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MINIREVIEW

Riboflavin storage in earthworm chloragocytes/eleocytes in an eco-immunology perspective

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Abstract

Earthworm immune-competent cells, celomocytes, are easily retrieved for *ex vivo* analyses. Celomocytes consist of amebocytes and species-specific numbers of chloragocyte-derived eleocytes, the latter accumulating free riboflavin in their chloragosome inclusions. Autofluorescent eleocytes are abundant in *Eisenia sp.*, *Allolobophora sp.*, *Dendrobaena sp.*, *Dendrodrilus sp.*, and *Octolasion sp.*, and their numbers and riboflavin contents are affected in species-specific ways by soil quality, as observed by flow cytometry and spectrofluorimetry. The most striking results were obtained in the case of epigeic *Dendrodrilus rubidus*; in unpolluted soil its riboflavin content was high, but when the earthworm was resident in metalliferous (Pb/Zn- or Ni-polluted) soils, or transferred experimentally from unpolluted to the polluted field soils the riboflavin content was significantly reduced. Such extreme alterations in a cohort of immune-competent cells were not observed in *E. andrei*, *D. veneta*, or *Al. chlorotica* transferred into metalliferous soils. Worms from these three species were also transferred to Zn/Pb/Cd-polluted and unpolluted soils from Southern Poland. It was observed that species-specific changes in riboflavin content occurred not only due to metal pollution, but also other edaphic factors, possibly including organic matter content/quality. Hypothetically, riboflavin status (storage/mobilization) may depend on parasite-immune system balance, which is disrupted by soil-derived stressors, including metals.

Key Words: earthworms; amebocytes/immunocytes; chloragocytes/eleocytes; riboflavin

Introduction

The prevailing view is that a high degree of homology between invertebrate and vertebrate immune systems is found for innate but not for acquired immunity, implying that invertebrates lack both specificity of responses to pathogen invasion and memory (Söderhäll, 2010). Little *et al.* (2005) cautioned against such a rigid dichotomous view on the basis that it is plausible that invertebrates may possess capacities analogous to specificity and memory but achieve these via mechanisms that are distinct from those of vertebrates. Nevertheless, the main focus in the burgeoning field of invertebrate immunology to date has been on innate responses to immunological challenges. A seminal review of innate immunity (Mydlarz *et al.*, 2006) concluded that there are a number of fundamental common pathways operating across invertebrate phyla; these pathways include the prophenoloxidase pathway, systemic phagocytic cells, cytotoxic effector responses, and antimicrobial molecules. There is, therefore, no question that innate immunity in all invertebrate taxa, irrespective of their preferred habitats and associated eco-physiology, involve processes that may be considered in the words of Handy *et al.* (2003) “essential to normal function at cell tissue or organism level”. For this reason it is unsurprising that a variety of immune parameters have been used not only in vertebrates (Duramad and Holland, 2011) but also in a wide range of terrestrial and aquatic taxa (Galloway and Depledge, 2001; Auffret *et al.*, 2006; Plytycz *et al.*, 2009a; Holmstrup *et al.*, 2010) to assess the adverse effects of environmental stressors, including inorganic and organic contaminants, with the ultimate aim of achieving sound risk assessments (Hagger *et al.*, 2006) and establishing regulatory frameworks (Handy *et al.*, 2003).

Contaminated field soils can present formidable risk assessment and management challenges not only because ‘real-world’ pollution events often comprise of mixtures of potentially

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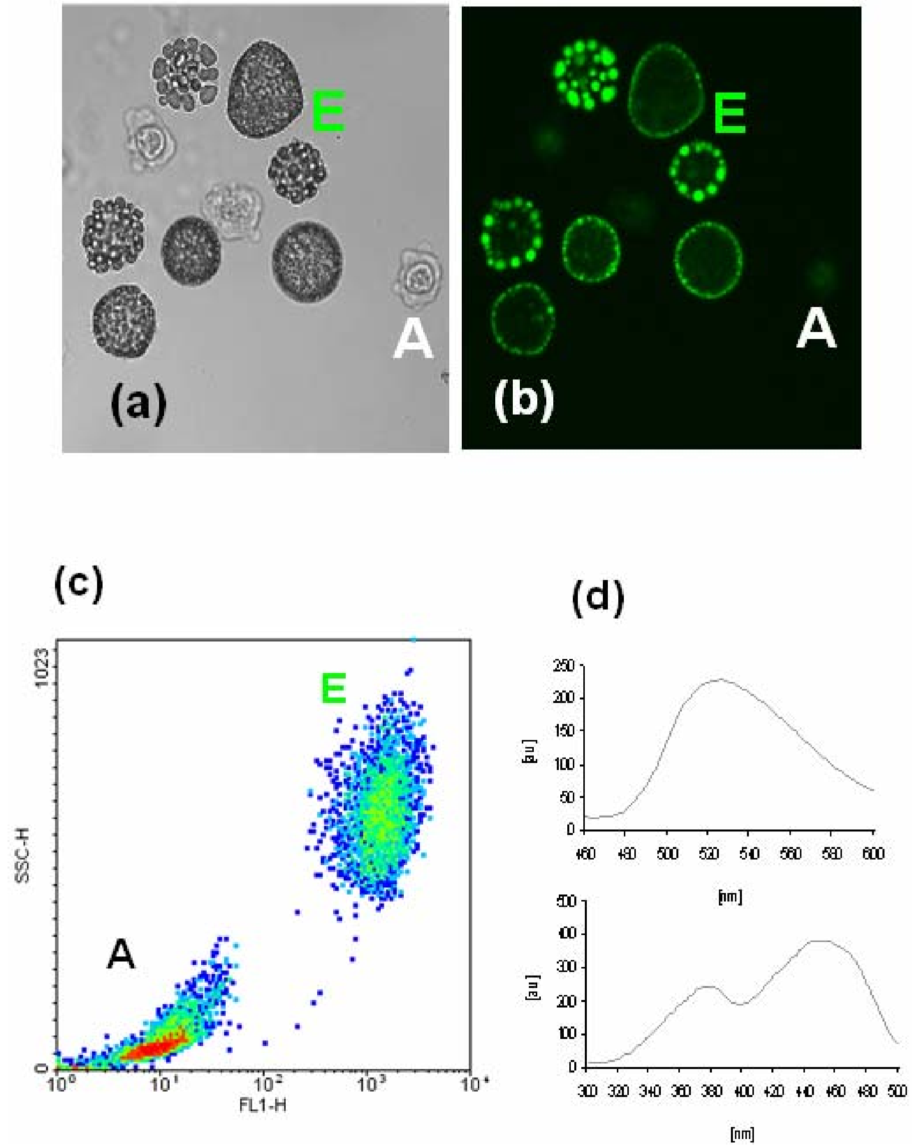

