

**Accessory publication****<sup>1</sup>H NMR-based metabolomic observation of a two-phased toxic mode of action in *Eisenia fetida* after sub-lethal phenanthrene exposure**

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**Earthworm maintenance prior to contact tests**

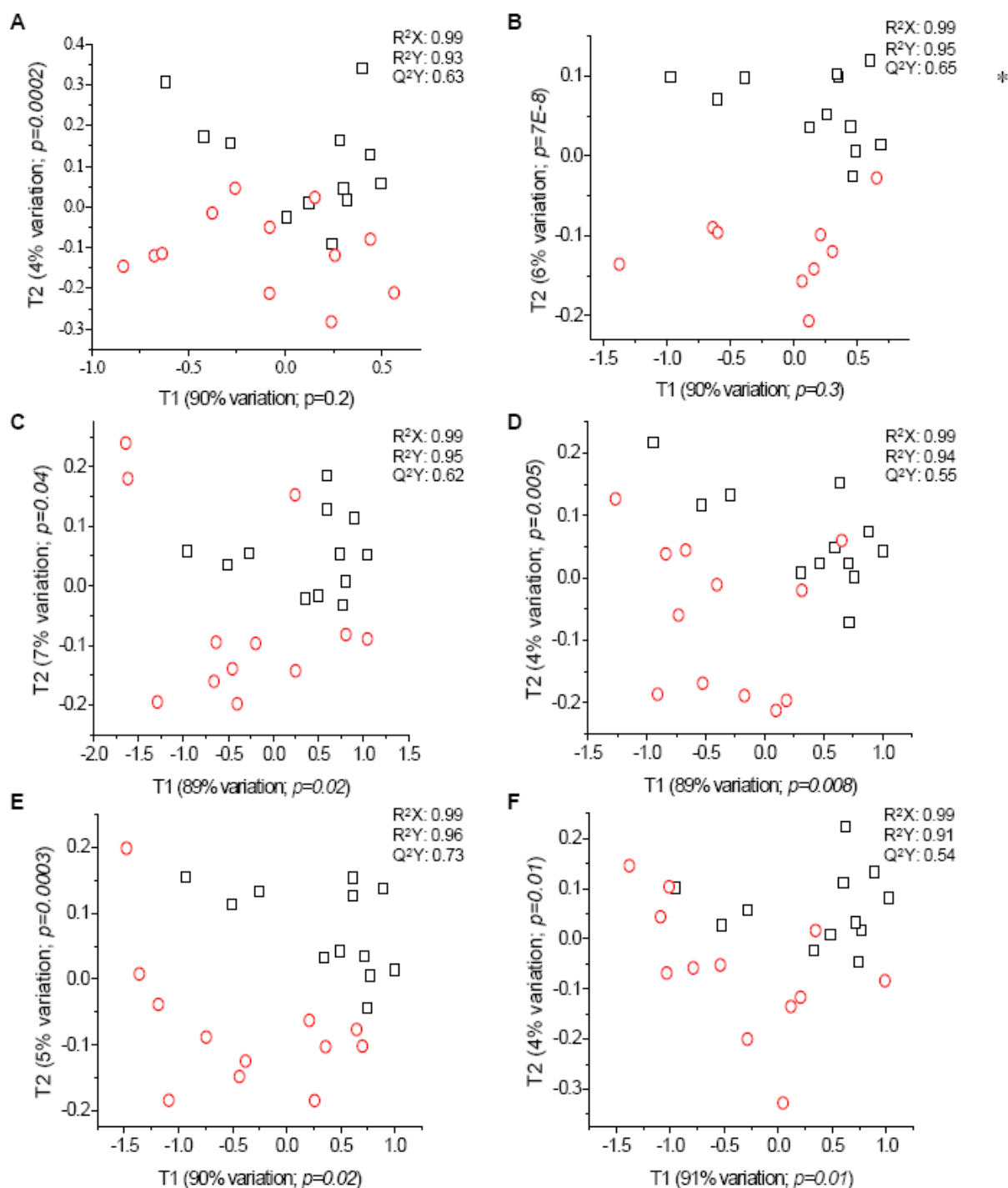
*E. fetida* were purchased from The Worm Factory (Kingston, ON, Canada). They were raised in earthworm bins containing sphagnum peat bedding (Magic Worm bedding, Magic Products, Amherst Junction WI, USA) at an approximate temperature of 24°C. The moisture content of the bedding was maintained at 67% (by weight). The earthworms were acclimated for several months to decrease variations in the <sup>1</sup>H NMR profile due to differences in diet and other environmental factors.<sup>[1]</sup> The worms were fed Magic Worm Food (Magic Products). Mature earthworms, indicated by a visible clitellum, were selected and depurated in groups of 10 in the dark for 96 hours in 500-mL glass jars containing moist Whatman 4 Qualitative filter papers with a diameter of 9 cm (Fisher Scientific, Whitby, ON, Canada) to empty their intestinal tracts<sup>[1]</sup>. The earthworms had an average mass of 0.62 g (±0.15 g; standard deviation) after depuration. The earthworms were then transferred to individual 120-mL amber glass jars containing pre-treated Whatman GF/A 4.70-cm diameter glass filter paper (Fisher Scientific).

