Supporting Information

The differences between the effects of a nano-formulation and a conventional form of atrazine to lettuce: Defense mechanisms and nutrient displacement

Juan Wu[1], Yujia Zhai[1*], Fazel Abdolahpur Monikh[1], Daniel Arenas-Lago[2], Renato Grillo[3], Martina G. Vijver[1], Willie J.G.M. Peijnenburg[1,4*]

[1] Leiden University, Institute of Environmental Sciences (CML), P.O. Box 9518, 2300 RA, Leiden, The Netherlands

[2] University of Vigo, Department of Plant Biology and Soil Science, As Lagoas, Marcosende, 32004, Ourense, Spain

[3] Department of Physics and Chemistry, School of Engineering, São Paulo State University (UNESP), 15385-000, Ilha Solteira, SP, Brazil

[4] National Institute of Public Health and the Environment (RIVM), P.O. Box 1, Bilthoven, The Netherlands

[*]Corresponding authors:

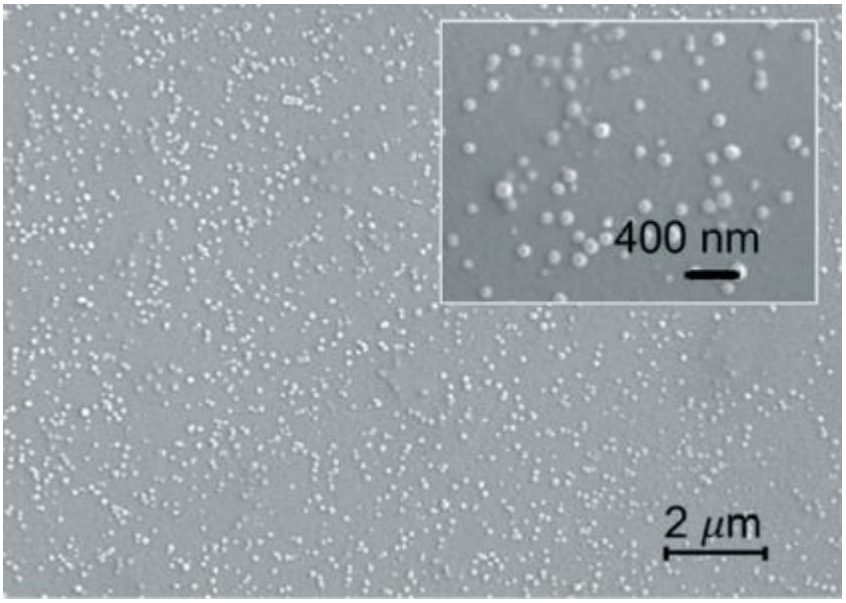
Yujia Zhai: y.zhai@tudelft.nl

Willie J.G.M. Peijnenburg: Peijnenburg@cml.leidenuniv.nl

S1. Nano-pesticides Synthesis[1]

Atrazine (ATZ) loaded poly- ε-caprolactone nano-capsules (NPATZ) were prepared according to the method reported by Grillo et al. (2012)[2]. This method involves the mixing of an organic phase (composed of 100 mg of polymer (poly- ε -caprolactone), 200 mg of triglycerides of capric and caprylic acids (Myritol 318), 50 mg of sorbitan monostearate surfactant (Span 60), 10 mg of atrazine and 30 mL of acetone) and an aqueous phase (composed of 60 mg of polysorbate 80 (Tween 80) and 30 mL of deionized water). The resulting suspension was maintained under magnetic stirring for 10 minutes. After this, the acetone was evaporated under reduced pressure using a rotary evaporator to a final volume of 10 mL. The concentration of herbicide was 1 mg mL^{-1}, The capsules were stored in amber flasks at room temperature (25°C).

The SEM images of the NPATZs show that the shape of the NPATZs is spherical and confirm that no particle agglomeration occurred. The median size of the NPATZs was around 100 nm as calculated from the SEM images. The hydrodynamic size of the NPATZs was 120 ± 10 nm and the zeta potential was −28 ± 4 mV.[1]



Scanning electron microscopy (SEM) picture of NPATZ used in this study.

