SUPPORTING INFORMATION

Toxicogenomic responses of the model legume *Medicago truncatula* to aged biosolids containing a mixture of nanomaterials (TiO$_2$, Ag and ZnO) from a pilot wastewater treatment plant

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Quantitative Reverse Transcription PCR (qRT-PCR). 4 genes in the shoots and 5 genes in the roots were selected from differentially expressed genes (DEGs) in the microarray analysis for qRT-PCR verification. Briefly, RNA was extracted using the same protocols as described above for the microarray samples. cDNA was transcribed from 800 ng of total RNA, using a high-capacity cDNA reverse transcription kit (Applied Biosystems) in a total volume of 20 µL per reaction. The qRT-PCR was performed in a 10 µL reaction mixture containing 5 µL of TaqMan Fast Universal PCR Master Mix , 0.5 µL of TaqMan gene expression assay consisted of forward and reverse primers and FAM^{TM} dye-labeled TaqMan probe, 3.5 µL of RNase-free water and 1 µL of a 1:5 dilution of each cDNA sample (Applied Biosystems). The primers and Taqman probes were designed using Applied Biosystem Primer Express software v3.0 and then synthesized by Applied Biosystems. The primer and probe sequence used in qRT-PCR assays were listed in the Supporting Information (Table S1). The following qRT-PCR reactions were performed on a StepOne Plus system (Applied Biosystems) for 20 s at 95 ℃, followed by 40 cycles of 95 ℃ for 1 s and 60 ℃ for 20 s. All samples were run in triplicates for each gene with reverse transcriptase minus (-RT) negative control to check for DNA contaminations. Actin 2 was selected as a reference gene for relative quantification, which showed stable expression within the microarray data and qRT-PCR confirmation. The qRT-PCR data was analyzed and normalized with the reference gene (Actin 2) using a GenEx software (Multi D). After normalization, fold changes in gene expression in bulk/dissolved and ENM treatments were calculated relative to controls.

Stability of the Reference Genes. An equivalent amount of total RNA from *Medicago truncatula* which exposed to control, bulk/dissolved and ENM treatment, was converted to cDNA and the cDNA diluted up to 5-fold for qRT-PCR. The amplification curves of Actin *2* gene under different treatments showed stable expression, indicating it can be used as a reference gene for relative quantification in our study.
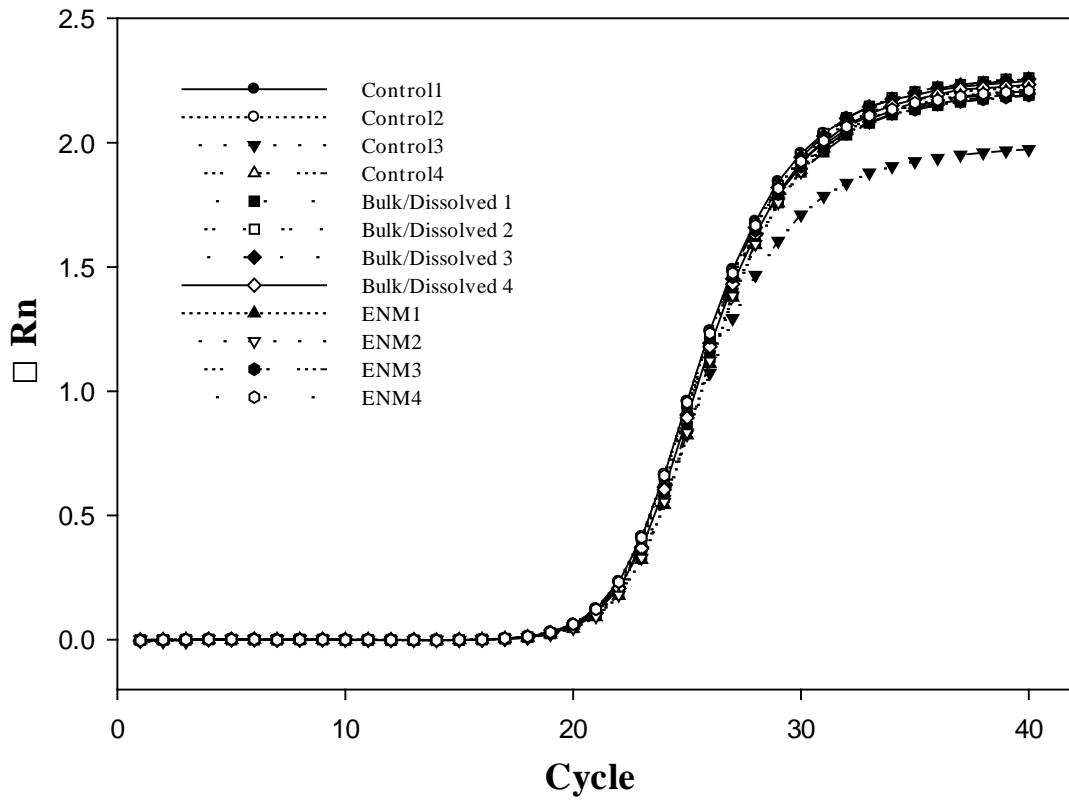

