiviação "*in situ*". De minério pobre e escórias foram produzidas cerca de 1,1 milhões de toneladas, que foram utilizadas para encher os túneis da lavra subterrânea e do céu aberto (em parte), aquando da cessação da exploração. Os depósitos de escórias restantes foram deixados em escombreiras nas imediações da exploração mineira (Santos Oliveira e Ávila, 2001; Neves et al., 1997).

Após o encerramento da mina, a empresa que detinha a concessão (ENU) tem vindo a implementar algumas medidas de segurança e recuperação ambiental para a área envolvente à mina. Em termos de segurança, foi colocada uma vedação em torno do céu e da lagoa de tratamento. De modo a minimizar o impacto do efluente aquático ácido (pH ≈ 3), causado pela lavagem do minério por H_2SO_4, a empresa procedeu à criação de uma estação de tratamento de águas. Esta estação procede ao bombeamento de águas provenientes do poço principal (antiga exploração da lavra subterrânea) e à sua neutralização com cloreto de bário ($BaCl_2$) (para valores pH ≈ 8) (Santos Oliveira e Ávila, 2001). O tratamento com cloreto de bário é usado também para induzir a precipitação de compostos de rádio bem como na remoção de outros elementos químicos perigosos (e.g. metais pesados). A precipitação dá então origem a um agregado de lamas potencialmente perigosas devido ao alto teor em metais e rádio (Pereira et al., 2004a; Santos Oliveira e Ávila, 1998). Por outro lado, o crescimento de vegetação ao longo do tempo tem vindo a minimizar o impacto visual dos "buracos" a céu aberto e das escombreiras adjacentes.

Actualmente, os potenciais riscos/perigos destes locais foram já reconhecidos pelas entidades responsáveis (Santos Oliveira et al., 2002). A alteração ambiental (e.g. destruição de *habitats* da fauna e flora), química (e.g. altos teores de metais nos solos e águas, com perda de viabilidade destes compartimentos), física (e.g. alteração da estrutura do solo com perda de capacidade de retenção dos contaminantes e de *habitat* qualificado para

organismos do solo) e paisagística (e.g. degradação da paisagem natural, e de bens patrimoniais) das minas abandonadas e/ou inactivas é um problema que requer intervenção urgente. Deste modo, estão a ser levados a cabo projectos de monitorização e reabilitação para algumas áreas mineiras classificadas como prioritárias após estudos realizados pela Direcção Geral do Ambiente e Instituto Geológico e Mineiro (actual INETI). Assim, e para as minas com riscos radiológicos identificados (ex: irradiação) torna-se indispensável dispor de um serviço de protecção e segurança radiológica. Tal situação pretende reduzir os riscos das actividades mineiras do urânio a níveis comparáveis aos de qualquer outra indústria extractiva de minérios. Consequentemente, a mina da Cunha Baixa tem sido alvo de inúmeros estudos e trabalhos, uma vez que se encontra classificada como área de intervenção prioritária (Santos Oliveira, 1997). Relatórios internos da ENU/EDM e do Instituto Geológico e Mineiro (actualmente Instituto Nacional de Engenharia e Tecnologia Industrial - INETI) apresentam dados que identificam e quantificam a contaminação dos solos, das águas superficiais e subterrâneas da mina da Cunha Baixa (Machado, 1998; Neves et al., 1997; Neves e Matias, 1999; Santos Oliveira et al., 1999). Além de inúmeros trabalhos relativos ao ambiente (essencialmente de caracterização química), a saúde pública não tem sido negligenciada. Recentemente, têm sido realizados estudos epidemiológicos, tanto nas populações adjacentes como nos antigos trabalhadores das minas, de modo a avaliar o grau de exposição e os perigos associados a este tipo de contaminação (e.g. poeiras, altos teores de radão, contaminação de águas e solos usados pelas populações) (Marinho Falcão et al., 2005). Por outro lado, a avaliação ecotoxicológica dos diversos compartimentos do ecossistema (ar, água, solo) tem sido descurada. Este tipo de avaliação é essencial e premente pois fornece dados mais realistas dos efeitos da contaminação e permite actuar em áreas específicas (mais preocupantes) com a facilidade de acompanhar as medidas de requalificação implementadas.

OBJECTIVOS

O trabalho de doutoramento, que culminou na elaboração desta dissertação, teve como principal objectivo gerar informação/dados ecotoxicológicos sobre a mina de urânio da Cunha Baixa (Mangualde – Viseu, Centro de Portugal), para posterior implementação de medidas de remediação e mitigação. No entanto, para que este objectivo geral fosse cumprido muitos outros objectivos específicos foram definidos, abrangendo essencialmente a avaliação de dois compartimentos (aquático e terrestre). Assim, e tendo sempre em vista o objectivo principal, definiram-se duas grandes áreas de intervenção para as quais foram delineados os seguintes objectivos específicos:

- Compartimento Aquático:

  - Avaliar a toxicidade aguda e crónica do sistema aquático (3 lagoas artificiais) criado pela mina; através de bioensaios com duas espécies de *Daphnia*.

  - Avaliar a toxicidade (aguda e crónica) de elutriados obtidos de sedimentos provenientes do sistema aquático (3 lagoas artificiais) da mina de urânio abandonada; através de bioensaios com duas espécies de *Daphnia*.

  - Avaliar a toxicidade da coluna de água e sedimento da lagoa artificial criada no antigo fosso de exploração de lavra subterrânea da mina de urânio da Cunha Baixa usando uma bateria de ensaios; através de bioensaios com duas espécies de *Daphnia*, uma alga e de um quironomídeo.

- Compartimento Terrestre:

  - Contribuição para a Etapa 1 de uma análise de risco ecológico para a mina de urânio da Cunha Baixa (Centro de Portugal): rastreio ecotoxicológico para o compartimento terrestre; avaliado através de bioensaios de evitamento com *Eisenia andrei*, Microtox® e bioensaios agudos com duas espécies de *Daphnia* com elutriados de solo.

  - Ensaio *in situ* com *Eisenia andrei* para avaliar a toxicidade do solo de uma mina de urânio abandonada; através do desenvolvimento de um bioensaio *in situ* com *Eisenia andrei* na avaliação da toxicidade do solo.

ESTRUTURA DA TESE

A presente tese encontra-se estruturada em sete capítulos de acordo com os objectivos específicos anteriormente mencionados. No entanto, inicialmente Capítulo I – Introdução, é feita uma integração da problemática de locais contaminados bem como um breve enquadramento do assunto no contexto nacional. O corpo da tese propriamente dito, do segundo ao sexto capítulo, encontra-se redigido em Inglês, na medida em que os capítulos se apresentam sobre a forma de artigos científicos submetidos ou aceites para publicação em revistas internacionais com arbitragem científica. Os capítulos II, III e IV apresentam o trabalho desenvolvido relativamente à avaliação da contaminação no compartimento aquático. Os capítulos V e VI debruçam-se sobre a problemática ambiental no compartimento terrestre. Por fim, o Capítulo VII (Considerações finais) pretende resumir e integrar toda a informação gerada com o trabalho desenvolvido fazendo uma demonstração do cálculo dos riscos para os dois compartimentos da área adjacente à mina da Cunha Baixa.

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CAPÍTULO II

Arch. Environmental Contamination and Toxicology (2007) 53(2): 207-213

"Toxicidade aguda e crónica do efluente aquático de uma mina de urânio abandonada"

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RESUMO

As minas abandonadas ou inactivas representam uma fonte significativa de impactos ambientais, químicos, físicos e estéticos. Em Portugal, este problema foi já diagnosticado e existem em desenvolvimento alguns projectos de remediação para estes locais. Dentre os casos mais preocupantes, a existência de lagoas abandonadas associadas à exploração mineira (para a sedimentação de sólidos, para neutralizar efluentes ou para a lavagem do minério) são comuns neste tipo de sistemas. Estas lagoas são por vezes fonte de contaminação das águas subterrâneas e solos adjacentes, por causa da sua falta de impermeabilização. A utilização destas águas na agricultura pode acarretar riscos para a população humana. O objectivo do presente estudo foi o de avaliar a toxicidade aguda e crónica de águas provenientes de lagoas artificiais de uma mina de urânio abandonada (Cunha Baixa, Mangualde, Centro de Portugal) em zooplanctontes. Neste trabalho, apenas o compartimento da coluna de água foi estudado e a sua toxicidade foi avaliada através de protocolos padronizados usando duas espécies de *Daphnia* (*D. longispina* e *D. magna*). Na caracterização toxicológica do sistema aquático da mina da Cunha Baixa foram usadas amostras provenientes de três lagoas: lagoa de referência (Ref), lagoa de tratamento do efluente mineiro (T) e lagoa do buraco da mina (M). Análises de metais realizadas às amostras de água demonstraram valores bastante elevados, em alguns casos, excedendo os valores legais estabelecidos (especialmente Al, Mn). Apenas se registou toxicidade aguda na lagoa M, com EC_{50}=28,4% e 50,4% para *D. longispina* e *D. magna*, respectivamente. A toxicidade crónica foi mascarada por um efeito estimulatório, que é comum em amostras naturais, devido à presença de bactérias que servem como fonte de alimento ou à presença de micronutrientes que favorecem o desempenho reprodutivo. Este efeito estimulatório pode levar a complicações na interpretação da toxicidade. Contudo, os efeitos negativos observados em parâmetros reprodutivos e populacionais para ambas as espécies dão azo a preocupação quanto aos eventuais riscos para as populações zooplanctónicas locais, potencialmente expostas a descargas do efluente tratado e não tratado da mina.

PALAVRAS-CHAVE

Mina de urânio abandonada, Metais, Toxicidade aquática, Testes de toxicidade, *Daphnia* sp.

"Acute and chronic toxicity of effluent water from an abandoned uranium mine"

ABSTRACT

Inactive or abandoned mines represent a significant source of environmental, chemical, physical and aesthetic impact. Among concerning situations, the occurrence of abandoned or semi-abandoned mine-associated ponds (for sedimentation of solids, for effluent neutralisation or for washing the ore) is a common feature in this type of systems. These ponds are a source of contamination for the groundwater resources and adjacent soils, because they lack appropriate impermeabilization. The use of this water for agriculture may also pose chronic risks to humans. In Portugal, these problems have been diagnosed and some remediation projects have been developed. The purpose of our study was to evaluate the acute and chronic toxicity of water samples collected from the aquatic system surrounding an abandoned uranium mine (Cunha Baixa, Mangualde, Central Portugal). The present study focuses on the water compartment, whose toxicity was evaluated by means of standard toxicity assays using two *Daphnia* species (*D. longispina* and *D. magna*). Three different ponds were used in the characterisation of the aquatic system from Cunha Baixa mine: a reference pond (Ref), a mine effluent treatment pond (T) and a mine pit pond (M). Metal analyses performed in the water samples from these ponds showed values that, in some cases, were much higher than Maximum Recommendable Values (MRV's) established (especially Al, Mn) on Portuguese legislation to waters for crop irrigation. Acute toxicity was only observed in the mine pit pond, with EC_{50} values of 28.4% and 50.4% for *D. longispina* and *D. magna*, respectively. The significant impairment of chronic endpoints, translated in reductions in the population growth rate for both species, gives rise to concerns regarding the potential risks for aquatic zooplanktonic communities, from local receiving waters, potentially exposed to point source discharges of the treated and non-treated effluent from Cunha Baixa uranium mine.

KEYWORDS

Abandoned uranium mine, Metals, Water toxicity, Toxicity assays, *Daphnia* sp.

INTRODUCTION

The generation of acidic waters bearing high concentration of heavy metals from mining is a great environmental concern. Beyond mining areas, many others sites all over the world (e.g. military bases, industrial zones and war zones) show significant levels of heavy

metals (Rahn et al. 1996, Bleise et al. 2003, Chen et al. 2004). Usually, mines have an extensive exploitation and, even when the activities cease, the impacts onsite and offsite continue. Consequently, all the surrounding compartments of the ecosystem (adjacent soils, groundwater, etc.) are exposed to very high concentrations of heavy metals (Pereira et al. 2004). Uranium mines, in particular, produce a large amount of tailings with a potential negative impact, resulting from both the abandoned radioactive material and high heavy metal concentrations (Lozano et al. 2000, 2002). Exposure to radioactivity and heavy metals poses serious threats to wildlife and humans (Bleise et al. 2003).

Uranium is a radioactive element that naturally occurs in the environment. Due to its instability, it rapidly decays to other elements (radon, lead, polonium and bismuth), which are equally toxic (Vera Tomé et al. 2002). The major concern associated with the occurrence of uranium in mining environments is direct inhalation or ingestion of particles by workers (Domingo 2001). However, information on the toxicity of effluents or natural waters contaminated with uranium to aquatic organisms is deficient, although some toxicity data have been derived from laboratory toxicity data (e.g. Poston et al. 1984, Franklin et al. 2000, Semaan et al. 2001, Hogan et al. 2005). Additionally, some authors (e.g. Franklin et al. 2000, Riethmuller et al. 2001, Charles et al. 2002, Özmen et al. 2004, Hogan et al. 2005) have assessed the factors which influence the toxicity of uranyl ion in natural waters, such as pH, hardness and the concentration of inorganic and organic ligands. Elless and Lee (1998) reviewed this issue, considering both the aquatic and soil compartments.

Uranium exploitation in Portugal began in 1913 and ended by the year 2000. During this period, the ore was mined in 62 different places, mostly located in the centre of Portugal, as open pit or underground mines. As a consequence of mining, a large amount of waste (about 13Mton) was produced and is currently deposited nearby the old mining sites. These wastes, composed of geological materials, are rich in hazardous chemicals and radioactive elements, promoting the transfer of these elements to the different environmental compartments (through leaching, erosion, etc.) (Santos Oliveira and Ávila 1998, Machado 1998, Pedrosa and Martins 1999). The Cunha Baixa mining area was defined as one of the most important uraniferous belts in Portugal. After cessation of the exploitation (ca. 1993), this mine was classified as deserving priority intervention. This classification resulted from environmental impact studies, which revealed water contamination due to the dispersion and accumulation of chemical elements originating from the mining area. Cunha Baixa mine is particularly close to the village, where inhabitants use water from the aquatic system near the mine for agriculture, domestic and animal drinking purposes (Pedrosa and Martins 1999).

The aim of this study was to characterize the aquatic system of the Cunha Baixa mining area in terms of its physico-chemistry and toxicity. This is one of the first studies to address the biological examination of water (through ecotoxicity assays) in this aquatic system. Ecotoxicological data were obtained from acute and chronic toxicity testing using two daphniids (Cladocera: Daphniidae): *Daphnia magna* Straus (standard test-organism) and *Daphnia longispina* O.F. Müller. Both cladocerans are key species in freshwater aquatic food chains and the last one provides a more relevant ecological evaluation since is a Portuguese native species.

MATERIALS AND METHODS

Study site

The uranium mine is located in Cunha Baixa (Mangualde, Viseu), in the centre of Portugal, and is included in the central uraniferous belt of the Iberian Peninsula (Santos Oliveira and Ávila 1998). The ore was extracted from 1967 to 1993 (Oliveira and Ávila 2001) and, after closure, the underground mine pit was filled with mine tailings and flooded with sulphuric acid. The extraction of ore left three temporary ponds: i) one upstream of the mine pit where exploration used to be performed in an open pit (reference pond - Ref); ii) a treatment pond (T) that receives the acid effluent that is pumped from the underground mine after it has been neutralized with lime and barium chloride; iii) and a third pond, which floods the underground exploration pits (M). In years of severe drought these ponds may disappear completely.

Collection of water samples

Water samples were collected from the three ponds with 1.5-20L plastic containers and transported to the laboratory. Samples were filtered through a Whatman GF/C filter (1.2µm porosity, 47mm diameter) and stored at 4ºC until the assays were performed (maximum storage time: one week). Samples for metal analysis were acidified with nitric acid to pH<2 to reduce adsorption phenomena and stored in a plastic container at 4ºC until the determinations were possible (see Chemical and microbiological analyses).

Daphnia culturing

Monoclonal cultures of *D. magna* (clone A, *sensu* Baird et al. 1989a) and *D. longispina* (clone EM7, *sensu* Antunes et al. 2003) were reared under a 16^{L}:8^{D}h cycle and a temperature of 20±2ºC. ASTM (1980) synthetic hard water medium was used as culture

medium and rearing procedures followed the recommendations of Baird et al. (1989b), Soares et al. (1992) and standard protocols (e.g. ISO 1996,2000, ASTM 1997, OECD 1998, 2000). A standard organic additive was added to the culture medium to provide essential microelements to daphniids (Baird et al. 1989b). Animals were fed the microalga *Pseudokirchneriella subcapitata*, which was cultured in Woods Hole MBL medium, in a semi-continuous 8L batch culture at a 24^{L}:0^{D} h regime (at 20±2 ºC). Algal ration was determined spectrophotometrically and daily supplied to the cladocerans (3.0×10^{5}cell/ml/day for *D. magna* and 1.5×10^{5} cell/ml/day for *D. longispina*). All experiments were initiated with neonates (<24-h-old), born between the 3rd and 5th broods, which were obtained from bulk group cultures.

Acute assays

Independent experiments were used to assess the acute toxicity of effluent water to both *D. magna* and *D. longispina*. Tests were performed in accordance with standard protocols (ISO 1996, ASTM 1997, OECD 2000), under the same temperature and photoperiod regimes as described for rearing procedures. A static design was employed, using 20 animals (randomly divided into four groups of five animals) per control (ASTM hard water medium alone) and per effluent water concentration (%). Test vessels (four per each treatment) consisted of glass beakers containing 100ml of test water effluent concentration. For each combination of species, 6-7 test concentrations were obtained by dilution with ASTM hard water medium. Range-finding bioassays were carried out to set appropriate dilutions (Ref and T: 0.0, 25.0, 50.0, 75.0, 100.0%) to obtain EC_{50} values with the best confidence interval. Final concentrations for M pond were: for *D. longispina* (20.7-41.6%, separated by a factor of 1.15), for *D. magna* (38.5-56.4% separated by a factor of 1.1). Oxygen concentrations and pH levels in test vessels were determined at 0, 24 and 48h to fulfil the test criteria (OECD 2000). All experimental treatments were checked for immobilised individuals at 24 and 48h, which were counted for posterior determination of EC_{50} values (see Statistical analysis).

Chronic assays

Independent experiments were used to assess the chronic toxicity of effluent water to both *D. magna* and *D. longispina*. Tests were conducted for 21 days in accordance with standard protocols (ASTM 1997, OECD 1998, ISO 2000), under the same temperature and photoperiod regimes as described for rearing procedures. A semi-static design was employed,

using 10 individualised animals randomly assigned to the control (Ctl) and to each effluent water concentration (Ref and T: 6.25, 12.50, 25.0, 50.00, 75.00 and 100.00%; M: 4.00, 6.00, 9.00, 13.50, 20.25 and 30.38%). Test vessels (10 per each treatment) consisted of glass beakers containing 50ml of effluent water concentration (including organic extract). The effluent concentrations were diluted with ASTM hard water medium and were chosen based on the previously-obtained acute EC_{50}. Daphniids were transferred to newly-prepared effluent water concentration every two days, and were fed daily with their respective *P. subcapitata* ration. Animals were daily checked for mortality and reproductive state and, if neonates had been released, they were counted and immediately discarded. The following parameters were registered: total number of offspring, number of broods, age at first reproduction (AFR), somatic growth rate, and rate of population increase (*r*). The somatic growth rate (expressed in day^{-1}) was estimated from the initial and final body size of the daphniids, according to the following expression:

$$growth\ rate = \frac{\ln(l_f) - \ln(l_i)}{\Delta t},$$

where l_f is the body size (in mm) of the test organism at the end of the test, l_i is the average body size (in mm) of a subsample (n=20) of neonates coming from the same batch of neonates that initiated the test, and Δt is the time interval (in days).

Survival and fecundity estimates were also used to compute the rate of population increase (*r*), which was iterated from the Euler–Lotka equation:

$$1 = \sum_{x=0}^{n} e^{-rx} l_x m_x,$$

where *r* is the rate of population increase (day^{-1}), *x* is the age class in days (0. . .n), l_x is the probability of surviving to age *x*, and m_x is the fecundity at age *x*. Pseudovalues of *r* were estimated using a jack-knifing technique described by Meyer et al. (1986), thus allowing hypothesis testing on this demographic parameter.

Chemical and microbiological analyses

Effluent water was analysed for selected metals using ICP-MS spectrometry (APHA et al. 1995). At the onset of the toxicity assays, a range of physical and chemical parameters were quantified in aliquots of the effluent water: pH, conductivity, total phosphorous - TP (persulfate digestion followed by ascorbic acid method; APHA et al. 1995), total nitrogen - TN (persulfate digestion followed by cadmium reduction method; APHA et al. 1995), calcium

and magnesium (both by titration with EDTA; APHA et al. 1995). Additionally, the total density of bacteria present in effluent water was quantified as colony forming units (CFU) of bacteria cultured in TSA (tryptic soil agar) plates.

Statistical analysis

EC_{50} values for immobilisation data (acute tests) were determined using probit analysis (Finney 1971). A one-way analysis of variance (ANOVA), followed by a Dunnett test (if applicable), was applied to each endpoint of the chronic assay to assess statistical differences between the different water effluent concentrations and the control (Ctl).

RESULTS

Water quality parameters of the studied ponds and dilution water (hard water culture medium) are presented in Tables 1 and 2. All pH levels were close to neutrality, except for site M, which was acidic (pH = 4.2). Conductivity values observed in T and M were much higher (around 1000µS/cm) than those recorded in Ref or the control (dilution water). Water from Ref exhibited a low conductivity value. In terms of their inorganic constituents, the samples from the aquatic system were also very different. Natural waters had higher levels of TP and TN than dilution water, but were more or less similar to each other. These sites also presented a higher level of calcium and magnesium than the dilution water, except for Ref, where very low levels of these minerals were found. Bacteria were found in all samples, including dilution water. Site T presented the highest level of CFU, while Ref and M, in decreasing order, presented the lowest levels of CFU. Metal analyses performed in the water samples from the three ponds showed values that, in some cases, were much higher than the maximum recommendable values to water for crop irrigation (MA 1998) (Table 2). In general, ponds T and M presented higher metal levels than Ref. This was noticeable for Zn, Co, Ni, Sr, and particularly for U and Mn. Al was also observed at high concentrations in M.

Table 1 – Physical, chemical and bacteriological water quality data for the three ponds (Ref, T and M) and dilution water (ASTM hard water medium).

	ASTM	Ref	T	M
pH	7.6-7.8	6.8	6.9	4.2
Cond (µS/cm)	550	52.6	1019	1005
TP (mg/L P)	0.02	0.06	0.03	0.04
TN (mg/L N)	0.11	0.60	1.80	1.20
Ca^{2+} (mg/L)	40.8	8.60	216	144
Mg^{2+} (mg/L)	18.0	1.56	103	51.0
CFU (ml^{-1})	600	143	1157	68

Table 2 – Average metal concentrations (µg/L, n=3 determinations) observed in the three ponds (Ref, T and M), analysed by inductively coupled plasma mass spectrometry (ICP/MS). MRV stands for Maximum Recommendable Values to waters for crop irrigation (MA, 1998). Values in bold show metal levels exceeding the MRV to waters for crop irrigation.

	Ref	T	M	MRV
U	<100	1177	1404	not available
Cd	<1.5	<1.5	2.9	10
Zn	21	138	572	2000
Ba	37	80	25	1000
Mn	8	**5137**	**3878**	200
Co	<5	45	**61**	50
Se	<25	<25	<25	20
Be	<0.5	3	27	500
Al	<50	<50	**5067**	5000
Ni	<6	83	116	500
Pb	<15	<15	<15	5000
Sr	13	399	273	not available
V	<5	<5	<5	100
Cr	<5	<5	<5	100

No acute toxicity (immobilisation) was found for Ref or T ponds for both *Daphnia* species. Water from pond M, however, caused a physiological impairment in both daphniids, with *D. magna* – EC_{50} = 50.4% (CI_{95}: 47.6% - 53.3%) – being less sensitive than *D. longispina* – EC_{50} = 28.4% (CI_{95}: 25.6% - 32.1%).

Results of chronic toxicity assays show the existence of long-term deleterious effects, but also stimulatory phenomena (Fig. 1 and Table 3). Water from Ref produced a significant stimulation for both species, by increasing the number of offspring produced, the somatic growth rate and the intrinsic rate of increase. An increase in the number of broods was also observed at the higher concentrations of the Ref water but only for *D. longispina*. Water

effluent from T produced a mixed effect: at low concentrations, occasional stimulation was observed (e.g. intrinsic rate of increase of *D. magna*), while toxic effects were observed in the two highest effluent concentrations (75 and 100%). Assays with water from M were performed using low concentrations (up to 30.38%) because of its acute toxicity (see above). For *D. magna*, these concentrations were sufficient to produce a negative effect on the life history responses, namely on the offspring and number of broods produced, as well as on the intrinsic rate of increase. However, no deleterious effects were observed for *D. longispina* at these concentrations even though these were acute levels (acute EC_{50} = 28%). Moreover, a stimulation of fecundity was observed for the lowest effluent concentrations. In fact, *D. longispina*'s intrinsic rate of increase was stimulated in all of the effluent concentrations, with the exception of the highest concentration (30.38%).

Table 3 - Summary table of the one-way analyses of variance applied to the life history responses of the daphniids. Age at first reproduction (AFR), number of broods, total offspring, somatic growth rate and rate of increase were analysed for the effect of water effluent concentration independently for each site.

