SUPPORTING INFORMATION

for the paper

Increased pollution-induced bacterial community tolerance to sulfadiazine in soil hotspots

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Material presented as part of supporting information consists of 11 pages (S1-S11) and includes:
5 appendices
6 figures
1 table
4 literature references

Experimental section

A1. Soil sampling and characteristics. Soil was sampled from one experimental field plot receiving spring amendments of municipal sewage sludge at a rate of 1100 kg N ha^{-1} for five consecutive years. Surface soil (0-20 cm depth; > 100 soil cores) were sampled and pooled to yield one composite soil sample. The soil contained (all wt/wt %) 18% clay, 16% silt, and 66% sand. The total organic C content was 1.70 % (wt/wt) and total N was 0.18% (wt/wt).

A2. Composition of artificial root exudates. The artificial root exudate (ARE) solution [1] contained a filter-sterilized mix of low molecular weight organics previously reported to occur in plant root exudates: D-fructose (50 mM, CAS no. 57-48-7), D-glucose (50 mM, CAS no. 50-99-7), sucrose (50 mM, CAS no. 57-50-1), succinic acid (25 mM, CAS no. 110-15-6), L-malic acid (25 mM, CAS no. 97-67-6), L-arginine (12.5 mM, CAS no. 74-79-3), serine (12.5 mM, CAS no. 312-84-5), and L-cysteine (12.5 mM, CAS no. 52-90-4). Analytical grade reagents of at least 99% purity were used. The artificial root exudate solution was mixed with appropriate volumes of sterile filtered solutions of either Milli Q water or SDZ (SDZ-Na in Milli Q water; CAS no. 547-32-0, > 98 % purity, Fluka, Switzerland).

A3. Graphical presentation of the experimental design for the PICT selection phase.

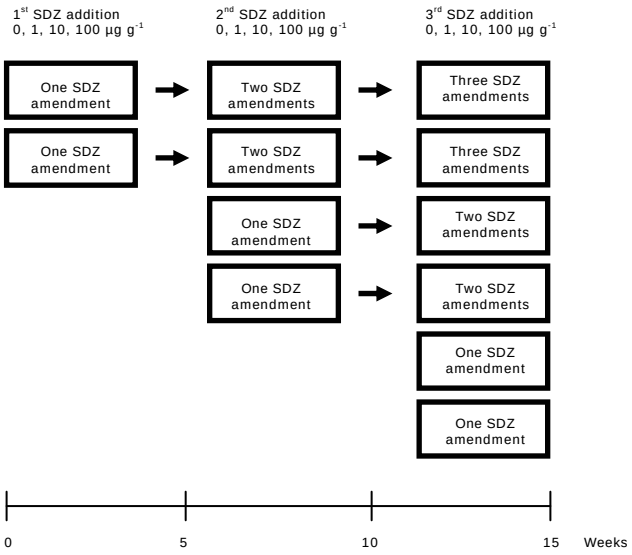


Figure S1. Experimental design. Determination of bacterial community tolerance to sulfadiazine (SDZ) was carried out after 10 and 15 weeks. SDZ dissipation was measured after 5, 10 and 15 weeks. Hotspot soil received weekly pulses of artificial root exudates (ARE). Bulk soil, corresponding soil without ARE. See text for details.

A4. Extraction of soil bacteria by Nycodenz density-gradient centrifugation and subsequent flow cytometric analysis of extracted bacteria. Moist soil (10 g) was mixed with 100 ml Davis Minimal Medium (DMM, Difco, Detroit, MI) without added C sources and blended in a water-cooled blender for 5 minutes [2]. Subsequently, 5 ml soil slurry from each soil microcosm was transferred to three 9-ml polyethylene centrifuge tubes (Ole Dich Instrument Makers, Hvidovre, Denmark). Following low-speed centrifugation (380 × g, 2 min, 22 °C), 4 ml of supernatant from each tube was carefully placed on top of a 2-ml Nycodenz cushion (8 g Nycodenz mixed with 10 ml Milli Q water, density of 1.3 g l^{-1}) in another 9-ml Ole Dich tube and subjected to high-speed centrifugation (10000 × g, 20 min, 4 °C). Following centrifugation, 3.6 ml of supernatant containing extracted soil bacteria was carefully sampled just above the Nycodenz cushion in each tube [2].

