TOOL WORKSHEET NO.13

Gene expression of metallothionein (MT) among earthworms (biomarker of soil cadmium contamination)

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DESCRIPTION OF THE INDICATOR

Name of the indicator: Gene expression of metallothionein among earthworms, indicator of metal contamination, especially cadmium.

Ecological role of the organism under test: Earthworms form one the most important soil taxons. They play a major role in the transformation of nutritive elements, the distribution of energy flows within terrestrial ecosystems and the increase of soil fertility. The numerous direct and indirect effects of earthworms' activities on water, nutrients and carbon cycle explain why, since the early works of Darwin (1883), earthworms are, under temperate and tropical climates, considered as the engineers of soil ecosystem (Lavelle & Spain, 2001). Besides, because of their biology, populations of earthworms can tell us about soil structure conditions, microclimatic conditions, nutritional situation and toxic elements held in soils (Christensen, 1988; Edwards & Bohlen, 1996; Edwards, 1998; Kautenburger et al., 2006). For these reasons, Lumbricidae have been adopted by the international community as **bioindicators** for the study of the potential ecological impact (Ecological Risk Assessment or ERA) of contaminants, such as pesticides, hydrocarbons and Metal Trace Elements (ETMs) from anthropic sources (see Spurgeon et al., 2003).

Figure 1: Lumbricus rubellus

Type of indicator: The emergence of molecular biology techniques applied to ecotoxicology enabled better understanding of the mechanisms of action of contaminants in living organisms and brought new knowledge, in particular "sequence data" in species very used in ecotoxicology. In this context, work on gene expression has developed. **Profiles of gene expression** represent in fact the first level of integration between environmental stress factors and the genome that, through protein synthesis, directs the response of organisms to external changes. The analysis of gene expression changes is a powerful tool, (1) to diagnose the existence of a stress within a population and (2) analyse mechanistically the response to a stress (Brulle et al., 2008).

In ecotoxicology, bioindicator and biomarker measurements can be conducted on collected organisms (sentinel species) directly from polluted sites, for biomonitoring purposes and the study of the "potential ecological impact" (Ecological risk assessment or ERA) of contaminants, such as pesticides, hydrocarbons and ETMs from anthropic sources.

The work consists in the measurement of the level of expression of the gene coding for Metallothionein (MT2), a metal-detoxicating protein, in earthworms collected on sites contaminated by ETMs. Previous works show that the measurement of the expression of the MT2 gene reveals to be a relevant bioindicator in a context of metallic pollution (Brulle et al., 2006; 2007b; 2008).

DESCRIPTION OF THE SAMPLING METHOD

Norms and/or protocols of reference

Reference publications: Brulle et al., 2006; 2007.

Collecting and sorting, important specifications

- The level of expression of MT2 is measured from a composite sample made of animals from a single species, collected on each sampling area.
- Identifying the species is paramount.
- Number of earthworms required from the same species: 12 per sampling area (the analysis of the level of expression of MT2 can be conducted for all species where the gene has been cloned and sequenced).

Description of steps at the laboratory

1. upon reception of the animals, make them fast 2 to 3 days in agar so as to empty their bowel.

2. for each set of earthworms, crush the animals in a liquid environment in Tri-ReagentTM (Euromedex) using a crusher (T8 ultraturax IKA).

3. freeze samples at -80°C until use.

4. when deciding on conducting measurements (technical necessity to group samples), defrost on ice.

5. individual extraction of RNA for each set of animals.

6. very precise dosage of the quantities of RNA extracted.

7. aliquote and refreeze RNA as it is extracted.

8. when all extractions are completed, defrost samples.

9. proceed with a second very precise dosage.

10. RT (reverse transcription = transcription of RNA in complementary DNA) of all the sets of RNA to dose.

11. real-time PCR to measure the gene expression of MT2 in duplicate or triplicate for each sample. On various sites, several measurements can be conducted for different sets of animals from the same species or from different species.

12. measurements of the gene expression of one or two housekeeping genes whose expression will serve as reference (chosen genes: β-actin and ribosomal protein S13). Measurements also realised in duplicate or triplicate.

13. statistical analysis and processing of results. We quantify the MT gene expression in relation to the level of expression of one or several housekeeping genes (genes of reference).

14. results reporting in histograms and interpretation based on statistics.

Estimated time: sampling: 1 day; Sample processing: 3 to 5 days depending on the number of samples.