Fig. 1 Celomocytes of *Eisenia andrei* in confocal microscope bright light (a) and blue light (b); A - amebocytes, E - eleocytes (phot. by Grzegorz Tylko). (c) Flow cytometric density plot of celomocyte sample from *E. andrei*; FL-1H - autofluorescence intensity; SSC-H - cell complexity/granularity; (d) Spectrofluorimetric analysis of parallel samples; top: emission spectrum with maximum (proportional to riboflavin content) at 525 nm; bottom: two-maxima of riboflavin-specific excitation spectrum.

interacting toxic chemicals, but also because only a fraction of the total concentration of a given contaminant is available to soil-dwelling organisms (Semple *et al.*, 2004). Estimating available fractions and modeling their deleterious impacts on target biota is not straightforward. For this reason, ectotoxicological risk assessment has been steadily moving away from reliance on geochemical measurements toward the direct assay of perturbations in functional parameters in sentinel organisms (Van Straalen and Roelofs, 2008; Roelofs *et al.*, 2008).

According to Römbke and Egeler (2009) oligochaete worms are the "most important organisms in soil ecotoxicology", a status attributable to a combination of factors including the ecological services that they render in a number of natural and disturbed temperate and tropical soils, as well as pragmatic considerations such as size, sensitivity, availability of standardized test methods. A number of earthworm biomarkers representing different levels of biological organization have been developed, optimized, and deployed (Gastaldi *et al.*, 2007; Guo *et al.*, 2009; Brulle *et al.*, 2010), including immunotoxicity biomarkers (Plytycz *et al.*, 2009a, 2011a; Homa *et al.*, 2010). The vast majority of earthworm biomarker studies have entailed laboratory exposures to experimentally spiked soils.

Table 1 Characteristics of several earthworm species from unpolluted sites in Wales (Cholewa *et al.*, 2006) and Krakow (*Kwadrans *et al.*, 2008) in respect of body weights, celomocyte numbers, among them percentages of eleocytes, and riboflavin content in celomocyte lysates, the latter being either undetectable (-), or present from low (x) to very high (xxxxx) amounts (Plytycz *et al.*, 2006). In bold - species with high percentages of riboflavin-loaded autofluorescent eleocytes

Species (alphabetically)	Body weight (g)	Celomocyte numbers (x 10^6)	Eleocytes (%)	Riboflavin content in celomyte lysates
Allolobophora chlorotica	**0,2**	**1,3**	**30-43**	**XX**
Aporrectodea caliginosa	0,9	0,4	1	-
Aporrectodea longa	1,9	1,6	0,2	-
Dendrobaena veneta*	**1,5**	**9,0**	**22**	**X**
Dendrodrilus rubidus	**0,2**	**1,1**	**21**	**X**
Eisenia fetida	**0,6**	**3,0**	**22**	**XXXX**
Lumbricus castaneus	0,2	0,3	0,5	-
Lumbricus festivus	1,0	2	0,05	-
Lumbricus rubellus	0,6	0,8	0,5	-
Lumbricus terrestris	4,0	1,9	0,5	-
Octolasion cyaneum	**1,5**	**1,5**	**22**	**XXX**
Octolasion tyrtaeum lacteum	**0,4**	**1,3**	**11**	
Octolasion tyrtaeum tyrtaeum	**0,7**	**3,4**	**35**	

Laboratory exposures of 'naïve' worms to contaminated field soils (*i.e.*, 'semi-field studies') or, much rarer, measurements of biomarkers in individual earthworms sampled from field populations inhabiting contaminated soils (*i.e.*, 'field studies') are less commonplace. This mini-review brings together some of our recent field and semi-field studies on metalliferous soils.