The suspensions of extracted soil bacteria were divided into two fractions. One fraction was preserved by addition of sterile-filtered formaldehyde (Poretics polycarbonate filter, 0.22 µm pore size) to a final concentration of 2 % (wt/vol) formaldehyde and immediately used to quantify the number of cells by automated flow cytometry. The remaining fraction was kept at 4 °C until termination of automated cell counts (same day), after which samples were individually diluted to yield cell suspensions of comparable cell density approximately 10^6 cells ml^{-1}. Automated flow cytometry cell counting was carried out with formaldehyde-fixed soil bacterial suspensions (900 µl) stained with 16 µl of a SyBR green I working solution (100-fold dilution in DMSO of chemical no. S-7567 from Invitrogen, Molecular Probes) for 15 minutes in the dark. Subsequently, known amounts of 2.15 µm SkyBlue fluorescent beads (10^5 beads ml^{-1}) were added to each microtube as internal cell quantification standards [3]. The total number of soil bacteria was finally counted on a FACS calibur flow cytometer (Becton Dickinson, San Jose, USA) equipped with an automated sample loader. FACSflow, FACSrinse and FACSclean (BD, Brøndby, Denmark) were used as low background flow solution and for cleaning, respectively. Samples were analyzed with detector voltages set at E01 for forward scatter (FSC), 385 V for side scatter (SSC) and 523 V for detector FL1 (Green fluorescence), compensation was omitted and threshold was set at 90 for both FSC and SSC. Samples were measured for 1 minute at a rate of 1000-3000 events per second.

For the detection of bacterial community tolerance, soil bacterial suspensions generated by the Nycodenz density gradient centrifugation method were divided into numerous aliquots (samples) and incubated with SDZ (400 mg l^{-1}) or without SDZ for 24 hours at 22 ˚C (PICT detection phase). All incubations were performed both in the absence and the presence of exogenously added C source (0.22 mM glucose) in order to make cells more susceptible to the bacteriostatic SDZ

antibiotic. Following incubation, esterase positive cells were quantified in all samples by flow cytometry using CFDA fluorescence probing as described previously [3] using the same flow cytometer and analytical conditions as described for automated flow cytometry cell counting as described above. Total cell counts were also performed in incubated samples by automated flow cytometry of formaldehyde-killed cells as described above.

