Supporting Information for

Plant Assimilation Kinetics and Metabolism of 2-Mercaptobenzothiazole Tire Rubber Vulcanizers by *Arabidopsis*

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Part 1: Additional Method Details

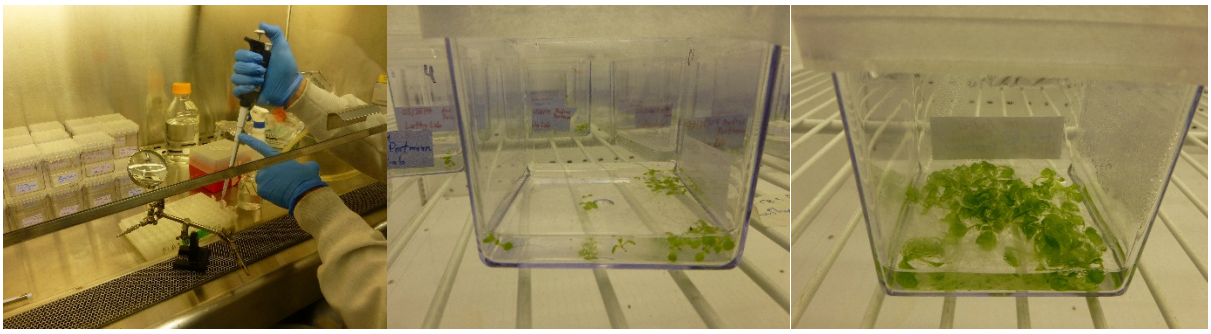


Figure S.1: Photos of planting procedure and plants. (left to right) a) Planting of seeds in sterile biological safety hood. b) Plants after 9 days of growth. c) Plants at harvesting day t = 3 d (after 18 days of growth in total).

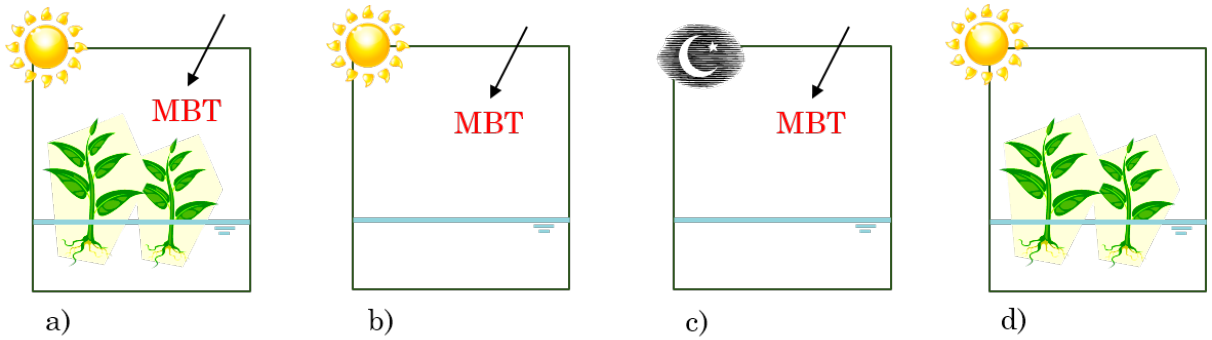


Figure S.2: Overview of treatment and controls. a) Treatment. b) Negative control. c) Dark control. d) Positive control.

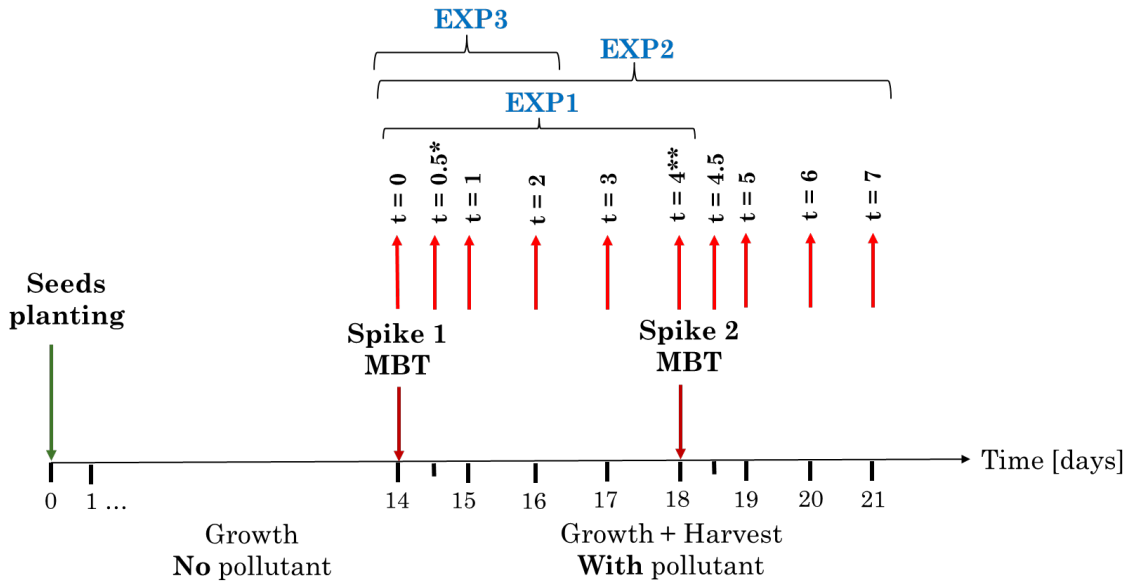


Figure S.3: Timeline of experiments and harvesting regimes. Red upward arrows signify harvesting / sampling. All samples were harvested with n=3 biological replicate boxes. *=no harvesting for EXP 1. **=no Spike 2 for EXP 1, harvesting only.

Method Detail 1: *Seed sterilization*. All procedures for seed sterilization were conducted over a flame and the bench and gloves were sterilized with a 70% ethanol spray solution to create a sterile working environment. 50 µL of seeds and 1 ml of seed sterilization solution were added to a 1.5 ml autoclaved tube. The seed sterilization solution consisted of 0.8 ml autoclaved water, 0.2 ml bleach (8.25% sodium hypochlorite, Clorox) and 10 µL Tween 20 surfactant (Polyoxyethylene sorbitan monolaurate, BioRad Laboratories Inc.). The tube was vortexed briefly and slowly inverted for 5 minutes. The supernatant was removed using an autoclaved pipet. 1 ml of sterile water was added to wash the seeds from the sterilization solution and again the supernatant was removed. The washing step needed to be repeated for a total of four times. The seeds were then stored at 4°C overnight to stratify.

Method Detail 2: *Hydroponic media preparation*. The media stock solution was prepared as follows: Per 1 liter Mili-Q water, 4.43 g MS Basal Medium (Murashige & Skoog Basal Medium with vitamins, Phyto Technology Laboratories), 0.5 g MES hydrate (Monohydrate 2-(N-morpholine)ethanesulfonic acid, Phyto Technology Laboratories) and 5 g Sucrose (J. T. Baker) were combined. While stirring the media with a stir bar, the pH was adjusted to 5.7 by carefully adding potassium hydroxide (KOH) to the media. The media was filter sterilized (0.22 µm Polyethersulfone, sterile, Corning) into a sterile glass container in the laminar flow biological safety hood.