Earthworm celomocytes

Evidently the earthworm immune system is functionally efficient (Bilej *et al.* 2011). Conveniently, the immunocompetent cells, celomocytes, of earthworms can readily be non-invasively retrieved for *ex vivo* examination, whilst the immune system subsequently has the capacity to fairly quickly recover its potency. This makes the earthworm immune system very attractive for studies on the adverse effects of various factors. The celomocytes of all earthworm species contain amebocytes, being according to the Ottaviani (2011) nomenclature classical immunocytes. In addition, some species possess a second and morphologically distinct cells freely floating in celomic cavity, *i.e.*, the eleocytes, which are considered to be mature, detached, chloragocytes. Eleocytes, but not amebocytes, exhibit autofluorescence under fluorescence and laser confocal microscopy which is confined to the characteristic granular inclusions (Figs 1a, b). Autofluorescent 'self-marking' predisposes these cells for analysis by flow cytometry (Fig. 1c). Studies by spectrofluorimetry has revealed that riboflavin (Koziol *et al.*, 2006) stored in the chloragosome granules of chloragocytes and eleocytes (Plytycz *et al.*, 2007) is one of fluorophores responsible for their autofluorescence (Fig. 1d) The percentages of autofluorescent eleocytes among celomocytes, and absolute amount of riboflavin stored within eleocytes are both species-specific parameters (Table 1). For example, both the frequency of eleocytes and their riboflavin content are high in the endogeic species *Allolobophora chlorotica*, whilst these granular cells are apparently absent or very uncommon in another endogeic species *Aporrectodea caliginosa* (Fig. 2) (Plytycz *et al.*, 2011a). Whether these inter-species differences reflect underlying differences in trophic-resource partitioning or in the biochemistry of these superficially similar, often sympatric, species is presently unknown.

Metal pollution affects earthworm celomocytes

Previous experiments indicated that celomocyte numbers and their composition can be significantly modified in response to soil metal contamination (Wieczorek-Olchawa *et al.*, 2003; Homa *et al.*, 2003) or by experimental exposures to metal-spiked soil (e.g., Kwadrans *et al.*, 2008; Dutkiewicz *et al.*, 2009; Podolak *et al.*, 2011) or metal-spiked filter paper (e.g., Olchawa *et al.*, 2007; Homa *et al.*, 2005, 2007, 2010). Recent observations have showed convincingly the superiority of eleocyte-rich *Al. chlorotica* and *Dendrobaena veneta* over *A. caliginosa* and *Lumbricus rubellus* (devoid of eleocytes) for investigation of immunotoxic effects of heavy metals (Plytycz *et al.*, 2011a). In these experiments adult worms of these four species were dermally exposed for 2 days to filter paper soaked with 1mM Ni, Cu, Zn, Cd, or Pb chlorides. The amebocytes of *A. caliginosa* and *L. rubellus* were subjected to flow cytometric measurements of *in vitro* neutral red uptake (NR) (loading method adapted from Weeks and Svendsen (1997) and reviewed by Svendsen *et al.*, 2004). However, the NR uptake assay was found to be technically demanding, requiring a strictly normalized incubation period for all samples

