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Supplementary information

Interaction between a nano-formulation of atrazine and the rhizosphere bacterial community: atrazine degradation and bacterial community alterations

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S1. Nano-pesticides Synthesis

Atrazine (ATZ) loaded poly-ε-caprolactone nano-capsules (NPATZ) were prepared according to the precipitation method, as described by Grillo et al. (2012)[1]. 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. Thus, the concentration of herbicide was 1 mg mL^{-1}, and it was stored in amber flasks at room temperature (25 ^{o}C).

S2. Release kinetics assays

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 results 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 type of 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 The system was maintained under magnetic stirring (350 rpm) at 30 ^{o}C. Aliquots were withdrawn at different time intervals and then analyzed by Varian Cary 50 Spectrophotometer.

S3. ATZ extraction and analysis

ATZ extraction from soil samples was carried out using the Navarro et al. (2000) procedure. [2] Briefly, ATZ was extracted by adding acetonitrile and dichloromethane to the soil, and sonicating the mixture. The suspensions were shaken mechanically in an Erlenmeyer flask for 2 h. The organic phase was filtered and evaporated to dryness. The residue was dissolved in acetone, and the solution was injected into an HPLC (Agilent 1100 series). 20 µl of sample was injected into a 4 by 250 mm Hypersil ODS column. We used acetonitrile/water as mobile phase at flow rate of 1 ml per minutes according to the method reported previously. [3]

S4. Composition of plant culture media

- *Solution A*: 94.5 g $Ca(NO_3)_2 \cdot 4H_2O$, 60.7 g KNO_3 dissolved in 1 L deionized water (100 times)
- *Solution B*: 49.3 g $MgSO_4 \cdot 7H_2O$, 11.5 g $NH_4H_2PO_4$ powder dissolved in 1 L deionized water (100 times)
- *Solution C*: 0.1004 g $Mn(NO_3)_2 \cdot 4H_2O$, 0.119 g $Zn(NO_3)_2 \cdot 6H_2O$, 0.005 g $CuSO_4 \cdot 5H_2O$, 0.002 g $MoNa_2O_4 \cdot 2H_2O$ dissolved in 1 L deionized water (400 times)
- *Solution D*: 0.07416 g H_2BO_3 dissolved in 500 mL deionized water (800 times), dissoved in hot water.
- *Solution E*: 1.1122 g $FeSO_4 \cdot 7\ H_2O$ dissolved in 500 mL deionized water (800 times)

The culture solution (pH was measured and adjusted at 6) is composed by adding 2.5 mL solution A, 2.5 mL solution B, 2.5 mL solution C, 1.25 mL solution D to one liter of deionized water.

S5. Physicochemical properties of the soil used in this study

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

• The pH was determined according to the method reported by Slattery et al. (1999).[4] 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).[5]

• The cation exchange capacity (CEC) and exchangeable cation content were determined according to the method reported by Hendershot and Duquette (1986).[6] 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
Cd		udl
Co		udl
Cr		1.41 (0.24)
Cu		2.24 (0.03)
Fe		8284 (435)
Mn		172.91 (5.84)
Ni		30.53 (10.61)
Pb		udl
Ti		357.7 (23.6)
Zn		8.97 (1.48)

CEC: Cation Exchange Capacity, udl: under detection limit

S6. Experimental design

The ATZ was dissolved in 5 ml acetone and further diluted to 50 ml using Milli-Q (MQ) water. To spike the soil, 15 ml of the ATZ or NPATZ suspensions were carefully and homogeneously dropped into the soil to reach a final concentration of 0.3, 1.5, or 3 mg per kg soil. The exposure to ATZ and NPATZ at each concentration was performed with three repetitive exposure durations separated by a two-week interval (week 2, 4 and 6 after exposure), representing a short-term, medium and long-term exposure scenario. [7] Control experiments were also performed similarly, however without exposure to ATZ or NPATZ. Another control experiment was carried out by exposing the plant samples to soil containing only the polymeric carriers (PNC) without the active ingredient (ATZ) at 1.5 mg kg^{-1} soil. In total eight treatments in triplicate with three exposure durations were set up, which resulted in 72 pots with three individuals planted in each pot. The plants were watered every two days and 10 mL of 1/4 Hoagland solution was added into the pots once a week. Plants and the associated rhizosphere soils in each pot were harvested after 2, 4 and 6 weeks. The soil loosely adhering to the plant roots were discarded by vigorous shaking the roots. The soil that was tightly adhered to the plant roots were collected as rhizosphere soils. All the experiments including the pre-growth of seedlings were performed in a climate room at a 20/16 °C day/night temperature and 60% relative humidity set to a 16 h photoperiod until the associated rhizosphere soils were collected.