Method Detail 3: *Plant tissue extraction*. Following harvest, each plant sample was padded dry by hand with a Kim-wipe (Kimberly-Clark) and transferred into a locking micro-centrifuge tube (Safe-Lock Tubes, 2 ml, Eppendorf). The moist plant tissue was freeze-dried overnight (approximately 12 hours) on a lyophilizer. A 1.0 mL solution of 1:1 water and methanol, a methanol-rinsed stainless-steel homogenizing ball, and 10 µL of internal standard (MBT-d_4; 10

mg/L) were added to the dry plant tissue. Each sample was vortexed briefly, then frozen at -80 °C for 15 - 20 minutes. Samples were then homogenized for 5 min at 30 Hz using a ball homogenizer, followed by vortexing for 2 min and then sonication for 10 min. The samples were then centrifuged at 10,000 × g for 15 min to separate plant tissue and supernatant. The supernatant was removed using a 3 mL syringe (Luer-Lok Tip, BD Falcon) and precision glide needle (0.8 mm x 40 mm, BD Falcon), and then filtered through a 0.2 μm PTFE syringe filter (13 mm, Fisherbrand). The aforementioned procedure (addition of 0.5 ml of the 1:1 water and methanol solution, vortexing, sonication and centrifugation) was repeated an additional two times to create a sequential extraction. For each sequential extraction, the supernatant was collected and filtered using the same syringe and filter into the sample vial to create a combined sample. The internal standard surrogate recovery method (using MBT-d4) was used to account for sample loss during the extraction procedure and matrix effects associated with the plant tissue (i.e., ionization suppression or enhancement). All reported results are normalized to internal standard surrogate recovery.

Method Detail 4: *Quality assurance and quality control*. Each time-point sample was harvested with three biological replicate boxes. Sampling of the MBT spiked media mastermix before addition to the plant boxes reliably established the initial value for the MBT mass balance. Internal standard addition (MBT-d_4) occurred for each sample analyzed via LC-MS/MS. A complete calibration curve was run with each sample set. Internal standard addition normalized quantification permitted accurate accounting for signal suppression/enhancement in electrospray ionization, matrix effects in the samples, and varying recovery from extraction processes (e.g., SPE, plant extraction). For direct injection samples, outliers were excluded if the recovery of the internal standard was less than 50% compared to their respective replicates while concurrently the signal (peak area counts) of MBT was more than 50% compared to the replicates. A total of 8 data points from all experiments were excluded from data sets based on the aforementioned quality screening procedure. Surrogate recovery (%) for included in data analysis for plant tissues was: ave±std dev=97±2 (min=93, max=99); values for hydroponic media were: ave±std dev=103±4 (min=91, max=108).

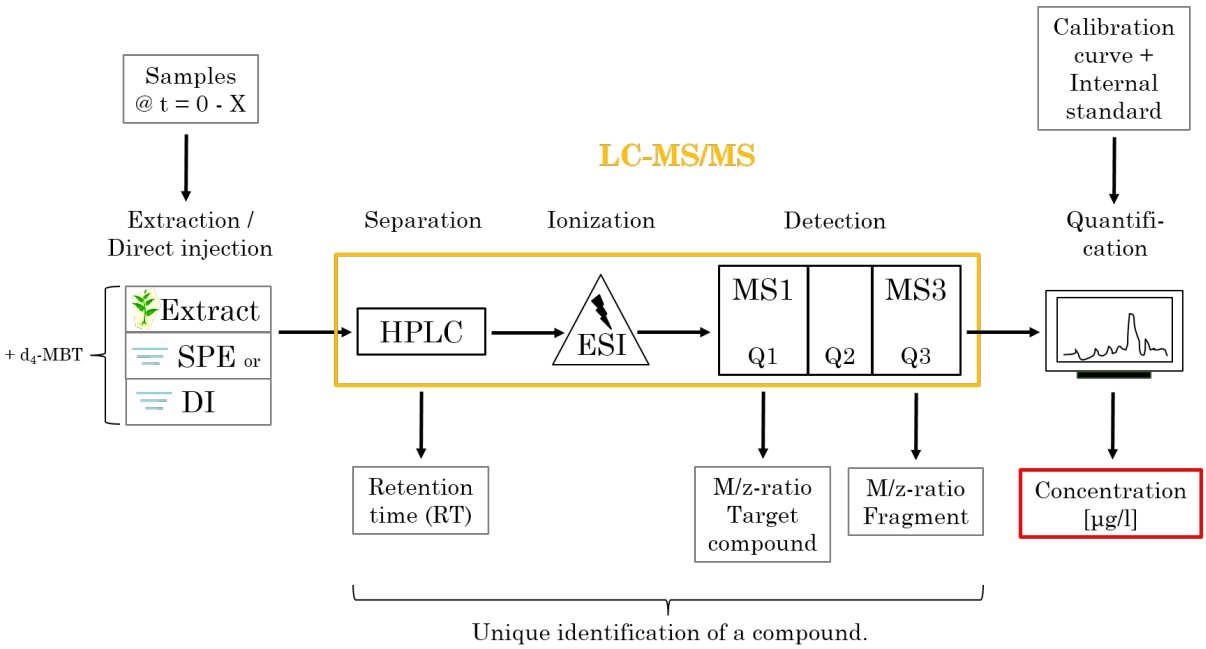


Figure S.4: Overview of the procedure for sample analysis.

Table S.1: MS parameters for the detection of MBT and the internal standard d4-MBT using ESI in the negative mode and MRM. RT: Retention time. M/z: Mass to charge ratio. F1: Fragment 1 (confirmed). F2: Fragment 2.

Analyte	RT [min]	M/z [-]	M/z F1 [-]	M/z F2 [-]
MBT	4.5	165.887	133.825	101.764
d_4-MBT (IS)	4.5	169.940	137.783	105.860

Ionization parameters of the MBT fragments as defined for the targeted MS/MS method can be read from Table S.2. Unique identification of the target compound (MBT) was determined when the LC-MS/MS retention time of the internal standard, M/z values matched in the first and third quadrapoles.

Table S.2: Ionization parameters of the fragments in the MS/MS. Abbreviations are as follows: DP: Declustering potential. FP: Focusing potential. EP: Entrance potential. CE: Collision energy. CXP: Collision cell exit potential.