Site	**Endpoint**	***D. longispina***			***D. magna***		
		F	**d.f.**	***P***	***F***	**d.f.**	***P***
Ref	AFR	1.055	6, 61	NS	0.929	6, 63	NS
	Number of broods	6.825	6, 61	<0.001	1.111	6, 63	NS
	Total offspring	55.448	6, 61	<0.001	5.332	6, 63	<0.001
	Somatic growth rate	51.539	6, 61	<0.001	13.350	6, 63	<0.001
	Rate of increase (*r*)	43.322	6, 63	<0.001	7.975	6, 63	<0.001
T	AFR	9.797	6, 57	<0.001	25.329	6, 55	<0.001
	Number of broods	3.230	6, 57	0.008	14.551	6, 55	<0.001
	Total offspring	4.004	6, 57	0.002	15.552	6, 55	<0.001
	Somatic growth rate	2.601	6, 57	0.027	7.815	6, 55	<0.001
	Rate of increase (*r*)	10.953	6, 63	<0.001	20.083	6, 59	<0.001
M	AFR	5.708	6, 63	<0.001	2.059	6, 62	NS
	Number of broods	2.000	6, 63	NS	19.805	6, 62	<0.001
	Total offspring	5.424	6, 63	<0.001	42.069	6, 62	<0.001
	Somatic growth rate	2.040	6, 63	NS	3.061	6, 62	0.011
	Rate of increase (*r*)	16.939	6, 63	<0.001	7.827	6, 62	<0.001

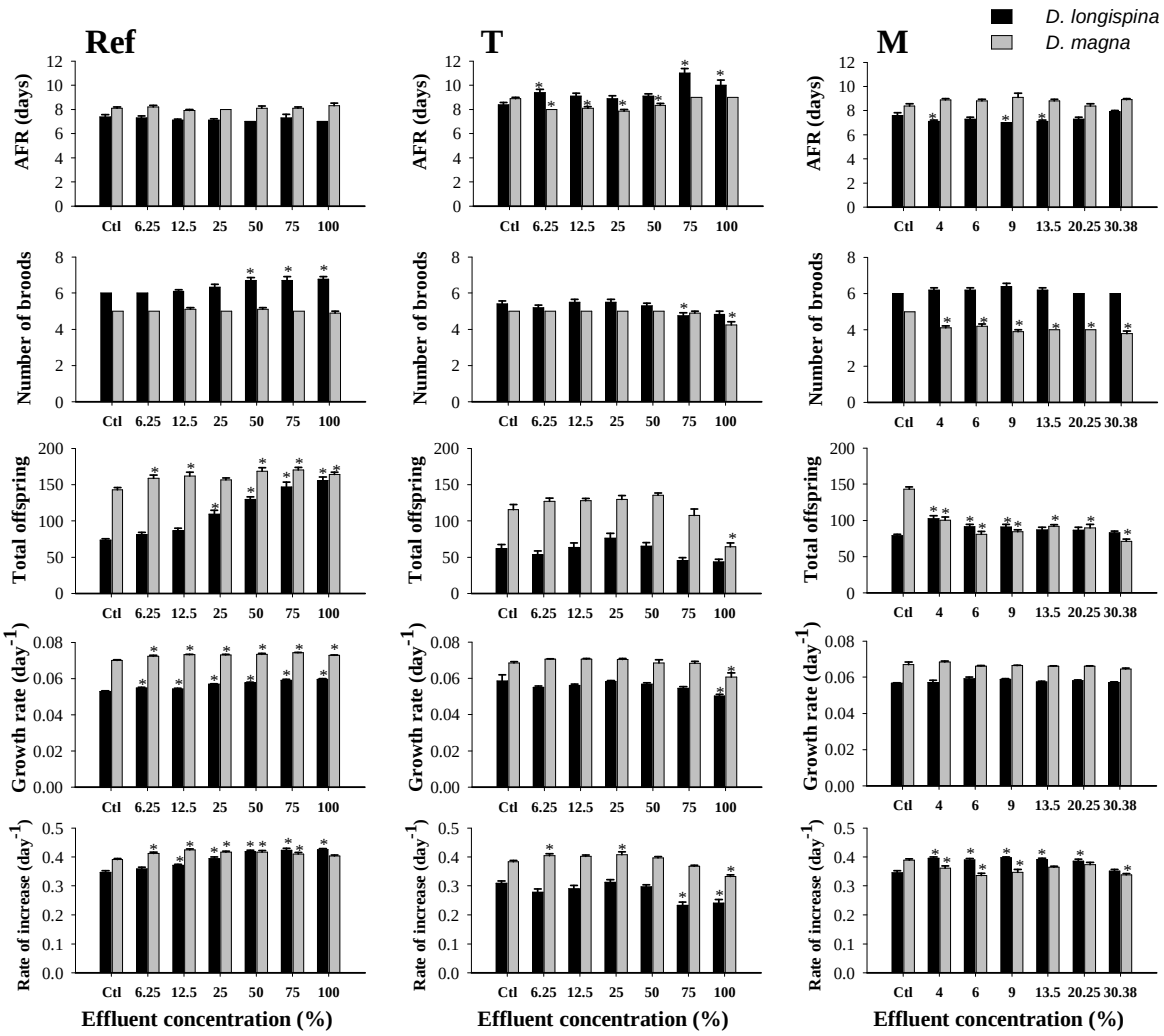

Figure 1 - Life history responses of *Daphnia longispina* and *Daphnia magna* exposed for 21 days to several concentrations of water from three mine ponds (Ref, T and M). The error bars correspond to the standard error and * represents statistically significant differences (Dunnett test, $P \leq 0.05$) between the different water effluent concentrations and the control (Ctl).

DISCUSSION

This work provides the first published data on the toxicity of natural waters from Cunha Baixa uranium mine. Toxicity data on uraniferous effluents are limited and most information is only available from laboratory assays with single-metal (uranium) solutions (*e.g.* Poston et al. 1984, Domingo 2001, Semaan et al. 2001, Sheppard et al. 2005). Poston et al. (1984) showed that the 48-h LC_{50} for *Daphnia magna* was between 1 and 10mg/L U. Semaan et al. (2001) observed acute toxicity values ranging between 160 and 390µg/L U for *Moinodaphnia macleayi* isolated from different field populations. Additionally, some works revealed that uranium has lower chemical toxicity when compared to other metals (Poston et al. 1984). In natural waters, uranyl ion [U(VI) – UO_2^{2+}] is the predominant form of U, but its

speciation is complex and highly dependable on pH and hardness (see Poston et al. 1984, Franklin et al. 2000, Riethmuller et al. 2001, Charles et al. 2002, Kuhne et al. 2002). Usually, a decrease in the toxicity of U has been reported concurrently to a decrease in pH (Franklin et al. 2000) and an increase in water hardness (Poston et al. 1984, Riethmuller et al. 2001, Charles et al. 2002), probably due to a reduction in the uptake of UO_2^{2+} at the cell membrane surface, due to a competition with ions with more affinity to cellular ionic channels (Riethmuller et al. 2001). The formation of U complexes with inorganic ligands (carbonate, phosphate or hydroxide ions) also decreases toxicity by reducing its bioavailability, since uranyl ion is its most toxic form (Fortin et al. 2004).

Mine effluents are usually rich in metals but also in inorganic compounds that affect their bioavailability and toxicity, as discussed above. In fact, our data showed that the highest concentrations of metals in the effluent water were observed concurrently with high concentration of Ca^{2+} and Mg^{2+} and H^+ (low pH). High levels of U and other metals (Al, Mn) were observed even in the treatment (T) pond, showing the ineffectiveness of the precipitation (with barium chloride) and neutralization of the effluent in reducing metal concentrations in solution (see also Poston et al. 1984). The mixture of metals present in the effluent, along with low pH, Ca^{2+} and Mg^{2+}, as well as the potential complex speciation of these metals (particularly U), demonstrates the complexity of studying these systems and understanding the potential hazards. Tables 1 and 2 clearly show the complexity of the mixture of metals present in the studied mine effluent. Al, Zn, U and Mn presented very high concentrations in the effluent water from T and M, therefore future mitigation measures should take this into account, since metals besides uranium are also particularly toxic at low pH values.

Toxicity results for *Daphnia* were also somewhat complex. First of all, no acute toxicity was observed in Ref and T. Acute toxicity in M was higher for *D. longispina* (EC_{50} =28.4%) than for *D. magna* (EC_{50} =50.4%). Other studies in our laboratory have also showed lowest tolerance of the native cladoceran species to different xenobiotics, when compared to *D. magna* (Antunes et al. 2004, Pereira et al. 2007). According to Van Leeuwen (1995), this can be partly explained by the faster diffusion of chemicals usually recorded in smaller-sized species, due to their high surface-to-volume ratios. Albeit high metal concentrations, T pond produced no acute toxicity when compared to M, suggesting that, although ineffective in precipitating metals, the treatment employed on the effluent neutralises the pH and reduces contaminant bioavailability. Still, deleterious chronic effects were felt in T at the highest concentrations (see below). The rise in pH caused by lime and the subsequent complexation phenomena with hydroxide and chloride ions are the most likely explanatory factors for the

reduced toxicity in T. At the same time, the high toxicity observed in M can not be fully attributed to low pH, because pH was close to neutrality in the majority of the tested concentrations of M (with the exception of the 100% treatment, where pH was 4.2). This neutralizing effect was due to the dilution water (ASTM hard water), which has a strong buffering capacity (a 60% dilution had a pH ≈ 6).

Chronic assays produced interesting profiles, although statistically confusing. A stimulatory effect on the life history responses of both species of daphniids was observed, a phenomenon that is relatively common when testing effluents (Sibley et al. 1997, Martinez-Madrid et al. 1999, Chapman 2000, Podemski and Culp 2001). This lead to an apparent hormetic curve (Calabrese 2002) for some endpoints in our study. A true hormetic effect is usually characterized by stimulation at low concentrations followed by strong inhibition at higher concentrations (Calabrese et al. 1987, 1998), an occurrence that was never clearly recorded for the endpoints analysed. The stimulatory effect could be due to the high concentration of bacteria (food source for *Daphnia*) in our samples, thus masking the effect of the contaminants at low concentrations. This was particularly evident in T and M where the intrinsic rate of increase (*r*) was stimulated at low concentrations, while decreasing in the highest concentrations. Additionally, it has been reported that low doses of radionuclides may induce hormesis (Calabrese et al. 1987), although we did not measure them in these ponds.

In Ref, the stimulatory effect was observed in all concentrations, leading to enhanced reproduction, growth and fitness (intrinsic rate of increase – *r*) in both species. In the treated effluent (T), almost all of the chronic endpoints for both species were significantly impaired at the highest concentrations, when compared to the control. Therefore, a delay in the timing of reproduction was observed, with a subsequent reduction in the number of broods for *D. longispina*, while a reduction in fecundity and growth rate was registered for *D. magna*. These deleterious effects produced significant decreases in the population growth rate of both species. Regarding the mine effluent (M), the only clear-cut deleterious chronic effect was observed in the fecundity (total number of offspring and total number of broods) of *D. magna* in M, where a reduction in the progeny was registered in all concentrations, relatively to the control. Although without a clear pattern, this impairment in fecundity was reflected on a significant reduction of the population growth rate at most concentrations. As previously discussed, *D. magna* is not a member of the receiving water communities and therefore may not be a good predictor of chronic effects on indigenous species. Surprisingly, stimulation in the fecundity and intrinsic rate of increase of *D. longispina* was recorded for the tested concentrations, with the exception of the highest ones. The factors behind this stimulation

have already been discussed and they were clearly responsible for masking toxic effects, since the acute EC_{50} for this pond (M) was 28.4%. In this case, an acute toxicological evaluation would have been preferable to predict the impact on potentially exposed populations. In brief, these results suggest that sublethal effects on the zooplankton communities of receiving waters should be expected if effluents (treated or untreated) are released in local freshwater resources with lower dilution capacity.

No previous aquatic toxicity data on the Cunha Baixa mine exist. It is therefore difficult to compare and discuss the effects of the uraniferous effluent to *Daphnia*. If we make the theoretical exercise of converting the obtained acute EC_{50}s (% effluent) in terms of uranium concentration (from Table 2, site M), it is possible to observe that the obtained EC_{50} equivalents do not differ much from the values obtained by Semaan et al. (2001), but are lower than the values attained by Poston et al. (1984) for uranium solutions. In this way, an EC_{50} of ≈ 700μg/L U and ≈ 399μg/L U would be obtained for *D. magna* and *D. longispina*, respectively. However, it is important never to forget that natural waters and mining effluents in particular, are complex mixtures of metals and other compounds (*see* Table 2). For this reason, interpretation of data concerning U is not as straightforward as simple mathematics. Further work is required to understand the intricate interactions between metals and other inorganic ions and organic ligands in complex mixtures. Other highly toxic metals for aquatic organisms, such as Al (Havens and Heath 1989, Kong and Chen 1995) were recorded at concerning concentrations, particularly in the mine effluent (pond M). Other metals, usually considered less toxic, can have their toxicity increased by the synergist action of other elements, when are part of complex mixtures (Otitoloju 2003).

Many mines (exploring uranium and other metals) are common in all of the NW of Portugal. An urgent intervention in some these mines was just identified by governmental institutions (Santos Oliveira et al. 1999). Cunha Baixa uranium mine was classified as one of the first priorities to receive intervention. However, there are still some difficulties to surpass before restoration, such as an effective evaluation of the ecological risks posed by this abandoned mine. Portuguese Quality Guidelines legally established for waters and soils are deficient for various metals, such as uranium, which poses a problem for environmental managers responsible for the decision-making processes on remediation measures. Therefore, additional site-specific information on the toxicity of the aquatic system (and also soil compartment) is required for the various trophic levels (bacteria, algae, macroinvertebrates, amphibians and fish), in order to assess the risks posed by point source discharges of this effluent in local freshwater resources. Some of these studies are already in progress within an

ecological risk assessment that is being performed for the area (e.g. Antunes et al. 2007). Although not focused on human health, this study also gives rise to concerns regarding human exposures via consumption of groundwater resources for drinking purposes, and the use of superficial waters for crop irrigation. These concerns are an additional justification to proceed with a more exhaustive ecotoxicological evaluation of the effluent water that is still being produced in the Cunha Baixa uranium mine, as well as the soil compartment of the surrounding area (current research in this field is now in progress in a funded research project from our team).

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Capítulo III

Journal of Soils and Sediments (aceite para publicação em 18/Agosto/07)

"Avaliação da toxicidade (aguda e crónica) em *Daphnia* de elutriados obtidos de sedimentos provenientes de uma mina de urânio abandonada"

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RESUMO

O sistema aquático da mina de urânio da Cunha Baixa comporta três lagoas artificiais: i) uma potencial lagoa de referência (Ref); ii) uma lagoa que inunda o buraco da mina (M), recebendo o efluente ácido resultante da lixiviação *in situ* do minério pobre; e iii) uma lagoa de sedimentação (T), onde o efluente ácido é neutralizado. Integrado na primeira etapa da análise de risco ecológico iniciada para este local, foi objectivo deste trabalho avaliar a potencial toxicidade dos sedimentos provenientes destas três lagoas. De modo a levar a cabo esta tarefa, foram produzidos elutriados com sedimentos recolhidos nas três lagoas em dois períodos distintos (Primavera e Inverno). A toxicidade aguda e crónica dos elutriados foi avaliada segundo protocolos padronizados, utilizando duas espécies de *Daphnia* (*D. magna* – espécie padrão e *D. longispina* – espécie índigena). Em oposição ao que seria previsto, baseado nas concentrações totais de metais, registou-se toxicidade aguda apenas para os sedimentos da lagoa M na Primavera (baixo valor de pH e valores elevados de metais) em ambas as espécies, tendo-se obtido valores de EC_{50} de 94,7% e 96,3% (*D. longispina* e *D. magna*, respectivamente). Nos ensaios crónicos, foram observados efeitos estimulatórios (no crescimento e fecundidade) para quase todas as concentrações dos três elutriados testados – excepto para as concentrações mais elevadas do elutriado de M. Algumas diferenças foram registadas nas respostas de *Daphnia*, tanto entre as espécies como entre períodos de amostragem (essencialmente no valor de pH). Embora se tenha registado toxicidade em M, na globalidade não foi observada toxicidade para os sedimentos do sistema aquático da mina, corroborando os resultados existentes. A toxicidade observada pode ser explicada pelas diferenças de pH, factor que actua indirectamente via mobilização dos contaminantes e directamente enquanto agente de stresse. Os fenómenos estimulatórios, característicos quando se trabalha com amostras naturais, funcionam na maior parte das vezes como factor de confusão. No entanto, algumas explicações são avançadas para explicar estes efeitos, mormente o papel estimulatório dos radionuclídeos (não medidos neste trabalho). Estes resultados e os de trabalhos anteriores parecem indicar que os sedimentos do sistema aquático da mina da Cunha Baixa desempenham um papel secundário na toxicidade na mina da Cunha Baixa, provavelmente funcionando mais como uma barreira do que como fonte de contaminantes para a coluna de água. De qualquer forma, a eventual remoção ou deposição dos sedimentos nesta área deve ser cuidadosamente ponderada, especialmente na lagoa M.

PALAVRAS-CHAVE

Elutriados, *Daphnia* sp., Minas de urânio, Toxicidade

"Evaluation of toxicity (acute and chronic) to *Daphnia* of elutriates prepared from sediments of an abandoned uranium mine"

ABSTRACT

Background, Aim and Scope. The superficial aquatic system of the Cunha Baixa uranium mine area is comprised by the flooded mine pit (M), which receives the acidic mine effluent resultant from *in situ* leaching of pore ore, a pond where this effluent is neutralised (T), and a potential reference pond (Ref). As part of the first tiers of an ecological risk assessment that is being performed in this area, the aim of this work was to evaluate the potential sediment toxicity of these ponds.

Methodology. To perform this work, elutriates were produced from sediments collected at the three ponds in two distinct seasons (Spring and Winter). Acute and chronic toxicity of elutriates was evaluated by standard assay protocols, using *Daphnia* spp. as test species (*D. magna* – standard species and *D. longispina* – native species).

Results. In opposition to what could be previewed based on total metal concentrations, results showed acute toxicity only in M site (low pH, high metal levels) in spring for both species with EC_{50}=94.7% and 96.3% (*D. longispina* and *D. magna* respectively). A stimulatory effect (in growth and fecundity) was observed in the chronic assays, for almost all of the tested concentrations of the three elutriates tested – except for the highest concentrations of the M elutriate. Some differences were observed in the responses of *Daphnia*, both between test species and seasons. Differences between the two sampling periods were also found for pH.

Discussion. Although some toxicity was observed in M, overall no toxicity was found for sediments of the aquatic system, corroborating previous results from our team (including whole-sediment tests). Differences in pH may explain the observed toxicity, acting both as a stressor and mobilizing contaminants. Stimulatory phenomena, typical when dealing with natural samples, worked as confounding factors. Several explanations should be considered for these stimulatory effects, however the role of radionuclides (not measured in this work) can not be ignored.

Conclusions. Supported by the results gathered in this study and in previous evaluations already performed, it is possible to state that in the present situation sediments plays a secondary role in the toxicity of the Cunha Baixa uranium mine, probably working more as

a barrier than as a source of contaminants to the water column. However, future reclamation works in this area should carefully consider the remobilisation of sediments, especially from pond M.

Recommendation and Perspectives. Radiochemical contamination is expected to be higher in field situations where radiation emitter isotopes are present in all the sediment compartment and overlying water, emitting radiations in all the directions. Thus whole-sediment and elutriate laboratory bioassays are not representative of long-term field exposures to radiation. Based on this supposition, these laboratory responses should be validated by field surveys of the benthic and planktonic freshwater communities of these ponds and freshwater receiving resources, at the higher tiers of the local ecological risk assessment.

KEYWORDS

Elutriates, *Daphnia* sp., uranium mine, toxicity

INTRODUCTION

Ecosystem contamination resultant from anthropogenic activities is a major ecological concern. Particularly, mining activities involve large environmental modifications in the landscape's outline, chemistry and biology. Abandoned or inactive mines are of particular concern and require continuous control and monitorization (Oliveira and Ávila, 2001). The major problem is the accumulation of tailings and pore ore with very high contents of heavy metals, which usually lead to the production of acid, metal-rich aquatic effluents. Cunha Baixa, Quinta do Bispo e Urgeiriça mines (Central, Portugal) were inserted in this problematic since they were used for radioactive ore exploration (mainly uranium), until 1993. These mining areas are presently classified as urgently needing intervention and their reclamation and environmental monitoring being of great public interest (Nero, 2003). The nature of the radioactive elements associated to their high migratory capacity increases their potential to be mobilized and transferred along the trophic chain, when physical and chemical conditions are favourable (Araújo et al. 1999; Chen et al. 2005; Pruvot et al. 2006). Mining activities are susceptible to cause serious disturbance in the trophic chains and, ultimately, this will reflect at the ecosystem level (Peplow and Edmonds, 2005).

The acid nature of the mine effluent usually results from sulphur (from metallic pyrites) oxidation. The low pH contributes for the increased velocity in the dissolution of toxic elements, favouring their transport in the shape of leachates (Rodrigues da Costa and Machado, 2000). Sediments act as sinks for these contaminants, due to complexation with organic materials, but pH shifts to acid in the effluent may remobilise them into the pore-water and subsequently into the overlying water column. The sediments are fundamental for the community's equilibrium, as they constitute a reservoir of organic and inorganic material, essential for many vital cycles (Wong et al. 1999). However, when disturbed they also act as a source of pollution to the ecosystems, a feature that is intimately connected with contaminant's bioavailability (Burton, 2002). This parameter depends on physical and chemical characteristics of pore-water (e.g. pH, alkalinity, hardness, redox potential) since these determine the solubility of chemical substances (Admiraal et al. 1995; Vaufleury and Pihan, 2002). The contaminant-sediment interactions are additionally complicated with the level of organic matter available (Allen et al. 1995) and with potential synergistic and antagonistic associations between contaminants (Pardos et al. 2000).

Because of the presence of complex mixtures of contaminants in sediments of mining areas and the potential for toxicological interactions among them, effects-based testing has been used to evaluate sediment toxicity (Bridges et al. 1996). Elutriates, in particular, are an indirect tool used to make a screening of the potential toxicity of sediments, and have the aim to make available the sediment-bound contaminants, thus liberating them to the aqueous phase were they become bioavailable for benthic and non-benthic aquatic organisms (Pardos et al. 2000). Many authors have used this tool to evaluate sediment toxicity in different organisms, from algae (Pardos et al. 1998) to bivalves (Geffard et al. 2002a) to cladocerans (Bridges et al. 1996). Primarily, elutriate sediment toxicity tests (ESTT) were developed as standard tools to simulate potential dredging hazards (USEPA, 1998). Several works provided some information corroborating the danger of dredged materials, using ESTT (Hyötyläinen and Oikari, 1999; Geffard et al. 2002b; Mucha et al. 2003).

The aim of this work was to evaluate the toxicity of elutriates produced from sediments of three ponds from the aquatic system of the inactive Cunha Baixa uranium mine. Ecotoxicological data were obtained from acute and chronic toxicity testing using two daphniids (Cladocera: Daphniidae): *Daphnia magna* (standard test-organism) and

Daphnia longispina (native species). The incorporation of indigenous *taxa* in ecotoxicological assessments is important to increase the ecological relevance of the data. The purpose of the study was to use elutriates as a tool to simulate the remobilisation of sediment-bound contaminants to the water column, thus assessing their potential toxicity to planktonic organisms from local freshwater resources, in case of mechanical disturbance (e.g. rainfall, human activities). This study and others (Antunes et al. 2007a,b) is part of the ecotoxicological evaluation of the aquatic system of the Cunha Baixa uranium mining area that are being performed within the first tiers of their ecological risk assessment.

MATERIAL AND METHODS

Study site

The uranium mine is located in Cunha Baixa (Centre of Portugal), and is included in the central uraniferous belt of the Iberian Peninsula between two systems of conjugate fissure (N40ºE and N70ºW with inclination the 75ºW) (Santos Oliveira and Ávila, 1998). The uranium mine was explored from 1967 until 1993 (Oliveira and Ávila, 2001). The mining activities left three temporary ponds: i) a reference pond (Ref), located upstream of the mine pit; ii) a treatment pond (T), which receives and neutralises (with lime and barium chloride) the acid effluent from underground tunnels; iii) the mine pit pond (M), which floods the underground exploration pit. In years of severe drought these ponds may disappear completely, but M pond can be surprisingly replenished by the acid mine effluent rising from due to the rise of the aquifer.

Elutriate preparation and characterisation

Sediments were collected from each pond in two moments: spring and winter. They were transported to the laboratory, sieved through a 2mm mesh and stored at 4ºC in the dark. Elutriates were prepared and tested within the following 8 weeks, as recommended by USEPA (1998). For the preparation of elutriates, a 1:4 (w/v) ratio of natural sediments to ASTM medium was used, which were shaken mechanically for 12hr at room temperature, followed by a 12hr deposition period. The overlying water (elutriate) and settled material were separated by decanting. Elutriates were filtered through a Whatman GF/C filter (1.2µm porosity, 47mm diameter) to remove suspended matters and stored at 4ºC until the assays were performed (maximum storage time: one week). In order to

produce a range of concentrations for the acute and chronic assays, elutriates were successfully diluted using the culture medium for *Daphnia* (see Toxicity assays).

Elutriates were prepared for metal analysis by immediate acidification with nitric acid to pH<2 (to minimize sorption of metals to the wall of the containers) and storage at 4ºC. Elutriates were analysed for selected metals using ICP spectrometry (APHA et al. 1995). At the onset of the toxicity assays, a range of physical and chemical parameters were quantified in aliquots of the elutriate samples: pH, conductivity, total phosphorous (persulfate digestion followed by ascorbic acid method; APHA et al. 1995), total nitrogen (persulfate digestion followed by cadmium reduction method; APHA et al. 1995), calcium and magnesium (both by titration with EDTA; APHA et al. 1995). Additionally, the total density of bacteria present in elutriates was quantified as colony forming units (CFU) of bacteria grown in TSA (tryptic soil agar) plates at 37ºC for 48hr.

Toxicity assays

Monoclonal cultures of *D. magna* (clone A, *sensu* Baird et al. 1989a) and *D. longispina* (clone EM7, *sensu* Antunes et al. 2003) were reared under a 16^{L}:8^{D} h cycle and a temperature of 20±2ºC. Daphniids were cultured in ASTM (1980) synthetic hardwater medium enriched with an organic additive (Baird et al. 1989b) and were fed with *Selenastrum capricornutum* (presently *Pseudokirchneriella subcapitata*) for several generations in our laboratory. All experiments were initiated with neonates (<24h old), born between the 3rd and 5th broods, which were obtained from group cultures.

Independent experiments were used to assess the acute toxicity of elutriates to both *D. magna* and *D. longispina*. Tests were performed in accordance with standard protocols (ISO, 1996; ASTM, 1997; OECD, 2000), under the same temperature and photoperiod regimes as described for rearing procedures. A static design was employed using 20 animals (randomly divided into four groups of five animals) per control and per elutriate concentration (%). Test vessels (four per each treatment) consisted of glass beakers containing 100ml of test elutriate concentration. For each combination of species, 6-7 test concentrations were obtained by dilution with ASTM hard water medium. Range-finding bioassays were carried out to set appropriate dilutions (Ref and T: 0.0, 25.0, 50.0, 75.0, 100.0%) to obtain EC_{50} values with the best confidence interval. Final concentrations for M pond in Spring sampling were: for *D. longispina* (78.75-95.72%, separated by a factor

of 1.05), for *D. magna* (94.06-98.86% separated by a factor of 1.01). Oxygen concentrations and pH levels in test vessels were determined at 0, 24 and 48h as quality criteria. All experimental treatments were checked for immobilised individuals at 24 and 48h, which were counted for posterior determination of EC_{50} values (using probit analysis – Finney, 1971).

Chronic assays were conducted for 21 days in accordance with standard protocols (ASTM, 1997; OECD, 1998; ISO, 2000), under the same temperature and photoperiod regimes as described for rearing procedures. A semi-static design was employed, using 10 individualised animals randomly assigned to the control (Ctl) and to each elutriate concentration (6.25, 12.50, 25.0, 50.00, 75.00 and 100.00%, except for M in Spring - 6.25, 12.50, 25.0, 50.00, 62.5 and 75.00%). Test vessels (10 per each treatment) consisted of glass beakers containing 50ml of test solution (including organic additive). Daphniids were transferred to newly-prepared elutriate concentrations every other day, and were daily fed with their respective *P. subcapitata* ration. Animals were checked daily for mortality and reproductive state and, if neonates had been released, they were counted and immediately discarded. The following parameters were registered: age at first reproduction (AFR), total number of offspring, somatic growth rate, and rate of population increase. The somatic growth rate was estimated from the initial and final body size of the daphniids, according to the following expression:

$$growth\ rate = \frac{\ln(l_f) - \ln(l_i)}{\Delta t}$$

where l_f is the body size (in mm) of the test organism at the end of the test, l_i is the average body size (in mm) of a subsample (n=20) of neonates coming from the same batch of neonates that initiated the test, and Δt is the time interval (in days). This daily growth rate was expressed in day^{-1}.