S2. Release kinetics assays[1]

The kinetic experiments were designed to assess the release profiles of the herbicide ATZ from the NPATZs in water and soil. All measurements were the results of five replicates. The experiments were run under dilution sink conditions. The ATZ released in water was expressed as a percentage, and the ATZ release is plotted as a function of time (minutes). In addition, the semi-empirical Korsmeyer–Peppas model was applied to the herbicide release curves in order to identify the release mechanism involved. The water experiment employed a system consisting of two

compartments (donor and receptor), maintained under gentle agitation. A cellulose membrane (Spectrapore, with 1000 Da molecular exclusion pore size) separated the nano-pesticides (1 mL) in the donor compartment from the receptor compartment containing 50 mL of water (pH=7). The system was maintained under magnetic stirring (350 rpm) at 30°C. Aliquots were withdrawn at different time intervals and then analyzed by a Varian Cary 50 Spectrophotometer.

S3. Physicochemical properties of the soil used in this study[1]

The physicochemical properties of the soil used in this study are reported in Table S1.

• The pH was determined according to the method reported by Slattery et al. (1999)[3]. The soil:water and soil:KCl ratio was 1:2.5 for both measurements.

• Organic carbon was analysed according to the method reported by Walkley and Black (1934)[4].

• The cation exchange capacity (CEC) and exchangeable cation content were determined according to the method reported by Hendershot and Duquette (1986)[5]. Al, Ca, K, Mg and Na were extracted with 0.1 M $BaCl_2$, and the concentrations were determined by Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES) (PerkinElmer Optima 4300 DV, PerkinElmer, Waltham, MA, USA).

• The total metal contents were extracted with a mixture of HNO_3 and HCl (1:3 v/v) in Teflon reactors under 10 bar, 180 °C and 35 min as operational conditions of the microwave oven. The concentration in the extracts was determined by ICP-OES as above.

Table S1. Soil characteristics (standard error)

	Units	**Soil**
pH_{H2O}	-	8.35 (0.04)
pH_{KCl}	-	7.43 (0.04)
Organic C	%	2.16 (0.09)
CEC	$cmol^{(+)}\ kg^{-1}$	0.386 (0.009)
Na^+		0.054 (0.001)
K^+		0.032 (0.001)
Ca^{2+}		0.062 (0.001)
Mg^{2+}		0.102 (0.002)
Al^{3+}		0.137 (0.003)
Element		**Total concentration***
As	$mg\ kg^{-1}$	udl [76]

Cd	udl [13]
Co	udl [190]
Cr	1.41 (0.24) [180 (Cr^{3+}) – 78 (Cr^{6+})]
Cu	2.24 (0.03) [190]
Fe	8284 (435) [niv]
Mn	172.91 (5.84) [niv]
Ni	30.53 (10.61) [niv]
Pb	udl [530]
Ti	357.7 (23.6) [niv]
Zn	8.97 (1.48) [720]

CEC: Cation Exchange Capacity, udl: under detection limit. []: Intervention values for soil remediation in Netherlands (niv: no intervention value). *The oxidative stress in lettuce (*Lactuca sativa* L.) caused by the metals in the soil is ruled out since the levels in the soil are below the intervention limits. (VROM - Ministry of Housing, Spatial Planning and the Environment (2013) Circular on target values and intervention values for soil remediation. The Netherlands).

S4. Culture media:

Composition of the Hoagland's solution

Chemicals	**mg/L**
$Ca(NO_3)_2 \cdot 4H_2O$	945
KNO_3	607
$MgSO_4 \cdot 7H_2O$	493
$NH_4H_2PO_4$	115
Micronutrients	
H_2BO_3	1.48
$Mn(NO_3)_2 \cdot 4H_2O$	1
$Zn(NO_3)_2 \cdot 6H_2O$	1.19
$CuSO_4 \cdot 5H_2O$	0.05
Mo $Na_2O_4 \cdot 2H_2O$	0.02
$FeSO_4 \cdot 7H_2O$	11.1

¼ Hoagland solution (pH is measured and adjusted at 6) is composed after 4 times dilution of the Hoagland's solution.

S5. Antioxidant enzyme assays

Enzyme Extraction. The tissue samples were homogenised in ice cold 50 mM sodium phosphate buffer (pH 7.8) containing 1 mM EDTA and 1% (w/v) polyvinylpyrrolidone (PVPP) at a 1:9 w/v ratio and using a pre-cooled ball mill. The extract was obtained after centrifugation (10,000 rpm, 20 min, 4 °C). The supernatant was used for enzyme activity assay.