Fragment (g/mol parent compound > g/mol fragment)	ID	Time [msec]	DP [Volts]	FP [Volts]	EP [Volts]	CE [Volts]	CXP [Volts]
MBT F1 (166 > 134)	2-McBTh_2	100	-36.00	-180.00	-10.00	-30.00	-13.00
MBT F2 (166 > 102)	2-McBTh_1	100	-36.00	-180.00	-10.00	-32.00	-7.00
d_4-MBT F1 (170 > 138)	d4-MBT1	100	-36.00	-140.00	-10.00	-32.00	-15.00
d_4-MBT F2 (170 > 106)	d4-MBT2	100	-36.00	-140.00	-10.00	-32.00	-7.00

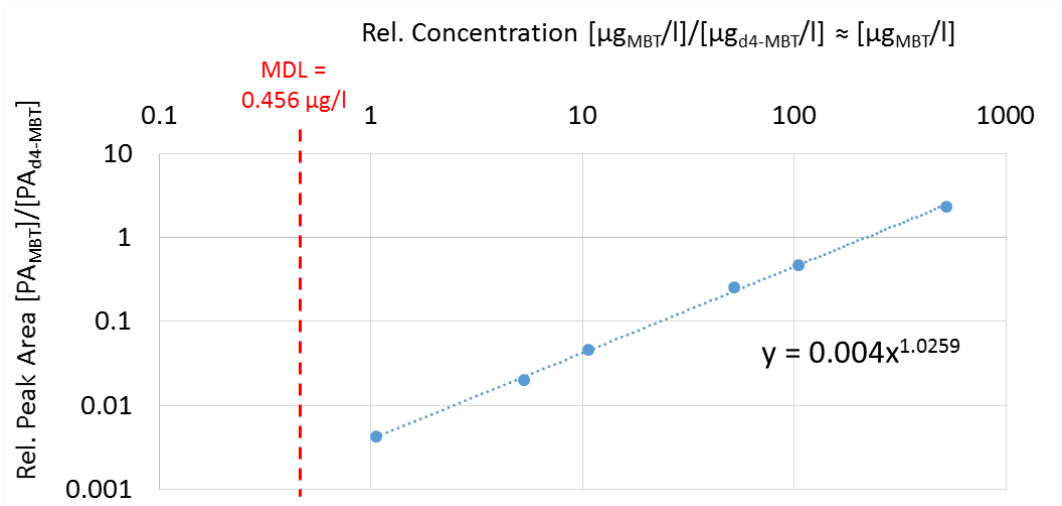


Figure S.5: Internal calibration curve (example). Note that only standards 6 (1 µg/L MBT) to 1 (500 µg/L MBT) were used for the calibration (linear range). The method detection limit (MDL) was determined to be 0.456 µg/l MBT.

Part 2 Supporting Results:

MBT Impacts on Plant Growth

To test if the plants grew equally well under the treatment condition (with MBT) compared to the positive control (without MBT), a paired t-test of the dry plant biomass was conducted (Figure S.6). No significant difference in the dry plant biomass between treatment and positive control was observed for EXP 1 over a four day time period (MBT C_0=approx. 25 µg/L; p=0.5507), or EXP 2 over a seven day time period (C_0 = 18 µg/L; p=0.9471). Thus, MBT is assumed to not impact overall plant growth for short durations at environmentally relevant concentrations at which most of the experiments were conducted. Other impacts on plant physiology, however, were not measured.

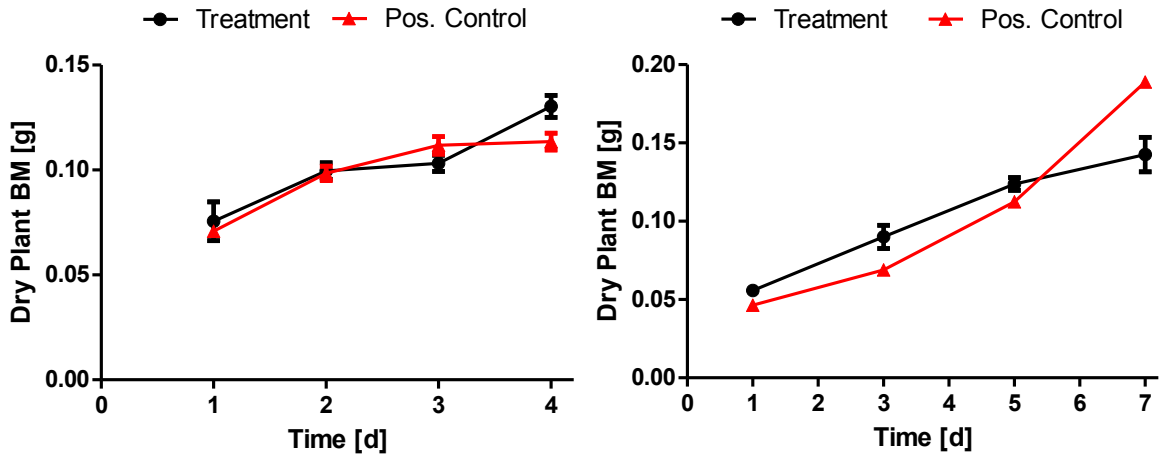


Figure S.6: Comparison of dry plant biomass weight between treatment and positive control. Error bars represent the standard error of the mean. Left: EXP 1: The spiked concentration of MBT corresponded approximately to 25 µg/L (not directly measured). Right: EXP 2: C_0 = 18 µg/L MBT. Note that there were no replicates for the positive control in EXP 2 and therefore no error bars. The statistical power is derived from the matched-pairs (temporally matched) experimental design.

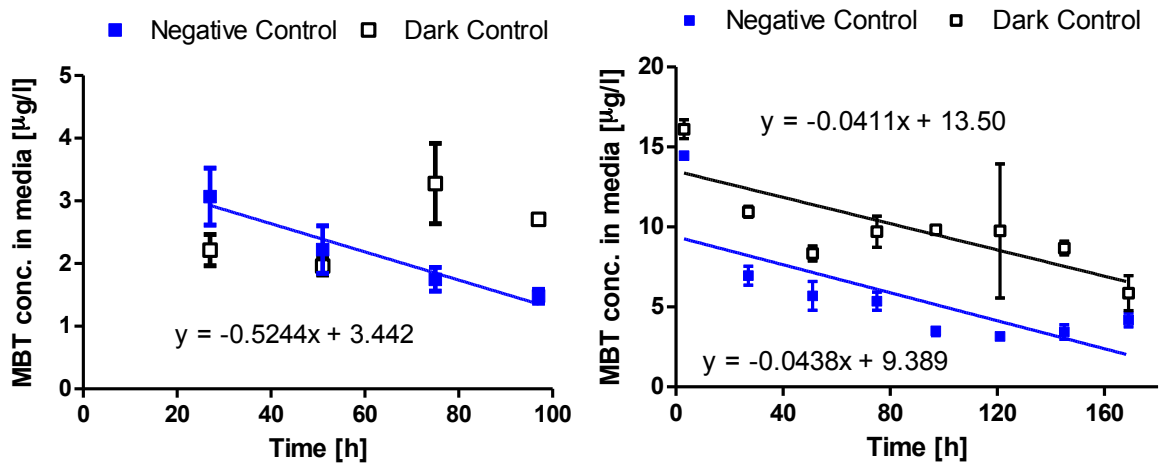


Figure S.7: Comparison of MBT concentration in media between negative control and dark control. Error bars represent the standard error of the mean. Left: comparison over 4 days and at a concentration level of approximately 25 µg/L. Right: comparison over 7 days and at a concentration of 18 µg/L.