Survival and fecundity estimates were used to compute the rate of population increase (r), which was iterated from the Euler–Lotka equation:

$$1 = \sum_{x=0}^{n} e^{-rx} l_x m_x$$

where r is the rate of population increase (day^{-1}), x is the age class in days (0. . .n), l_x is the probability of surviving to age x, and m_x, is the fecundity at age x. Standard errors for r were estimated using a jack-knifing technique described by Meyer et al. (1986). Data from

each endpoint were analysed using a one-way analysis of variance (ANOVA), followed by a Dunnett test (if applicable), in order to determine significant effects of elutriate concentration on the life history of *Daphnia*.

RESULTS AND DISCUSSION

Physical, Chemical and Bacteriological parameters

The variation in the physical, chemical and bacteriological parameters of elutriates from the three ponds and ASTM hard water medium is presented in Table 1. In general, the pH of elutriates was close to neutrality, being more acidic in spring in M (pH≈5). High levels of conductivity and Ca^{2+} were observed in pond T in both sampling periods when compared to the other sites. Values of TP observed were low and similar in all ponds at the two sampling periods. T and M elutriates presented low values of TN, except in Ref-winter (14mg/L). High values of bacteria were found in all elutriates produced from natural sediments, being generally higher in winter. Overall, elutriates did not present high levels of metals (Table 2), with the exception of Mn, which exceeded the Maximum Recommendable Value for the three ponds in the winter and for M in spring. Sr and U (chiefly), elements for which no established legal limits exist, also seemed to show high total concentrations in the elutriates from the three ponds.

Table 1 - Physical, chemical and bacteriological elutriate quality data for the three ponds (Ref, T and M) and dilution water (ASTM hard water medium).

	ASTM		Ref		T		M	
	Spring	Winter	Spring	Winter	Spring	Winter	Spring	Winter
pH	7.6–7.8	7.6–7.8	≈ 7.6	≈ 6.6	≈ 7.7	≈ 7.5	≈ 5	≈ 7.5
Cond (µS/cm)	550	550	≈ 500	≈ 340	≈ 900	≈ 750	≈ 390	≈ 522
TP (mg/L P)	0.02	0.02	0.07	0.33	0.09	0.05	0.08	0.03
TN (mg/L N)	0.11	0.07	1.20	14.00	0.40	1.50	1.20	1.80
Ca^{2+} (mg/L)	40.8	29.8	34.4	27.6	101.4	135.2	25.2	79.,0
Mg^{2+} (mg/L)	18.0	20.3	11.0	5.4	40.1	34.0	86.7	12.0
CFU (ml^{-1})	$6x10^2$	$2x10^2$	$4x10^4$	$3x10^5$	$2x10^4$	$1x10^4$	$6x10^3$	$6x10^4$

Table 2 - Average metal concentrations in μg/L (n=3) observed in elutriates from the three ponds (Ref, T and M), analysed by inductively coupled plasma mass spectrometry (ICP/MS) in the two sampling periods (S – spring; W - winter). MRV stands for Maximum Recommendable Values to waters for crop irrigation (MA, 1998) (NA = not available). Bold values show metal levels exceeding the MRV to waters for crop irrigation.

	Ref		**T**		**M**		
	S	W	S	W	S	W	**MRV**
U	**1225**	<100	**3200**	**1033**	**269**	**4254**	NA
Cd	<0.1	<0.1	<0.1	<0.1	0.52	<0.1	10
Zn	29	42	15	39	114	25	2000
Ba	36	34	23	39	26	58	1000
Mn	112	**2377**	18	**675**	**1160**	**1114**	200
Co	<0.05	9	0.72	10	19	10	50
Be	<0.05	<0.05	<0.05	0.6	2.4	0.6	500
Al	84	<1	40	<1	246	<1	5000
Ni	1.5	<6	2.7	<0.2	25	20	500
Sr	109	67	517	236	58	113	NA

Acute toxicity

In fact, one of the main advantages of elutriate toxicity tests is the possibility given for comparison with water quality standards (Giesy et al., 1990) and once more it was realized that chemical data alone cannot be used for toxicological prediction. Total uranium concentrations in all the elutriates were well above the EC_{50} for reproduction endpoints (0.52 mg/l), reported in the review made by Sheppard et al. (2005) of the U toxicity data for freshwater invertebrates. However, no acute toxicity was found in ponds Ref and T, in both sampling periods. In pond M, no toxicity was observed in winter, but in spring acute toxicity was observed for both species: *D. longispina* – EC_{50} = 94.7% (CI_{95}: 80.1% - 110.9%); *D. magna* – EC_{50} = 96.3% (CI_{95}: 95.6% - 97.0%). Still, significant immobilisation only occurred at elutriates concentrations close to 100%. Considering only the physical and chemical parameters, namely pH (≈5.0), the values differed between the two sampling periods. Acidification is one of the principal causes of aquatic contamination, not only for the toxic effects, but also due to its capacity to mobilize other contaminants (Lopes et al. 1999). Thus, contaminants may become accessible and be transferred through the trophic chain (Araújo et al. 2002; Chen et al. 2005; Pruvot et al. 2006). The chemical treatment used in T demonstrated a positive outcome, as no acute toxicity was observed in the corresponding elutriate. However, in M the water was acidic, due to the rise of the mine effluent yielded by past *in situ* leaching of pore ore with

sulphuric acid. This mine effluent rich in heavy metals from the flooded mine fills the pond when the aquifer is recharged. This increase in acidity of the pond water may also cause remobilization of heavy metals associated to the sediment (Oliveira et al. 1999). Uranium, manganese and strontium were the principal elements occurring in anomalous levels in Cunha Baixa uranium mine (namely in sediments and water). Additionally, the concentration of Cd reported in spring was above the chronic limits for *Daphnia magna*, as reported by USEPA (2001) at different hardness values. Soucek et al. (2000) already described that a complex mixture of heavy metals in samples from mines present high toxicity values, most of the time associated with low pH values.

Chronic toxicity

Chronic toxicity data showed the existence of reproductive adverse effects, but also stimulatory phenomena (Figures 1-3 and Table 3).

Table 3 - Summary table of the one-way analyses of variance applied to the life history responses of the daphniids. Age at first reproduction (AFR), total offspring (TO), somatic growth rate (SGR) and rate of increase (*r*) were analysed for the effect of water effluent concentration independently for each site. NS = non significant.

Site	Endpoint	*D. magna*						*D. longispina*					
		Spring			Winter			Spring			Winter		
		F	d.f.	*P*	F	d.f.	*P*	F	d.f.	*P*	F	d.f.	*P*
Ref	AFR	0.43	6, 63	NS	0.61	6, 60	NS	0.64	6, 53	NS	0.32	6, 62	NS
	TO	1.32	6, 63	NS	7.74	6, 60	<0.001	44.0	6, 53	<0.001	26.1	6, 62	<0.001
	SGR	2.80	6, 3	0.018	7.11	6, 60	<0.001	20.3	6, 53	<0.001	15.4	6, 62	<0.001
	r	0.39	6, 63	NS	2.41	6, 63	0.037	3.31	6, 63	0.007	5.91	6, 63	<0.001
T	AFR	0.31	6, 62	NS	1.61	6, 62	NS	2.36	6, 57	0.041	0.40	6, 60	NS
	TO	8.57	6, 62	<0.001	5.08	6, 62	<0.001	20.9	6, 57	<0.001	10.7	6, 60	<0.001
	SGR	11.5	6, 62	<0.001	1.32	6, 62	NS	30.5	6, 57	<0.001	7.26	6, 60	<0.001
	r	3.07	6, 63	0.011	1.15	6, 63	NS	5.19	6, 63	<0.001	1.85	6, 63	NS
M	AFR	4.22	6, 60	0.001	14.2	6, 62	<0.001	9.23	6, 61	<0.001	6.58	6, 60	<0.001
	TO	5.74	6, 60	<0.001	32.8	6, 62	<0.001	16.0	6, 61	<0.001	50.8	5, 51	<0.001
	SGR	4.16	6, 60	0.001	24.9	6, 62	<0.001	4.37	6, 61	<0.001	26.5	5, 51	<0.001
	r	4.23	6, 63	0.001	20.4	6, 62	<0.001	23.4	6, 63	<0.001	25.7	6, 63	<0.001

Elutriate from Ref produced a significant stimulation for both species in the two sampling periods (Figure 1), with an increased production of offspring in the highest

concentrations, except for *D. magna* in spring. Statistically higher somatic growth rates, when compared to the control, were also observed in the two sampling periods in the highest concentrations, except for *D. magna* in winter. The intrinsic rate of increase was significantly stimulated only for *D. longispina* in the winter.

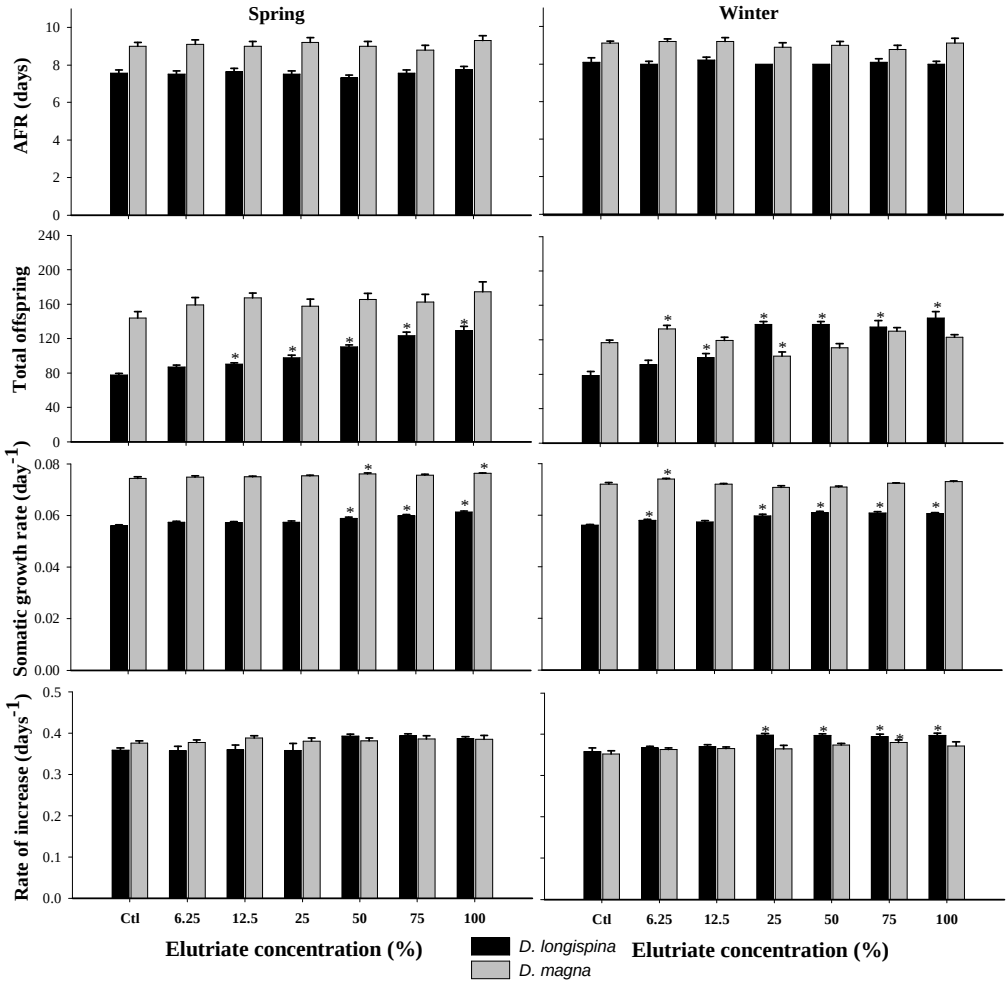

Figure 1 - Life history responses of *Daphnia longispina* and *Daphnia magna* exposed for 21 days to several concentrations of water from pond Ref in the two periods (Winter and Spring). The error bars correspond to the standard error and *represents statistically significant differences (Dunnett test, P≤0.05) between the different water effluent concentrations and the control (Ctl).

Similarly to Ref, stimulation of reproductive and growth parameters was observed in T, mostly in spring for both species (Figure 2 and Table 3). In winter, however, a significant decrease of total offspring was observed only for *D. longispina*. Nonetheless, an apparent stimulation was recorded in the somatic growth rate of the same species exposed to elutriate concentrations above 12.5%. In spite of the toxic effect observed in the number of offspring produced, no statistical differences were observed for the rate of increase in

winter. Hence, it seems that this adverse effect at the individual level may be compensated by other strategies that reduced the effect at the population level.

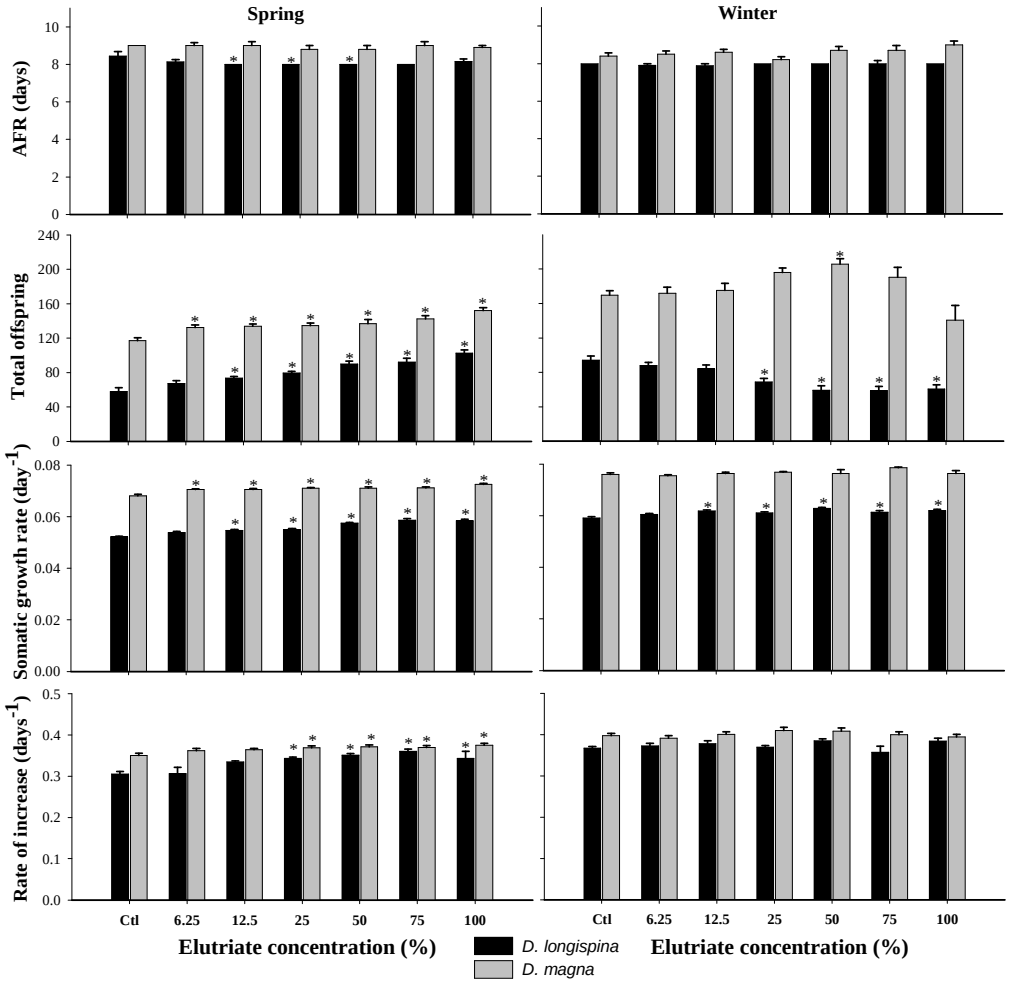

Figure 2 - Life history responses of *Daphnia longispina* and *Daphnia magna* exposed for 21 days to several concentrations of water from pond T in the two periods (Winter and Spring). The error bars correspond to the standard error and *represents statistically significant differences (Dunnett test, P≤0.05) between the different water effluent concentrations and the control (Ctl).

Assays with elutriate from pond M revealed a response pattern more difficult to interpret, for both species (Figure 3 and Table 3). First of all, the winter elutriate (100% concentration) was acutely toxic to all *D. longispina* organisms that died after the release of the third brood. However, this did not compromise the population growth rate (see *r*), since this parameter integrates both reproduction and survival, and the size of the first brood was large (in average 54 neonates per female). The life history parameters of both daphniids showed an overall hormetic-like curve, but without reaching a significant toxic effect in the highest concentrations. Earlier reproduction was observed for *D. magna* in

spring sampling and in winter for *D. longispina*, for intermediate concentrations, while the highest concentration tested caused a significant delay in the reproduction of *D. longispina* (in spring) and *D. magna* (in winter). In spite of the hormetic-like curve, significant stimulatory effects were recorded in the intrinsic growth rate of increase of both species, at almost all the spring and winter elutriate concentrations. The only exception was observed in *D. magna*, where *r* was significantly reduced at 100%, comparatively to the control.

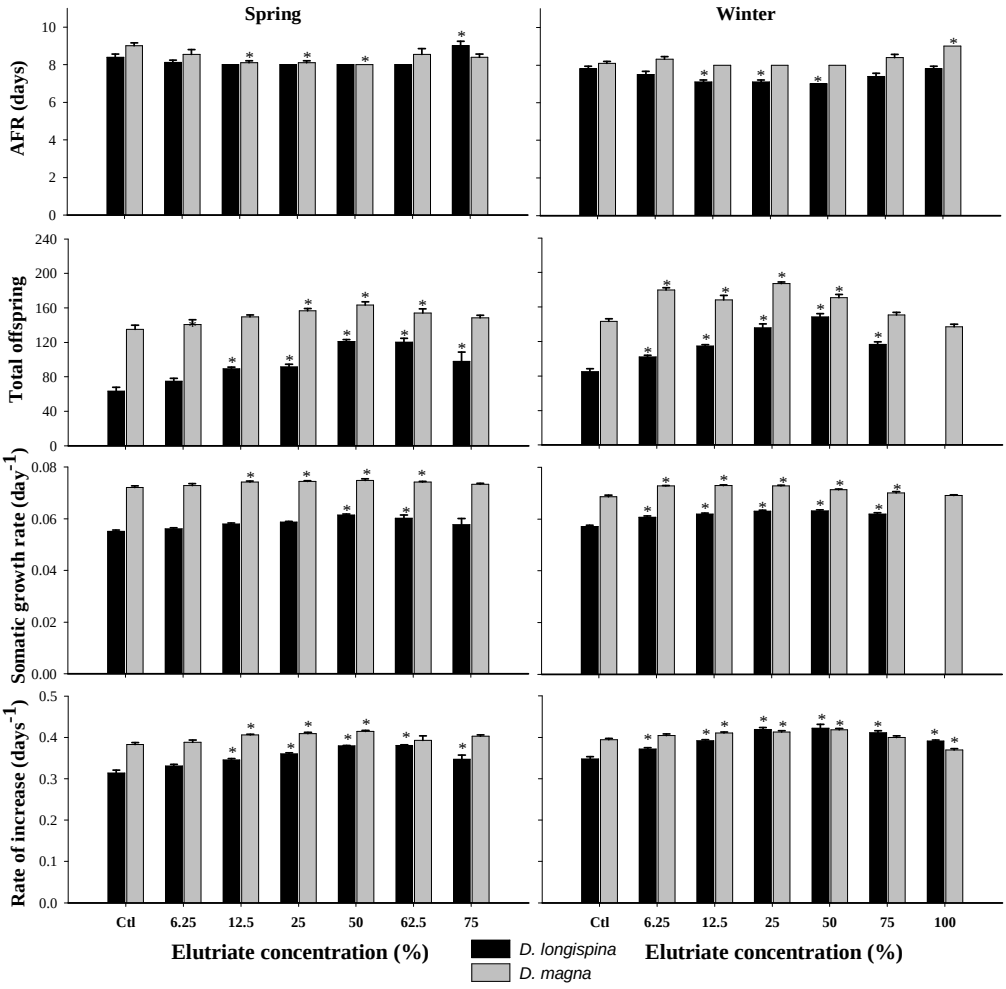

Figure 3 - Life history responses of *Daphnia longispina* and *Daphnia magna* exposed for 21 days to several concentrations of water from pond M in the two periods (Winter and Spring). The error bars correspond to the standard error and *represents statistically significant differences (Dunnett test, P≤0.05) between the different water effluent concentrations and the control (Ctl).

Sediments accumulate contaminants and serve as source of pollution to the ecosystems they are connected to. Metals, as one example, tend to sorb onto organic and inorganic materials that eventually settle in depositional areas. Thus, the sediment may accumulate excessive quantities of contaminants that directly and indirectly disrupt the

ecosystem, causing significant contamination and loss of desirable species (Burton, 2002). During the course of time, contaminants accumulate in the sediments and usually form biologically unavailable complexes, in equilibrium with the surrounding aquatic environment. Any physical disturbance (dredging, strong winds, heavy rainfall) caused in the sediments may alter this equilibrium and produce a release of contaminants to the water column. A consequent increase in toxicity may be verified, as contaminants are made bioavailable. In order to evaluate the potential consequences of these phenomena, it is common to simulate re-suspension events with elutriates and/or extractable pore-water, which are then tested with aquatic organisms (e.g. toxicity assays with *Daphnia* sp.). Nevertheless, some authors verified that assays with pore-water were more realistic that elutriates, and it was demonstrated that pore-water was more toxic than corresponding elutriates (Ankley et al. 1991; Sibley et al. 1997). On the other hand, other authors considered that elutriate and solid-phase tests combined are a valuable tool because the combination shows when the sediment is toxic and if it is releasing dissolved contaminants to the overlying water (Nebeker, et al. 1984). Regarding this particular subject, a whole sediment toxicity test with *Chironomus riparius* for pond M revealed no toxic effects (Antunes et al. 2007a). Therefore, the biological effects recorded in this study for sediments elutriate from the same pond may be explained by differences in species sensitivity or by the release of unavailable sediment-bound contaminants, during elutriate preparation.

In spite of expected negative effects in terms of life-history parameters in contaminated sediments, in general our results demonstrated the opposite. This pattern was already observed in other works, which recorded an increase in total offspring and adult final biomass in *D. magna* when exposed to elutriate (Martinez-Madrid et al. 1999). Occasionally, an apparent stimulatory effect was observed, as previously described for natural samples (Chapman, 2000). On the other hand, studies with natural samples also indicate that the presence of suspended particles namely in elutriates, may confound toxicity evaluations in cladocerans (Bridges et al. 1996). Nevertheless, in locals with poor contamination and a high bioavailability of oligoelements, a general stimulatory effect may be observed in the growth of the organisms (Sibley et al. 1997). In our work, we observed high contents of bacteria in all elutriates, which can work as a way to mask the toxic effects associated to the sediments. Since *Daphnia* feed on suspended particles of organic

matter (Lampert, 1987; Ojala et al. 1995), bacteria and some algae (Ojala et al. 1995; Michels and De Meester, 1998), this increment in bacteria may produce the stimulatory effect described (at low concentrations), masking potential toxic effects at high concentrations. In fact, in elutriate from pond M, a decrease in the rate of increase was observed only in the highest concentration, but a stimulation of this parameter had been recorded in the previous elutriate dilutions. This was also the case for the reproductive delay observed in *D. longispina* and *D. magna* (spring and winter, respectively) at the highest concentration. Other authors have mentioned the stimulatory effects of low concentrations of radionuclides (Calabrese et al. 1987). Although these elements were not measured, their existence mainly in the M and T ponds is unquestionable and their contribution to the stimulatory effects recorded on daphniid growth and reproduction endpoints can not be ignored. Therefore, it is possible to conclude that elutriate from pond M contained bioavailable contaminants able to yield toxic effects on freshwater planktonic organisms. However, many confounding variables that can not be controlled seem to be present in natural samples, thus masking toxicity.

CONCLUSIONS

Once more sediment elutriates have found to be a powerful tool to screen the potential toxicity of contaminated sediments, in the first tiers of the ecological risk assessment of this uranium mine area. The three elutriates tested demonstrated a stimulant effect on cladocerans, either in growth or in reproductive and demographic parameters. No acute toxicity was found in Ref and T pond. These results indicate that sediment-bound contaminants do not pose environmental concern in terms of acute toxicity. However, this was not the case in pond M, where acute toxicity was observed in spring, probably heightened by low pH that has increased the mobilisation of metals to the aqueous phase of the sediments. In light of our results, and also those concerning water toxicity, as observed by Antunes et al. (2007b), it is possible to state that, in the present situation, sediments play a secondary role on the toxicity of the Cunha Baixa mine effluent, probably working more as a barrier than as a source of contaminants to the water column. Thus, we may say that the main source of contaminants seems to be the groundwater, rather than the sediments, which worked as deposit of mine tailings. However, future reclamation measures should

carefully considered the remobilisation of sediments from pond M, since particle-bound contaminants may become bioavailable to exert their toxic effects.

RECOMMENDATIONS AND PERSPECTIVES

Radiochemical contamination is expected to be higher in field situations where radiation emitter isotopes are present in all the sediment compartment and overlying water, emitting radiations in all the directions. Thus whole-sediment and elutriate laboratory bioassays are not representative of long-term field exposures to radiation. Based on this supposition, these laboratory responses should be validated by field surveys of the benthic and planktonic freshwater communities of these ponds and freshwater receiving resources, at the higher tiers of the local ecological risk assessment.

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Capítulo IV

Science of the Total Environment (2007) 374(2-3): 252-259

"Avaliação toxicológica da coluna de água e sedimento de uma mina de urânio abandonada usando uma bateria de ensaios"

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RESUMO

A mina da Cunha Baixa, Mangualde (Portugal) teve uma exploração intensiva de urânio desde 1967 até 1993, com grande produção de minério pobre. Esta exploração deixou milhões de toneladas de escória a céu aberto na área envolvente, perto de zonas habitacionais. A contaminação da área (compartimento aquático e terrestre) representa um sério risco para os humanos e vida selvagem. Este trabalho apresenta uma primeira abordagem integrada da caracterização dos potenciais riscos e compartimento ambiental de destino dos contaminantes no sistema aquático na mina da Cunha Baixa. Assim, definiu-se como objectivo principal do presente trabalho avaliar a toxicidade aguda da água e sedimento de uma lagoa formada no antigo buraco da mina, em dois períodos de tempo distintos (Primavera e Outono). Concentrações elevadas de metais foram encontradas nas amostras de água (nomeadamente Mn, Fe, Al, U, Sr). Foi utilizada uma bateria de bioensaios (crescimento algal, imobilização em *Daphnia*, crescimento e sobrevivência em *Chironomus riparius*) para avaliar a toxicidade aguda nos diferentes compartimentos. Os resultados obtidos revelaram que o sedimento não apresentou toxicidade, ao contrário da água. A toxicidade da água foi mais elevada no Outono, quando o efluente se apresentou mais ácido, quando comparado com a Primavera. Nos ensaios de toxicidade com amostras de água, a sensibilidade relativa das espécies utilizadas foi *Daphnia longispina* > *Pseudokirchneriella subcapitata* > *Daphnia magna*. Este trabalho faz parte de um estudo mais abrangente, envolvendo a caracterização química e ecotoxicológica do compartimento aquático da mina da Cunha Baixa, estando integrado na primeira etapa da Análise de Risco Ecológico em curso para a área.