Superoxide dismutase (SOD). 50 µL of the supernatants of the enzyme extract and 2.95 ml of 0.05 M phosphate buffer (pH 7.8) containing 13 mM L-methionine, 100 nM EDTA-Na_2, 75 µM NBT and 2 µM riboflavin were mixed in a cuvette and placed in the plant growth chamber with light intensity 250 µmol $m^{-2}s^{-1}$ (4000 lux) for 20 min. Blank A consisted of the assay mixture plus the enzyme extract, and was placed in the dark while Blank B included all components of the assay mixture except the enzyme extract, and was placed in light. The reaction stopped when the lamp was switched off and the tubes were placed in darkness. Reduction of NBT was recorded at 560 nm. One unit of the enzyme activity was defined as the amount of enzyme required to result in a 50 % inhibition of the rate of NBT reduction at 560 nm.[6]

Peroxidase (POD). 50 µL of enzyme extract was mixed with reaction buffer containing 2.75 mL/(1.75 mL) of 50 mM sodium phosphate buffer (pH 7.0) and 0.1 mL of 4 % guaiacol in a cuvette and 0.1 mL of 1 % H_2O_2 was used to initiate the reaction. Increased absorbance was recorded at 470 nm for 2 min. One unit of enzyme activity was defined as the amount of the enzyme which caused a change of 0.001 in absorbance per minute.[7]

Catalase (CAT). 100 µL of enzyme extract was placed in a quartz cuvette with 1.9 mL/(2.9 mL) of 15 mM H_2O_2 in phosphate buffer (50 mM, pH=7), and the absorbance was recorded at 240 nm for 3 min. The H_2O_2 extinction coefficient was 23.148 mM^{-1} cm^{-1}.[7]

Ascorbate peroxidase (APX). 100 µL of enzyme extract was placed in a quartz cuvette with 886 µL of 0.1 M phosphate buffer at pH=7.4 and 4 µL of 25 mM ascorbate were placed in a quartz cuvette. Decreased absorbance was monitored at 290 nm over a period of 4 min at 30-seconds intervals after initiating the reaction with 10 µL of 17 mM H_2O_2 at 290 nm. The activity was calculated using an extinction coefficient of 2.8 $mM^{-1} \cdot cm^{-1}$.[8]

Polyphenol oxidase (PPO) was extracted in the same buffer as stated in the POD extraction. The reaction mixture consisted of 200 µL of enzyme extract and 2.8 mL of 10 mM catechol. PPO activity was recorded by measuring its ability of oxidizing catechol at 410 nm.[7]

Glutathione S-transferase (GST) was extracted in 50 mM phosphate buffer (pH 7.5) containing 1 mM Ethylenediaminetetraacetic acid (EDTA) and 1 mM dithiothreitol (DTT). 1-Chloro-2,4-dinitrobenzene (CDNB) was used to conjugate with thiol group of glutathione (GSH) and form GS-DNB conjugate. The increase of absorbance recorded at 340 nm for 5 min represents GST activity.[7]

Table S2. The statistical results of the tested enzymes in Lactuca sativa exposed to NPATZ and ATZ with concentrations ranging from low, medium, to high and different exposure durations (short-, medium- and long-term). The different letters indicate significant differences between treatments within the same exposure duration by one-way ANOVA and Duncan's honestly significant difference tests at $\alpha < 0.05$ (capital letters for shoot tissues and lower-case letters for root tissues).