Equation S.1: Second order reaction model for MBT degradation in media. Note that the dimensions of the rate constant K are $[T^{-1}]$ instead of the usual $[LM^{-1}T^{-1}]$ for a second order reaction due to the fact that the model was fitted to the relative concentration data (C/C_0).

$$-\frac{d[Y]_t}{dt} = K\,([Y]_t)^2 \;\rightarrow\; [Y]_t = Y_0\frac{1}{1+KY_0t}$$

$[Y]_t$: Relative concentration of MBT in media after time t, C/C_0
Y_0: Relative concentration of MBT in media at time zero, C_0/C_0
K: Degradation rate constant, $[h^{-1}]$
t: Time, [h]

Table S.3: Model parameters (best-fit values) and goodness of fit measures for the fitting of a second-order degradation model to the C/C_0 treatment data from spike 1 and spike 2.

The standard error (n=3 biological replicates each at n=5 sampling time points) of the rate constant K and intercept is provided in parenthesis. The upper and lower boundaries of the 95% confidence interval for the modeled half-life are also provided, as well as the model goodness of fit.

Second-order degradation model		Initial MBT concentration in the media (Spike 1)				Spike 2
		C1: C_0 = 1973 µg/L	C2: C_0 = 147 µg/L	C_0 = 18 µg/L	C3: C_0 = 1.59 µg/L	C_0 = 36 µg/L
Model parameters (best-fit values)	Intercept Y_0 (± std err)	1.013 (±0.0677)	1.000 (±0.0375)	0.9990 (±0.0261)	0.9965 (±0.0640)	1.000 (±0.0053)
	Rate constant K $[h^{-1}]$ (±std err)	0.4302 (±0.1001)	1.910 (±0.6343)	0.8691 (±0.1030)	0.4228 (±0.1030)	8.932 (±1.146)
	Half-life $t_{1/2}$ [h] [95% Confidence Interval: (Lower, Upper)]	2.2947 (1.5466, 4.6751)	0.5236 (0.3038, 1.8943)	1.1518 (0.9200, 1.5366)	2.3735 (1.5893, 4.6232)	0.1120 (0.0880, 0.1538)
Goodness of fit	R^2	0.9256	0.9775	0.9853	0.9153	0.9995
Negative Control Modeled parameter	Rate Constant $[h^{-1}]$ (±std err)	~ 2.220×10^{-16} (± <0.0001)	~ 2.220×2^{-16} (± <0.0001)	0.04711 (±0.005953)	0.01823 (±0.006229)	~ 2.220×2^{-16} (± <0.0001)

The transpiration rate was estimated in a manner similar to LeFevre et al. (2015, ES&T) by subtracting the evaporation rate from the evapotranspiration rate; the corresponding values for spike 1 and spike 2 are shown in Table S.4 All rates were the average over the duration of one day. The detected mass loss was calculated based on measured data (LC-MS/MS) and compared with the expected loss, yielding a factor. Please note that this is an *approximate estimation* for transpiration; more advanced approaches such as pressure chamber methods would be required to determine transpiration more precisely. The objective of these measurements were to determine if the ratio of MBT mass loss to transpiration water mass was substantially greater than unity.

Table S.4: Calculation of transpiration rate 1 (spike 1, EXP 2) and 2 (spike 2, EXP 2), expected MBT uptake due to transpiration and detected MBT mass loss from media (based on measured data).

	Measured Water loss rates	Expected MBT uptake by transpiration:		Detected MBT loss from media:		Factor:	
	[mL/d]	[µg/d]	[µg/3d]	[µg/d]	[µg/3d]	t = 1d	t = 3d
Evaporation rate	-0.1898						
EvapoTranspiration rate 1	-0.7685						
EvapoTranspiration rate 2	-0.5461						
Transpiration rate 1	-0.5787	0.0104	0.0312	0.4435	0.4519	42.6	14.5
Transpiration rate 2	-0.3563	0.0128	0.0385	0.8873	0.8875	69.2	23.1

LC-MS/MS Chromatograms

LC-MS/MS chromatograms of a plant tissue sample that was exposed to MBT (Figure S.8), hydroponic media from a magenta box that had been exposed to MBT and that contained plants (Figure S.9), and a negative control that contained media and MBT but no plants (Figure S.10). The initial MBT concentration was 1973 µg/L in all examples. In all cases, the internal standard (MBT-d_4) and the MBT in the sample were within 0.03 min retention time.