PALAVRAS-CHAVE

Mina de urânio abandonada, efluente ácido, toxicidade aquática, toxicidade de sedimentos, bateria de bioensaios.

"Evaluation of water column and sediment toxicity from an abandoned uranium mine using a battery of bioassays"

ABSTRACT

Uranium mining activities in Cunha Baixa, Mangualde (Portugal), were extensive between 1967 and 1993, with high production of poor ore. Ore exploitation left millions of tons of tailings in the surrounding area, close to human houses. Contamination of the area (water and soil compartment) presently represents a serious hazard to humans and wildlife. The aim of this work was to evaluate the acute toxicity of water and sediments from a pond that floods a uranium mine pit, in two periods (spring and autumn). High contents of metals were found in water samples (chiefly Mn, Fe, Al, U, Sr). A battery of assays was applied to screen the acute toxicity of the different compartments using algae, crustaceans and dipterans. Results showed that the sediments were non-toxic, unlike the superficial water. Water toxicity was higher in the autumn, when the effluent was more acidic, compared to spring. In the water toxicity assays, the relative sensitivity of the test species used was *D. longispina* > *P. subcapitata* > *D. magna*. The present study is part of the chemical and ecotoxicological characterisation of the aquatic compartment performed in the tier 1 of the Ecological Risk Assessment of the Cunha Baixa mining area.

KEYWORDS

Abandoned uranium mine, Acid effluent, Metals, Water toxicity, Sediment toxicity, Battery of bioassays

INTRODUCTION

Several Ecological Risk Assessment (ERA) schemes of contaminated sites follow a tiered approach in which progression through a series of tiers results in a greater improvement in the quantity and quality of data gathered and also a progressive reduction in uncertainty (Weeks et al., 2004). In this framework, after the conceptualization of a site specific model (Tier 0) in which chemicals of potential concern (COPCs) are identified, their concentrations in the different environmental compartments are compared with guidance values, for each chemical individually, and risks are added (Tier 1). However, complex mixtures of pollutants (e.g. metals, pesticides, PAHs) are a widespread concern

for all ecosystems. When these mixtures are present, false-negatives or false-positives may be obtained in this chemically-based Tier 1 because potential synergistic or antagonistic effects are not accounted for. Thus, time- and cost-effective toxicity tests may be a useful tool at this step to reduce uncertainty and the possibility of an incorrect exclusion of sites for a more detailed evaluation (Weeks et al., 2004). Given the relevant role of toxicity tests in ERA, it is essential to gather as much ecotoxicological data as possible. One of the ways to do so is to use a comprehensive battery of toxicity tests, integrating different levels of the trophic chain, as well as different environmental compartments. Slabbert and Venter (1999), Castillo et al. (2000) and Costan et al. (1993) already supported the use of a battery of assays using different key species in the trophic chains to evaluate the toxicity of effluents.

The aquatic environment usually represents the final destination of contaminants from problematic areas, where they can affect local biota, directly or indirectly (Fleeger et al., 2003). Algae, crustaceans, insect larvae and fish are the most commonly used test species in aquatic ecotoxicology, being recommended by several authors (Nebeker et al., 1984; OECD, 1984, 2000a,b; Taylor et al., 1991; Environment Canada, 1992; USEPA, 1994; Ingersoll et al., 1995; ISO, 1996; ASTM, 1997, 2000). Primary producers are the first link of aquatic systems and adverse impacts on them may have important consequences for the health of the whole aquatic ecosystem (Franklin et al., 2000). Growth inhibition tests with unicellular algae (e.g. *Chlorella*, *Chlamydomonas*, and *Pseudokirchneriella*) have long been used to evaluate the bioavailability and toxicity of water-borne contaminants (Franklin et al., 2000; Baun et al., 2002; Charles et al. 2002). Although some species have been successfully used by other authors for toxicity screening complex metal mixtures, including acid mine drainage effluents (Moreira-Santos et al., 2004), information on uranium effluent waters is scarce (Franklin et al., 2000; Charles et al., 2002; Hogan et al., 2005). Primary consumers (like cladocerans) are filter-feeding organisms and can be useful indicators of the bioavailability of particle-bound contaminants (Hyne et al., 1993). *Daphnia* (Crustacea, Cladocera), for example, is one of the most widely used organisms in Ecotoxicology. Benthic macroinvertebrates live or depend on the sediment, and many are detritivorous, being responsible for the recycling of organic matter but also for the transfer of contaminants to the water column. Chironomid larvae are in constant contact with the sediment (Peck et al., 2002) and are commonly used

as test organisms in the assessment of sediment toxicity (larval growth and survival bioassay). As for algae, most metal toxicity studies for cladocerans and chironomids refer to single-metal exposures and, although some studies have successfully used these organisms in the toxicity evaluation of complex effluents (Castro et al., 2003; Hickey et al., 1998; Pereira et al., 2000; Song et al., 2001), few of them addressed uranium mine effluents (Poston et al., 1984; Franklin et al., 2000; Hogan et al., 2005; Sheppard et al., 2005).

As part of the Tier 1 of the ERA of Cunha Baixa uranium mine (Viseu, Central Portugal), the aim of this study was to evaluate the toxicity of sediments and water from an effluent pond, which floods the underground exploration mine pit, using a battery of ecotoxicological bioassays. This is one of the first studies addressing an ecotoxicological evaluation of the aquatic system of a uranium mine, integrating both environmental compartments (water and underlying sediments). A parallel physical and chemical characterization was also carried out (including total metal concentrations). In order to account for seasonal fluctuations, analyses and toxicity bioassays were performed in two distinct periods: spring and autumn. Effluent water toxicity was evaluated by means of a growth inhibition bioassay with the microalga *Pseudokirchneriella subcapitata* and an immobilisation bioassay with two species of cladocerans (*Daphnia magna* and *D. longispina*). Sediment toxicity was assessed in whole-sediment bioassays with *Chironomus riparius* (larval growth and survival). Additionally, sediment toxicity was indirectly screened in extracted elutriates, using an acute bioassay with *Daphnia* spp.

MATERIAL AND METHODS

Study site

The abandoned uranium mine is located in the small village of Cunha Baixa (Mangualde, Viseu), in the centre of Portugal, and is included in the uraniferous belt of the Iberian Peninsula (Santos Oliveira and Ávila, 1998). The ore was extracted from 1967 to 1993 (Oliveira and Ávila, 2001) and the underground mine pit was filled with poor ore and flooded with sulphuric acid to extract uranium. The extraction of ore left a temporary pond, which floods the underground exploration pit. This pond is subjected to high variations in water level (including drought), as a consequence of the groundwater level of the aquifer. A more detailed description of the aquatic system of the Cunha Baixa mine is presented

elsewhere (Antunes et al., *in press*). The aquatic environment (pond and underground effluent) comprises a complex mixture of metals under low pH (Pedrosa and Martins, 1999; Antunes et al., *in press*).

Collection and preparation of samples

Water and sediment samples were collected from the pond in plastic containers and transported to the laboratory, where they were stored at 4ºC in the dark. Water samples were filtered through a Whatman GF/C filter (1.2µm porosity, 47mm diameter) until bioassays were performed (maximum storage time: one week). For algal bioassays, water was additionally filtered through a cellulose nitrate filter (0.2µm porosity, 47mm diameter). Samples for metal analysis were acidified with nitric acid to pH<2 to reduce adsorption phenomena and stored in plastic containers until the determinations were possible (see *Chemical analyses*).

In the laboratory, sediment samples were visually checked and visible indigenous fauna and large debris (leaves, etc.) removed with forceps. Sediment was then stored at 4ºC in the dark for a period of two weeks until it was used, either for elutriate preparation (see following lines) or for whole-sediment tests (see *Sediment bioassays*). Elutriates were prepared with a 1:4 (w/v) ratio of sediment to ASTM medium (see below), and shaken mechanically for 12h at room temperature, followed by a 12h deposition period. The overlying water (elutriate) and settled material were separated by decantation. Elutriates were filtered through a Whatman GF/C filter (1.2µm porosity, 47mm diameter) and stored (at 4ºC, in the dark) until the assays were performed (maximum storage time: one week).

Cultures of organisms

The microalga *P. subcapitata* is currently recommended as a standard species for algal toxicity tests (Environment Canada, 1992; OECD, 1984; USEPA, 1994). It was maintained in nonaxenic batch cultures with Woods Hole MBL medium, at 20±2ºC and with a 16^{L}:8^{D}h photoperiod. At the start of new cultures, algae were harvested while still in the exponential growth phase (5–7d old) and inoculated in fresh medium. Monoclonal cultures of *D. magna* (clone A, *sensu* Baird et al., 1989a) and *D. longispina* (clone EM7, *sensu* Antunes et al., 2003) were reared under a 16^{L}:8^{D}h cycle and a temperature of 20±2ºC. ASTM (1980) synthetic hard water medium was used as culture medium and

rearing procedures followed the recommendations of Baird et al. (1989b), Soares et al. (1992) and standard protocols (e.g. ASTM, 1997; ISO, 1996, 2000; OECD, 2000a). A standard organic additive was added to the culture medium to provide essential microelements to daphnids (Baird et al., 1989b). Animals were fed with *P. subcapitata*, which was cultured as described above. Algal ration was determined spectrophotometrically and daily supplied to the cladocerans (3.0×10^5cells/ml/day for *D. magna* and 1.5×10^5cells/ml/day for *D. longispina*). Midges (*C. riparius*) were maintained in an enclosed transparent plastic box containing all the apparatus necessary to complete the complete life cycle of the chironomids and large enough to allow swarming and copulation of emerged adults (OECD, 2000b). Cultures were maintained at 20±2ºC, with a 14^L:10^Dh cycle. At the start of a new culture, approximately 30 first-instar larvae (3–4days post-hatch) were introduced into a new glass beakers containing ASTM hard water and sea sand (0.1–0.4mm particle size range; supplied by Merck Co.). An *ad libitum* suspension of ground Tetramin® (Tetrawerke®, Germany) was then added as the single food source. Each beaker was gently aerated. Seven days later, larvae were either used in tests or transferred to new culture beakers with fresh media, food and sand until emergence occurred. Adults fed on a sucrose solution wetted paper, placed inside the culture unit. Fresh laid egg masses were transferred onto small plastic Petri dishes with culture medium for a period of 3–4 days, until eclosion occurred. The newborn larvae (1^{st} instar) were then used to start a new culture.

Water column bioassays

The laboratory experiments followed the OECD (1984) guidelines for algal growth inhibition tests. The algae were exposed during a 96h period to several dilutions of the water effluent in MBL medium. Assays were conducted in 100ml glass vials filled with 40ml of test medium, using a total of three replicate vials per effluent dilution. Negative controls (optimal growth) consisted of algae grown on MBL, under the same test conditions. Test vials were randomly incubated in an orbital shaker, bearing an initial cell density of 10^4cells.mL^{-1} (inoculated from the exponential-growing batch culture). Range-finding bioassays were carried out to set appropriate dilutions (spring: 0, 25.0, 50.0, 75.0, 100%; autumn: 0, 20.0, 30.0, 40.0, 50.0%) to obtain EC_{50} values with the best confidence interval. In order to exclude any potential effects from nutrient deficiency due to the

dilutions, additional controls were tested: i) a 50% MBL-control corresponded to the alga grown in 20ml distilled water added to 20ml MBL; ii) a 75% MBL-control corresponded to the alga grown in 30ml distilled water added to 10ml MBL). At the end of the assay, algal biomass (chlorophyll *a*) was quantified and expressed as a proportion (%) relatively to the negative (100% MBL) control.

Independent experiments were used to assess the acute toxicity of mining waste water to both *D. magna* and *D. longispina*. Tests were performed in accordance with standard protocols (ASTM, 1997; ISO, 1996; OECD, 2000a), under the same temperature and photoperiod regimes as described for rearing procedures. All assays were initiated with neonates (<24h-old), born between the 3rd and 5th broods, obtained from the bulk group cultures. A static design was employed, using 20 animals (randomly divided into four groups of five animals) per control (ASTM medium) and dilutions of the effluent water. Test vessels (four per each treatment) consisted of glass beakers containing 100ml of test water. For each combination of species and season six to seven test concentrations were obtained by dilution with ASTM medium. Range-finding bioassays were carried out to set appropriate dilutions to obtain EC_{50} values with the best confidence interval. Final concentrations were: for *D. longispina* (spring: 14.0-75.0%, separated by a factor of 1.4x; autumn: 8.0-50.0% separated by a factor of 1.3x); for *D. magna* (spring: 70.6-100.0% separated by a factor of 1.06x; autumn: 20.0-59.72% separated by a factor of 1.2x). Oxygen concentrations and pH levels in test vessels were determined at 0h, 24h and 48h to fulfil the test criteria (OECD, 2000a). All experimental treatments were checked for immobilised individuals at 24h and 48h, which were counted for posterior determination of EC_{50} values (see *Statistical analyses*).

Sediment bioassays

Whole-sediment laboratory bioassays with *C. riparius* followed, with some modifications, the standard 10-day larval growth test (ASTM, 2000; Ingersoll et al., 1995; Nebeker et al., 1984; Taylor et al., 1991). All major SETAC recommendations on bioassay experimental design (test vessel, sediment and overlying water) were followed (Hill et al., 1993). Three treatments were used: 1) a negative control (CTL), consisting of artificial substrate (sea sand) + ASTM hard water; 2) contaminated sediment + ASTM hard water and 3) contaminated sediment + water from the mine pit pond. Eight replicate test vessels

(250ml glass flasks, with 5.5cm in diameter), with 10 organisms each, were used in all treatments. Sediment and overlying water were added the day before starting the test. A 2cm layer of sediment was carefully placed at the bottom of the beakers and water was slowly added (to minimize sediment disturbance) up to 8cm depth, yielding a sediment:overlying water depth ratio of 1:4, as recommended by OECD (2000b). After a 24h settlement period with gentle aeration, 2^{nd}-3^{rd} instar chironomids (approx. 7-10d-old) were added (day 0). While adding the chironomids, aeration was stopped for a 30min period, allowing larvae to burrow. After this short time, gentle aeration was restarted and kept continuously throughout the test. Food (ground Tetramin®) was added in a single 0.5mg/larva /day dose at day 0 and every two days henceforth. At day 10, organisms were collected by sieving the test sediment through a 500-µm mesh. The larvae recovered from each replicate flask were pooled and weighed after drying at 105ºC for 12h in an oven (dry weight) and igniting in a muffle furnace at 450ºC for 6h (ash weight). This allowed the calculation of the ash-free dry weight (AFDW), which was used as a measure of chironomid growth (biomass). Mortality, pupation and number of emerged adults were also determined at the end of the test. Physical and chemical parameters (pH, dissolved oxygen, and conductivity) were measured on days 0 and 10 of the experiment for validation purposes.

Additionally, toxicity tests using sediment elutriate (see *Collection and preparation of samples*) were performed with *D. magna* and *D. longispina* to evaluate indirect sediment toxicity. The bioassays followed the same methodology described for the evaluation of effluent water toxicity (see *Water column bioassays*). Dilutions were performed with ASTM medium and ranged between 0 to 100%, separated by a factor of 1.4x, for both seasons and species, except for the *D. longispina* bioassay in autumn (61, 67, 74, 81 and 90%).

Chemical analyses

Effluent water was analysed for selected metals using ICP spectrometry (APHA et al., 1995). At the onset of the toxicity assays, several physical and chemical parameters were also quantified in aliquots of the effluent water: pH, conductivity, total phosphorous (persulfate digestion followed by ascorbic acid method; APHA et al., 1995), total nitrogen (persulfate digestion followed by cadmium reduction method; APHA et al., 1995).

Sediment organic matter content was determined as the percentage of initial dry weight of sample remaining after igniting in a muffle furnace at 450ºC for 8h (adapted from Buchanan and Kain, 1971; SPAC, 2000).

Statistical analyses

EC_{50} values for *Daphnia* immobilisation data and algal growth inhibition were determined using probit analysis (Finney, 1971). Larval dry weights, pupation and survival were analyzed for differences with two-way analysis of variance (ANOVA), followed by Tukey HSD multiple comparison tests, when applicable. Pupation and survival data were arcsine transformed prior to the ANOVA (Zar, 1996).

RESULTS

The variation in the physical and chemical parameters of the effluent water and sediment from the Cunha Baixa uranium mine pond is presented in table 1. Water was acid, showing the lowest pH value in autumn (pH=3.44). In opposition, conductivity and TN levels were higher in the autumn when compared to spring. Low concentrations of TP in the water and organic matter in the sediment were measured, and they were quite similar between seasons. Total metal concentrations were higher in autumn that in spring for all measured metals except Cd and Ba (Table 1). Several metals, especially Al, Mn and Fe, were measured at concentrations that exceeded Maximum Recommendable Values – MRV for crop irrigation established by Portuguese legislation (MA, 1998) (Table 1). Although no guidance values exist for uranium (Sheppard et al., 2005), the concentrations recorded seemed to be environmentally preoccupant as well, especially in the autumn.

Effluent water was acutely toxic to both algae and daphnids (Table 2). The toxic effect of the effluent water was not due to depletion of nutrients because controls with different concentrations of MBL (100%, 75% and 50%, *see* Material and Methods section) performed similarly. A significant increase in toxicity (i.e. a reduction in the EC_{50} values) was observed in the autumn for all species, comparatively to spring. The relative sensitivity of test organisms to the water samples was identical in both sampling periods: *D. longispina* > *P. subcapitata* > *D. magna* (from most to less sensitive). No acute toxicity was found for sediment elutriates, except for *D. longispina* in the autumn with EC_{50} = 75.2% (CI_{95}: 71.3% - 79.5%).

Table 1 – Physical and chemical water quality data for the pond and average metal concentrations (μg/L, n= 3 determinations). Percentage of organic matter for sediments is also presented. Maximum Recommendable Values (MRV) to waters for crop irrigation (MA, 1998) are shown in the shadowed column. Values in bold show metal levels exceeding the MRVs.

		Spring	**Autumn**
pH		5.67	3.44
Conductivity (μS/cm)		1153	1778
TP (mg/L P)		0.06	0.04
TN (mg/L N)		2.2	5.4
O.M. (%)		0.75	0.79
Zn	2000	569	680
Cd	10	9	2.8
Be	500	20	43
Al	5000	495	**9070**
Mn	200	**7016**	**11865**
Cu	200	17	44
Sr	not available	352	498
V	100	0.1	0.6
Cr	100	0.4	0.8
Fe	5000	1692	**10657**
Co	50	49	**117**
B	300	5	79
Ni	500	116	193
As	100	1	2.6
Se	20	0.3	1.4
Ba	1000	29	17
Pb	5000	1	3
U	not available	552	1842

Table 2 – Summary of EC_{50} values and corresponding 95% confidence intervals (CI_{95}) obtained with the alga and crustacean bioassays for effluent water.

	Water	
	Spring	**Autumn**
P. subcapitata (EC_{50} – 96h)	41.6 < **60.4** < 82.8	25.49 < **27.0** < 28.42
D. longispina (EC_{50} – 48h)	41.6 < **49.3** < 60.2	16.95 < **20.5** < 24.25
D. magna (EC_{50} – 48h)	80.5 < **83.6** < 87.2	26.75 < **35.8** < 50.78

Growth of *C. riparius* was stimulated in the natural sediment, but no toxic effects were found. However, in the autumn no surviving larvae (100% mortality) were retrieved

from the sediment in the treatment where natural sediment and water were combined (Table 3). Correspondingly, very low pH (3.5) was observed in this treatment, unlike all other combinations of treatments and seasons (Table 3). High conductivities were consistently observed in the treatments where effluent water was used as overlying water, but not in the case of ASTM. Within each experimental treatment, conductivity, oxygen and pH levels were fairly stable throughout the 10d bioassay (Table II.3).

Table 3 – Ash free dry weight (AFDW), survival and pupation rates of *Chironomus riparius* larvae at the end of the bioassay (after 10 days of exposure). Different letters (a,b) represent significant differences between treatments (P≤0.05). The shaded columns represent the range of physical and chemical parameters measured. [1]ANOVA – Spring: F = 9.8; d.f. = 23, 2; *P* < 0.001; Autumn: F = 43.7; d.f. = 15, 1; *P*<0.001. [2]ANOVA – Spring: F = 3.4; d.f. = 23, 2; *P* = 0.052; Autumn: F = 203.9; d.f. = 23, 2; *P*<0.001. [3]ANOVA – Spring: F = 2.1; d.f. = 23, 2; *P* = 0.142; Autumn: F = 1.0; d.f. = 23, 2; *P*=1.

	AFDW[1] (mg/Ind)	**Survival[2]** (%)	**Pupation[3]** (%)	**Cond** µS/cm	**pH**	**O_2** mg/L
Spring						
Ctl	0.97±0.07[a)]	100±0.0	31.25	589-632	8.0-8.3	7.3-9.1
M-ASTM	1.14±0.10[b)]	98±1.6	42.36	572-610	8.1-8.4	7.4-9.3
M-Effluent	0.96±0.09[a)]	91±4.0	25.72	1161-1228	7.4-7.9	7.5-9.1
Autumn						
Ctl	0.57±0.06[a)]	89±3.5[a)]	0.00	562-598	7.9-8.1	8.2-8.6
M-ASTM	0.80±0.07[b)]	96±2.6[a)]	0.00	563-605	7.3-7.6	8.7-9.5
M-Effluent	-	0.00[b)]	0.00	1706-1758	3.5-3.7	9.2-9.8

DISCUSSION

Although no specification exists about which species were tested to derive Portuguese MRVs, total metal concentrations for Al, Mn, Fe and Co were well above these regulatory values. Relatively high concentrations of Sr were also observed, but they were below the toxic effect concentrations reported for aquatic organisms (ATSDR, 2004). Additionally, the U concentration recorded in autumn was 3 to 12 times above the LC_{50} or EC_{50} for some cladocerans and other aquatic invertebrates, as reviewed from the literature by Sheppard et al. (2005). Uranium (U) is a matter of concern in the area, not only for its presence in the water (mainly groundwater) and soils, but also because of its decay, which generates considerable levels of radioactivity in the area (ATSDR, 1999). Data on U toxicity are scarce and several studies have shown that its toxicity is dependent on many variables, mostly pH and carbonate content (Poston et al., 1984; Franklin et al., 2000;

Charles et al., 2002; Sheppard et al., 2005). Uranium is considered to be one of the most important and potential ecotoxicological concerns to the freshwater biota (Reithmuller et al., 2001; Charles et al., 2006), and there is a need to establish legal threshold values. However, no guidance values for U exist in Portuguese legislation. The high levels of Mn, Fe, Al and U observed in our preliminary chemical evaluation suggest that the effluent water of Cunha Baixa mine may present a hazard to exposed aquatic freshwater organisms and to humans exploring freshwater resources in the area.

When looking at the toxicity profiles of the water and sediment samples from the mine pit pond, it is clear that the tested effluent was acutely toxic to the biota. Relatively to the water column, *D. longispina* revealed to be the most sensitive *taxon*, followed by the microalga and only then by *D. magna*, in both sampling periods. As already pointed out by Chapman (2000), tests performed with the same effluent commonly show differences in response depending on which water flea species is tested. Hence some care is needed when using only *D. magna* as a standard test organism. Several studies have demonstrated that this species is usually more resistant to pollutants than smaller-sized, and more widespread cladoceran species, such as *D. longispina* (Antunes et al., 2004; Marques et al., 2004). Moreover, *D. longispina* is a native species from Portuguese freshwaters, hence providing more ecologically relevant information on potential toxic effects.

Our results have shown a high degree of variability of the Cunha Baixa aquatic system, since large differences were observable in the pH and toxicity of the mine effluent between spring and autumn. In general, the EC_{50} values for all test organisms decreased two-fold in the autumn, relatively to spring, indicating higher toxicity in autumn. This increase in toxicity was accompanied by a decrease in the pH of the effluent in the autumn. Nevertheless, the low pH (translated in a high concentration of H^+) did not seem to produce a significant toxic effect, because the effluent concentrations tested were near neutrality, because of the buffering capacity of the dilution waters (ASTM hard water for cladocerans and chironomids, and MBL for algae). However, Goodfellow et al. (2000) reported the occurrence of a toxic ion imbalance, physiologically intolerable for test species, yielded by the mixture of the effluents with dilution waters. This should not be ignored. Furthermore, one can not exclude that the more acidic pH may have promoted a higher bioavailability of metals in the water effluent.

Complex effluents, particularly in mine areas, possess a dynamic equilibrium with large fluctuations in the quality of the water. These fluctuations may be swift or slow, and are usually associated with climatic conditions, namely precipitation, and hydrological regimes. This chemical instability is bound to reflect itself on the toxicity of the mine effluent (Kelly, 1988). It is important to bear this in mind when evaluating the toxicity of these complex mixtures, by taking into account a seasonal component of toxicity. This seasonality was recorded for Cunha Baixa uranium mine effluent, but it did not seem to be directly determined by precipitation but by the rise of contaminated groundwater (due to past *in situ* leaching activity), when the aquifer was replenished. This fact explains the increase in toxicity in the autumn, when a dilution effect by rain would be expected. The reduced depth of the contaminated groundwater of the Cunha Baixa mine area promotes this type of chemical fluctuations and is of additional concern in terms of remediation, because groundwater discontinuously supplies the mine pit pond with high concentrations of metals and H^+ ions. Mason (1996) considers that mine effluents whose water comes from underground sources tend to be more acidic and with higher loads of metals, being more difficult to control.

Although assays showed substantial deleterious effects of water column on the test organisms, this was not true for sediments. Whole-sediment test with *C. riparius* clearly demonstrated that the toxicity was mainly attributable to the water column, particularly due to low pH. Again, toxicity was observed only in the autumn, concordantly to what had been observed for the water samples. Sediment toxicity (100% mortality) was only observed when the mine effluent was used as overlying water, while no toxicity was observed with ASTM hard water. These findings suggest that sediment-bound contaminants may be present in non-bioavailable forms. Elutriate tests confirmed that a small fraction of these contaminants can become available in the water column by mechanical disturbance, although this was only shown for *D. longispina* in the autumn.

Our study showed that the effluent of the Cunha Baixa mine was acutely toxic to the tested organisms, although its degree of toxicity varied with season. Heavy metal ions present in the water (associated with low pH) seemed to be the most likely cause of the observed toxicity, while contaminants in the sediments of the abandoned mine pit seemed to be unavailable to the biota. Abandoned uranium mining areas generate complex mixtures containing U and other metals, whose effects on the environment are still to be

fully understood. Additional data regarding sublethal effects, combining laboratory and *in situ* approaches, must be collected in these areas to adequately quantify the risks. This information is also particularly important for the Portuguese case, as there are about 58 abandoned radioactive mine areas widespread in the North part of the country (Nero et al., 2003). Some of these mines (including Cunha Baixa mine) were considered as requiring urgent intervention. Thus, chemical and toxicological information is required to make decisions about reclamation/mitigation measures to be undertaken with the purpose of protecting indigenous wildlife and human health.