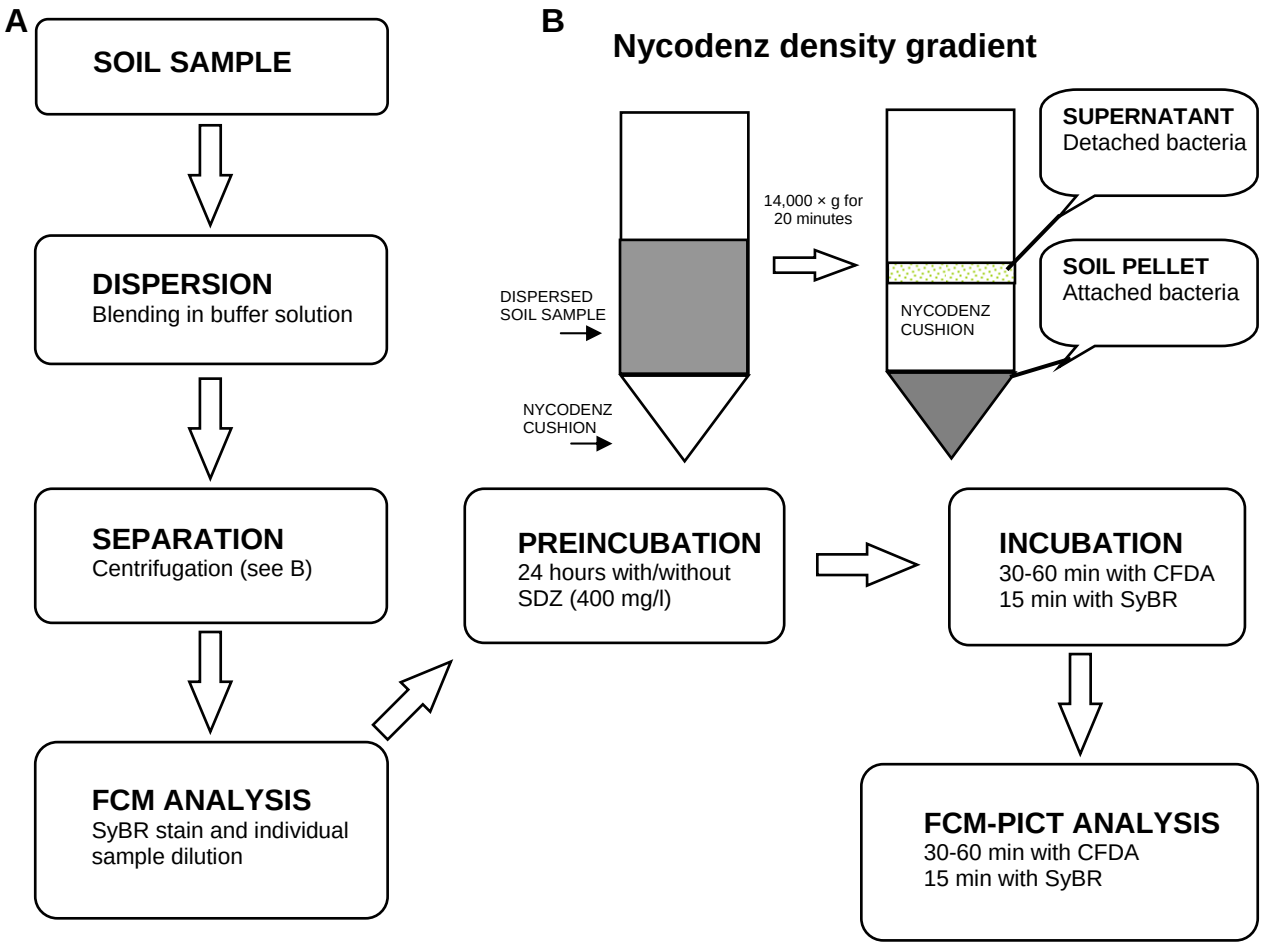


Figure S2. Flow chart of the used flow cytometry pollution-induced community tolerance protocol (FCM-PICT). A) A general flow chart for the entire protocol. The preincubation, incubation and FCM-PICT analysis were performed according to Stepanauskas and co-workers [3]. B) The separation step, where particle-detached bacteria are physically separated from soil particles by Nycodenz density gradient centrifugation. The entire layer above the Nycodenz cushion was sampled.

A5. Solid phase extraction and subsequent chemical analysis of extracted SDZ. The analytical method used has previously been described and validated by Jacobsen et al. [4]. In brief the extraction of SDZ from soil was performed by pressurised liquid extraction (PLE), using an ASE 200 system from Dionex (Sunnyvale, CA, USA). Approximately 10 g of soil sample was mixed with 10 g of Ottawa sand and added to the extraction cell. The extraction buffer consisted of a 1:1 (v/v) mixture of methanol and 0.2 M citric acid buffer with pH adjusted to 4.7 with NaOH. The automated PLE program was as follows: Two separate extraction steps at 1500 psi and room temperature each using 30 ml extraction buffer with a static time of 10 min and 3 min, for the first and second step respectively. Each extraction step was followed by a flushing of the extraction cell. PLE extracts (60 ml) containing approximately 30 ml methanol, were diluted with Milli-Q water to a methanol content below 10%.

Clean up was performed using a combination of SAX cartridges (strong anion exchange, 500 mg sorbent, 6 ml cartridge, Isolute, IST, Mid Glamorgan, UK) and Oasis HLB cartridges (poly(divinylbenzene–co-*N*-pyrrolidone), 200 mg sorbent, 6ml cartridge, Waters, Milford, MA, USA). The SAX cartridge was placed on top of the HLB cartridge and both columns were conditioned first with 2 ml methanol and then 2 ml 0.04 M citric acid buffer (pH 4.7). The diluted PLE extracts were passed through both SPE-columns at approximately 5 ml min^{-1} and after extraction the columns were washed with 2 ml 0.04 M citric acid buffer (pH 4.7) and 2 ml 0.1 M potassium acetate and dried under vacuum for 15 min. Then the SAX cartridge was removed and SDZ was eluted from the HLB-sorbent with 2 ml methanol.