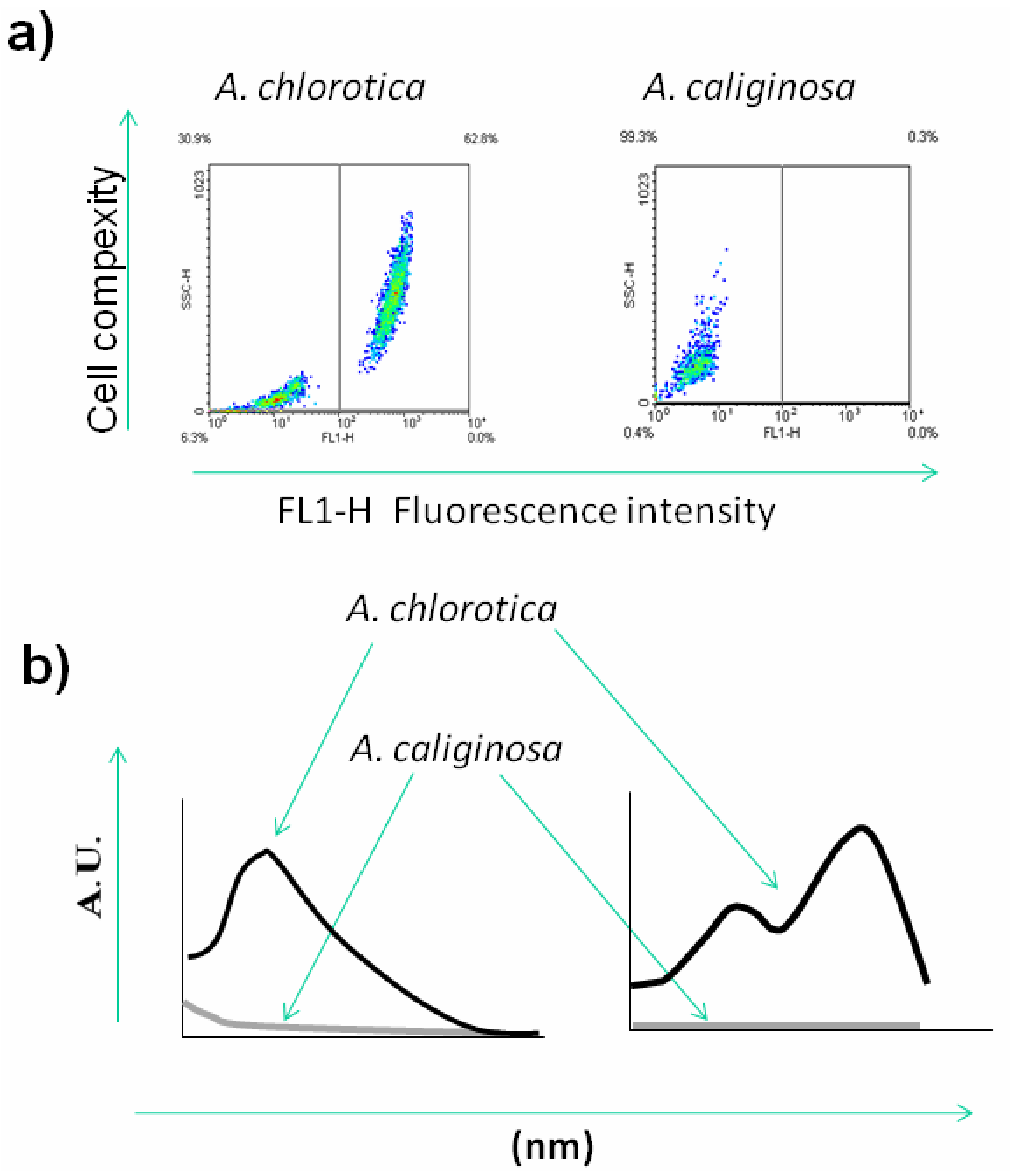

Fig. 2 Analysis of celomocytes of *Allolobophora chlorotica* and *Aporrectodea caliginosa*, (a) flow cytometry, and b) spectrofluorimetry (based on Plytycz *et al.*, 2011a).

to yield useful comparative data. In contrast, the riboflavin content in celomocyte lysates of eleocyte-rich species appeared to be a robust and convenient immune-function biomarker of environmental stress (Plytycz *et al.*, 2011a).

In *Al. chlorotica*, the number of celomocytes, the percentage of eleocytes, and the amount of riboflavin were significantly decreased in Cu-exposed worms; these cytometric parameters were less adversely affected by Ni, Zn, Cd, and were unaffected by Pb at the wide range of concentrations (Plytycz *et al.*, 2011a). As seen in Figure 3, a decrease of riboflavin content in samples from metal-exposed *Al. chlorotica* corresponds strongly with a decreased number of riboflavin-storing eleocytes. Any generalizations and extrapolations may, however, be misleading as further studies have revealed that this relationship does not seem to hold under different experimental conditions (see below, and Plytycz *et al.*, 2009b, 2011b).