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Capítulo V

Science of the Total Environment (aceite para publicação em 27/Julho/07)

"Contribuição para a Etapa 1 da análise de risco ecológico para a mina de urânio da Cunha Baixa (centro de Portugal): II. Rastreio ecotoxicológico do solo"

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RESUMO

Este estudo apresenta os primeiros dados ecotoxicológicos respeitantes ao solo da área adjacente à mina de urânio da Cunha Baixa. O objectivo principal deste trabalho foi o de categorizar os solos da área com base nos perfis toxicológicos obtidos com uma bateria de ensaios (elutriados de solo avaliados com ensaios agudos em Microtox® e *Daphnia*; amostras de solo testadas em Microtox® e ensaios de evitamento com *Eisenia andrei* Bouché). Este estudo é parte integrante da etapa 1 da análise de risco ecológico em curso para o local. Não foi observada toxicidade aguda para os 10 locais escolhidos, utilizando os ensaios agudos com Microtox® e *Daphnia*. Por outro lado, as respostas comportamentais de *E. andrei* revelaram ser um parâmetro extremamente sensível, permitindo discriminar solos moderadamente e altamente tóxicos. Os solos que exibiram elevada toxicidade corresponderam a áreas sujeitas a escorrências ou a lamas de deposição provenientes do efluente aquático, enquanto que os solos que não apresentaram toxicidade foram os mais afastados da área de exploração mineira. Os dados obtidos nos ensaios de evitamento vêm reforçar avaliações anteriores dos riscos para este local, baseadas em dados químicos, e sustentar decisões relativamente ao prosseguimento para a etapa 2.

PALAVRAS-CHAVE

Mina de urânio, rastreio de toxicidade no solo, *Eisenia andrei*, *Daphnia* sp., Microtox®

"Contribution for Tier 1 of the Ecological Risk Assessment of Cunha Baixa Uranium Mine (Central Portugal): II. Soil ecotoxicological screening"

ABSTRACT

This study presents the first ecotoxicological data concerning the soils of the area surrounding the Cunha Baixa uranium mine. Our main goal was to categorise soils from the area based on their toxicity profiles using a battery of cost- and time-effective bioassays (elutriate approach – Microtox® and *Daphnia* acute tests; whole-soil approach – Microtox® and avoidance assays with *Eisenia andrei*), as a part of tier I of an ongoing Environmental Risk Assessment. No acute toxicity was found for any of the 10 sites/soils using Microtox® or *Daphnia*. On the contrary, the behavioural response of *E. andrei* was found to be an extremely sensitive endpoint, allowing the discrimination of highly to moderately toxic soils based on their toxicity profiles (as a function of soil concentration). Soils exhibiting highest toxicity corresponded to areas subjected to runoffs or sludge deposition from the aquatic effluent, while non-toxic soils were farthest to the mine. Data obtained in avoidance assays strengthen the previous evaluation of risks based on chemical data and supported decisions about proceeding for tier 2.

KEYWORDS

Uranium mine, soil toxicity screening, *Eisenia andrei*, *Daphnia* sp., Microtox®

INTRODUCTION

In the Tier 1 of a site-specific assessment, toxicity can be measured directly by exposing test organisms to contaminated environmental samples or to pore water or leachates derived from these (O'Halloran, 2006). Thus, bioassays contribute to integrating the combined effect of chemical mixtures present at a polluted site, and help to evaluate the risks associated with the exposure to bioavailable fractions of contaminants (Fernandez et al., 2005).

Earthworms are commonly used as test-organisms in terrestrial ecotoxicology because of their burrowing habits and importance in soil habitat function. In addition, they are relevant indicators of environmental change (Capowiez et al., 2003; Römbke et al., 2005). Earthworms are highly mobile in the soil and possess the ability to detect and avoid

contaminated areas (Yeardley et al., 1996). Avoidance tests with earthworms have already been implemented as a rapid screening tool for contaminated soil samples (Hund-Rinke et al., 2003; Loureiro et al., 2005b; Lukkari and Haimi, 2005). Other simple bioassays have long been used as screening tools for soil toxicity, adapted from aquatic ecotoxicological practices, namely Microtox® (Abbondanzi et al., 2003; Loureiro et al., 2005a; Weeks and Comber, 2005) and elutriate assays with algae or daphniids (Robidoux et al., 2004). Complementary, microbial bioassays (soil respiration, enzymatic activities) and bait-lamina tests have also been employed in the first step of soil toxicity evaluation (Abbondanzi et al., 2003; Brohon et al., 2001; Robidoux et al., 2004). The use of a battery of simple assays constitutes a cost- and time-effective tool, which is able to cover a variety of effects across different *taxa* in the framework of tier 1 of an ecological risk assessment (see e.g. Abbondanzi et al., 2003; Brohon et al., 2001; Loureiro et al., 2005a; Robidoux et al., 2004; van Gestel et al., 2001).

Following the tiered approached of the Britain framework for the Ecological Risk Assessment of contaminated sites (Weeks and Comber, 2005) in the absence of biological data, this work aimed at screening the soil toxicity of the area surrounding the Cunha Baixa uranium mine (Mangualde, Central Portugal). Data gathered with this study will reinforce the previous evaluation of risks based on chemical data, performed by Pereira et al. (in press). Our main goals were to help identifying potentially hazardous soil samples and to provide a toxicological categorisation of sites within the Cunha Baixa mining area. Soil toxicity screening was carried out using three different approaches: a) avoidance assays performed with contaminated soils, using the earthworm *Eisenia andrei* Bouché; b) acute (immobilisation) assays performed with soil elutriates (aqueous phase), using two cladocerans (*Daphnia magna* – standard species and *Daphnia longispina* – indigenous species); c) Microtox® assays with aqueous (elutriate) and solid (soil) phase samples.

Materials and methods

Study site

The uranium mine is located in Cunha Baixa (Centre of Portugal), and is included in the central uraniferous belt of the Iberian Peninsula between two systems of conjugate fissure (Machado, 1998). The ore was exploited between 1967 and 1993 (Neves and Matias, 1998) and the exploitation left a small aquatic system composed of artificial

temporary ponds (see e.g. Antunes et al., 2007a; b) and an underground effluent. Soil contamination results from the deposition of mine tailings and sludge from the effluent treatment pond and from runoffs from the aquatic system. For this work, ten sites were chosen in the area surrounding the old mine exploitation (Figure 1). Sites B and D corresponded to locations where sludge from a sedimentation pond are cyclically dumped, while site F was near a runoff from this pond. Site E was purposely chosen due to its proximity to household agriculture fields. Other sites (Figure 1) were randomly distributed along a distance gradient to the mine pit (A nearest and J farthest to the mine pit). A more detailed description of these sites can be obtained elsewhere (Pereira et al., in press).

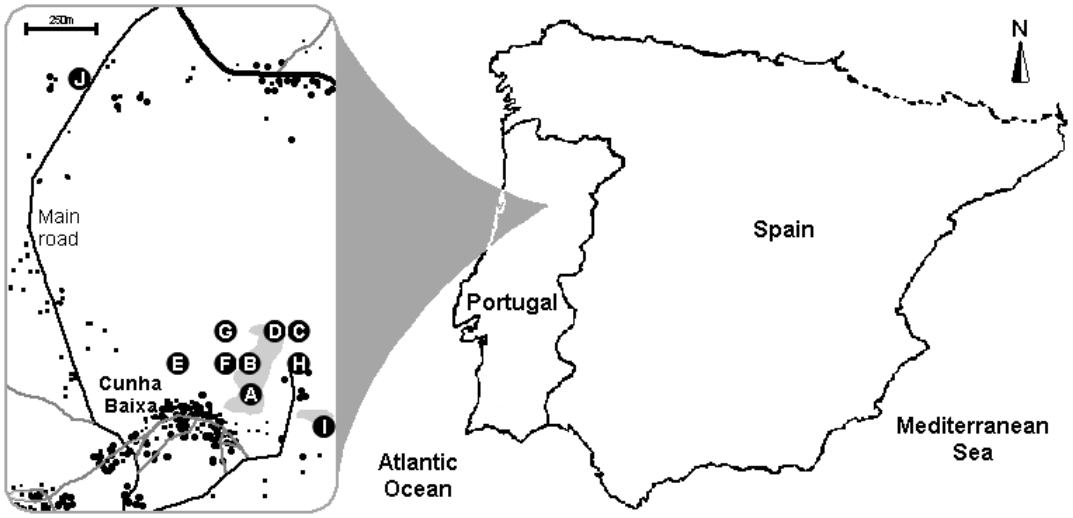

Figure 1 – Schematic representation of study area with sampling sites (A-J). Black and grey lines represent main and secondary roads, respectively, while black circles represent houses. Grey areas represent the abandoned mining areas.

Soil collection and characterisation

After discarding the superficial layer (plant debris and humus), the first 20 cm of soil were collected as composite samples from at least two different locations within each site. On site, samples were hand-mixed and coarse materials, such as plant roots or stones, were removed. Samples were then stored in opaque plastic bags and, later, they were sieved to discard the >2 mm fraction and then transported to the laboratory and stored at 4ºC, until analysis was possible, always within a one week time frame. For the determination of soil chemical properties (pH, conductivity, moisture, water holding capacity and organic matter content), replicate samples were randomly collected at each site, as described above. Physical, chemical and metal (extracted with artificial rain water during 1 week) analyses in soils were performed according to Pereira et al. (in press). A

Principal Component Analysis (PCA) was performed to relate samples based on soil physical and chemical parameters. Prior to ordination, environmental variables were standardized to reduce the relative influence of scale (ter Braak, 1995).

Soil avoidance assays

Adult clitellate earthworms (*Eisenia andrei*) came from a synchronised culture reared in a large container, under controlled environment (temperature 20±2ºC; photoperiod $16h^{L}$:$8h^{D}$). The assays were performed in plastic test chambers (area = 425 cm^2) and followed ISO (2005) recommendations. The test principle is to assess whether organisms distribute at random between uncontaminated and contaminated soil or if, on the contrary, they avoid contaminated soil (Hund-Rinke and Wiechering, 2001; ISO, 2005; Yeardley et al., 1996). Thus, assays were performed under a dual-choice design, where half of each container was filled with different test soil concentrations (%) and the other half was filled with a standard control soil (Standard LUFA 2.2, Agricultural Research Centre, Speyer, Germany). The soil dilutions (in a total of five: 12.5, 25.0, 50.0, 75.0 and 100%) were obtained by diluting (w/w) the natural soils with the control soil, thus producing a final design of 10 sites x 5 test soil vs. control soil combinations x 3 replicate containers. In each replicate container, 10 adult earthworms (weight: 0.3-0.6 g) were placed on the border line separating the two soils (Figure 2A), after filling each half of the container with 200 g of either test or control soil (roughly corresponding to 325 cm^3 of each soil). Before adding the test organisms, soil humidity was adjusted to 40% of the water holding capacity (WHC). The assay was carried out for 48hr under the same environmental conditions as described for the culture (see above).

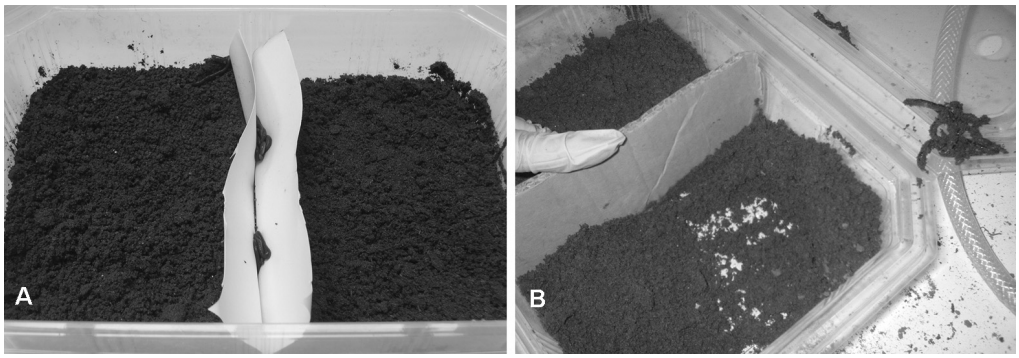

Figure 2 – Avoidance assay procedures: A – beginning of the assay, illustrating the dual-choice design (separator is removed before adding the earthworms); B – end of the assay, showing recovery of test organisms.

At the end of the assay, the number of worms present in each of the soils (natural and control) was counted (Figure 2B). Worms stretching across the border line separating the two soils were counted as 0.5 individuals for both soils, independently of the length of the body present in each soil. Results were expressed as net response (varying from -1 to 1), using a similar approach to Amorim et al. (2005):

$$\text{net response} = \frac{C - T}{N},$$

where C is the number of organisms in the control soil, T is the number of organisms in the test (natural) soil, and N is the total number of surviving organisms. Positive values thus account for avoidance of test soil, while neutral or negative responses represent indifference or preference towards test soil. We assume a null (i.e. zero) net response in dual-control experiments, as demonstrated by Yeardley et al. (1996). For each dual-choice experiment, mean net response was computed (n = 3) and independent t-tests were used to determine if net response was significantly higher than zero (H_0: $\mu \leq 0$; H_1: $\mu > 0$). Additionally, given the highly skewed distribution of the data and the underlying binomial nature of the response, independent *G*-tests (Zar, 1996) were also used to determine significant deviations to a hypothesized 1:1 ratio (control *vs* test soil), by pooling counts (frequencies) from the three replicates. For comparative purposes, we also used the habitat function threshold proposed by Hund-Rinke & Wiechering (2001) and recommended by ISO (2005), which states that habitat function of the soil is limited (i.e. compromised) when > 80% of earthworms avoid the test soil. In terms of net response, this corresponds to a value of 0.6 [from the above expression: net response = (80-20)/100]. In order to test for linear trends in net response across soil concentration, we further analysed the avoidance data with one-way ANOVAs (independently for each soil/site) followed by tests for trends using orthogonal polynomials (Quinn and Keough, 2002). A significance level of 0.05 was used in all analyses.

Soil elutriate assays

Soil elutriates were prepared and tested within the following 8 weeks of sampling, as recommended by USEPA (1998). For the preparation of elutriates, a 1:4 (w/v) ratio of natural soils to ASTM medium (see below) was used and they were shaken mechanically for 12hr at room temperature, followed by a 12hr deposition period. The overlying water

(elutriate) and settled material were separated by decanting. After that, elutriates were centrifuged (5000rpm during 10min) and filtered through a Whatman GF/C filter (1.2μm porosity, 47mm diameter) to remove suspended matter and stored at 4ºC, in the dark, until the assays were performed (maximum storage time: one week).

Standard Microtox® assays were performed using solid-phase (soil) and liquid-phase (elutriates) procedures according to standardised protocols (Microbics Corporation, 1992). Additionally, immobilisation assays with *Daphnia* were also performed to assess the toxicity of soil elutriates. Neonates of *D. magna* (clone A, *sensu* Baird et al., 1989a) and *D. longispina* (clone EM7, *sensu* Antunes et al., 2003) were obtained from laboratory cultures, which have been reared for several generations under standard procedures (see e.g. Antunes et al., 2003; 2004; 2007a; b). Daphniids were cultured in ASTM (1980) synthetic hardwater medium enriched with an organic additive (Baird et al., 1989b) and were fed with *Pseudokirchneriella subcapitata*. Experiments were initiated with neonates (< 24-hrs-old), born between the 3rd and 5th broods, without providing food (algae) or organic additive. Acute tests were performed in accordance with standard protocols (ASTM, 1997; ISO, 1996; OECD, 2000) under the same environmental conditions as described for earthworms. A static design was employed using 20 animals (randomly divided into four groups of five animals) per control and per elutriate concentration (%). Test vessels (four per each treatment) consisted of glass beakers containing 100ml of elutriate concentration. Range-finding bioassays were carried out to set appropriate dilutions (0.0, 25.0, 50.0, 75.0, 100.0%) to obtain EC_{50} values with the best confidence interval for all soil elutriates. Soil elutriate concentrations were established using ASTM medium as dilution water. Vessels were checked for immobilised individuals at 24 and 48h, which were counted for posterior determination of EC_{50} values (using probit analysis - Finney, 1971).

RESULTS

The PCA ordination based on soil physical and chemical properties produced a good separation of tested soils. Soils B and D occurred in association, opposing all other soils (Figure 3). These two soils were characterized by high values of pH (≈ 8), conductivity (B = 2263 μS/cm, D = 603 μS/cm), alkalinity (B = 1.18 mg CaCO3/g soil, D = 1.73 mg CaCO3/g soil), moisture (B = 48.2%, D = 32.7%), WHC (B = 126.0%, D =

123.5%) and a high uranium (U) concentrations, especially for D soil (109.2 mgU/Kg soil). All other soils were distributed along opposing gradients of trace metals, hardness and organic matter (OM). Soils E, G, H and J presented a positive association essentially with Mn (30.7, 25.0, 39.8 and 53.3 mg Mn/kg soil, respectively), but also with Zn, Cd and OM. Soils A and C (and, to a lesser extent, also I and F) were mostly associated to high values of Al (A = 205.3 mgAl/kg soil, C = 251.3 mgAl/kg soil) and hardness (A = 11.04 mg $CaCO_3$/g soil, C = 7.38 mg $CaCO_3$/g soil). The eigenvalues for the first two PCA axes were 0.416 and 0.205, which explained 62.1% of the total variation in physical and chemical parameters among the soil samples.

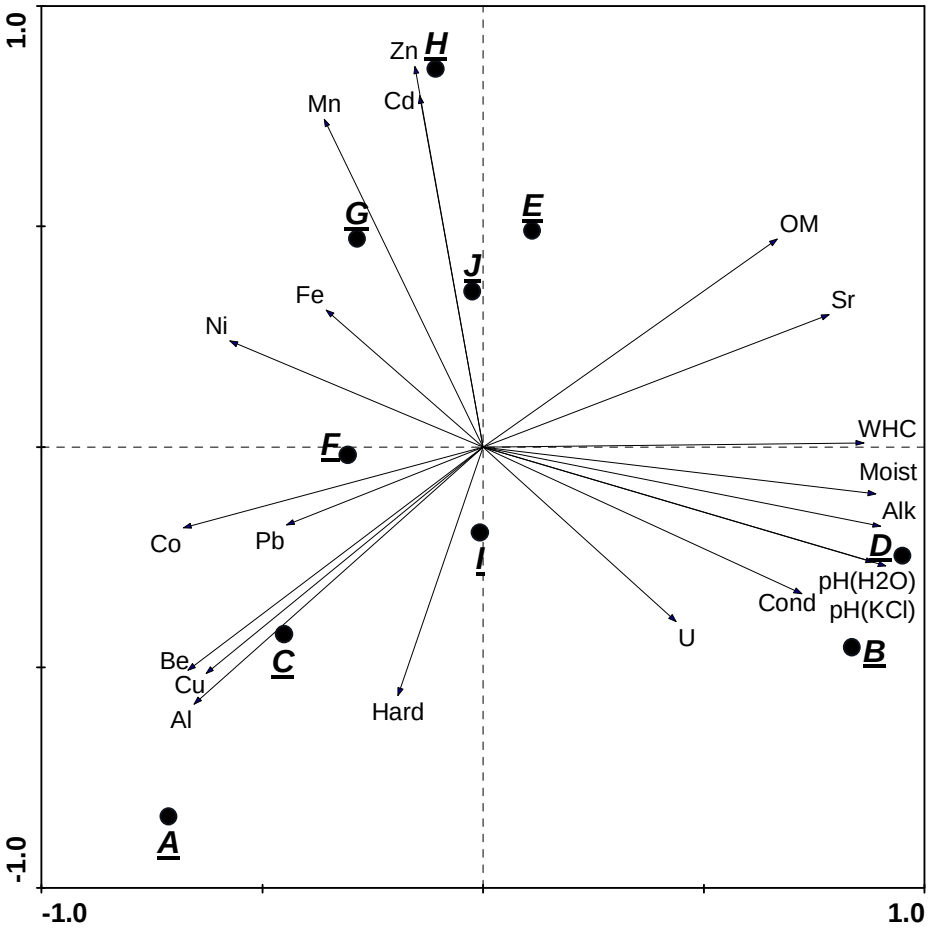

Figure 3 – PCA biplot of soils from the Cunha Baixa area, based on physical and chemical characteristics. Abbreviations: Alk – alkalinity; Cond – conductivity; Hard – hardness; Moist – moisture; OM – organic matter; WHC – water holding capacity.

With a few exceptions (1-2 fatalities), all earthworms were found alive in the dual-choice test containers at the end of the avoidance assays. Earthworm response differed

greatly between sites and, in some cases, the net response was concentration-dependent (see below and Figure 4). Significant positive deviations from 0 (*t*-tests and *G*-tests) in earthworm response roughly corresponded to values above the theoretical threshold of habitat function compromise (see Figure 4).

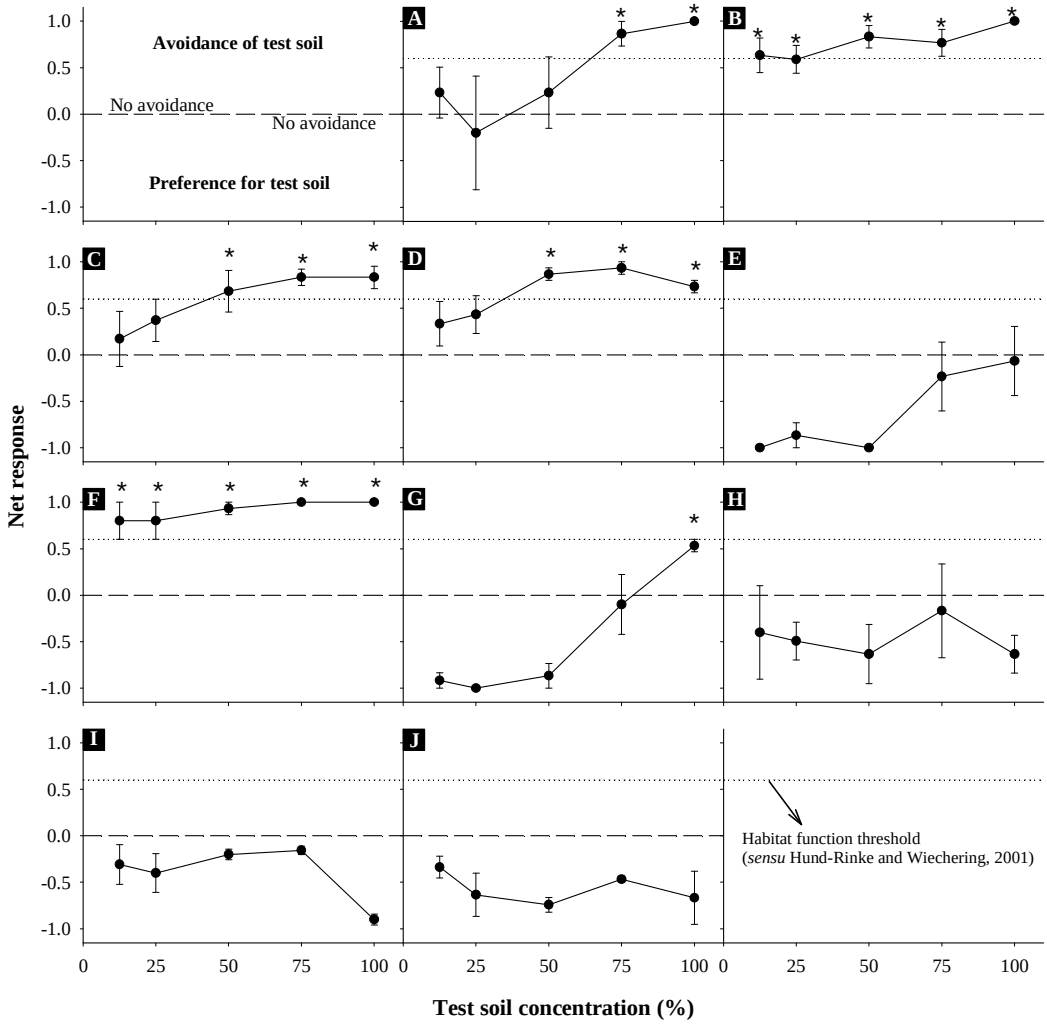

Figure 4 – Net response of *Eisenia andrei* when exposed to dual combinations between different soil concentrations and control soil (LUFA 2.2). Results are expressed as average values ± standard error. A positive response represents avoidance from test soil and values >0.6 are equivalent to Hunde-Rinke and Wiechering's (2001) habitat function threshold. Statistically significant deviations from zero (0) are shown (*) for each test soil concentration (*t*-tests, $P \leq 0.05$).

Table 1 – Summary table of one-way ANOVAs applied to the avoidance data (net response), including linear trend contrasts for each soil.

Source of Variation	**MS**	**d. f.**	***P***
Soil A concentration	0.74	4	0.168
Linear contrast	2.03	1	**0.041**
Residual	0.37	10	
Soil B concentration	0.08	4	0.284
Linear contrast	0.25	1	0.060
Residual	0.06	10	
Soil C concentration	0.26	4	0.158
Linear contrast	0.96	1	**0.020**
Residual	0.13	10	
Soil D concentration	0.21	4	0.065
Linear contrast	7.53	1	**0.021**
Residual	0.07	10	
Soil E concentration	0.60	4	0.052
Linear contrast	1.88	1	**0.009**
Residual	0.18	10	
Soil F concentration	0.03	4	0.668
Linear contrast	0.11	1	0.175
Residual	0.05	10	
Soil G concentration	1.34	4	**0.000**
Linear contrast	4.33	1	**0.000**
Residual	0.08	10	
Soil H concentration	0.11	4	0.889
Linear contrast	0.01	1	0.907
Residual	0.42	10	
Soil I concentration	0.25	4	**0.041**
Linear contrast	0.25	1	0.079
Residual	0.06	9	
Soil J concentration	0.08	4	0.520
Linear contrast	0.07	1	0.399
Residual	0.09	10	

There was a very good correspondence between the two statistical approaches (*t*-tests and *G*-tests) used to detect significant avoidance of test soil. The only exception was for soil D at 25%, where the *G*-test detected significant differences whereas the t-test did not (Figure 4). According to the toxicity profiles observed, we categorised soils as follows:

i) highly toxic soils (B and F), which were significantly avoided by earthworms even at low concentrations (≤ 25%); ii) toxic soils (A, C and D), where earthworm avoidance was observed at test-soil concentrations above or equal to 50%; iii) moderately toxic soils (G), where significant avoidance was only recorded in undiluted soil exposures (i.e. soil concentration = 100%); iv) non-toxic soils (E, H, I, J), where no avoidance behaviour was recorded at any concentration. In both extremes (B and F *vs* H, I and J), we found no concentration-dependent relationship in net response (linear trend contrasts, Table 1), since earthworms exhibited a fairly consistent response in all soil concentrations (Figure 4). For most soils (A, C, D, E, G), however, significant monotonic relationships between earthworm response and soil dilution were found (Table 1), as avoidance behaviour increased with increasing soil concentration (see above and Figure 4).