**Calculation of  $Q^2Y$  of partial least squares regression models**

The PLS model was fully cross-validated using leave-one-out cross-validation (LOOCV).<sup>[2,3]</sup> In each iteration of LOOCV one sample (validation data) from the set of samples (the training set) that were used to build the PLS model is left out of the model, forming a new model. The  $x$ -value of the validation data is then used to predict the  $y$ -value ( $\hat{y}$ ). The predicted value is then subtracted from the known value ( $y$ ) and a residual is obtained. This procedure is repeated until all of the samples in the training set are left out once. The  $Q^2Y$  is then calculated according to the following equation<sup>[2]</sup>:

$$Q^2Y = 1 - \frac{\sum(y_i - \hat{y}_i)^2}{\sum(y_i - \bar{y})^2}$$

where,  $\hat{y}_i$  is the predicted value of  $y_i$  of given sample  $i$  by the PLS model derived during LOOCV in which sample  $i$  was omitted from the model as the validation data.  $\bar{y}$  is the mean value of  $y$  for all of the samples.



**Fig. A1.** PLS-DA scores plots of T1 (first PLS component) v. T2 (second PLS component) for  $^1\text{H}$  NMR spectra of *E. fetida* aqueous buffer tissue extracts showing the separation of control worms ( $\square$ ) from exposed worms ( $\circ$ ) at phenanthrene concentrations of: (a)  $0.025 \text{ mg cm}^{-2}$  (1/64th of  $\text{LC}_{50}$ ); (b)  $0.05 \text{ mg cm}^{-2}$  (1/32th of  $\text{LC}_{50}$ ); (c)  $0.10 \text{ mg cm}^{-2}$  (1/16th of  $\text{LC}_{50}$ ); (d)  $0.20 \text{ mg cm}^{-2}$  (1/8th of  $\text{LC}_{50}$ ); (e)  $0.40 \text{ mg cm}^{-2}$  (1/4th of  $\text{LC}_{50}$ ); and (f)  $0.80 \text{ mg cm}^{-2}$  (1/2 of  $\text{LC}_{50}$ ). The  $P$ -values were obtained from  $t$ -tests that compared the scores of the control and exposed worms for each component. \*Three phenanthrene exposed earthworms were identified as outliers in the DModY plot and removed from the model.

**References**

- [1] S. A. E. Brown, A. J. Simpson, M. J. Simpson, Evaluation of sample preparation methods for nuclear magnetic resonance metabolic profiling studies with *Eisenia fetida*. *Environ. Toxicol. Chem.* **2008**, *27*, 828. doi:10.1897/07-412.1
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