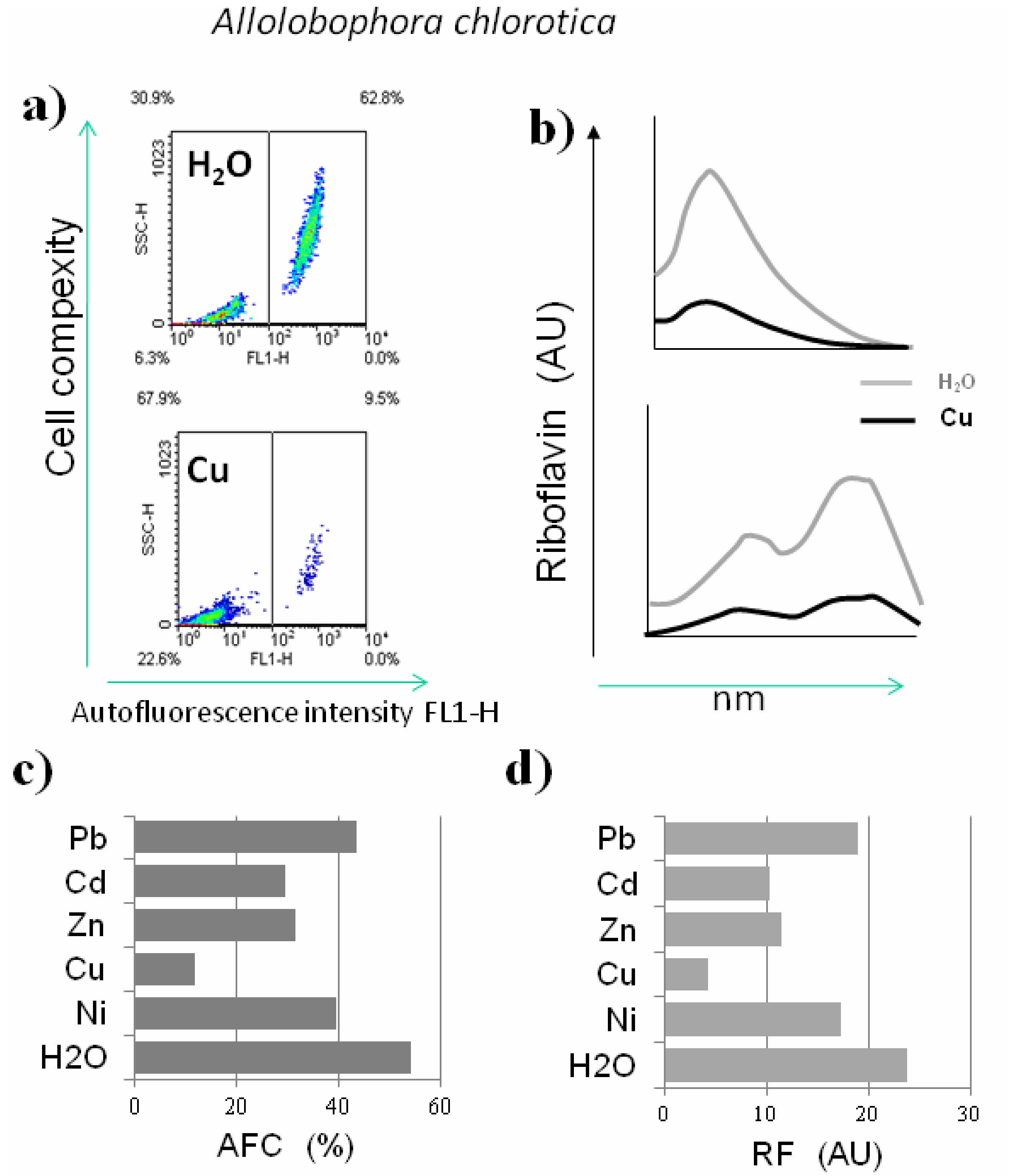

Fig. 3 Analysis of coelomocytes of *Allolobophora chlorotica* after 2-day exposure to filter papers soaked with 1mM metal chlorides; (a) flow cytometry; (b) spectrofluorimetry; (c) mean percentages of autofluorescent eleocytes (AFE); (d) mean riboflavin (RF) contents in coelomocyte lysates (in arbitrary units, AU) (based on Homa *et al.*, 2010). Description in the text.

Riboflavin depletion in celomocytes of *Dendrodrilus rubidus* is a biomarker of metal soil contamination

One of the aims of the series of subsequent experiments was to apply flow cytometric assessment of autofluorecent elecytes and/or spectrofluorimetric quantification of riboflavin storage in eleocytes as putative biomarkers of natural soil quality. In Wales, unpolluted parkland at Cardiff is inhabited by several earthworm species, while only litter-inhabiting epigeic species (almost exclusively *Dendrodrilus rubidus* and *L. rubellus*) live in the shallow, heavily polluted soils associated