Acute assays other than earthworm avoidance were less sensitive to soil toxicity and failed to detect deleterious effects on both whole-soil (Microtox®) and elutriate exposures (*Daphnia* and Microtox®). A reduction in survival was only observed for *D. longispina* in soils B and D (40% mortality), at a concentration of 100% elutriate. However, no EC_{50} could be calculated.

DISCUSSION

Scientists and environmental consultants often bear the responsibility of alerting regional and governmental entities of the problematic of local contamination and its potential hazard to ecological receptors, including humans. Abandoned contaminated sites, such as mines or industrial facilities, are fairly common and many exhibit high levels of environmental contamination. High concentrations of heavy metals have been determined in water (Antunes et al., 2007b; Machado, 1998; Pedrosa and Martins, 1999), sediments (Antunes et al., 2007a; Antunes et al., in press) and, more recently, soils (Pereira et al., in press) from the Cunha Baixa uranium mining area. It is difficult to infer the potential impacts of such complex mixtures on local natural communities, especially if we acknowledge that interactions between contaminants (e.g. synergism) may produce deleterious effects, even at very low concentrations of the individual constituents (O'Halloran, 2006; Warne, 2002). At this stage, ecotoxicological assessments with field-collected samples, represent a much more useful and realistic tool in the first tier of ERA.

This study provides the first evaluation of soil toxicity in the area surrounding the abandoned uranium mine of Cunha Baixa. *A priori,* assays with earthworms were expected to be ecologically more relevant, since neither *Vibrio fischeri* nor *Daphnia* are soil organisms, although acute assays (using whole-soil or elutriate exposures) with these *taxa* are commonly used in soil toxicity screening (Abbondanzi et al., 2003; Loureiro et al., 2005a). Our data have shown that *E. andrei* was much more sensitive than Microtox® or *Daphnia* spp., since the latter bioassays failed to detect significantly toxic effects of the tested soils and elutriates. A similar outcome (i.e. higher sensitivity of earthworm avoidance) has been described by other authors (e.g. Schaefer, 2004; Sheehan et al., 2003). On one hand, this discrepancy may result from the intrinsically lower sensitivity of the endpoints used (immobilisation/luminescence), relatively to behaviour (avoidance). Environmental concentrations of contaminants are usually below acute levels, being their impacts mainly related with chronic exposures. On the other hand, the methods used to assess the toxicity on *Daphnia* and Microtox® rely on some sort of contaminant mobilisation to the aqueous phase (more intense in the case of elutriates). The absence of acute toxicity for *Daphnia* and Microtox® can thus be explained by a low mobilisation of soil contaminants to the aqueous phase. Although Pereira et al. (in press) have confirmed metal mobilisation in artificial rain water (mainly U and Al), the concentration of metals that became solubilised during elutriate preparation were not be sufficient to produce toxic effects, as seen here. However, we can not exclude an even more reduced mobilisation (than rainwater) to the ASTM medium (elutriate assays) and to the 3.5% NaCl solution (used in the Microtox® solid-phase assay) to perform soil extracts, due to their higher content in inorganic salts. According to Peijnenburg and Jager (2003), different chemical interactions, mainly influenced by pH, organic ligands, dissolved organic matter, ionic strength and the activity of competing cations, such as Ca^{2+}, determine the partitioning of metals between the solid and the aqueous phase of soils. In spite of its name, in the Microtox® solid-phase assay, the bacteria is not exposed to the solid fraction of soils, but also to an extract of soil. Hence, results from the three aqueous phase-assays were consistent, leading us to conclude that the soil retention function of these soils was not compromised.

At the light of the data obtained from the earthworm avoidance assays, soils nearest to the mine pit presented the highest toxicity. The worst cases were observed in sites near

deposits of pore ore and sludge from the effluent treatment pond (B, C, D), as well as the pine tree forest (F) near the mine pit, receiving aqueous runoffs from this pond. This pond continuously receives acid effluent from the underground tunnels of mine, which is neutralised and contaminants precipitated (see Antunes et al., 2007a; 2007b). Sludge accumulates and is periodically dumped in the mine area. Soil toxicity (avoidance) data suggest this is a hazardous practice with potential consequences to edaphic fauna. Soil A (within the mine pit) was also found to be toxic, while soils farthest to the mine pit were not significantly avoided by earthworms, with the exception of soil G. Among these, soil J was the most distant to the mine pit and it exhibited the lowest metal concentrations. However, good correspondence between toxicity profiles and soil association based on extracted metal concentrations (PCA diagram, Figure 3) was seldom found for the tested soils. In part, this is due to the fact that the total amount of a metallic element in a soil sample is not indicative of its bioavailability or potential toxicity, which in turn is significantly influenced by several abiotic parameters (e.g. organic matter, pH, soil texture, cation exchange capacity, concentration of competing ions and nutrient status) (Arnold et al., 2003). Again, this emphasizes the usefulness of toxicological assessments at the early stage of an ERA program. Avoidance assays showed a greater sensitivity and identified a larger number of sites potentially posing risks to edaphic communities, when compared to the chemically-based assessment (*see* Pereira et al., in press). Consequently, B, F, and G soils (also in the mine surroundings) can not be excluded from the site specific assessment without more ecotoxicological data from laboratory and *in situ* tests, which will be performed in the following tiers of the process (e.g. assays with plants, *in situ* assays with earthworms). Due to their behaviour and morphology, earthworms integrate exposures to both the aqueous phase and the solid phase of the soil compartment (via soil ingestion or dermal contact), which may contribute to the greater sensitivity of avoidance assays with *Eisenia* sp. (Schaeffer, 2004; Römbke et al., 2005). Since no effects were observable in the acute assays using extraction procedures to the aqueous phase (see above), the toxicity of soils A, B, C, D, F and G may derive mainly from the solid phase of the soil. The ecotoxicological data here presented suggest that the soil quality guideline values (SQGVs) used by Pereira et al. (in press) to support risk evaluation in these same sites were probably established based on less sensitive endpoints, leading to an underestimation of risks. Another potential reason for the underestimation of risks could result from the occurrence

of synergistic/additive effects between contaminants, which could not have been accounted for in this preliminary evaluation. Our results clearly demonstrate that the use of SQGVs alone in soil risk assessment (tier 1) can not be recommended. Furthermore, there is a clear need for producing more protective and uniform (from country to country) baseline benchmarks.

Many works have already described the high sensitivity of earthworms to contaminants, such as metals (e.g. Lukkari et al., 2005; Lukkari and Haimi, 2005; Yeardley et al., 1996) and pesticides (e.g. Amorim et al., 2005; Capowiez et al., 2006; Slimak, 1997). Hund-Rinke et al. (2003) and Römbke et al. (2005) have described in detail the major advantages of using earthworms in terrestrial ecotoxicology. Earthworms are highly mobile in soil and have the ability to detect and avoid contaminated areas (Van Zwieten et al., 2004; Yeardley et al., 1996). This avoidance behaviour can be rapidly and easily quantified and, as shown here, can produce a primary categorisation of areas/soils in a site-specific manner, for the subsequent phases of ERA. However, a vast array of approaches in the expression and statistical analysis of avoidance data is evident from the literature, both in *Eisenia* (Natal da Luz et al., 2004; Hund-Rinke et al., 2003; Hund-Rinke et al., 2005; Loureiro et al., 2005b; Schaefer, 2003; Schaefer, 2004; Van Zwieten et al., 2004; Yeardley et al., 1996), other earthworm species (Amorim et al., 2005; Booth et al., 2004; Capowiez and Berard, 2006; Lukkari and Haimi, 2005; Slimak, 1997) and edaphic arthropods (Aldaya et al., 2006; Natal da Luz et al., 2004; Loureiro et al., 2005b). Here, we use net response (Amorim et al., 2005), which we consider an intuitive parameter, due to its simple scale varying between avoidance (+1) and preference (-1) towards test soil (zero representing no avoidance). Since it is an index, rather than a proportion, it can be analysed with parametric inferential statistics, such as *t*-tests. As seen here, independent t-tests can be performed for each dual-choice set to assess significant deviations from zero (no avoidance). Given the overall agreement between t-tests (applied to net response) and *G*-tests (applied to frequency data) observed in our study, we recommend the use of the former (t-test), because it is a more simple and widespread procedure. Furthermore, some authors find *G*-tests to be too powerful in some situations (see Quinn and Keough, 2002; Zar, 1996 and references therein). Skewness of data and power of *t*-tests can be improved by increasing the number of replicates (n = 3 in our study).

CONCLUSIONS

In the Cunha Baixa uranium mine, further research should be carried out to improve toxicity information about contaminated soils in order to better assess the ecological risks posed by this area. No ecological data exists for the area giving sufficient evidence to support conclusions about the harmful impacts on local ecological receptors. As it was supported by Weeks and Comber (2005), when screening tests such as Microtox® show no toxicity but SQGVs indicate risks, then the site specific assessment should proceed for tier 2. In our study this conclusion was reinforced by the results of the avoidance bioassay with *E. andrei*. Hence, the evaluation should proceed mainly in A-D and F sites with a battery of chronic assays with sublethal endpoints for a proper assessment of the long-term ecological risks of contaminated soils. Soils I and J have proved to be natural reference soils that can be used in substitution of the LUFA 2.2 soil, increasing the ecological relevance of the bioassays performed within this mining area.

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Chapter VI

Manuscript submitted to publication

"Ensaio in situ com *Eisenia andrei* para avaliar a toxicidade do solo de uma mina de urânio abandonada."

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RESUMO

Dados anteriores sugerem a existência de potenciais riscos para os organismos edáficos da área adjacente à mina da Cunha Baixa (Mangualde, Portugal), o que suportou a decisão de prosseguir para uma avaliação ecotoxicológica *in situ*, no âmbito da análise de risco ecológico para locais contaminados. Deste modo, definiu-se como principal objectivo deste estudo desenvolver metodologias para um bioensaio *in situ* com *Eisenia andrei*, que foi levado a cabo em vários locais na área de estudo. Na prática, a aplicação do ensaio *in situ* a uma situação real de contaminação teve um propósito bivalente: por um lado, pretendeu-se validar os procedimentos do bioensaio *in situ*; por outro lado, era pretendido que os resultados obtidos viessem reforçar os dados das anteriores avaliações dos riscos efectuadas com ensaios laboratoriais. De modo a desenvolver um bioensaio eficaz do ponto de vista logístico e económico, a exposição *in situ* dos organismos foi curta (48 hrs). Os parâmetros analisados incluíram a avaliação de biomarcadores de stress oxidativo juntamente com o conteúdo em metais nos solos e em *E. andrei*. O bioensaio *in situ* decorreu sob diferentes condições experimentais, simulando condições naturais (solo local) vs. controlo (solo LUFA), bem como diferentes tipos de irrigação (água da chuva artificial vs. efluente ácido diluído - simulando possíveis escorrências). No final do ensaio, todos os organismos foram recuperados de todas as câmaras de teste. A maior porção de variação dos dados foi explicada pelas diferenças entre tipos de solo (solo natural vs. LUFA), ao passo que a água de irrigação pouco contribuiu para a variação total. Foi também observada uma grande heterogeneidade entre os locais escolhidos, dentro da área da mina abandonada. Os biomarcadores não foram um parâmetro tão sensível quanto seria de esperar, mas o conteúdo em metais nos tecidos de *E. andrei* sugeriu uma potencial preocupação relativamente à bioacumulação de alguns elementos metálicos que ocorreram na zona.

PALAVRAS-CHAVE

Bioensaio *in situ*, toxicidade do solo, *Eisenia andrei*, bioacumulação de metais, biomarcadores de stress oxidativo, mina de urânio abandonada

"*In situ* bioassay with *Eisenia andrei* to assess soil toxicity in an abandoned uranium mine"

ABSTRACT

Previous data suggested the existence of potential risks to soil organisms in the area surrounding the Cunha Baixa uranium mine (Mangualde, Portugal), which has supported the decision to proceed for an *in situ* evaluation, within the framework of the ecological risk assessment of contaminated sites. Hence, the goal of the present study was to develop an *in situ* bioassay methodology with *Eisenia andrei*, deploying it in several locations of the study area. Our objectives were two-fold: i) on one hand, we intended to validate the *in situ* soil bioassay procedures; ii) on the other hand, the data gathered will reinforce the previous evaluation of risks based on laboratorial assays, in a site-specific and ecologically relevant manner. To promote cost- and time-effectiveness of the assay, the *in situ* exposure was short (48 hrs) and the endpoints analysed included sensitive sub-lethal endpoints (oxidative stress biomarkers) along with soil and earthworm metal content (to address the bioaccumulation potential of the studied soils). The *in situ* bioassay was carried out under different experimental conditions, simulating local (natural soil) vs. control conditions (LUFA soil), as well as irrigation with artificial rain water vs. irrigation with diluted acidic effluent (mimicking possible runoffs). Complete recovery of test organisms from all sites and treatments combinations was achieved. Most of the variation in the data was due to differences between soil types (natural vs. LUFA soil), rather than irrigation water. Large heterogeneity was found between sites within the abandoned mine area. Data on biomarkers did not fully work here as sensitive parameters, but earthworm metal burdens suggested a potential concern in terms of bioaccumulation of some metallic elements.

KEYWORDS

In situ bioassay, soil toxicity, *Eisenia andrei*, metals, oxidative stress biomarkers, abandoned uranium mine

INTRODUCTION

During the last five decades, and around the globe, uranium extractive industry generated considerable volumes of wastes often containing elevated concentrations of toxic

metals and radionuclides. Cunha Baixa uranium mine (Mangualde, Central Portugal) is such an example, with uranium exploitation being carried out between 1967 and 1993 (Santos Oliveira and Ávila, 2001). Hazards to human health and wild life in mine areas are complex, and they simultaneously derive from dusts, high levels of heavy metals and low pH values, both in water and in soils (Lopes et al., 1999; Pruvot et al., 2006). In order to quantify the risks of such hazards, chemical analyses of environmental samples are usually employed, neglecting the assessment of biological effects. The importance of biologically based endpoints in Environmental Risk Assessment (ERA) is vital, and additional importance should be given to the realism of environmental exposures for toxicity assessments. Ecological relevance can thus be increased with the use of native species and *in situ* bioassays. Many examples are available in the specialized literature of *in situ* assays in the aquatic compartment (water and sediment), showing their successful applicationn to environmental contamination scenarios (e.g. Castro et al., 2004; Moreira-Santos et al., 2004; Pereira et al., 2000). On the other hand, less attention has been given to soils, and published studies or standard protocols focusing on *in situ* soil bioassays are scarce. Thus, it seems important to develop these methodologies for the soil compartment in order to provide tools that allow more realistic assessments of soil toxicity. This might allows us to reduce the uncertainty in the assessment of risks, which are mainly derived from differences in the complexity of mixtures and in the bioavailability of contaminants between environmental and laboratorial scenarios. Uranium mines are an excellent example of the potential applicability of this type of assays, since laboratorial exposures will most certainly underestimate the additional effects of *in situ* radioactivity, and the combined effects of variables such as temperature, organic matter, fluctuations in soil humidity or other confounding factors that are not included in laboratory based toxicity assays.

Earthworms are common in a wide range of soils and have been largely used in assays, since they have been considered the most suitable bioindicators for evaluating hazardous chemicals in soils (Paoletti, 1999; Cortet et al., 1999; Römbke et al., 2005). Until now, acute (mortality) and chronic toxicity (reproduction, avoidance) assays are available to evaluate soil toxicity in OECD countries (OECD, 1984, 2004). Although these bioassays produce relevant data (e.g. Spurgeon et al., 2004; Férnandez et al., 2005) additional information is needed, in particular concerning the biological effects of

contaminants on sub-lethal endpoints (Saint-Denis et al., 1999), in order to perceive the mechanisms of action of contaminants in the ecosystem. Moreover, the detection of toxic effects at a lower level of biological organization (e.g. biomarkers) can serve as an early warning signal for potential effects at higher levels. Biomarkers, such as oxidative stress enzymes (e.g. catalase, superoxide dismutase, glutathione peroxidase), are well documented indicators of exposure to heavy metals, whose biological effects produce sharp changes in these enzymes' activities (Atli et al., 2006; Fatima and Ahmad, 2005). Earthworms and plants have been commonly used as models for biomarker studies in the soil compartment, since they respond quickly to ecosystem changes (e.g. Atli et al., 2006; Saint-Denis et al., 2001). However, no methodologies exist for conducting *in situ* assays with these organisms, particularly with native species.

Soil chemical characterization and toxicity screening in the Cunha Baixa uranium mine area has suggested the existence of potential risks to soil organisms (Antunes et al., 2007a, 2007b; Pereira et al., in press) and has supported the decision to proceed for an *in situ* evaluation, following the application of the Britain tiered approach framework for the ecological risk assessment of contaminated sites (Weeks and Comber, 2005). Accordingly, this work aimed at developing an *in situ* assay with earthworms to evaluate sub-lethal endpoints such as specific biomarkers. The assay was carried out in the area surrounding the Cunha Baixa uranium mine (Mangualde, Central Portugal) and data gathered will reinforce the previous evaluation of risks based on laboratorial assays. The *in situ* assay with *Eisenia andrei* also tested different experimental conditions at several sites with different degrees of contamination: i) natural vs. artificial (LUFA 2.2) soil; ii) irrigation with artificial rain water (simulating rainfall) vs. irrigation with diluted acidic effluent (mimicking possible runoffs). Several biochemical markers of oxidative stress in exposed earthworms and metal body burdens were evaluated as a means of assessing the bioavailability and bioaccessibility of contaminants that produce toxic effects.

MATERIAL AND METHODS

Study area and site selection criteria

The uranium mine is located in Cunha Baixa (Centre of Portugal), and is included in the central uraniferous belt of the Iberian Peninsula between two systems of conjugate fissure (N40ºE and N70ºW with inclination the 75ºW) (Santos Oliveira and Ávila, 1998).

The ore was explored from 1967 until 1993 (Santos Oliveira and Ávila, 2001). When the exploration ended, the underground mine pit was filled with mine tailings and flooded with sulphuric acid, creating an artificial pond (M). This pond is subjected to high variations in water level (including drought), as a consequence of the groundwater level of the aquifer. A more detailed description of the aquatic system of the Cunha Baixa mine is presented elsewhere (Antunes et al., 2007a). Water samples from the mine pit were shown to be acutely toxic to freshwater organisms (Antunes et al., 2007a, 2007b). Several impacts of ore exploitation are also present in the surrounding soils, through deposition of tailings and sludge (the latter still ongoing). A previous study has evaluated contamination and toxicity profiles of several soils adjacent to the mine (Antunes et al., in press; Pereira et al., in press]. Bearing this information, four sites were chosen (A, B, F, and I *sensu* Antunes et al., in press) for conducting an *in situ* assay with *Eisenia andrei* in the present study. Site A is located near the mine pit, site B is a deposition site for sludge from an effluent treatment pond, and site F is under the influence of runoffs from this pond (where neutralization and metal precipitation is cyclically performed – see Antunes et al., 2007a). Soils from these sites displayed high toxicity in avoidance assays with *E. andrei* and high values of metals (namely U, Mn and Sr). Site I (farthest from the mine pit) was additionally chosen as a reference site, based on its metal content and absence of toxicity to *E. andrei* (Antunes et al., in press; Pereira et al., in press).

In situ *assay procedure*

Test-chambers were specially designed to carry out the *in situ* assay with earthworms (*E. andrei* were obtained from laboratory-reared batch cultures – see Antunes et al., in press). The chambers consisted of 1-L plastic buckets (12 cm height and 10 cm in diameter), with lids, bearing two openings, one at the top (for ventilation) and another at the bottom (to drain excess water) (Fig. 1A) and to allow the contact between the soil inside the chamber with local soil. Both openings were covered with 300 μm nylon mesh, using white thermal glue (supplied by Elis-Taiwan, Taiwan, ref. TN122/WS, with a chemical composition of 50% ethylene-vinyl-acetate copolymer, 45% synthetic hydrocarbon, and 5% polyethylene wax), which has been shown to be non-toxic to cladocerans (Pereira et al., 2000). In the field, test chambers were filled with either local soil (collected in the area, integrating the upper 10 cm of soil, excluding surface litter) or

LUFA 2.2 standard soil (controls). To ensure survivability of earthworms during the *in situ* exposure, soils were moistened prior to the beginning of the assay. Two types of water were used for this purpose: a) artificial "rain water" [$NaNO_3$ 4.07 $mg \cdot L^{-1}$, NaCl 3.24 $mg \cdot L^{-1}$, KCl 0.35 $mg \cdot L^{-1}$, $CaCl_2 \cdot 2H_20$ 1.65 $mg \cdot L^{-1}$, $MgSO_4 \cdot 7H_2O$ 2.98 $mg \cdot L^{-1}$, $(NH_4)_2SO_4$ 3.41 $mg \cdot L^{-1}$ – Lægdsmand et al., 1999], or b) water from pond M diluted to 50% with artificial "rain water" (to neutralize the pH of the natural water sample). Thus, in each site (A, B, F, and I), four experimental *in situ* treatments were carried out: *NsRw* = natural soil + artificial "rain water" (n = 4 test chambers); *NsMw* = natural soil + pond M diluted at 50% with artificial "rain water" (n = 4); *LuRw* = LUFA + artificial "rain water" (n = 2); *LuMw* = LUFA + pond M diluted at 50% with artificial "rain water" (n = 2). The *in situ* assay began with the introduction of seven earthworms in each of the test chambers, followed by the placement of the chambers inside a depression made in the local soil (Fig. 1B). Chambers were buried in such a way that the top of the chambers (i.e. lid with aeration opening) was levelled to the adjacent soil surface. No food or additional water was added to the test chambers, which were covered with a black plastic bag to prevent direct sunlight.

After 48hrs, test chambers were removed from the field and earthworms and soils were recovered. Test organisms from each chamber were divided in two sets: i) five organisms were frozen in liquid nitrogen for posterior quantification of biochemical markers (*see* 2.5 sub-section) and ii) two worms were frozen at -20ºC to analyse metal body burdens (*see* 2.4 sub-section) to assess bioaccumulation. Before freezing, both sets of recovered earthworms were immersed in distilled water for 5-10 min to wash external soil particles and to allow partial excretion of gut contents (mostly soil particles), which could influence metal analyses. Test soils were transported in the original test chambers to the laboratory, where they were immediately characterized in terms of their physical and chemical properties and potential mobile fractions of metals (*see* 2.3 sub-section).

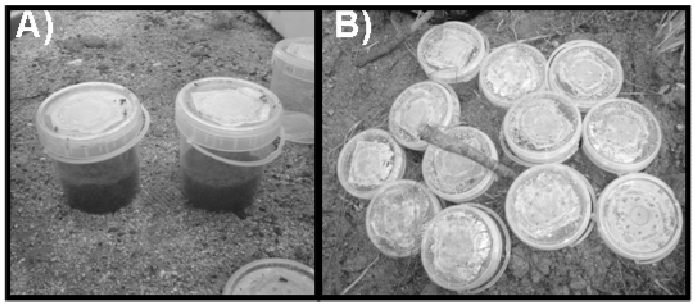

Figure 1 – *In situ* assay: A) test chambers with soil and earthworms (*E. andrei*) prior to B) deployment in the field.

Soil characterization

For the determination of soil chemical properties (pH, conductivity, organic matter content, and potential mobile metal fractions) a sub-sample of each test soil was used. Soil pH (H_2O) and conductivity were measured in a soil-water suspension (1:5 w/v extraction ratio), according to the method described in FAOUN (FAOUN, 1984). Conductivity was measured in the same suspension, which was left to rest overnight in order to allow the bulk of soil to settle (SPAC, 2000). Organic matter content was determined by loss on ignition at 450ºC, during 8 hrs (SPAC, 2000). For the determination of potential mobile (i.e. potentially bioavailable) metal fractions of soil, 15 g of soil were mixed with 200 ml of artificial "rain water" (*see* above) and stirred twice a day. After two days (to mimic the duration of the *in situ* bioassay), one aliquot of supernatant was collected for metal analysis and filtered through a Whatman GF/C filter (1.2 μm porosity, 47 mm diameter). Filtered samples were then acidified with nitric acid to pH<2 to reduce adsorption phenomena and stored in a plastic container at 4ºC until determinations were possible. Samples were analysed for selected metals using ICP spectrometry (APHA-AWWA-WEF, 1995). Additionally, another aliquot of supernatant was used for titrimetric determination of hardness and alkalinity (APHA-AWWA-WEF, 1995).

Earthworm metal body burdens

Test organisms were thawed at room temperature and dried at 60ºC until constant weight. Each set of two earthworms (corresponding to each replicate test chamber) was then placed in teflon vials, to which 1 ml of 65% suprapur nitric acid was added (acidic metal extraction). Samples were left overnight in a hot (60ºC) sand bath until all tissues were digested. After cooling, 0.5 ml of 30% suprapur hydrogen peroxide was added and the mixture was reheated overnight in the hot sand bath. After cooling, ultra-pure water was added to a total volume of extract of 20 ml. Extracts were analysed for selected metals using ICP spectrometry (APHA-AWWA-WEF, 1995). To obtain the metal bioconcentration factor (BCF) in earthworms, we calculated the ratio between the metal content in earthworms (in $\mu g \cdot g^{-1}$ dry weight) and in soil ($\mu g \cdot g^{-1}$ soil) in the *NsRw* (natural soil + artificial "rain water") treatments, for each site (I, A, B, and F):

$$\mathbf{BCF} = \frac{[\mathbf{metal}]_{\mathbf{earthworm}}}{[\mathbf{metal}]_{\mathbf{soil}}}$$

For calculation purposes, we used the standard pseudo total-metal concentrations extractable with aqua regia (from Pereira et al., in press) as the metal content of soils.

Biomarkers

Oxidative stress was evaluated individually (in each of the 5 worms of each replicate test chamber) through enzymatic determinations (Catalase – CAT, Glutathione peroxidase – GPx) and lipid peroxidation assays (TBARS). Organisms, which had been previously frozen in liquid nitrogen (see above), were thawed and homogenised in ice-cold phosphate buffer (50 mM, pH = 7.0 with 0.1% TRITON X-100). Homogenates were centrifuged at 10000g for 10 min and supernatants were divided into four aliquots, one for each determination (CAT, GPx, and TBARS) and a spare one. Aliquots were stored at -80ºC until determinations were possible.

Catalase (CAT) activity was determined in homogenates by the spectrophotometric method described by (Aebi, 1984). This method involves the monitoring of the consumption of hydrogen peroxide, at a wavelength of 240 nm ($\varepsilon = 0.0394\ mM^{-1} \cdot cm^{-1}$), for a period of 30 secs. Glutathione peroxidase (GPx) activity was determined according to Flohé and Günzler (1984), following the oxidation of NADPH at a wavelength of 340 nm ($\varepsilon = 6.22\ mM^{-1} \cdot cm^{-1}$), when GSSG is reduced back to GSH by glutathione reductase. GPx activity was monitored using both hydrogen peroxide (0.255 mM) and cumene hydroperoxide (0.7 mM) as independent substrates, corresponding respectively to selenium-dependent glutathione peroxidase [GPx(H_2O_2)] and total glutathione peroxidase [GPx(cumene)]. Enzymatic activities were determined in triplicate and were expressed in μmol (Cat) or nmol (GPx) of substrate hydrolyzed per minute per mg of sample protein (see protein quantification below).