Measured parameters

- Level of expression of the gene coding for MT
- The level of expression can vary up to 10 times on sites highly contaminated by metals

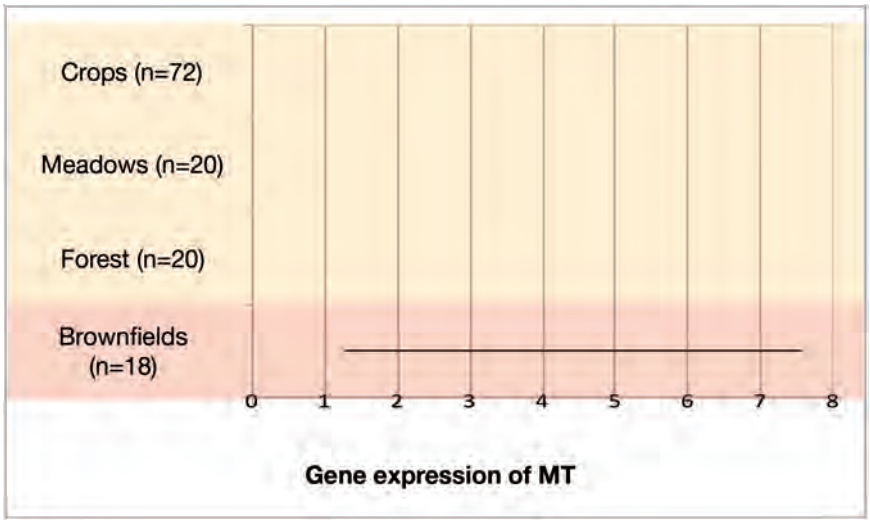


Figure 2: Variation range of MT expression measured only on contaminated sites of the Bioindicateur 2 program.

INTERPRETATION OF RESULTS

Relative quantification of the level of expression

For each sample, the level of targeted gene expression has been compared with β-actin (β-act) gene expression, which is expressed constitutively (Ricketts et al. 2004). The expression ratio (R) has been calculated according to the formula: $R = (EGC)^{CPGc}/(Eact)^{CPact}$. Another control gene expressed constitutively and coding for ribosomal protein S 13 (RiboS13) was used during our experiments. To calculate the amplification efficiency (E) of each effector, calibration curves have been generated by using dilution series (1:10, 1:100, 1:1000, 1:10000) of a cDNA cocktail made of a mix of cDNA (one per condition) of our experiment. Calibration curves have been generated using LightCycler® 480Software release 1.2.0.0625. They are based on cross-point values and log value of the dilution of cDNA. Efficiency of real-time PCR has been calculated from the slope of the calibration curve using the following equation: $E = 10^{(-1/slope)}$.

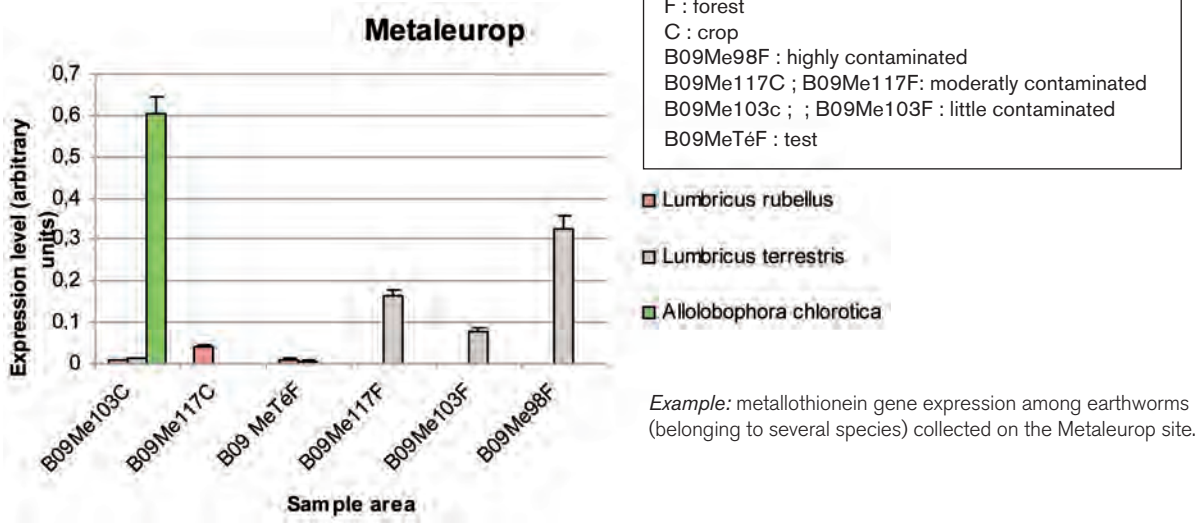
A **global frame of reference** may be determined. The lower the metallic contamination, the lower the level of expression of mt2 gene seems to be, sometimes undetectable. Note however, for several species, that data is very limited.