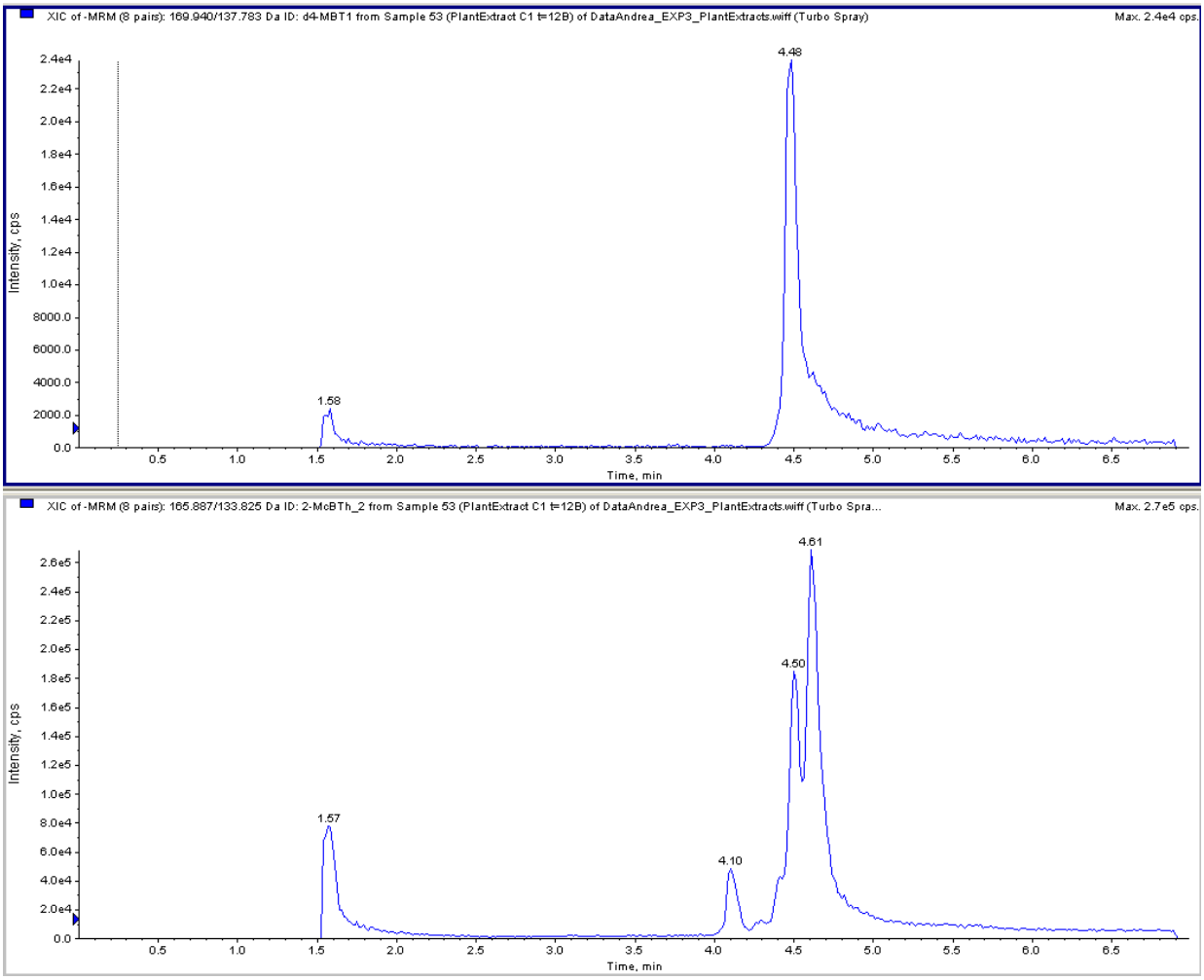


Figure S.8: LC-MS/MS chromatogram of a treatment plant sample (plant tissue extrant from sample plants exposed to MBT) from t=12h; C_0=1973 µg/L MBT (EXP 3). Y-axis: Intensity (cps). X-axis: Time (min). Top: Internal standard MBT-d_4 (RT=4.48 min). Bottom: Parent compound MBT (RT=4.50 min), Metabolites M1 (RT=4.10 min) and M2 (RT=4.61 min). The HPLC diverter switched to the mass spec at 1.5 min, thus the initial peak. Sample was taken at t=12h.

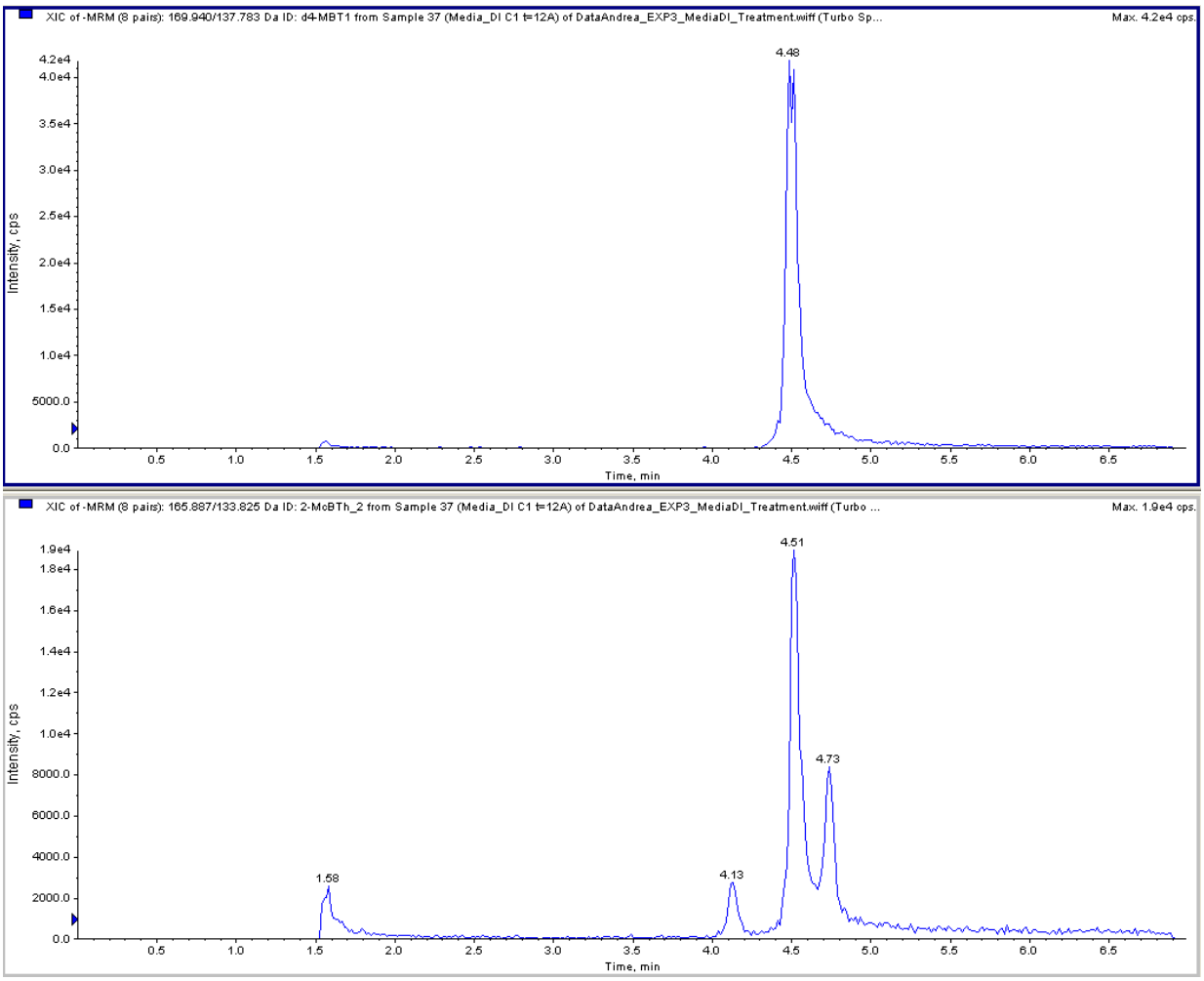


Figure S.9: LC-MS/MS chromatogram of a treatment media sample (hydroponic media exposed to MBT and plants) from t=12h; C_0=1973 µg/L MBT (EXP 3). Y-axis: Intensity (cps). X-axis: Time (min). Top: Internal standard MBT-d_4 (RT=4.48 min). Bottom: Parent compound MBT (RT=4.51 min), Metabolites M1 (RT=4.13 min) and M3 (RT=4.73 min). The HPLC diverter switched to the mass spec at 1.5 min, thus the initial peak. Sample was taken at t=12h.