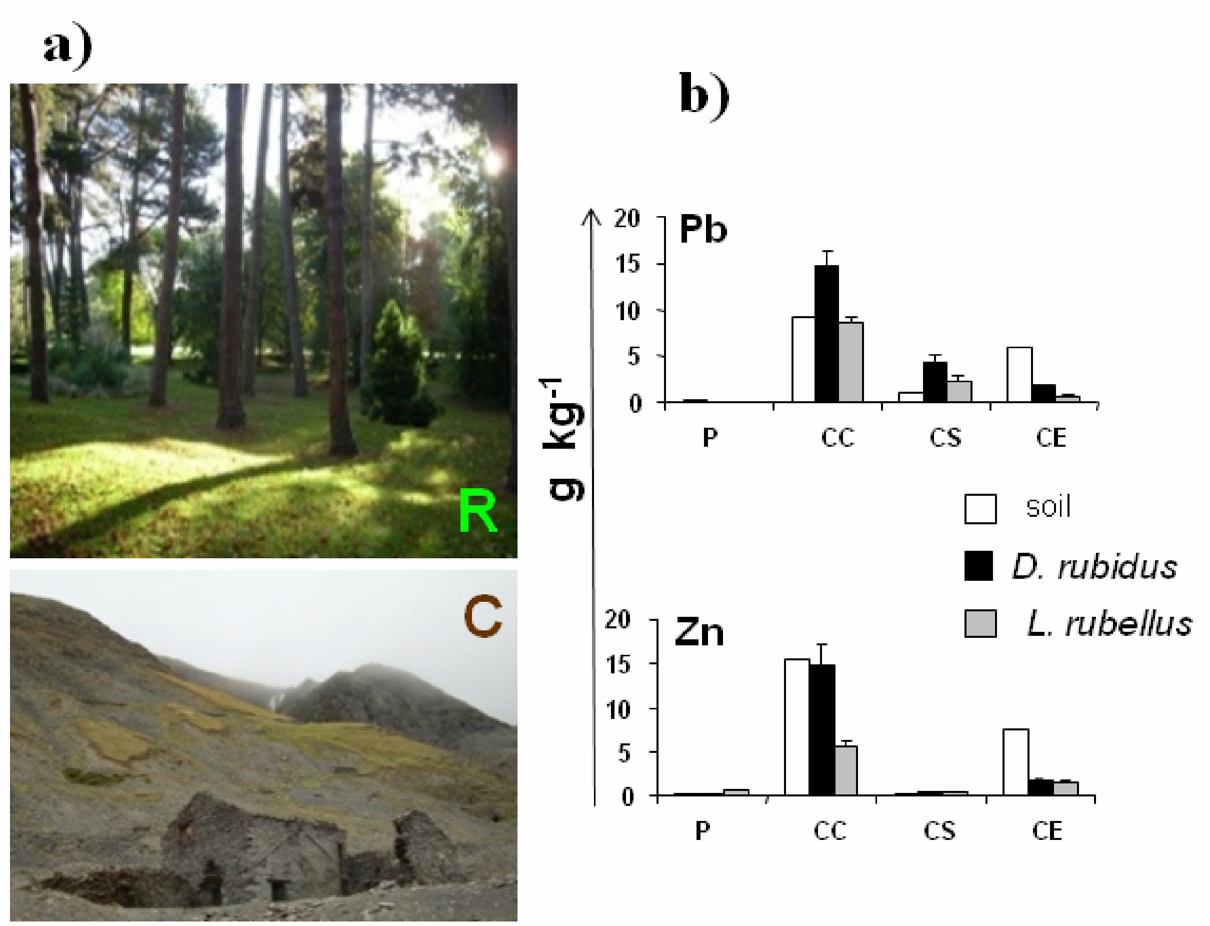

Fig. 4 Photos (a) of the reference site R (Pontcanna) and post-industrial site C (Cwymstwyth) from Wales inhabited by several earthworm species (R) and a few of them (C), sampled for experiments on *Dendrodrilus rubidus* and *Lumbricus rubellus*. (b) Contents of Zn and Pb in soil (empty bars) and whole bodies of *D. rubidus* (black bars) and *L. rubellus* (grey bars) from the reference R site and from three metal polluted sub-sites: CC, CS and CE (based on Plytycz *et al.*, 2009b, 2010a).

with and abandoned Zn/Pb mine at Cwymstwyth (see sites R and C in Fig. 4a). The earthworms resident at the metalliferous soils tolerate the metals not by exclusion but by accumulative immobilization within certain tissues (Fig. 4b).

D. rubidus contains a dense population of autofluorescent eleocytes both in worms from unpolluted reference and metal-polluted sites (Plytycz *et al.*, 2009b, 2010b). In contrast, riboflavin content was uniformly very low in conspecifics inhabiting Zn/Pb-polluted field sites (Plytycz *et al.*, 2009b) and in soils aerially polluted with Ni from a long-established and still active Ni smelter (Plytycz *et al.* 2010b). As illustrated in Fig. 5a, a cluster of complex granular cells exhibiting strong autofluorescence (*i.e.*, eleocytes) are very abundant in the sample from earthworms inhabiting an unpolluted habitat and in counterparts from Zn/Pb-contaminated soil. In spite of this, Fig. 5b shows that spectra characteristic for riboflavin are obtainable only from control (*i.e.*, unpolluted) earthworm samples but not in samples derived from earthworms from the Zn/Pb metalliferous soil. These observations indicate that other fluorophores, probably lipofuscins, are responsible for eleocyte autofluorescence in worms from metal-contaminated sites. It is interesting to postulate that the so-called ‘ageing pigment’ lipofuscin (Yin, 1996) accumulates in the presumptive metal-mediated stressed cells of earthworms from polluted soils as a consequence of enhanced cytological damage and membrane turnover (Regoli, 1992). On the other hand, there is a body of evidence that the presence of lipofuscin can itself under certain circumstances mediate cell damage, including the loss of lysosomal membrane integrity (Brunk and Terman, 2002).

Growing evidence reveals that cryptic/sibling speciation (genetically distinct, but morphologically very similar or indistinguishable organisms) is widespread amongst the clitellate annelids (Erséus and Gustafsson, 2009), including some earthworm ‘species’ (King *et al.*, 2008; Pérez-Losada *et al.*, 2009; Stürzenbaum *et al.*, 2009). One of the important implications of these findings is the possibility that the genetically distinct ‘lineages’ (the