Additional information such as climate, soil use, type of soil does not seem required. Nevertheless, several parameters like pH, texture, CE, content in Organic Matter can influence bioavailability of metals and may affect the level of expression of MT2, metal-detox protein.

EXAMPLE OF APPLICATION

It is possible to consider data depending on sites or on species from all sites where it is collected.

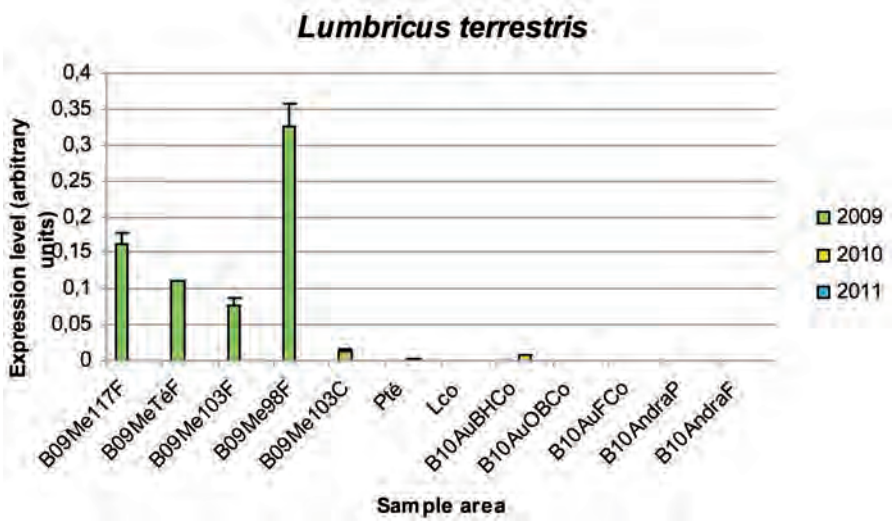
Data from sites



Example: metallothionein gene expression among earthworms (belonging to several species) collected on the Metaleurop site.

Final results show more or less strong metallothionein (MT) expression depending on species (Figure 2). Among Lumbricus terrestris, a species found on most modalities of the site, MT expression is very high compared to other workshop sites under study. It is at its highest on Me98F, the most contaminated modality, and responds on a declining basis along the contamination gradient.

Data from species



Example: metallothionein gene expression among Lumbricus terrestris on the three sites where the species was collected.

Here too, we observe a higher level of expression on animals collected on the sites most contaminated by metals.

INTERESTS AND LIMITS OF THE INDICATOR

+ integrates all factors modulating or likely to modulate bioavailability of soil metallic contaminants (pH, pedological parameters, climate...).
+ most earthworms seem to respond similarly.
+ speed of analysis.
+ ease of interpretation.
− difficulty of the choice of a reference site.
− specificity of response (metallic contamination and specific sensitivity to cadmium).
− measurements cannot be conducted on all species of earthworms since it requires preliminary cloning of the MT2-coding gene.
− difficult to compare measurements conducted on different species.

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PUBLICATIONS : ***Brulle** et al., 2006. Environmental Science and Technology; **Brulle** et al., 2007. Comparative Biochemistry and Physiology C; **Brulle** et al., 2008. Ecotoxicology and Environmental Safety; **Brulle** et al., 2008. Developmental and Comparative Immunology; **Bernard** et al., 2010. Ecotoxicology and Environmental Safety; **Brulle** et al., 2011. Science of the Total Environment.*

***Pérès, G., F. Vandenbulcke, M. Guernion**et al., 2011. The use of earthworms as tool for soil monitoring, characterization and risk assessment. Example of a Bioindicator Programme developed at National scale (France). Pedobiologia 54, 77–87.*

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