Figure S1. The quantitative real-time PCR (qRT-PCR) amplification curves of Actin 2 gene expression in control, bulk/dissolved and ENM treatments

Table S1. Taqman primers and probes for confirming individual gene expression

genes	probeset	primers and probe sequences 5' to 3'		amplicon size / bp	PCR efficiency	R^2
reference gene						
Actin 2		Forward	CTGTGCCAATCTATGAGGGTTATG	63	98.52%	0.999
		Reverse	GACCAGCAAGATCCAAACGAA			
		Probe	ACTCCCACATGCCATC			
shoots (4 genes)						
IFR	Mtr.410.1.S1_s_at	Forward	CACGCCTTTACCGGTTACTTCT	66	96.46%	0.999
		Reverse	ATCCCGAGGAGGATCAGTGA			
		Probe	ACGTAACTTGGCTCAACT			
F3H	Mtr.13960.1.S1_at	Forward	GGCTTCGTATGGTCCAACTTCT	70	95.61%	0.998
		Reverse	AGTCCATTTTCAAGTTCCCAATCA			
		Probe	ACAGCTACACTGGCCCA			
GST	Mtr.43621.1.S1_at	Forward	TGCATTTTCTTTTGAGGTTCCA	70	95.04%	0.999
		Reverse	CAGGGACGGGTAAGACAGAAAC			
		Probe	TTATGTACGACGTCCGTTGC			
P450	Mtr.12616.1.S1_at	Forward	AGCACTCACCTTCCAATAACCAA	66	94.76%	0.996
		Reverse	CCATGAACACCATTGCAAATTC			
		Probe	ACTACCGTCTCTACTCTAT			
roots (5 genes)						
MTP	Mtr.37075.1.S1_at	Forward	CAATCTGCACCTCCTGAACTTCT	70	95.18%	0.999
		Reverse	TGCGCTTAATTTGAGAGTGCAT			
		Probe	CAGAAGCTAACGTATCTTGTCAT			
MTR	Mtr.17288.1.S1_at	Forward	ATGCCGCACAATCTTTTCCT	67	98.61%	0.999
		Reverse	CGCGCTTGGTACCAGGTATT			
		Probe	CACTCTGCTCTAGTGCTA			
PEROX	Mtr.14635.1.S1_at	Forward	GCAAGCAGAGATGCAGCAAA	67	91.06%	0.999
		Reverse	TTGCGATGAGTTGTGAGAAGTTAAA			
		Probe	ATAAATCTTCCACCGGCATT			
NADPH	Mtr.11719.1.S1_at	Forward	CATTAAATGTGGAGGAGCTTGGA	67	91.87%	0.999
		Reverse	ACCGATAGGCGAAGGATTTGA			
		Probe	ACAGAAGCTCTACCAGTTG			
Acc_Oxidase	Mtr.46283.1.S1_at	Forward	TGGGAACCACAAAACATTCTGATA	72	94.96%	0.999
		Reverse	GGAGGCCACCAATATGATCATC			
		Probe	TTGTTTTCTCACATTGCTTC			

Table S2. Quantitative real-time PCR (qRT-PCR) confirmation of fold changes in expression level for 9 representative genes determined by microarrays analysis.