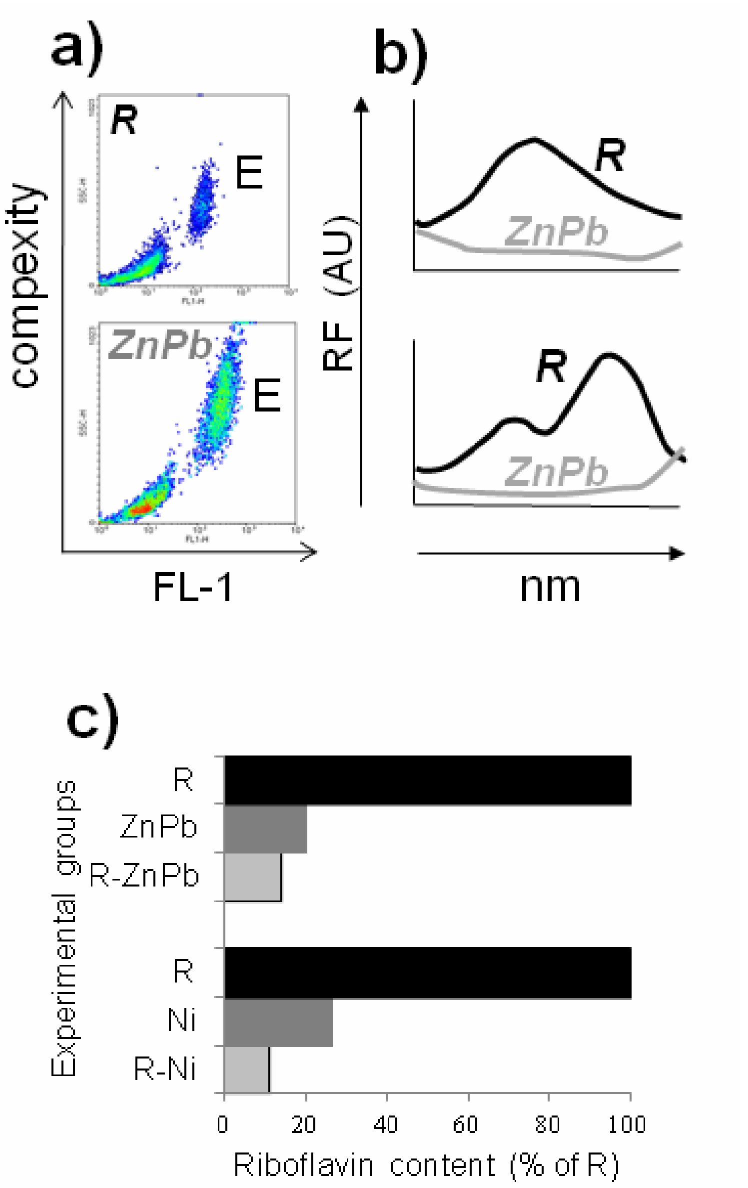

Fig. 5 Main results of experiments on celomocytes of *Dendrodrilus rubidus* from Wales, from the unpolluted reference site R and abandoned Zn/Pb site C (see Fig. 4); (a) analysis by flow cytometry in respect of percentages of autofluorescent eleocytes; (b) analysis by spectrofluorimetry in respect of riboflavin (RF) content; (c) riboflavin content (in arbitrary units) from the reference site R (considered as 100 %; black bars), or Zn/Pb-polluted or Ni-polluted (dark bars) or transferred for 4 or 6 weeks from the reference soil to the polluted soil: R-Zn/Pb or R-Ni (grey bars); (based on Plytycz *et al.*, 2009b, 2010b).

term being used advisedly instead of ‘cryptic species’ to cover the possibility of a lack of reproductive isolation and, therefore, conformity with the biological definition of ‘species’) are differentially responsive to environmental contaminants (Morgan *et al.*, 2007). A striking example of this micro-evolutionary phenomenon is seen in the differences in PAH tolerance and metabolism amongst sibling species within the species complex of the polychaete *Capitella capitata* (Bach *et al.*, 2005). We were alert to the possibility that the endogeic and cosmopolitan earthworm species *D. rubidus* also forms a species complex, the members of which may be differentially susceptible to metal-mediated stress. As a device designed to minimize the possible confounding effects of cryptic speciation we performed a laboratory-based reciprocal transfer experiment involving the chosen reference and metalliferous field soils as well as their resident earthworms. We observed that the riboflavin content in celomocyte lysates prepared from *D. rubidus* sampled initially from the unpolluted