		Root						Shoot					
		SOD	APX	CAT	POD	GST	PPO	SOD	APX	CAT	POD	GST	PPO
Short-term	CK	A	AB	A	A	A	A	a	a	ab	ab	a	a
	NPC	A	AB	A	AB	A	AB	a	a	a	ab	a	a
	NPATZ_L	AB	AB	AB	AB	A	AB	a	a	ab	ab	ab	a
	NPATZ_M	B	BC	B	BC	B	C	a	a	b	ab	c	a
	NPATZ_H	B	C	B	C	B	D	a	a	ab	c	ab	a
	ATZ_L	AB	A	A	AB	A	AB	a	a	ab	a	ab	a
	ATZ_M	AB	AB	A	AB	A	BC	a	a	ab	ab	bc	a
	ATZ_H	AB	C	B	C	A	C	a	a	ab	bc	ab	a
Medium-term	CK	A	AB	AB	A	AB	A	ab	a	a	a	a	a
	NPC	A	A	A	A	A	A	ab	a	a	a	a	a
	NPATZ_L	A	ABC	B	AB	BC	A	ab	a	a	ab	a	a
	NPATZ_M	A	BC	C	B	ABC	A	ab	a	ab	a	a	a
	NPATZ_H	B	C	C	C	C	B	ab	a	b	c	a	a
	ATZ_L	A	AB	AB	AB	A	A	a	a	a	a	a	a
	ATZ_M	A	AB	B	AB	AB	A	ab	a	a	b	a	a
	ATZ_H	A	BC	C	AB	AB	A	b	a	ab	d	a	a
Long-term	CK	A	A	A	A	A	A	a	ab	a	a	a	a
	NPC	A	A	A	AB	A	AB	a	ab	ab	a	a	a
	NPATZ_L	A	B	BC	ABC	AB	AB	ab	ab	ab	ab	a	bc
	NPATZ_M	A	B	CD	CDE	B	B	ab	a	ab	bc	a	abc
	NPATZ_H	B	C	D	E	C	C	c	c	c	c	b	d
	ATZ_L	A	AB	AB	BC	AB	AB	a	a	ab	ab	a	ab
	ATZ_M	A	AB	BC	BCD	A	AB	a	ab	ab	ab	a	cd
	ATZ_H	A	BC	BC	DE	AB	AB	b	b	b	c	c	cd

CK: control check, control plants without exposure to chemicals. NPC: exposure to a polymeric carrier without the ATZ (control). NPATZ-L: exposure to a low concentration of NPATZ. NPATZ-M: exposure to a medium concentration of NPATZ. NPATZ-H: exposure to a high concentration of NPATZ. ATZ-L: exposure to a low concentration of ATZ. ATZ-M: exposure to a medium concentration of ATZ. ATZ-H: exposure to a high concentration of ATZ.

Table S3. The TFs of macro-and micronutrients in *Lactuca sativa* exposed to NPATZ and ATZ with concentrations ranging from low, medium, to high and different exposure durations (short-, medium- and long-term). Data are mean ± SE (n = 3). The different letters indicate significant differences between treatments within the same exposure duration by one-way ANOVA and Duncan's honestly significant difference tests at $\alpha < 0.05$.

		TFs						
		K	Mg	B	Fe	Zn	Mn	Cu
Short-term	CK	1.6±0.1a	0.44±0.08a	0.68±0.18a	0.16±0.04a	0.42±0.08a	0.62±0.13ab	0
	NPC	1.3±0.3a	0.46±0.06a	0.35±0.07abc	0.13±0.10a	0.40±0.06a	0.67±0.08ab	0
	NPATZ_L	1.3±0.1a	0.58±0.06a	0.61±0.20ab	0.20±0.04a	0.63±0.02a	0.78±0.15ab	0
	NPATZ_M	1.36±0.01a	0.45±0.03a	0.51±0.07abc	0.19±0.04a	0.87±0.17a	0.95±0.19a	0
	NPATZ_H	1.26±0.03a	0.53±0.03a	0.36±0.05abc	0.16±0.03a	1.00±0.4a	0.64±0.02ab	0
	ATZ_L	1.7±0.2a	0.45±0.10a	0.31±0.03bc	0.12±0.02a	0.83±0.12a	0.56±0.03b	0
	ATZ_M	1.3±0.1a	0.51±0.06a	0.27±0.05bc	0.19±0.04a	0.74±0.19a	0.48±0.02b	0
	ATZ_H	1.6±0.6a	0.51±0.16a	0.24±0.04c	0.18±0.02a	0.67±0.21a	0.53±0.09b	0
Medium-term	CK	0.67±0.06a	0.39±0.04a	0.30±0.06a	0.07±0.002a	0.48±0.12ab	0.57±0.06a	0
	NPC	1.80±0.2ab	0.55±0.08ab	0.41±0.21a	0.07±0.011a	0.39±0.01ab	0.93±0.28ab	0
	NPATZ_L	1.90±0.2b	0.56±0.05ab	0.69±0.20a	0.10±0.007a	0.34±0.07a	0.89±0.16ab	0
	NPATZ_M	2.00±0.3b	0.92±0.19b	0.69±0.08a	0.21±0.026ab	0.47±0.04ab	1.80±0.7b	0
	NPATZ_H	2.00±0.5b	0.58±0.14ab	0.91±0.28a	0.24±0.061b	0.35±0.03ab	1.70±0.3b	0
	ATZ_L	1.60±0.1ab	0.48±0.06a	0.33±0.08a	0.12±0.046ab	0.62±0.12b	0.61±0.07a	0
	ATZ_M	1.90±0.5b	0.63±0.15ab	0.27±0.11a	0.17±0.08ab	0.35±0.10ab	0.86±0.16ab	0
	ATZ_H	1.80±0.6ab	0.67±0.20ab	0.80±0.25a	0.07±0.01a	0.35±0.05ab	0.59±0.11a	0
Long-term	CK	0.61±0.31a	0.28±0.03a	0.58±0.12a	0.11±0.02a	0.32±0.03a	0.78±0.24a	0
	NPC	1.10±0.1a	0.41±0.05a	0.73±0.25ab	0.13±0.01a	0.33±0.04a	0.81±0.17a	0
	NPATZ_L	0.85±0.16a	0.51±0.11a	0.50±0.04a	0.11±0.04a	0.15±0.03b	0.61±0.22a	0
	NPATZ_M	1.30±0.2a	0.63±0.14ab	0.28±0.14a	0.11±0.01a	0c	0.78±0.13a	0
	NPATZ_H	1.20±0.4a	0.60±0.04ab	1.17±0.02b	0.18±0.004ab	0c	0.84±0.04a	0
	ATZ_L	1.30±0.2a	0.42±0.05a	0.57±0.07a	0.17±0.02ab	0.16±0.06b	0.73±0.03a	0
	ATZ_M	1.10±0.5a	0.55±0.04a	0.33±0.11a	0.19±0.001ab	0.13±0.07b	0.73±0.001a	0
	ATZ_H	1.50±0.8a	1.10±0.4b	0.24±0.02a	0.25±0.07b	0c	0.68±0.17a	0