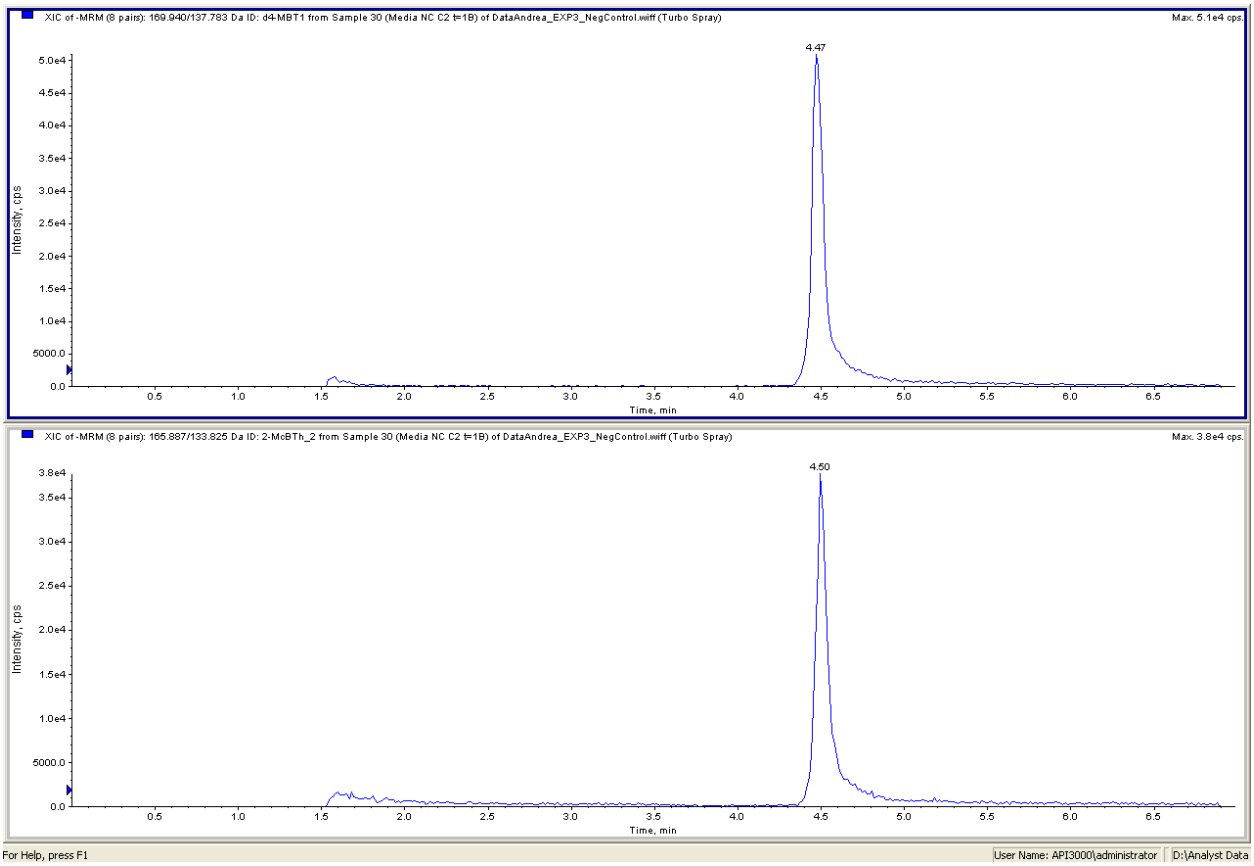


Figure S.10: LC-MS/MS chromatogram of a negative control media sample (hydroponic media containing MBT and no plants) from t=1d; C_0=1973 µg/L MBT (EXP 3). Y-axis: Intensity (cps). X-axis: Time (min). Top: Internal standard MBT-d_4 (RT=4.70 min). Bottom: Parent compound MBT (RT=4.72 min). Sample was taken at t=27h (~1d).

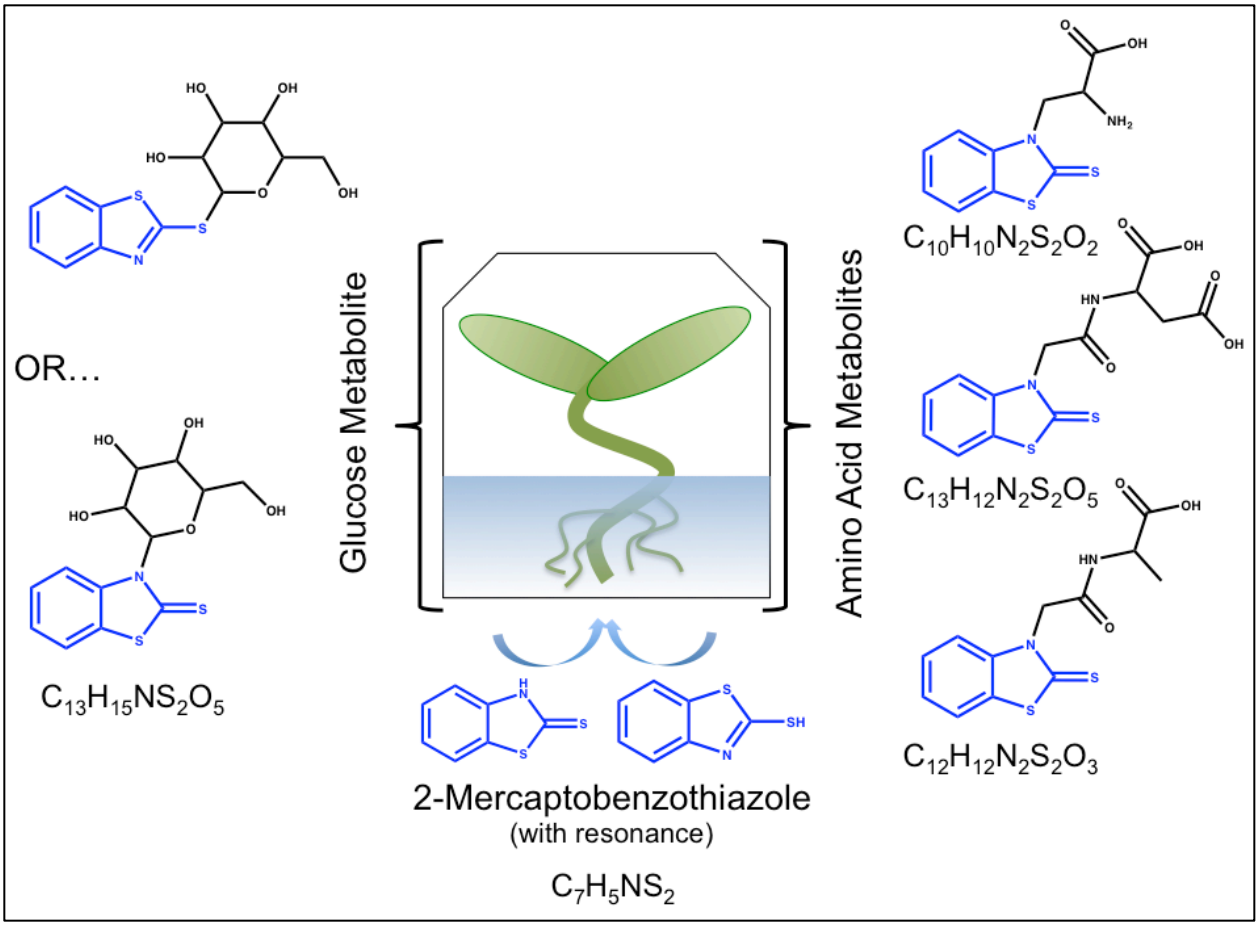
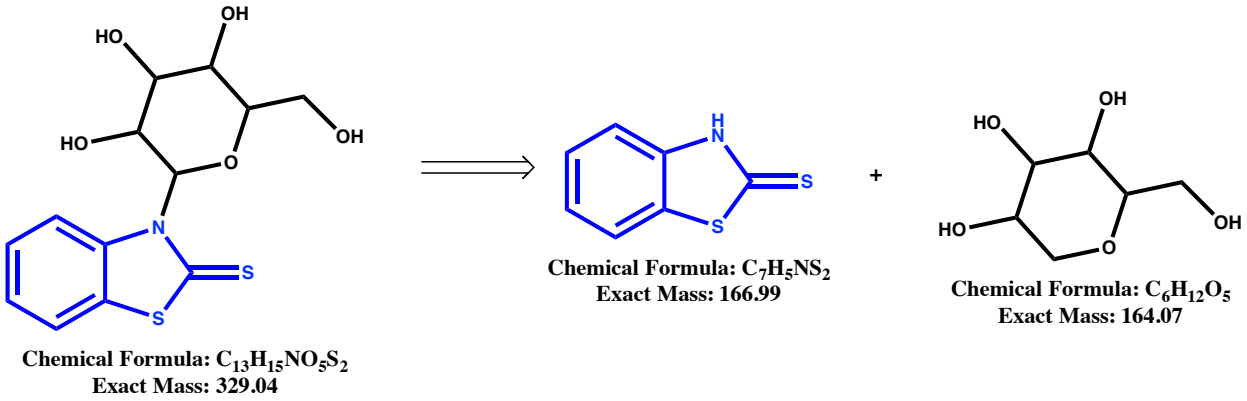