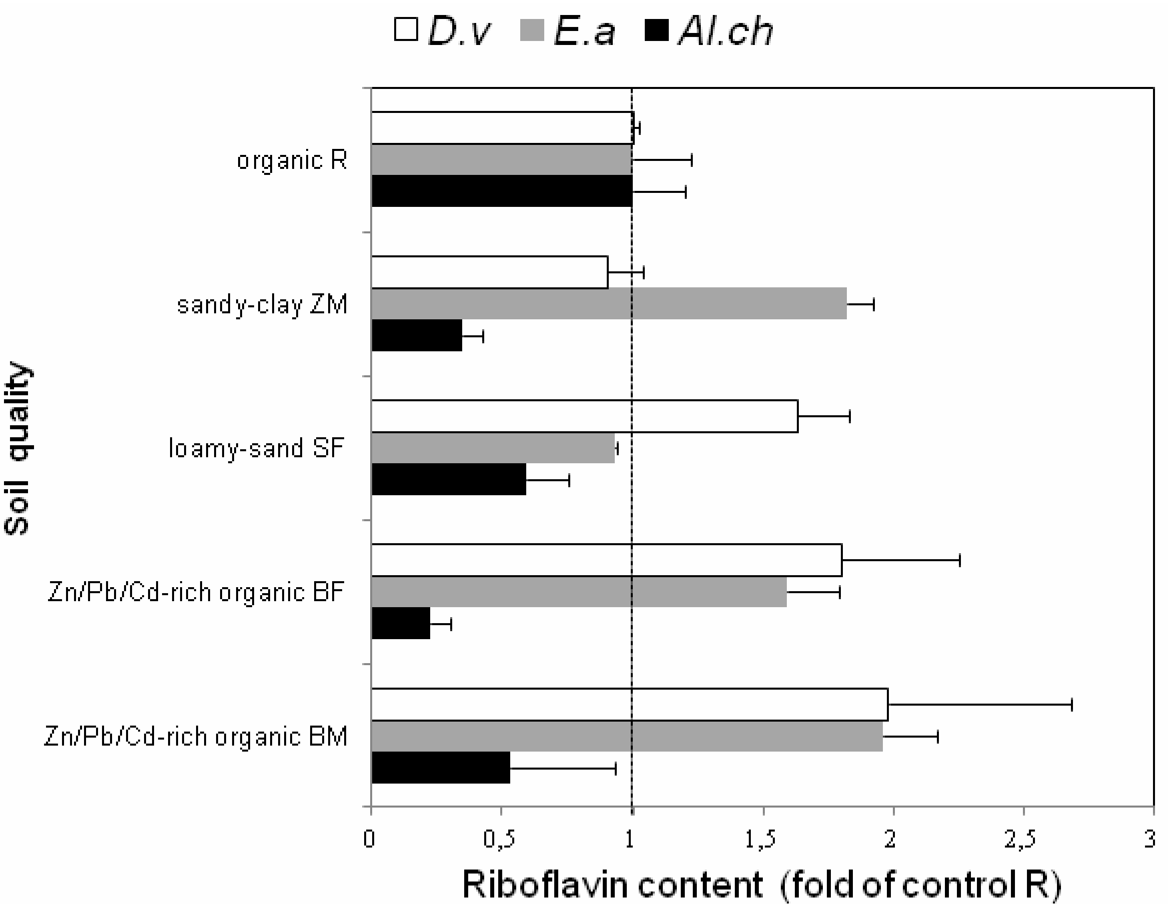

Fig. 6 Main results of experiments on *Dendrobaena veneta* (Dv, empty bars), *Eisenia andrei* (Ea, grey bars) and *Allolobophora chlorotica* (Ach, black bars) maintained for 4 weeks in metal-free natural soil samples (R, ZM, SF) or soil samples heavily polluted mainly with lead, zinc, or cadmium (BF, BM) from Southern Poland); Fold of riboflavin content in coelomocyte lysates when content in the R sample was considered as 1 (based on Plytycz *et al.*, 2011b).

soil dropped significantly after maintenance for several weeks in Zn/Pb- or Ni-polluted soils (Fig. 5c) (Plytycz *et al.*, 2009b, 2010b).

Riboflavin amount in celomocytes is not a universal biomarker of soil quality

Eisenia andrei, *D. veneta*, and *Al. chlorotica* have been exposed in another experiment to metalliferous field soils samples from Wales UK (Piotrowska *et al.*, 2009) and Southern Poland (Plytycz *et al.*, 2011b). Celomocytes and riboflavin content were affected by soil quality in species-related ways. For example, it was evident according to these immuno-toxicity variables that *Al. chlorotica* is much more sensitive to soil metal pollution intensity than the two composting species examined in the studies (Piotrowska *et al.*, 2010, Podolak *et al.*, 2011; Plytycz *et al.*, 2011b). Moreover, it was apparent that riboflavin depletion in *Al. chlorotica* celomocytes occurs not only in metal-polluted soil samples but also in metal-free sandy-clay or loamy-sand natural soil samples. In contrast, the riboflavin content increased in the eleocytes of *E. andrei* and/or *D. veneta* transferred from the lab soil to metal polluted soil samples and also to the unpolluted sandy clay or loamy-sand soils (Fig. 6) (Plytycz *et al.* 2011b). In conclusion, various edaphic factors other than elevated metal concentrations can effectively act as stressors, and this may lead to species-specific alterations in riboflavin metabolism.

Putative role of riboflavin storage in earthworm chloragocytes/eleocytes

Riboflavin plays an important role in immunity of both plants (Dong and Beer 2000; Zhang *et al.*, 2009) and animals (Araki *et al.*, 1995; Osame *et al.*, 1995; Verdrengh and Tarkowski 2005; Bertello *et al.*, 2006), and plays a role in bacterial quorum sensing (Rajamani, 2008). In particular, riboflavin has antioxidative effects (Seekamp *et al.*, 1999; Iwanaga *et al.*, 2007), antinociceptive and anti-inflammatory activity in mammals (Bertollo *et al.*, 2006; Mazur *et al.*, 2008), and at high doses riboflavin possesses pronounced anti-septic properties (Toyosawa *et al.*, 2004; Kodama *et al.*, 2005). Therefore an immunomodulatory role of riboflavin in human immunity is highly appreciated

(Iwanaga *et al.*, 2007; Yazdanpanah *et al.*, 2009; Damian *et al.*, 2010). In vertebrates, flavin deficiencies lead to diseases such as glossitis, cheilosis, and organic acidurias (Powers, 2003). The potential adaptive advantages of possessing riboflavin-storing immune-competent cells with high bactericidal potency are evident to soil-dwelling earthworms, with their highly permeable integument rendering them potentially vulnerable to invasion by pathogenic bacteria.

We have shown that riboflavin storage is universal in earthworm species, as it was detected both in attached chloragocytes forming the chloragogen tissue and in chloragocyte-derived eleocytes (Mazur *et al.*, 2011). Moreover, we have shown that riboflavin acts as a chemoattractant for celomocytes of several earthworm species, both in eleocyte-rich species (*Al. chlorotica*, *E. andrei*, *D. veneta*) and in species that are devoid of free-floating eleocytes (*L. terrestris*, *L. rubellus*, *A. caliginosa*) (Mazur *et al.*, 2011). This property of riboflavin is probably of adaptive value to earthworm species through the targeted recruitment of immune-competent celomocytes to the loci of pathogen invasion, and subsequently to facilitate their destruction and disposal by encapsulation within so-called ‘brown bodies’ (Valembois *et al.*, 1992, 1994; Wieczorek-Olchawa *et al.*, 2003). Hypothetically, riboflavin status (storage/mobilization) may depend on parasite-immune system balance, which is disrupted by soil-derived stressors, including metals.

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