CK: control check, control plants without exposure to chemicals. NPC: exposure to a polymeric carrier without the ATZ (control). NPATZ-L: exposure to a low concentration of NPATZ. NPATZ-M: exposure to a medium concentration of NPATZ. NPATZ-H: exposure to a high concentration of NPATZ. ATZ-L: exposure to a low concentration of ATZ. ATZ-M: exposure to a medium concentration of ATZ. ATZ-H: exposure to a high concentration of ATZ.
The Cu content in plants shoots in all treatments were undetectable regardless of the exposure duration.

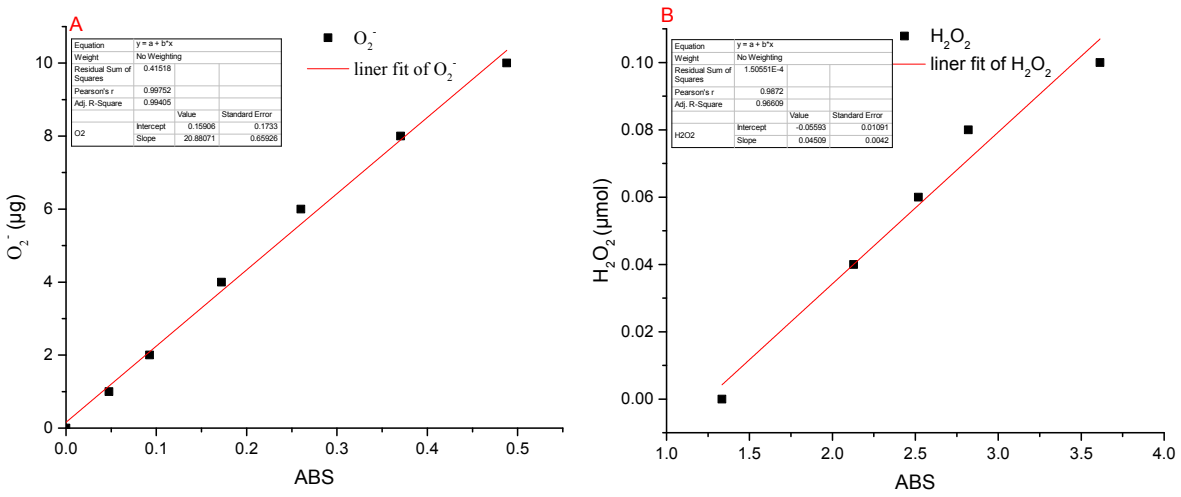


Figure S1. The calibration curve of O_2^- and H_2O_2 (x-axis represents the absorbance).

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