The extent of lipid peroxidation was measured by the quantification of thiobarbituric acid reactive substances (TBARS), according to the protocol described in Buege and Aust (1978). This methodology is based on the reaction of lipid peroxidation by-products, such as malondialdehyde (MDA), with 2-thiobarbituric acid (TBA). The amount of TBARS was measured spectrophotometrically as a single determination, at a

wavelength of 535 nm (ε = 156 $mM^{-1} \cdot cm^{-1}$), and results were expressed as nmol of MDA equivalents per mg of sample protein (see below).

Protein concentration of each sample was determined in triplicate, according to the spectrophotometric (wavelength = 595 nm) method of Bradford (1976) adapted to microplate. All biomarkers (catalase, GPx, and TBARs) were expressed as function of the protein content of the corresponding sample. For statistical purposes, and to reduce the variability of biochemical determinations, we averaged the biomarker values of the 5 organisms in each replicate test chamber.

Statistical analyses

To assess the relative contribution of the experimental factors (soil type and water type), we conducted a two-way ANOVA for each site independently. All parameters were tested for significant differences between treatments, using a significance level of 0.05 in all analyses. The two-way ANOVA approach allowed us to assess whether the observed effects (metal body burdens and oxidative stress biomarkers) were due to exposure and ingestion of contaminated soil particles. We expected large differences between soil types (natural soil *versus* LUFA 2.2), since the latter is a contaminant-free standard reference soil. Additionally, this approach allowed us to test the influence of an effluent runoff (worst-case scenario) in the tested variables (soil chemistry, metal body burdens and oxidative stress biomarkers in *E. andrei*) *versus* the contribution of rainfall to mobilize soil metals. We also conducted a one-way ANOVA, followed by a Tukey test (when applicable), on the data concerning the natural treatment (*NsRw* – local soil + artificial rain water), in order to determine differences between sites/soils (I, A, B, and F). In the case of metal contents in soil and earthworms, these univariate tests were preceded by a MANOVA.

RESULTS

Soil chemical properties

Table 1 presents the variation in soil chemical parameters measured inside the test chambers, for the different treatments. We found significant variation in the pH levels between the soil samples from each site (one-way ANOVA for *NsRw* treatment among soils: $F_{3, 15} = 681.73$; $P < 0.000$). Soils/sites I and A were acidic, while sites F and B were

closer to neutrality. Significant variation between sites was also found for soil OM ($F_{3, 15}$ = 9.75; P = 0.002) and hardness of soil extracts ($F_{3, 14}$ =105.14; P < 0.001). Soil I presented a much higher content in OM than the other soils, especially A and F, where a very low percentage of OM was observed. Variation in hardness was mainly due to the high levels recorded in site B, which is a sludge deposition site. Low variation between sites and/or experimental data was observed for alkalinity and conductivity and we chose not to present these data.

Soil type (natural *versus* LUFA 2.2) gave the largest contribution to the variation in soil chemical data due to the experimental treatments conducted at each site. Natural soils often differed significantly from the standard reference soil, both in terms of pH, OM and hardness (Table 1). Only sporadically we observed a significant effect of water type (artificial rain water *versus* mine effluent) on the soil chemical parameters or on the differences between soil types (i.e. water x soil type interaction) (Table 1).

Table 1 – Soil chemical parameters (mean ± se) for each combination of site (I, A, B, and F) and experimental treatment. The corresponding two-way ANOVA summary is also shown for each parameter. Experimental treatments correspond to combinations of natural (Ns) or LUFA 2.2 soil (Lu) moistened with artificial rain water (Rw) or mine effluent water (Mw). NS stands for non significant.

Site	treatment	**pH**	**ANOVA pH**	**OM** (%)	**ANOVA OM**	**Hardness** ($mg \cdot L^{-1}$ $CaCO_3$)	**ANOVA hardness**
I	*NsRw*	4.86±0.05	Soil type P<0.001 Water type NS Interaction P=0.041	13.41±3.81	Soil type P<0.001 Water type NS Interaction P=0.041	23.00±2.12	Soil type P<0.001 Water type P<0.001 Interaction P<0.001
	LuRw	5.80±0.03		7.87±3.61		27.00±3.00	
	NsMw	4.84±0.05		13.90±2.43		43.25±2.06	
	LuMw	5.58±0.02		4.16±0.11		83.00±1.00	
A	*NsRw*	4.99±0.01	Soil type P<0.001 Water type P=0.006 Interaction NS	0.66±0.10	Soil type P<0.001 Water type NS Interaction P=0.041	32.67±1.76	Soil type P=0.001 Water type NS Interaction NS
	LuRw	5.68±0.04		4.04±0.01		75.00±13.00	
	NsMw	4.78±0.02		0.72±0.04		44.50±3.86	
	LuMw	5.59±0.08		4.13±0.06		83.00±7.00	
B	*NsRw*	7.59±0.04	Soil type P<0.001 Water type NS Interaction NS	3.89±0.37	Soil type P<0.001 Water type NS Interaction P=0.041	205.50±15.95	Soil type NS Water type NS Interaction NS
	LuRw	6.12±0.29		4.11±0.06		153.00±3.00	
	NsMw	7.62±0.03		5.05±0.35		181.25±14.63	
	LuMw	6.19±0.05		4.00±0.02		147.50±37.50	
F	*NsRw*	6.16±0.07	Soil type NS Water type NS Interaction NS	0.92±0.01	Soil type P<0.001 Water type NS Interaction P=0.041	25.75±3.57	Soil type NS Water type NS Interaction NS
	LuRw	5.74±0.17		3.99±0.10		42.50±1.50	
	NsMw	6.41±0.29		1.36±0.07		41.25±8.96	
	LuMw	5.78±0.18		3.97±0.15		46.00±14.00	

Metal content in test soils

Of the 10 metals analysed, Be and Cd were below detection limit in most soil samples. Sites I, A, B, and F differed significantly in their metal content (MANOVA Pillai's trace = 3.00; $F_{(30,\ 9)}$ = 204.9; P < 0.001). Univariate test statistics showed significant differences for all the elements, except Pb (Table 2). Soils from site I were characterised by high Al and Mn concentrations, but overall low levels of other elements. Site A was characterised by high Be, Cd, Mn, Ni, U and Zn concentrations, while in site B high doses of Sr and U were recorded along with overall low concentrations of other metals. Soils from site F displayed low or intermediate metal levels. These evidences can be visualised in Figure 2 in the *NsRw* treatment, which presents metal contents in the tested soils.

Higher concentrations of Al, Fe and Pb were found in LUFA 2.2 than in local soils, while the opposite was true for Mn and U and for most elements in site A (Figure 2). The largest contribution to the variation in soil metal content was usually provided by soil type (see two-way ANOVA summaries in Figure 2). Only sporadically we observed a significant effect of water type (*Rw versus M*w) on the soil chemical parameters or on the differences between soil types (i.e. water x soil type interaction). When this occurred, the use of mine water (*Mw*) did not necessarily correspond to an increase in the metal concentration in soil, except for Sr in site I, the only situation where the addition of *Mw* was responsible for a consistent increase of soil metal content in both soil types (Figure 2).

Table 2 - Univariate F statistics for metal content in soil and earthworms from the in situ assay (see test for additional explanation). Significant effects of site on both variables are in bold letter.

	Soil ($\mu g \cdot g^{-1}$)			**Earthworms** ($\mu g \cdot g^{-1}$)		
	F	d.f.	*P*	*F*	d.f.	*P*
Al	40.8	3, 10	**<0.001**	3.50	3, 12	**0.050**
Be	40649	3, 10	**<0.001**	106	3, 12	**<0.001**
Cd	1325	3, 10	**<0.001**	0.22	3, 12	0.884
Fe	22.7	3, 10	**<0.001**	2.20	3, 12	0.141
Pb	0.48	3, 10	0.704	2.90	3, 12	0.079
Mn	111	3, 10	**<0.001**	67.7	3, 12	**<0.001**
Ni	25.8	3, 10	**<0.001**	89.4	3, 12	**<0.001**
Sr	635	3, 10	**<0.001**	0.63	3, 12	0.608
U	215	3, 10	**<0.001**	11.3	3, 12	**0.001**
Zn	14.5	3, 10	**0.001**	19.4	3, 12	**<0.001**

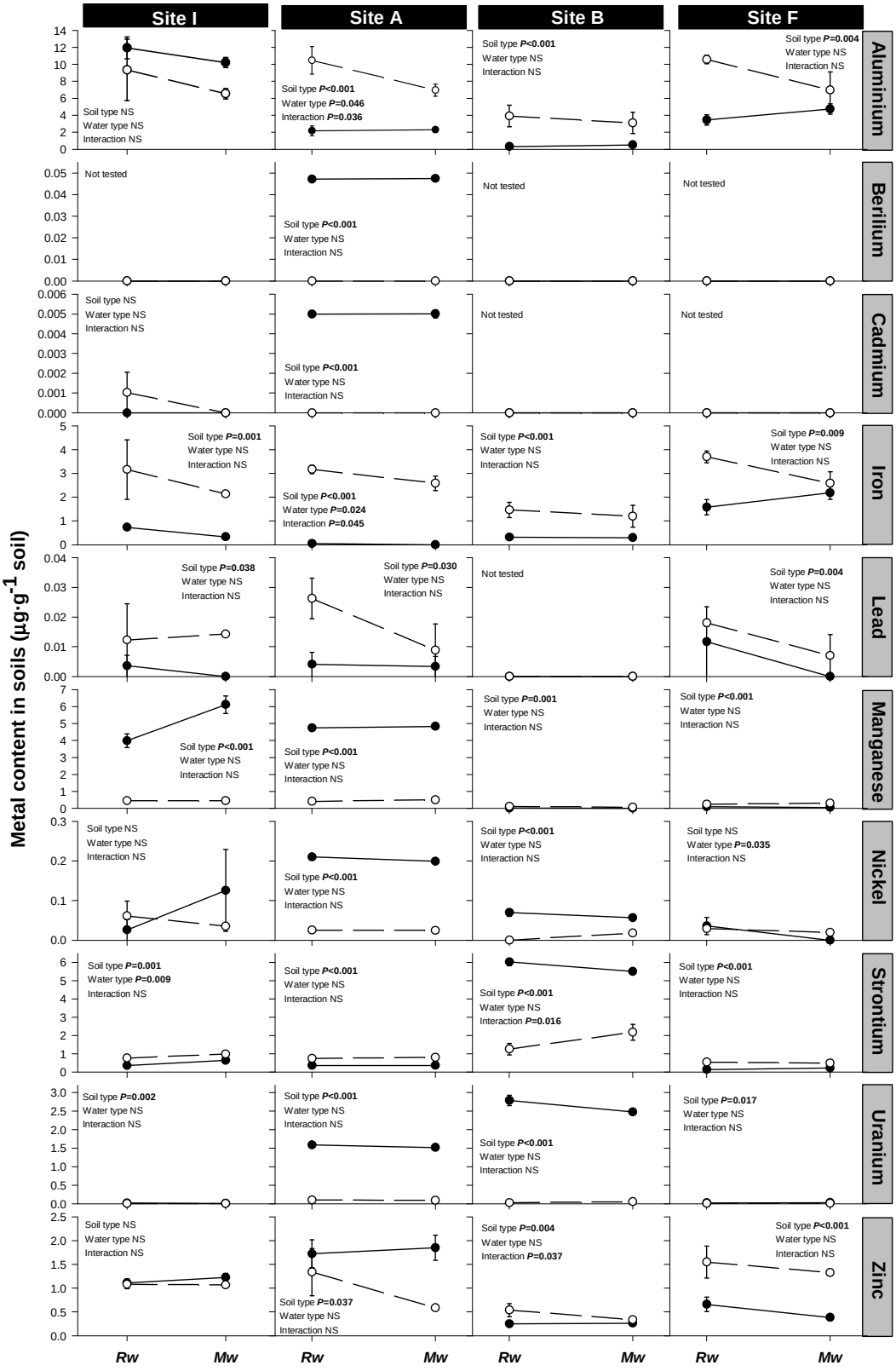

Figure 2 – Metal content (mean ± se) in soils for each combination of site (I, A, B, and F) and experimental treatments. The corresponding two-way ANOVA summary is also shown for each metal, within each site. Experimental treatments correspond to combinations of natural (Ns - ●) or LUFA 2.2 soil (Lu - ○) moistened with artificial rain water (Rw) or mine effluent water (Mw). NS stands for non significant.

Metal body burdens in E. andrei

Earthworms exposed to soils from the different sites (I, A, B, and F) differed significantly in their metal burdens (MANOVA with Pillai's trace = 2.805; $F_{(30, 15)} = 7.2$; $P < 0.001$). However, accordind to univariate statistical tests, this was true for Al, Be, Mn, Ni, U and Zn, but not for Cd, Fe, Pb and Sr (Table 2). Earthworms exposed to soils from site I and A displayed the highest metal burdens for Al and U (respectively), but other elements were recorded in comparable or lower concentrations than other sites. The highest body burdens in Be, Mn, Ni and Zn were observed in earthworms exposed to soil from site B. Earthworms from site F were characterised by low body burdens in all elements (relatively to the other sites), except for U. *Post-hoc* Tukey tests statistically supported these findings (see also Figure 3).

Like for soil metal content, the major contribution to the variation in metal body burdens was given by soil type (see two-way ANOVA summaries in Figure 3). As expected, metal body burdens were generally higher in local soils (*Ns*), comparatively to LUFA 2.2 (*Lu*). Sr and Pb were consistent exceptions in almost all sites, being higher in earthworms from *Lu* (Figure 3). The effect of soil was independent of the type of water used to moisten the test soils. Furthermore, significant effects of water type on metal body burdens were only observed in four occasions (Cd and Zn in site A; Sr and Pb in site F – Figure 3), suggesting an overall low contribution of water from the mine effluent (*Mw*) as an additional source of metal bioconcentration in *E. andrei*.

Using the metal body burdens of *E. andrei* in the *NsRw* treatment, we calculated the bioconcentration factors (BCFs), which are presented in Table 3. Overall, earthworms displayed none (BCF ≤ 1) or low (BCF ≤ 3.7) bioconcentration of metals, when exposed to most sites/soils. Cd was the exception, since it displayed high BCFs in three (I, A and F) of four studied sites, revealing a high potential for transference along the trophic chain. Two other metals (Sr and Zn) also displayed high BCFs (BCF > 20), both in site A. In fact, earthworms exposed to soil from site A have shown to be particularly prone to bioconcentration of these metallic elements, as revealed by high BCFs for Cd, Sr and Zn, and intermediate BCFs for all other elements, including U (Table 3).

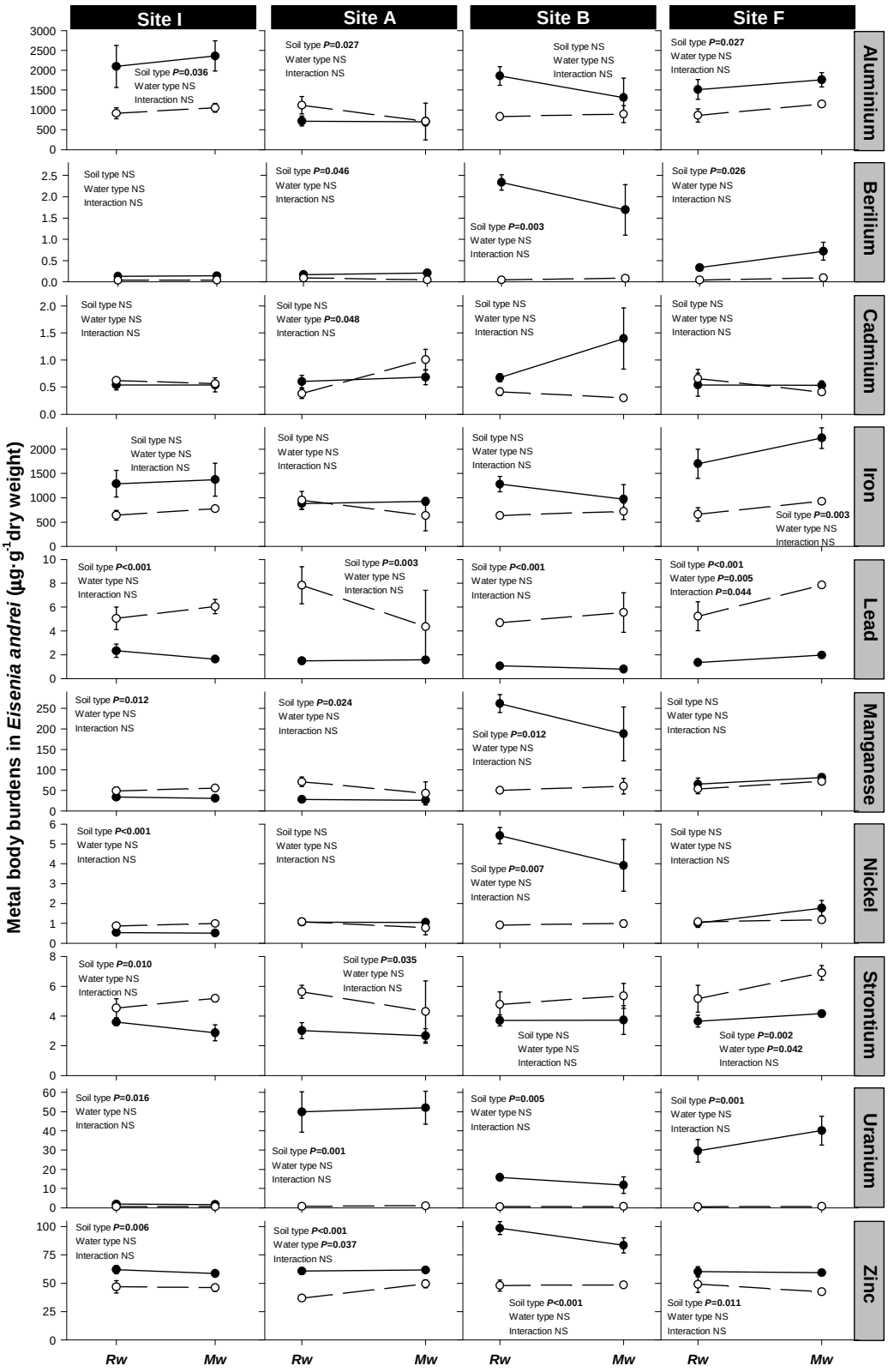

Figure 3 – Metal body burden (mean ± se) in E. andrei for each combination of site (I, A, B, and F) and experimental treatments. The corresponding two-way ANOVA summary is also shown for each metal, within each site. Experimental treatments correspond to combinations of natural (Ns - ●) or LUFA 2.2 soil (Lu - ○) moistened with artificial rain water (Rw) or mine effluent water (Mw). NS stands for non significant.

Table 3 – Bioconcentration factors (BCF) in E. andrei after in situ exposure, calculated for each site (I, A, B, and F). Shadowed cells represent BCF values concordant with low (1<BCF<10; light grey) and intermediate (10<BCF<100; dark grey) bioconcentration values.

	I	A	B	F
Al	0.5	1.3	0.1	0.1
Be	0.1	1.8	0.1	0.1
Cd	18.9	87.0	0.4	13.9
Fe	0.5	1.4	0.1	0.2
Pb	0.3	1.5	0.2	0.2
Mn	0.1	3.3	0.1	0.2
Ni	0.3	3.6	0.1	0.3
Sr	0.5	20.6	2.1	1.5
U	0.1	3.7	0.1	0.2
Zn	2.2	21.9	0.4	1.4

Oxidative stress biomarkers in E. andrei

Significant differences were found among sites in the *NsRw* treatment for TBARS (one-way ANOVA: $F_{(3, 12)} = 5.4$; $P = 0.014$) and total GPx (one-way ANOVA: $F_{(3, 12)} = 63.5$; $P < 0.001$), but not for Cat (one-way ANOVA: $F_{(3, 12)} = 3.4$; $P = 0.055$) and Se-dependent GPx (one-way ANOVA: $F_{(3, 12)} = 1.0$; $P = 0.420$). Site I was responsible for the differences observed in TBARS and total GPx, as earthworms exposed to soil from this site displayed significantly higher values than those exposed to other sites/soils (*post-hoc* Tukey tests, $P \leq 0.05$) – see also Figure 4. At a first glance, these findings seem to be contradictory to the data collected on the soils' metal content and bioaccumulation potential of the studied sites.

Variation in biomarker data seemed to be mostly random throughout the experimental treatments (Figure 4), since in most cases no differences were found between soil type used (*Ns versus Lu*), water type (*Rw versus Mw*), or their interaction, as seen in Figure 4. Actually, we only found statistically significant differences in two occasions, both at site I: TBARS (significant effect of water type) and Se-dependent GPx (significant soil x water type interaction). Thus, it seemed that the use of effluent water caused a significant increase in lipid peroxidation (TBARS – Figure 4), while the responses of earthworms were inconsistent between soil types for Se-dependent GPx (Figure 4).

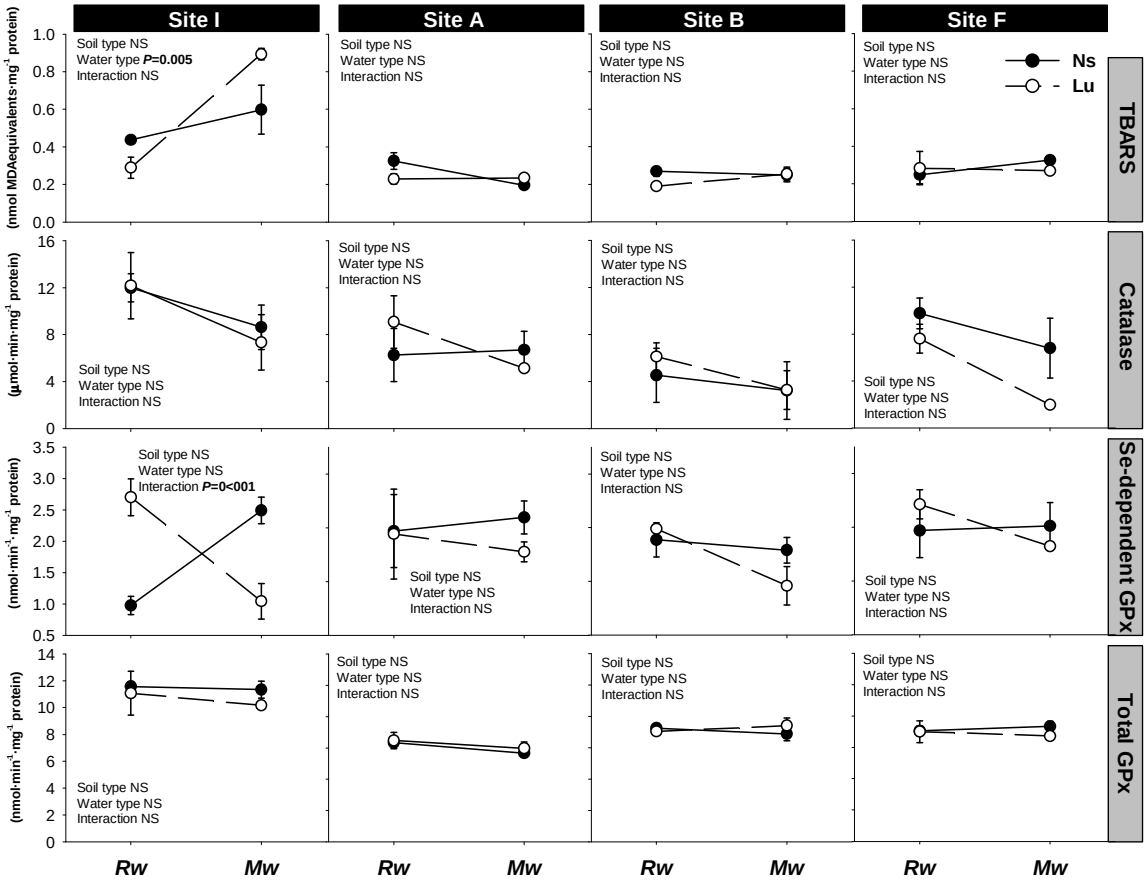

Figure 4 – Oxidative stress biomarkers (mean ± se) in E. andrei for each combination of site (I, A, B, and F) and experimental treatments. The corresponding two-way ANOVA summary is also shown for each metal, within each site. Experimental treatments correspond to combinations of natural (Ns - ●) or LUFA 2.2 soil (Lu - ○) moistened with artificial rain water (Rw) or mine effluent water (Mw). NS stands for non significant.

DISCUSSION

In situ bioassay

Data gathered in this study report the first application of an *in situ* assay with earthworms to assess soil toxicity in a contaminated area. *In situ* assays have become popular tools in aquatic toxicology (e.g. Castro et al, 2004, Pereira et al., 2000; Moreira et al., 2006) and their use as providers of site-specific toxicological information is recommended in the latter tiers of ERA (Weeks and Comber, 2005; Moreira et al., 2006). However, standard *in situ* bioassay procedures remains a chimera for soil ecotoxicologists. Here, we described a simple and reproducible procedure to conducting *in situ* bioassays with *Eisenia andrei*, which was successfully applied to an actual contamination situation, comprehending different locations (sites/soils) and experimental conditions within each site (soil type and irrigation water). Earthworms play a key role in many soil processes

(e.g. soil aeration, decomposing organic matter, soil microbial activity) and are in full contact with the soil (aqueous and solid phase), which makes them relevant indicators of environmental change (Paoletti, 1999; Cortet et al., 1999; Römbke et al., 2005; Arnold et al., 2003]. Moreover, earthworms construct burrows which promote a direct contact with contaminants via distinct routes (ingestion of contaminated soil or food particles, passive adsorption of the metal dissolved in interstitial water through the body wall, ingestion of contaminated soil solution) (Arnold et al., 2003). Hence, we have considered them a good model species for the development of *in situ* assays to assess soil contamination.

The *in situ* bioassay chambers were successfully deployed and produced a 100% recovery rate of the organisms. The time scale of responses can be measured across a hierarchical level of biological systems, although this may vary according to the environmental stressor (Winston et al., 2002). Since we intended to promote a short-term *in situ* exposure (48hrs), the choice of rapid-responding endpoints seemed appropriate (namely oxidative stress biomarkers). However, we do not exclude the possibility of employing other sensitive parameters, since the choice of the tested enzymological biomarkers was also a matter of feasibility. Thus, other parameters can be incorporated into the *in situ* design, as long as the exposure time is adjusted accordingly. Here, we also quantified metal body burdens in earthworms, which could be viewed as a risky option, given the short-term exposure time. As will be discussed later, the latter choice produced more consistent results than the biochemical approach.