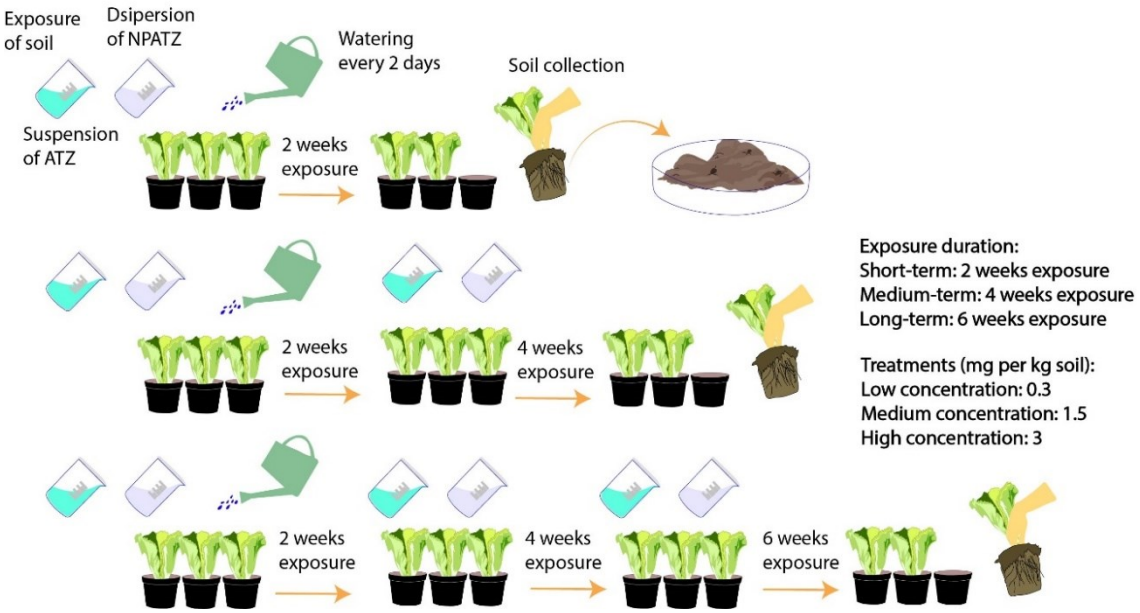

Figure S1. Schematic illustration of the experimental set-up. The soil was spiked with atrazine (ATZ) and ATZ nano-pesticides (NPATZ) and used to expose the *L. sativa* rhizosphere (3 replicates and 7 plants per treatment) at nominal concentrations of 0.3, 1.5 and 3 mg kg^{-1} soil for short-term (2 weeks), medium-term (4 weeks) and long-term (6 weeks) exposure. In the short-term exposure, the plants were exposed once at the beginning of the exposure experiment and harvested after 2 weeks. For the medium-term exposure, the plants were exposed at the beginning and after two weeks. The plant's rhizospheres were harvested after 4 weeks. For the long-term exposure, the plants were exposed at the beginning, after 2 weeks and after 4 weeks. The plant's rhizospheres were harvested after 6 weeks of exposure. The plants were watered every two days in all treatments. Control experiments in which the plants were exposed to water or to polymeric carriers without ATZ were also performed.

S7. DNA concentration

Table S2. Concentration of DNA extractions in each sample.

Time	Treatments	Sample name	DNA concentration (ng/µl)
Short-term exposure	CK	Y19070801	24.8
		Y19070802	19.9
		Y19070803	18.3
	ATZ_L	Y19070804	22.0
		Y19070805	40.5
		Y19070806	17.3
	ATZ_M	Y19070807	25.8
		Y19070808	51.6
		Y19070809	45.9
	ATZ_H	Y19070810	69.2
		Y19070811	54.4
		Y19070812	19.7
	PNC	Y19070813	25.8
		Y19070814	36.2
		Y19070815	19.8
	NPATZ_L	Y19070816	35.4
		Y19070817	22.4
		Y19070818	19.7
	NPATZ_M	Y19070819	19.6
		Y19070820	29.4
		Y19070821	12.4
	NPATZ_H	Y19070822	43.0
		Y19070823	16.1
		Y19070824	27.8
Medium-term exposure	CK	Y19070825	14.5
		Y19070826	19.2
		Y19070827	23.8
	ATZ_L	Y19070828	15.9
		Y19070829	18.7
		Y19070830	23.5
	ATZ_M	Y19070831	22.2