Figure S.11: Summary of hypothesized plant metabolites of 2-mercaptobenzothiazole are based on plant metabolites of benzotriazole from the literature and formulae generated from accurate mass LC-QTOF-MS data. The formulae were present in MBT treated samples analyzed (n=3) and absent from no-MBT exposure controls (n=3). The bonding site of in the glycosylated MBT may be in one of two different locations; this compound is presumed to be the more polar metabolite (M1) in Fig 4. Amino acid conjugation with MBT is also hypothesized (right). Please note that the hypothesized formulae are only based on targeted accurate mass formula searches and are not as rigorous as full metabolomics-based approaches and the proposed structures were examined with only limited MS/MS fragmentation analysis; other isomers besides those proposed may exist.

Figure S.2: Sample MS/MS fragmentation for hypothesized metabolite $C_{13}H_{15}NO_5S_2$.

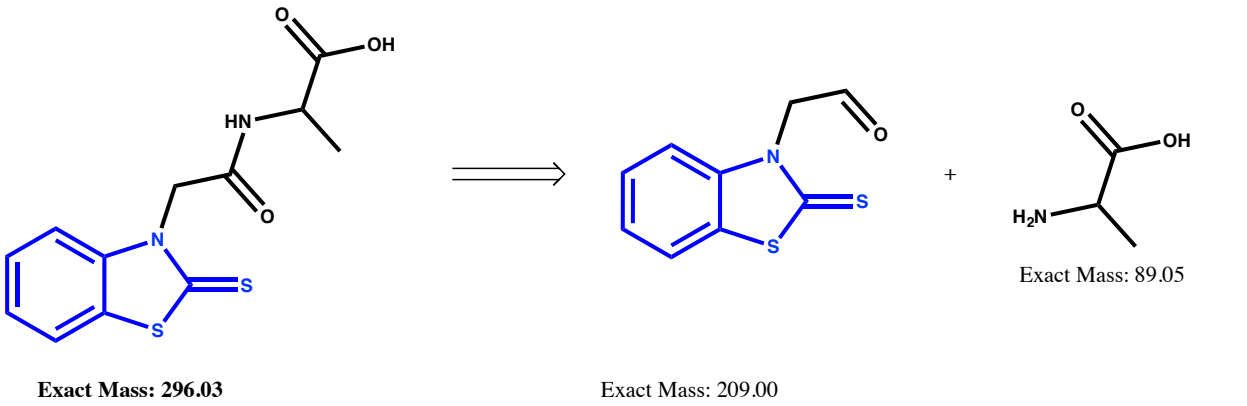


Chemical Formula: $C_{13}H_{15}NO_5S_2$
Exact Mass: 329.04

Chemical Formula: $C_7H_5NS_2$
Exact Mass: 166.99

Chemical Formula: $C_6H_{12}O_5$
Exact Mass: 164.07

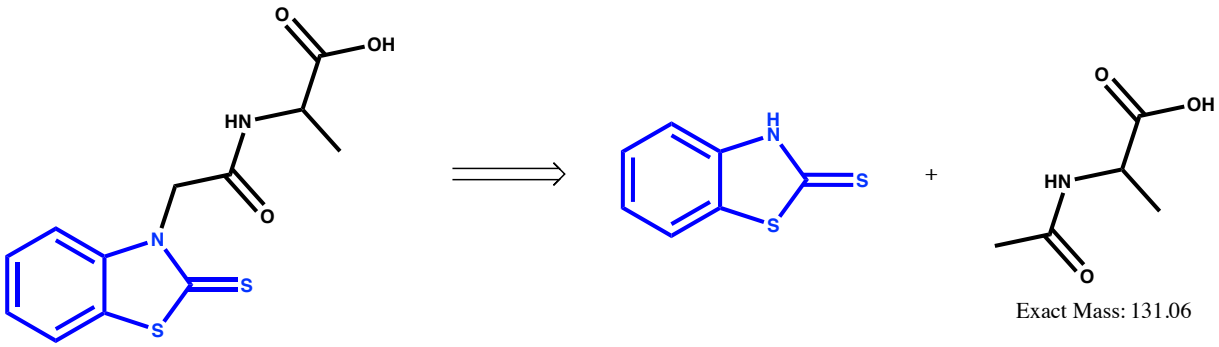
Figure S.3: Sample MS/MS fragmentation for hypothesized metabolite $C_{12}H_{12}N_2O_3S_2$.