gene name	fold change			
	ENM *vs* control		bulk/dissolved *vs* control	
	microarray	qRT-PCR	microarray	qRT-PCR
shoots (4 genes)				
IFR	$1.93 \pm 0.18^*$	$3.43 \pm 0.91^*$	1.07 ± 0.05	1.79 ± 0.41
F3H	$2.00 \pm 0.21^*$	$2.69 \pm 0.44^*$	1.17 ± 0.01	1.68 ± 0.26
GST	$2.34 \pm 0.29^*$	$7.37 \pm 1.17^*$	1.23 ± 0.04	$2.77 \pm 0.27^*$
P450	$2.66 \pm 0.10^*$	$2.70 \pm 0.24^*$	1.59 ± 0.63	$1.64 \pm 0.18^*$
roots (5 genes)				
MTP	$14.32 \pm 4.03^*$	$9.02 \pm 1.92^*$	$-1.15 (0.97 \pm 0.14)$	$-1.01 (0.99 \pm 0.37)$
MTR	$12.68 \pm 1.3^*$	$11.29 \pm 1.81^*$	$-1.25 (0.80 \pm 0.15)$	1.08 ± 0.53
PEROX	$42.20 \pm 3.83^*$	$23.92 \pm 2.08^*$	$-1.53 (0.65 \pm 0.06)$	1.08 ± 0.10
NADPH	$27.25 \pm 3.76^*$	$12.27 \pm 1.06^*$	$-1.55 (0.64 \pm 0.27)$	$-1.39 (0.72 \pm 0.07)$
ACC_Oxidase	$56.85 \pm 13.46^*$	$64.07 \pm 10.19^*$	$-1.47 (0.68 \pm 0.18)$	1.56

Note: ENM, engineered nanomaterial. When comparing two groups, expression ratio ENM or Bulk/Dissolved (A) and Control (B), if A <B, the expression ratio and fold change are both A/B (up-regulation); if A<B, the expression ratio is still A/B, but fold change is –B/A (down-regulation). Microarray data represent the mean $\pm$ SEM (standard error of the mean) of $n = 3$ pooled specimens; qPCR data represent the mean $\pm$ SEM of $n = 5$ individual shoots ($n = 3$ individual pooled roots). The asterisk denotes a significant difference from the control ($P < 0.05$). For down-regulated genes, data in parentheses represent the average of expression ratio with SEM.

Table S3. Metabolic pathways significantly over-represented ($P < 0.05$, *FDR* correction) in shoots of *Medicago truncatula* exposed to ENM (engineered nanomaterial) treatment.

pathways	no. of Enzymes included in *M. truncatula*	no. of Differentially Expressed Enzymes	*P*-value
Drug metabolism-cytochrome P450	6	5	0.0053
Metabolism of xenobiotics by cytochrome P450	4	4	0.0053
Flavonoid biosynthesis	10	6	0.0055
Isoflavonoid biosynthesis	3	3	0.0247
Starch and sucrose metabolism	33	10	0.0247

Supporting Information File S1 summarize the data of down-regulated and up-regulated differentially expression genes (DEGs) identified (based on greater than ±1.5-fold changes with $P < 0.05$) in the shoots or roots of *Medicago truncatula* following ENM (engineered nanomaterial) or bulk/dissolved exposure.

Supporting Information File S2 summarize the results of gene ontology (GO) categories significantly enriched with all differentially expression genes (DEGS) in tissues (shoots and roots) of *Medicago trucnatula* exposed to ENM (engineered nanomaterial) or bulk/dissolved treatments that compared to controls.

Supporting Information File S3 summarize the results of KEGG pathways significantly enriched with all differentially expression genes (DEGs) in tissues (shoots and roots) of *Medicago truncatula* exposed to ENM (engineered nanomaterial) or bulk/dissolved treatments that compared to controls.

Supporting Information File S4 summarize the results of the genes that were identified as significantly differentially expressed after applying FDR of 0.05 and 0.1 in tissues (shoots and roots) of *Medicago truncatula* exposed to ENM (engineered nanomaterial) or bulk/dissolved treatments that compared to controls.