		Y19070832	32.2
		Y19070833	24.1
	ATZ_H	Y19070834	16.0
		Y19070835	44.4
		Y19070836	28.3
	PNC	Y19070837	24.0
		Y19070838	28.8
		Y19070839	14.6
	NPATZ_L	Y19070840	9.6
		Y19070841	13.8
		Y19070842	15.2
	NPATZ_M	Y19070843	33.2
		Y19070844	17.6
		Y19070845	14.7
	NPATZ_H	Y19070846	22.4
		Y19070847	20.0
		Y19070848	28.0
Long-term exposure	CK	Y19070849	58.3
		Y19070850	33.7
		Y19070851	38.9
	ATZ_L	Y19070852	34.8
		Y19070853	42.6
		Y19070854	50.2
	ATZ_M	Y19070855	51.3
		Y19070856	49.3
		Y19070857	52.5
	ATZ_H	Y19070858	46.1
		Y19070859	48.8
		Y19070860	38.8
	PNC	Y19070861	35.9
		Y19070862	48.4
		Y19070863	51.9
	NPATZ_L	Y19070864	47.4
		Y19070865	63.6
		Y19070866	59.4
	NPATZ_M	Y19070867	58.6
		Y19070868	47.5
		Y19070869	42.4
	NPATZ_H	Y19070870	69.6
		Y19070871	69.6
		Y19070872	69.2

Table S3. Sample details of each sequencing library submitted to NCBI database.

Time	Treatments	Sample_name	Biosample_accession	Accession
Short-term exposure	CK	Y19070801	SAMN14271685	SRR11236976
		Y19070802	SAMN14271686	SRR11236975
		Y19070803	SAMN14271687	SRR11236964
	ATZ_L	Y19070804	SAMN14271688	SRR11236953
		Y19070805	SAMN14271689	SRR11236942
		Y19070806	SAMN14271690	SRR11236931
	ATZ_M	Y19070807	SAMN14271691	SRR11236920
		Y19070808	SAMN14271692	SRR11236909
		Y19070809	SAMN14271693	SRR11236906
	ATZ_H	Y19070810	SAMN14271694	SRR11236905
		Y19070811	SAMN14271695	SRR11236974
		Y19070812	SAMN14271696	SRR11236973
	PNC	Y19070813	SAMN14271697	SRR11236972
		Y19070814	SAMN14271698	SRR11236971
		Y19070815	SAMN14271699	SRR11236970
	NPATZ_L	Y19070816	SAMN14271700	SRR11236969
		Y19070817	SAMN14271701	SRR11236968
		Y19070818	SAMN14271702	SRR11236967
	NPATZ_M	Y19070819	SAMN14271703	SRR11236966
		Y19070820	SAMN14271704	SRR11236965
		Y19070821	SAMN14271705	SRR11236963
	NPATZ_H	Y19070822	SAMN14271706	SRR11236962
		Y19070823	SAMN14271707	SRR11236961
		Y19070824	SAMN14271708	SRR11236960
Medium-term exposure	CK	Y19070825	SAMN14271709	SRR11236959
		Y19070826	SAMN14271710	SRR11236958
		Y19070827	SAMN14271711	SRR11236957
	ATZ_L	Y19070828	SAMN14271712	SRR11236956
		Y19070829	SAMN14271713	SRR11236955
		Y19070830	SAMN14271714	SRR11236954
	ATZ_M	Y19070831	SAMN14271715	SRR11236952
		Y19070832	SAMN14271716	SRR11236951
		Y19070833	SAMN14271717	SRR11236950
	ATZ_H	Y19070834	SAMN14271718	SRR11236949
		Y19070835	SAMN14271719	SRR11236948
		Y19070836	SAMN14271720	SRR11236947
	PNC	Y19070837	SAMN14271721	SRR11236946
		Y19070838	SAMN14271722	SRR11236945
		Y19070839	SAMN14271723	SRR11236944
	NPATZ_L	Y19070840	SAMN14271724	SRR11236943
		Y19070841	SAMN14271725	SRR11236941
		Y19070842	SAMN14271726	SRR11236940
	NPATZ_M	Y19070843	SAMN14271727	SRR11236939
		Y19070844	SAMN14271728	SRR11236938
		Y19070845	SAMN14271729	SRR11236937
	NPATZ_H	Y19070846	SAMN14271730	SRR11236936
		Y19070847	SAMN14271731	SRR11236935
		Y19070848	SAMN14271732	SRR11236934
Long-term exposure	CK	Y19070849	SAMN14271733	SRR11236933
		Y19070850	SAMN14271734	SRR11236932
		Y19070851	SAMN14271735	SRR11236930
	ATZ_L	Y19070852	SAMN14271736	SRR11236929
		Y19070853	SAMN14271737	SRR11236928