The *in situ* design comprised different treatments, simulating rainfall (using artificial rain water - *Rw*) and runoff (using the mine effluent water - *Mw*) events on the soil compartment. The use of a standard reference soil (LUFA 2.2) allowed us to perceive the relative contribution of the type of irrigation water. Clearly, the mine effluent provided a reduced contribution to the potential mobile fractions of metals in soils, as well as in the bioaccumulated fractions of metals in earthworms (*see* also below). Our results suggest that potential runoffs will have reduced influence in the increment of risks posed by local contaminated soils. Still, evidence from TBARS and CAT (*see* next paragraphs) also suggest a sub-lethal effect of the mine effluent water. Nevertheless, the major contribution to metal content, both in soils and in earthworms, was due to soil type, showing that some contaminants of concern are available (and apparently bioaccessible, *see* further in the discussion) in natural soils. For a few elements (Al, Fe, Pb), LUFA exhibited higher

mobile fractions of metals than natural soils, but levels were not of environmental concern (Pereira et al., in press).

Biomarker responses

In general terms, the battery of biomarkers chosen showed not to be sensitive to the *in situ* exposure to contaminated soils, at least for the exposure duration considered in this study. Although it is hard to make inferences from biological responses in highly complex environments, such as mining areas, we would expect some sort of oxidative stress damage and/or induction of anti-oxidation defences. However, the most consistent pattern observed was that of no effects either between soil or water types. Since biochemical markers are at a low level of biological organization they are supposed to respond to stressors within hours (Winston et al., 2002). Furthermore, we did find high metal burdens in earthworms, sometimes at levels suggesting bioaccumulation (*see* below). This high dosage of metals in the earthworms' body is likely to endure cellular damage, such as lipid peroxidation. Although other detoxification and/or protection mechanisms may constitute cellular defences (e.g. metallothioneins), anti-oxidative stress defences should constitute an important barrier of defence against reactive oxygen species (ROS) normally produced by metabolic processes involving oxygen as final acceptor of electrons in the mitochondrial respiratory chain. The exposure to compounds capable of exerting oxidative stress accelerates the overall process of ROS production, ending in exertion of oxidative stress by surpassing the total antioxidant capacity of the organism (Nunes et al., 2006). At the light of such theoretical background, our findings are difficult to explain. However, we may justify the overall lack of responsiveness due to the fact that the antioxidant defence mechanism depends upon the expression of enzymes. Since these agents are proteins, it is absolutely necessary to undergo genetic expression at the nucleus, which is not an immediate response to oxidative insult. As mentioned above, it is thus expectable that other mechanisms of antioxidant defence may play a major role, both through specific mechanisms (e.g. scavenging of metals by metallothioneins), or by unspecific responses to the presence of free radicals (e.g. scavenging vitamins, such as vitamin C or E). Only after the complete exhaustion of this complex defensive apparatus, it is expectable that metals may exert oxidative toxic damage (causing an increased expression of CAT, GPx and damaging cellular lipids), which may not occur after short periods of exposure (such as the

one used in the *in situ* assay). An alternative hypothesis is the potential refractory behaviour of the antioxidant mechanism of *E. andrei* towards metal contamination. As summarized in Nunes et al. (2006), a large number of reasons (e.g. absence of specific receptors involved in the proliferation of subcellular organelles with strong ROS productions, such as peroxissomes) may underlie the biological lack of response of several animal species. Due to the absence of explanatory data concerning the physiology of this particular species of worm, is it thus impossible to state if the absence of response is a normal physiological feature of *E. andrei*.

Although statistically non-significant, the exposure of the selected test organisms to metals caused a fairly consistent (across sites and treatments) decrease in the activity of the biomarker catalase. This effect was most noticeable between water types (*Rw* vs. *Mw*), irrespective of soil type. Previous works involving plants (Pandey and Sharma, 2002) and fish (Vutukuru et al., 2005; Atli and Canli, 2007) corroborate our findings. These studies have shown a decrease of CAT activity in the presence of metals (e.g. Fe, Zn), which is believed to be a consequence of interference of the absorbed metallic ions with the synthesis of metal-bearing enzymes (such as catalase), showing a crucial interplay between anthropogenic contamination by metallic species and this oxidative stress biomarker. In our study, the strongest reduction of CAT was followed by a statistically significant increase of TBARS content in the tissues of test organisms from site I, establishing a possible relationship between biological effects and oxidative damage caused by the irrigation of soils with the mine effluent (*Mw*). Apparently, the effect of mine water addition was only perceivable in the soil/site farthest from the mining area, which had been previously labelled as a potential reference soil (this study, Antunes et al., in press; Pereira et al., in press).

Metal bioaccumulation

This study focused on the potential of earthworms as a biological indicator of pollutant exposure, combining chemical and biological investigations on the status of contaminated soils in an abandoned uranium mine. Site I was characterised by the highest percentage of OM (a relevant variable, as along with pH, in earthworm activities – Römbke et al., 2005) and, overall, the lowest levels of metal content. Taking these characteristics into account, we expected low levels of bioaccumulation. Contrarily, site A

presented the lowest values of OM and pH, essential characteristics for higher bioavailability of most metal elements (namely Be, Cd, Mn, Ni and Zn) (Férnandez et al., 2005; Lukkari et al., 2004). The other two sites (B and F) had quasi-neutral pH values and low percentage of OM. This feature represents an ideal scenario for U and Sr mobilization from the soil into the aqueous phase (Férnandez et al., 2005; Lukkari et al., 2004), which can be observed namely in site B (Table 1 and Figure 2). However, these considerations may not be as straightforward as stated above, since these soils display a complex mixture of contaminants (whose interactive effects are unknown). When working with natural soils, this is worsened because it is not feasible to quantify all the contaminants present in the samples and some sort of *a priori* selection must be made.

All tested soils showed a potential mobilization of metals towards the biota, but each soil displayed a singular behaviour in terms of the bioaccumulation potential of metals in earthworms. Chemical data obtained in our study reinforced site I as being a reference, confirming our previous findings (Antunes et al., in press; Pereira et al., in press). Still, high levels of a few metals (namely Al, Mn) were recorded, which do not seem to be bioavailable for organisms. This situation is well illustrated in Table 3, where Al and Mn recorded the lowest values of BCF for site I. Extraction of metals using artificial rain water revealed that they were mobilizable from the soil (Fig. 2). It is important to perceive that metal extraction (mobilization to an aqueous phase) produces some uncertainty, and several authors have alerted that each extraction method results in various intensities of mobilized metals (Gupta et al., 1996; Sterckeman et al., 1996). Furthermore, mobilizable metals may not necessarily be bioaccessible for organisms. Bioaccessibility and bioavailability are terms used for monitoring contaminants in organisms, but they have different meanings. Peijnenburg and Jager (2003), in their work about bioavailability and bioaccessibility, define these terms according to the fraction of the total amount of contaminant: “bioavailability is a fraction of the total amount of a chemical present in a specific environmental compartment”, while “bioaccessibility is a fraction of the total amount of a chemical present in ingested food (i.e. the fraction that, after ingestion, may be mobilized by the gut fluids)”. The use of earthworms as test organisms is particularly relevant and helpful in soil toxicology, since they ingest soil particles, being exposed to all contaminant forms, both solid and aqueous, while their digestive juice (pH is variable in the different compartments of the gastrointestinal tract)

may increase the mobilization of contaminants from soil (Arnold et al., 2003; Peijnenburg and Jager, 2003). We must stress out that the digestive juice of earthworms is approximately neutral in pH (Peijnenburg and Jager, 2003) and such conditions, in our case, may induce an increase of bioaccessibility of uranium, unlike for other elements.

Taking into account these definitions, it is possible to state that contaminants were not bioaccessible in site I. The highest mobilizable concentrations of metal (Be, Cd, Mn, Ni, U and Zn) were recorded in A (Fig. 2). However, in this site, earthworms presented high metal contents only for U (Fig. 3). This means that most metals were bioavailable to earthworms, but they were not bioaccessible, except for U. Site B showed the highest metal content for U and Sr (Fig. 2), but metals other than these (Be, Mn, Ni, and Zn) were found to occur in high concentrations in earthworms (Fig. 3). Sites B and A were both characterised by high levels of U in soils (B>A), but the U content in earthworms produced contradictory results (A>>B). A possible explanation for this is due to the fact that site B was located at a sludge deposition site from the treatment pond, where contaminants were precipitated and complexated. In this manner, metals (namely U and Sr) may be present in forms not bioaccessible for organisms. Site F scored the lowest values for most metals, but earthworms presented high burdens of Fe and U. Calculation of BCFs clearly showed that site A can be viewed as the site posing most concerns (BCFs>1 for all elements), while Cd was the metal with higher BCFs (consistently in most sites). Mariño and Morgan (1999) have shown that Cd can be strongly accumulated by earthworms, since it is a non-essential element that is difficult to eliminate.

CONCLUSIONS

Overall, our results demonstrated that the *in situ* toxicity assay, based on the exposure of *E. andrei* to contaminated soils moistened with different water types, was sensitive and responsive, generating ecologically relevant data to be incorporated in risk assessment studies. The developed test-chamber and *in situ* procedure revealed to be suitable for exposing and retrieving *E. andrei*, in a cost-effective and simple way. It is, however, necessary to optimize this *in situ* assay to different time exposures according to the different endpoints intended to measure (e.g. bioaccumulation, survival, reproduction, growth, EC_{50}). This will further validate this tool for future implementation in tier 3 of ecological risk assessment and will allow the assessment of longer exposures to

contaminants in the tested endpoints (e.g. biomarkers, which did not fully work here as sensitive parameters to environmental contamination). In our case, contaminants seemed to be available to earthworms via soil particles, since irrigation water gave a less significant contribution to soil and earthworm metal contents. Metal content in soil and earthworms revealed to be good indicators of soil toxicity and, in spite of the reduced duration of the *in situ* exposure, the assay produced some indications of potential concern regarding metal bioaccumulation in earthworms exposed to site-specific conditions.

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Capítulo VII

CONCLUSÕES

No decurso do presente estudo é notório a obtenção de informação ecotoxicológica referente à mina de urânio da Cunha Baixa. Assim, e de acordo com os objectivos estabelecidos inicialmente o presente trabalho possui dados relevantes para uma análise de risco ecológico que contribuem para um cálculo preliminar dos riscos relativamente aos compartimentos aquático e terrestre. No entanto, a falta de valores de referência legislados para o contexto nacional, para diversos contaminantes (nomeadamente, metais), dificulta a interpretação dos dados da caracterização química. Deste modo, a análise de risco ecológico tem que avançar até etapas, mais demoradas, mais dispendiosas e com desenhos experimentais mais complexos que poderiam ser dispensadas. Assim, é perceptível a pertinência na obtenção de valores de referência para os diversos contaminantes do solo (essencialmente metais) de modo a inferir, *a priori*, (Etapa 1 da ARE) os potenciais riscos de locais contaminados. Não obstante o facto de existirem critérios de qualidade para os compartimentos aquático e terrestre, definidos por outros países europeus, a sua transferência e aplicação directa não é aconselhável, na medida em que estes foram obtidos com base em critérios políticos específicos, em diferentes tipos de utilização dos recursos, e, por vezes, com base em dados toxicológicos para espécies nativas (revisão de valores efectuada em Pereira et al., in press).

Por conseguinte, quando existe necessidade de obter dados mais relevantes para a avaliação dos potenciais riscos de um determinado local contaminado, a prossecução para etapas mais complexas da ARE é premente. Assim, possuindo dados ecotoxicológicos, obtidos com amostras naturais, é possível calcular os riscos que uma dada área apresenta. Há vários autores que apresentam diferentes metodologias para calcular os riscos para locais contaminados (e.g. Laurence, 2001; Van Vlaardingen et al., 2003; Fox, 2006; Jensen e Mesman, 2006). Laurence (2001) apresenta um modelo em que classifica os factores de risco associados ao encerramento de uma mina e assim deduz os riscos. Para tal, parte do princípio que os riscos se resumem ao somatório de todos os riscos individuais, assumindo que estes se dividem em: riscos ambientais, riscos para a saúde humana, riscos para a comunidade social, riscos no uso final do solo, riscos financeiros e legais e, finalmente, riscos técnicos. Em cada um destes tipos de risco apresenta diversos parâmetros que classifica numa escala de 1 a 10 (1 corresponde a um risco reduzido e 10 ao risco mais elevado). No entanto, este método é demasiado simplista e subjectivo, uma vez que a classificação não é feita com base em valores medidos ou obtidos, mas sim de forma

qualitativa. Jensen e Mesman (2006) apresentam uma nova metodologia para o cálculo dos riscos, partindo de dados expressos em diversas unidades de medida, sub-divididos em três linhas de evidência distintas (química, toxicológica, e de inventariação biológica), que são convertidos numa escala a variar entre 0 e 1 e posteriormente aplicados, de forma integrada, no cálculo dos risco. No entanto, o modelo apresentado por Jensen e Mesman (2006) está direccionado exclusivamente para o cálculo dos riscos para o compartimento terrestre (solo). Van Vlaardingen et al. (2003) apresentam um modelo matemático que só pode ser aplicado a dados correspondentes ao mesmo parâmetro e à mesma unidade de medida. Para o efeito prevê o cálculo da mediana de cada parâmetro (e.g. mediana de todos os valores de EC_{50} obtidos para *Daphnia magna*, mediana de todos os valores de EC_{50} obtidos para *Pseudokirchneriella subcapitata*). Os valores calculados para as diferentes medianas são utilizados para construir uma curva de distribuição de sensibilidade das espécies, da qual se extrapola o valor de HC_5. Este valor corresponde à concentração do agente de stress que garante a protecção de 95% das espécies (Aldenberg et al., 2002). Deste modo, na aplicação deste modelo quanto maior o número de parâmetros/espécies avaliados, menor é o erro no cálculo dos riscos. Adicionalmente, para aplicação deste modelo matemático devem usar-se, preferencialmente, parâmetros sub-letais (e.g. LOEC ou NOEC).

CÁLCULO DOS RISCOS

As tabelas 1 e 2 descrevem, de forma resumida, os dados ecotoxicológicos obtidos para os dois compartimentos estudados neste trabalho. Note-se que os dados relativos à caracterização físico-química bem como ao conteúdo em metais dos dois compartimentos (aquático e terrestre), fundamentais para uma avaliação preliminar dos riscos, efectuada na etapa 1 (Quadro 2) da ARE, não estão aqui apresentados uma vez que estão pormenorizadamente descritos em tabelas nos capítulos da tese e em Pereira et al. (in press). Assim, com base nos dados obtidos apresenta-se uma simulação do cálculo dos riscos para os dois compartimentos (aquático e terrestre) de acordo com as metodologias descritas por (Van Vlaardingen et al., 2003; Jensen e Mesman, 2006). Contudo, é preciso ressalvar que, apesar da credibilidade dos dados obtidos na presente tese, a incerteza dos valores calculados é elevada, dada a informação existente ainda ser reduzida.

Para o compartimento aquático fez-se o cálculo dos riscos apenas para o local M, com os valores dos ensaios agudos, uma vez que só foi possível determinar EC_{50s} para este local, para praticamente todas as espécies utilizadas, nas diversas situações (Tabela 1 e Quadro 1). Uma vez que a caracterização ecotóxicológica para o compartimento aquático se baseou essencialmente na obtenção de valores de EC_{50}, expressos em percentagem do efluente mineiro, recorreu-se ao modelo matemático proposto por Van Vlaardingen et al. (2003) (Quadro 1).

Tabela 1 – Resumo dos resultados obtidos com os ensaios ecotoxicológicos desenvolvidos para o compartimento aquático do local de estudo (*ver* Capítulos II, III e IV).

LAGOA REF	LAGOA T	LAGOA M
Inibição de crescimento algal (*P.subcapitata*) 96h		
		Outono *P. subcapitata* EC_{50}=27.0%(LC_{95}: 25,5 %-28,4%) Primavera *P. subcapitata* EC_{50}=60,4%(LC_{95}: 41,6%-82,8%)
Morte/Imobilização de dafnídeos 48h (água)		
Não foi detectada toxicidade	Não foi detectada toxicidade	*D. magna* EC_{50}=50,4%(LC_{95}:47,6%-53,3%) *D. longispina* EC_{50}=28,4%(LC_{95}:25,6%-32,1%)
		Outono *D. magna* EC_{50}=35,8%(LC_{95}:26,8%-50,8%) *D. longispina* EC_{50}=20,5%(LC_{95}:17,0%-24,3%) Primavera *D. magna* EC_{50}=83,6%(LC_{95}:80,5%-87,2%) *D. longispina* EC_{50}= 49,3%(LC_{95}:41,6%- 60,2%)
Reprodução de dafnídeos 21dias (água)		
D. magna LOEC=6,25%↑ ::: NOEC=25% *D. longispina* LOEC=25%↑ ::: NOEC=12,5%↑	*D. magna* LOEC=100%↓ ::: NOEC=75% *D. longispina* LOEC=ND ::: NOEC=100%	*D. magna* LOEC=4%↓ ::: NOEC=ND *D. longispina* LOEC=4%↑ ::: NOEC=13,5%
Morte/Imobilização de dafnídeos 48h (elutriados de sedimentos)		
Inverno Não foi detectada toxicidade Primavera Não foi detectada toxicidade	Inverno Não foi detectada toxicidade Primavera Não foi detectada toxicidade	Inverno Não foi detectada toxicidade Primavera *D. magna* EC_{50}=96,3%(LC_{95}: 95,6% - 97,0%) *D. longispina* EC_{50}=94.7%(LC_{95}: 80,1% - 110,9%)
Reprodução de dafnídeos 21dias (elutriados de sedimentos)		
Inverno *D. magna* LOEC=6,25%↑ ::: NOEC=12,5% *D. longispina* LOEC=12,5%↑ ::: NOEC=6,25%↑ Primavera *D. magna* LOEC=ND ::: NOEC=ND *D. longispina* LOEC=12,5%↑ ::: NOEC=6,25%↑	Inverno *D. magna* LOEC=50%↑ ::: NOEC=25% *D. longispina* LOEC=25%↓ ::: NOEC=12,5%↑ Primavera *D. magna* LOEC=6,25%↑ ::: NOEC=ND *D. longispina* LOEC=12,5%↑ ::: NOEC=6,25%	Inverno *D. magna* LOEC=6,25%↑ ::: NOEC=75% *D. longispina* LOEC=6,25%↓ ::: NOEC=ND Primavera *D. magna* LOEC=25%↑ ::: NOEC=12,5% *D. longispina* LOEC=12,5%↑ ::: NOEC=6,25%
Sobrevivência e crescimento larvar de quironomídeos 10dias		
		Outono PSLC *todos os organismos morreram Primavera PSLC=0,96mg/Ind

↑, ↓ = representam se o efeito foi positivo ou negativo, respectivamente
PSLC = peso seco livre de cinzas

Quadro 1.
Exemplo de cálculo dos riscos para o local M do compartimento aquático:
(*adaptado de* Van Vlaardingen et al., 2003)

Determinação do HC5

1) Cálculo da mediana de todos os valores de EC_{50} determinados para cada espécie (% de efluente):

	Mediana
Pseudokirchneriella subcapitata	43,7
Daphnia magna	50,4
Daphnia longispina	28,4

2) Inserção dos valores das medianas no programa matemático ETX 2000 1.409 (Agosto de 2003)

3) Valores de HC_5 debitados pelo programa:

HC5 results		These values are calculated using Table 1 from Aldenberg and Jaworska (2000)	
Name	*Value*	*^{10}log(value)*	*Description*
LL HC_5	4.035E+00	6.058E-01	lower estimate of the HC_5
HC_5	**2.225E+01**	**1.347E+00**	**median estimate of the HC_5**
UL HC_5	3.280E+01	1.516E+00	higher estimate of the HC_5
sprHC_5	8.129		spread of the HC_5 estimate

Assim, **22,25%** é a concentração do efluente a partir da qual mais de 5% das espécies do compartimento aquático estão ameaçadas.

NOTA: No entanto, é necessário ressalvar mais uma vez, que quanto maior o número de valores de entrada, mais correcto será o valor do cálculo dos riscos. Apesar deste valor indicar riscos, outros parâmetros sub-letais (e.g. LOEC, ensaios crónicos) deverão ser utilizados preferencialmente para efectuar o cálculo dos riscos.

No Quadro 2, a título exemplificativo, apresentam-se os dados do cálculo dos riscos para o compartimento terrestre, em alguns dos locais de estudo, através da integração de duas linhas de evidência (química e ecotoxicológica).

Tabela 2 – Resumo dos resultados obtidos com os ensaios ecotoxicológicos desenvolvidos para o compartimento terrestre da área adjacente ao local de estudo (*ver* Capítulo V).

Local	**Ensaio de evitamento com *E. andrei* = NR**	**BCF em *E. andrei* após ensaio in situ**	**Biomarcadores medidos em *E. andrei* após exposição *in situ***
A	1,0	Be = 1,8 Al = 1,3 Mn = 3,3 Fe = 1,4 Ni = 3,6 Zn = 21,9 Sr = 20,6 Cd = 87,0 Pb = 1,5 U = 3,7	TBARS = $2{,}71 \times 10^{-3}$ µM/min/mg protein Catalase = 107,3 µM/min/mg protein Se-dependente GPx = 1,088µmol/min/mg protein GPx total = 5,862 µmol/min/mg protein
B	1,0	Sr =2,1	TBARS = $2{,}24 \times 10^{-3}$ µM/min/mg protein Catalase = 135,6 µM/min/mg protein Se-dependente GPx = 1,390 µmol/min/mg protein GPx total = 6,665 µmol/min/mg protein
C	0,8		
D	0,7		
E	-0,6		
F	1,0	Zn = 1,4 Sr = 1,5 Cd = 13,9	TBARS = $2{,}07 \times 10^{-3}$ µM/min/mg protein Catalase = 149,0 µM/min/mg protein Se-dependente GPx = 1,426 µmol/min/mg protein GPx total = 6,508 µmol/min/mg protein
G	0,5		
H	-0,6		
I	-0,9	Zn = 2,2 Cd = 18,9	TBARS = $3{,}64 \times 10^{-3}$ µM/min/mg protein Catalase = 153,3 µM/min/mg protein Se-dependente GPx = 0,417 µmol/min/mg protein GPx total = 10,670 µmol/min/mg protein
J	-0,7		

Quadro 2.
Exemplo de cálculo dos riscos:
(*adaptado de* Jensen e Mesman, 2006)

Química:
Extracção química dos contaminantes
Dados de metais quantificados através da extracção com água da chuva artificial após uma semana e analisados em ICP-MS (Capítulo V e Pereira *et al*. submetido). Exercício de simulação apenas para dois locais (A e I) e para um metal (U).

	Ref (Local I)	**Local A**
R1 Urânio (mg/Kg de solo seco)	0,18	41,27
R2 SSL ou SQGV	5	5
R3 = R1/R2	0,036	8,254
R4 Classificação do risco **= 1-(1/(1+R3))**	0,035	0,892
R5 Correcção para concentrações de base **= (R4-R4Ref)/(1-R4Ref)**	0	0,888
R6 Risco combinado **= 1-((1-R5)$_1$*(1-R5)$_2$*…(1-R5)$_n$)** 1, 2, …, n = cada metal analisado	0	0,967

Resultados da linha de evidência química para os diferentes locais da área de estudo

	A	**B**	**C**	**D**	**E**	**F**	**G**	**H**	**I**	**J**
R6	**0,967**	-0,743	**0,869**	**0,881**	-0,255	0,485	-0,184	0,282	0	0,497

Locais em que a caracterização química classificou como de risco: **A**, **C** e **D**

SSL – soil screening values ou SQGV – soil quality guideline values (valor mais baixo disponível revisto por Pereira et al., in press).

Quadro 2 (cont.):

Toxicologia:
Resposta positiva nas amostras de referência ou controlo
Dados relativos ao ensaio de evitamento com *E.andrei* em % de preferência.
Exercício de simulação para dois locais de estudo (A e G) em função do local de referência (I) (Capítulo V).

	Ref (Local I)	Local A	Local G
Dados	91,7	0	23,3
R1 = (100-x)/100	0,083	1	0,767
R2 = (x-Ref)/(1-Ref)	0	1	0.746

	A	**B**	**C**	**D**	**E**	**F**	**G**	**H**	**I**	**J**
R2	**1**	**1**	**0,909**	**0,855**	0,418	**1**	**0,746**	0,109	0	0,091

Dados relativos ao ensaio agudo com os elutriados do solo com *Daphnia* em % de sobrevivência. (Não se registou toxicidade; todos os dados são = 100% de sobrevivência).

	A	**B**	**C**	**D**	**E**	**F**	**G**	**H**	**I**	**J**
R2 *D. magna*	-0,091	-0,091	-0,091	-0,091	-0,091	-0,091	-0,091	-0,091	-0,091	-0,091
R2 *D. longispina*	-0,091	-0,091	-0,091	-0,091	-0,091	-0,091	-0,091	-0,091	-0,091	-0,091

Linha de evidência toxicológica

	Ref (Local I)	Local A	Local G
E. andrei	0	0,999	0,746
***Daphnia* magna**	-0,091	-0,091	-0,091
Daphnia longispina	-0,091	-0,091	-0,091
R1 = log (1-x)			
E. andrei	0	-3	-0,594
D. magna	0,038	0,038	0,038
D. longispina	0,038	0,038	0,038
R2 = média (R1) em cada local	0,025	-0,975	-0,173
R3 = 1-(10^R2)	-0,060	0,894	0,328

Integração da linha de evidência toxicológica

	A	**B**	**C**	**D**	**E**	**F**	**G**	**H**	**I**	**J**
R3	**0,894**	**0,894**	**0,524**	0,443	0,115	**0,894**	0,328	-0,020	-0,060	-0,027

Locais em que a caracterização toxicológica classificou como de risco: **A**, **B**, **C** e **F**.

Quadro 2 (cont.):

Integração dos riscos:

	A	**B**	**C**	**D**	**E**	**F**	**G**	**H**	**I**	**J**
Química **(R6)**	**0,967**	-0,743	**0,869**	**0,881**	-0,255	0,485	-0,184	0,282	0	0,497
Toxicol. **(R3)**	**0,894**	**0,894**	**0,524**	0,443	0,115	**0,894**	0,328	-0,020	-0,060	-0,027
R1 = Log (1-x)										
Química	-1,482	0,241	-0,883	-0,923	0,099	-0,288	0,073	-0,143	0	-0,299
Toxicol	-0,975	-0,975	-0,322	-0,254	-0,053	-0,975	-0,173	0,009	0,025	0,011
R2 = média (R1) em cada local	-1,228	-0,367	-0,603	-0,589	0,023	-0,631	-0,050	-0,068	0,013	-0,144
Integração dos riscos										
R3 = 1-(10^R2)	**0,941**	**0,570**	**0,750**	**0,742**	-0,054	**0,766**	0,108	0,144	-0,029	0,282

Locais para os quais existem riscos através da integração dos dados químicos e ecotoxicológicos: **A**, **B**, **C**, **D** e **F**.

Em suma, o cálculo dos riscos para a área adjacente à antiga exploração mineira da Cunha Baixa revelou que para o:

- Compartimento aquático: o local M apresenta um valor de HC_5 de 22,25%, ou seja, a partir de concentrações superiores (> 22,25%) do efluente mineiro, mais de 5% das espécies expostas ao compartimento aquático encontram-se ameaçadas;

- Compartimento terrestre: os locais A, B, C, D e F apresentam riscos elevados para as espécies edáficas.

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