Analysis of the resulting SPE extracts was performed using a liquid chromatography tandem mass spectrometry (LC-MS/MS) method. The LC system consisted of an Agilent 1100 series HPLC system (Agilent Technologies, Palo Alto, CA, USA), equipped with a degasser, a cooled autosampler (4 ºC) and a cooled column oven (13 ºC). MS detection was done using a Sciex API 3000 triple quadropole mass spectrometer (Applied Biosystems, Foster City, CA, USA) and equipped with an ESI source (Turbo Ionspray). Collection and treatment of data were performed using Analyst software (Applied Biosystems). The instrument was operated in the positive ion mode and detection was obtained using MRM (multiple reaction monitoring) detection with m/z 251.3/156.0 as precursor mass and product ion mass, respectively.

LC separations were achieved using an Xterra MS-C18 analytical column (100 mm × 2.1 mm, particle size 3.5 µm, from Waters) and gradient elution consisting of increasing content of mobile phases B over time. The compositions of mobile phases were: A: 5 % methanol and B: 95%

methanol both containing 80 mM formic acid. The flow rate was 0.2 ml min^{-1} and injection volume was 5 µl.

It became necessary to dilute the samples with the highest concentrations of SDZ in order to use the same calibration curve for the concentration range 5–500µg l^{-1} for all the samples. SDZ stock solutions were prepared in methanol, wrapped in tinfoil and stored at 5 ºC for a maximum of 1 month.

Results

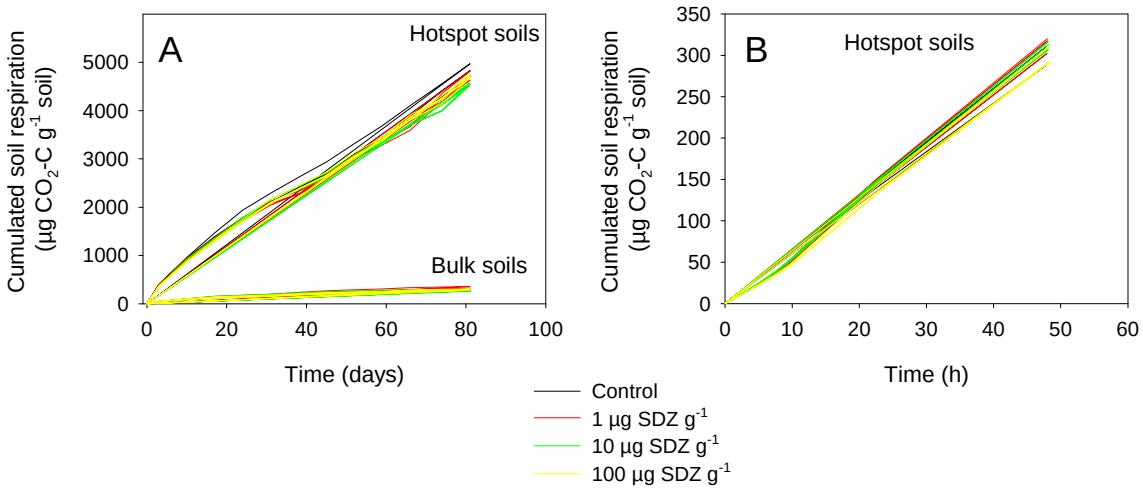


Figure S3. Impacts of SDZ exposure level on cumulated soil respiration. CO_2 accumulation curves derived from individual soil microcosms are shown with three replicate accumulation curves per treatment. Panel A) Impacts of SDZ exposure level on soil microbial respiration in bulk soil and hotspot soil microcosms during the first 81 days of the experimental period (same data as depicted in Figure 1). Panel B) Impacts of SDZ exposure level on short-term soil microbial respiration in hotspot soil microcosms. Accumulated CO_2 was measured four times during the first 10 hours and again after approximately 24 and 48 hours. Hotspot soil, soil receiving weekly pulses of artificial root exudates (ARE). Bulk soil, corresponding soil without ARE.