		Y19070854	SAMN14271738	SRR11236927
	ATZ_M	Y19070855	SAMN14271739	SRR11236926
		Y19070856	SAMN14271740	SRR11236925
		Y19070857	SAMN14271741	SRR11236924
	ATZ_H	Y19070858	SAMN14271742	SRR11236923
		Y19070859	SAMN14271743	SRR11236922
		Y19070860	SAMN14271744	SRR11236921
	NP	Y19070861	SAMN14271745	SRR11236919
		Y19070862	SAMN14271746	SRR11236918
		Y19070863	SAMN14271747	SRR11236917
	NPATZ_L	Y19070864	SAMN14271748	SRR11236916
		Y19070865	SAMN14271749	SRR11236915
		Y19070866	SAMN14271750	SRR11236914
	NPATZ_M	Y19070867	SAMN14271751	SRR11236913
		Y19070868	SAMN14271752	SRR11236912
		Y19070869	SAMN14271753	SRR11236911
	NPATZ_H	Y19070870	SAMN14271754	SRR11236910
		Y19070871	SAMN14271755	SRR11236908
		Y19070872	SAMN14271756	SRR11236907

S8. Data analysis

Quality control was performed by filtering the chimeras, singletons, chloroplasts, mitochondria, archaea and eukaryotes. The sampling depth heterogeneity was corrected by performing rarefaction. Qualified sequences were clustered into Operational Taxonomic Units (OTUs) based on the 97% sequence similarity level. [8] The taxonomic assignment was conducted using the UCLUST consensus taxonomy classifier. Sequences were aligned with Python Nearest Alignment Space Termination (PyNAST) to build a phylogenetic tree. QIIME then assembled a table of OTU abundances in each sample with taxonomic identifiers for each OTU. The core OTUs were selected based on the NCBI annotated ATZ-degrading bacterial genera, with presence in 100% of all the replicates for each treatment. Afterwards, phylogenetic investigation of communities by reconstruction of unobserved states (PICRUSt) [9] was used to translate the 16S rRNA gene amplicon data sets into predicted metagenomes to predict the functional capabilities of bacterial communities (PICRUSt v1.0.0 pipeline in QIIME). The predicted metagenomes were further annotated using Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways. [10] The core functional genes were selected based on the KEGG pathway of ATZ degradation, with presence in 100% of all the replicates for each treatment. The correlation between the core OTUs and functional genes was assessed

based on person correlation analysis with significance $p<0.05$. Analyses were conducted using R v3.3.2, Paleontological Statistics (PAST, v3.14), and Statistical Analysis of Metagenomic Profiles (STAMP, v2.1.3).

S9. Spiking the soil with ATZ and NPATZ

The ATZ was dissolved in 5 ml acetone and further diluted to 50 ml using Milli-Q (MQ) water. An aliquot of the NPATZ was dispersed in MQ water and was used as 1 g L^{-1} stock dispersion. Spiked soil samples were prepared by carefully dropping the dispersion of NPATZ or solution of ATZ into soil surface to reach a final concentration of 1 mg per kg soil. The soil was homogenized by thoroughly mixing the soil for 20 minutes at room temperature.

S10. Bacterial genera and functional genes associated with ATZ and NPTAZ degradation

The bacterial genera and functional genes involved in the degradation pathways of ATZ and NPATZ are given in Figure S2. For short-term exposure, *Pseudomonas* and *Mycobacterium* and functional genes of *atzB*, *atzC*, *atzD*, *atzE* and *atzF* were involved in both ATZ and NPATZ degradation from hydroxyatrazine to CO_2. As the exposure time increased to medium-term exposure, the degradation of ATZ and NPATZ differentiated with each other at the stage of degradation from carboxybiuret to CO_2 (*atzE* and *atzF* were mainly used by *Pesudomonas* and *Acetobacter* for ATZ degradation, and *Janthinobacterium*, *Mycobacterium* and *Arthrobacter* for NPATZ degradation). After long-term exposure, the ATZ degradation pathway is different from NPATZ degradation pathway, with more bacterial genera involved in the processes from N-isopropylammelide to carboxybiuret of NPATZ degradation pathway.

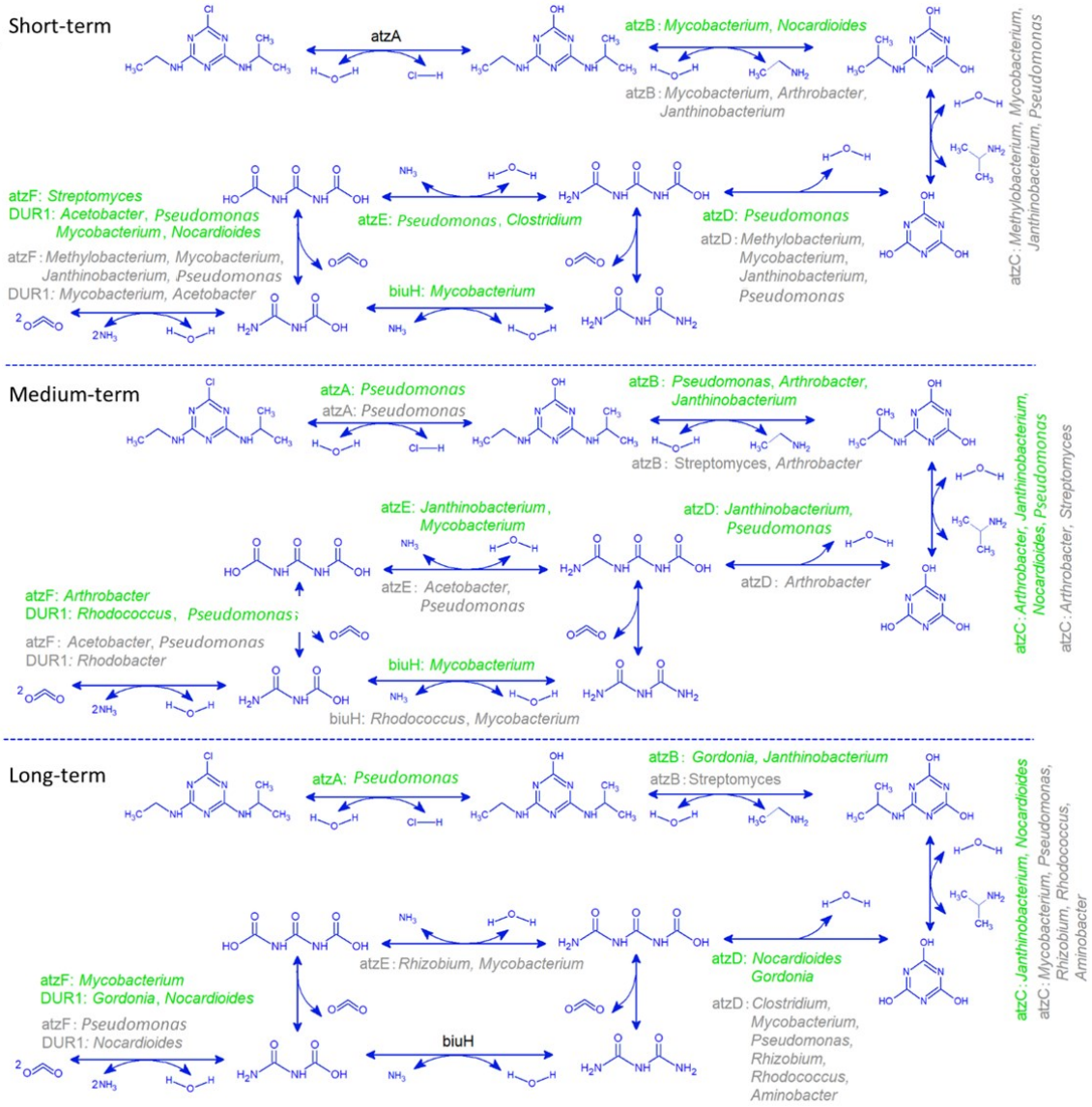

Figure S2. Bacterial genera and functional genes related with ATZ and NPTAZ degradation. Bacterial genera and functional genes in green are related with ATZ degradation. Bacterial genera and functional genes in grey are related with NPATZ degradation. Functional genes in black represent no significant positive correlation with bacterial genera.

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