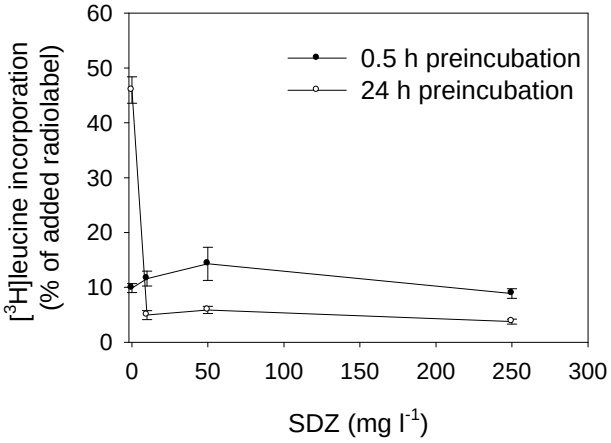


Figure S4. Optimization of ^{3}H-leucine incorporation assay conditions for detection of pollution-induced community tolerance (Leu-PICT assay). Mean ± standard deviation (n = 3).

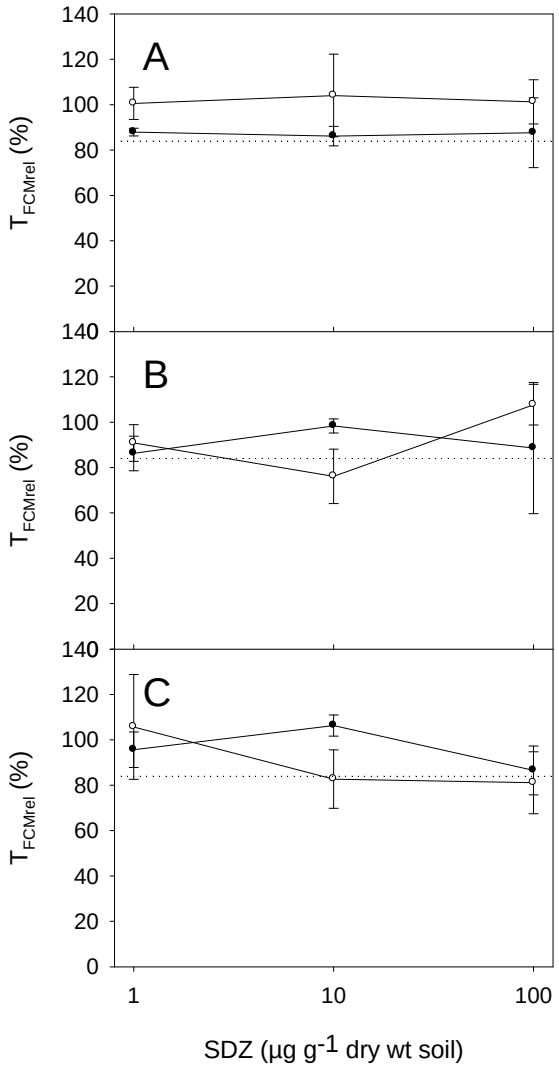
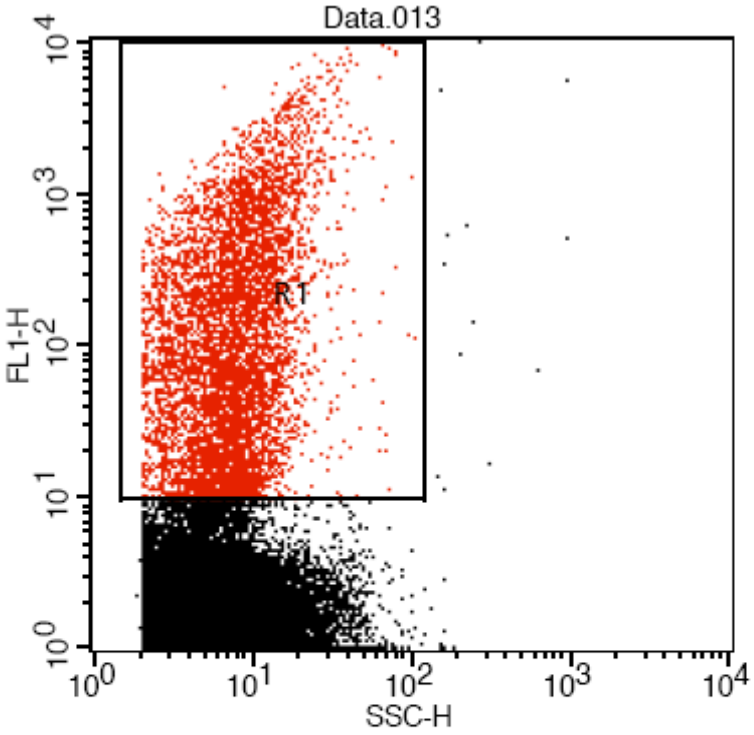


Figure S5. Determination of pollution-induced community tolerance (PICT) by flow cytometry viability probing of single-cell carboxyfluorescein diacetate esterase activities five weeks after preceding amendment with sulfadiazine (SDZ) in soil microcosms subjected to one (A), two (B) or three (C) repeated 5-week SDZ exposure periods. Dotted line represents background community tolerance to SDZ in control soil treatments without SDZ during the PICT selection phase.



Gated Events: 69510 Total Events: 69510

X Parameter: SSC-H (Log) Y Parameter: FL1-H (Log)

Region	Events	% Gated	% Total
R1	6692	9.63	9.63

Figure S6. Representative flow cytometric dot plot for Nycodenz extracted soil bacterial community stained with carboxyfluorescein diacetate (CFDA). FL1-H, green fluorescence. SSC-H, side scatter. R1, gated cells scored as CFDA positive (i.e. cells expressing esterase activity).

Table S1. Solid phase extractable sulfadiazine (SDZ) in soil microcosms.

SDZ treatment ($\mu g\ g^{-1}$)	No. of SDZ amendments	Total spiked SDZ ($\mu g\ g^{-1}$)	Extractable SDZ [a]	
			Bulk soil ($\mu g\ g^{-1}$)	Hotspot soil ($\mu g\ g^{-1}$)
1	1 [b]	1	0.07 ± 0.03	0.05 ± 0.03
1	2 [c]	2	0.07 ± 0.02	0.09 ± 0.09
1	3 [d]	3	0.08	0.06
10	1 [b]	10	0.53 ± 0.12	0.24 ± 0.03
10	2 [c]	20	1.05 ± 0.38	0.49 ± 0.04
10	3 [d]	30	1.24	0.83
100	1 [b]	100	25.8 ± 6.5	20.4 ± 4.3
100	2 [c]	200	45.8 ± 4.2	46.2 ± 0.8
100	3 [d]	300	50.7	5.6

[a] SDZ analyzed following solid phase extraction. Values are given as mean ± standard deviation.

[b] Soil sampled after one 5-week incubation period; $n = 3$

[c] Soil sampled after two 5-week incubation periods; $n = 2$

[d] Soil sampled after three 5-week incubation periods; $n = 1$

Literature Cited

1. Griffiths BS, Ritz K, Ebblewhite N, Dobson G. 1999. Soil microbial community structure: Effects of substrate loading rates. *Soil Biol Biochem* 31:145-153.

2. Hesselsøe M, Brandt KK, Sørensen J. 2001. Quantification of ammonia-oxidizing bacteria in soil using microcolony technique combined with fluorescence *in situ* hybridization (MCFU-FISH). *FEMS Microbiol Ecol* 38:87-95.

3. Stepanauskas R, Glenn TC, Jagoe CH, Tuckfield RC, Lindell AH, Mcarthur JV. 2005. Elevated microbial tolerance to metals and antibiotics in metal-contaminated industrial environments. *Environ Sci Technol* 39:3671-3678.

4. Jacobsen AM, Halling-Sorensen B, Ingerslev F, Hansen SH. 2004. Simultaneous extraction of tetracycline, macrolide and sulfonamide antibiotics from agricultural soils using pressurised liquid extraction, followed by solid-phase extraction and liquid chromatography-tandem mass spectrometry. *J Chromatogr A* 1038:157-170.