Exact Mass: 296.03

Exact Mass: 209.00

Exact Mass: 89.05



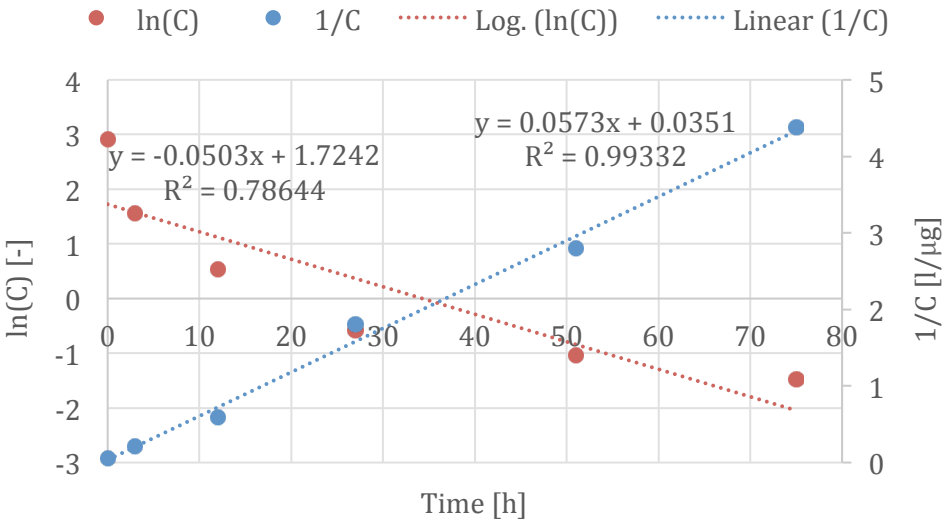
Exact Mass: 296.03

Exact Mass: 166.99

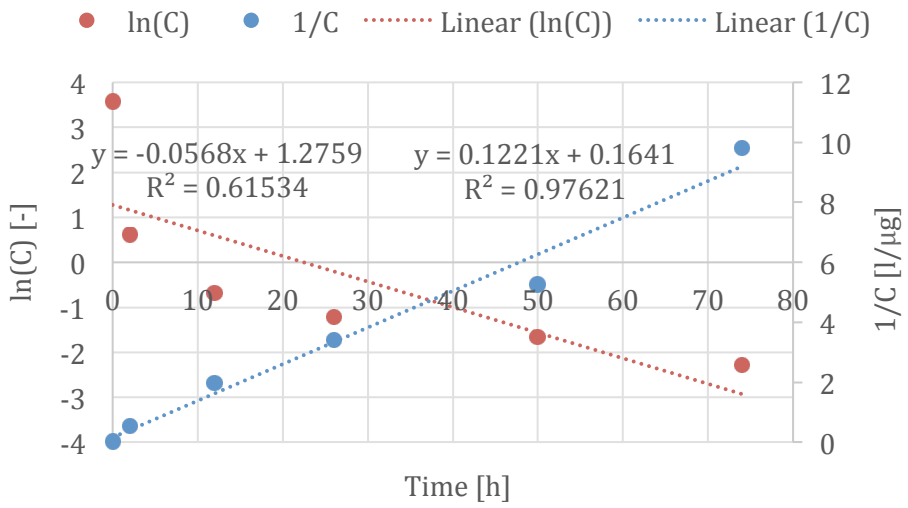
Exact Mass: 131.06

Figure S.14: Linear regression plots for changing concentration with time for four different initial concentrations used to determine best approximation of reaction rate model (Eqn. S.1). By plotting the linear regression of the natural log of the concentration data and the inverse of concentration the data through time and comparing the goodness of fit, one can determine which reaction rate best describes the observed phenomena. Based on the data collected, the linearized 1/C data typically exhibited greater goodness of fit, suggesting that a second order rate better describes the kinetics. Second order kinetics were also observed by Macherius et al. (2012) for triclosan assimilation into carrot cells.

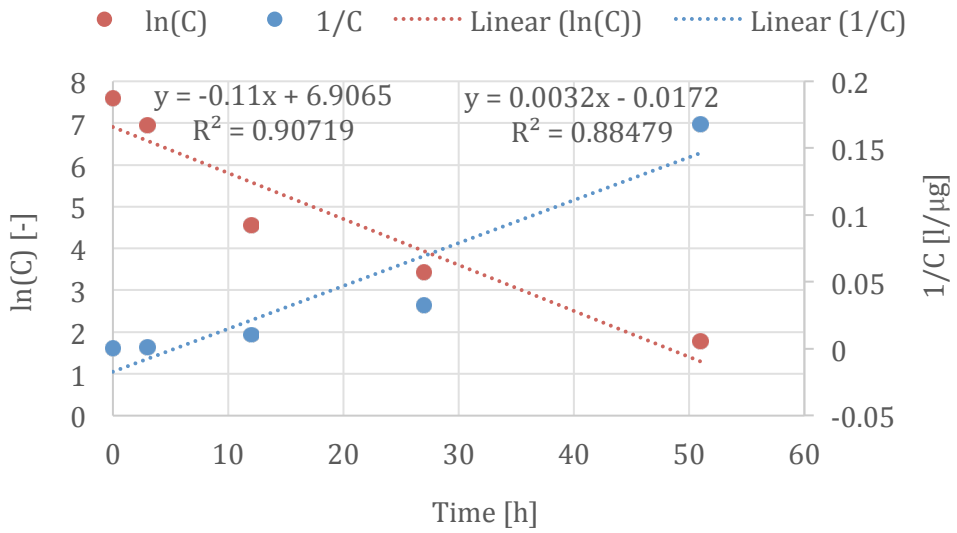
Macherius, A.; Eggen, T.; Lorenz, W.; Moeder, M.; Ondruschka, J.; Reemtsma, T. Metabolization of the Bacteriostatic Agent Triclosan in Edible Plants and its Consequences for Plant Uptake Assessment. *Environ. Sci. Technol.* **2012**, *46*, 10797-10804.



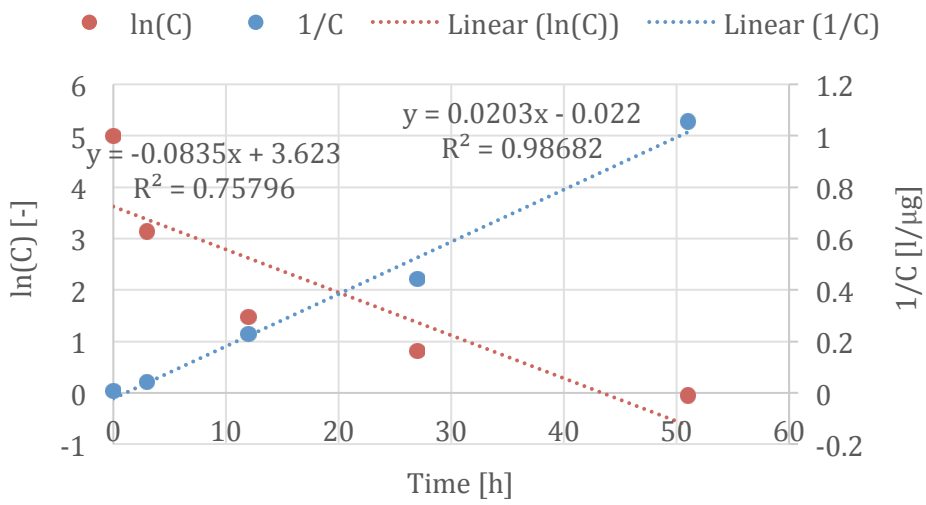
Linear regression of MBT media concentration data; C_0=18 µg/L MBT



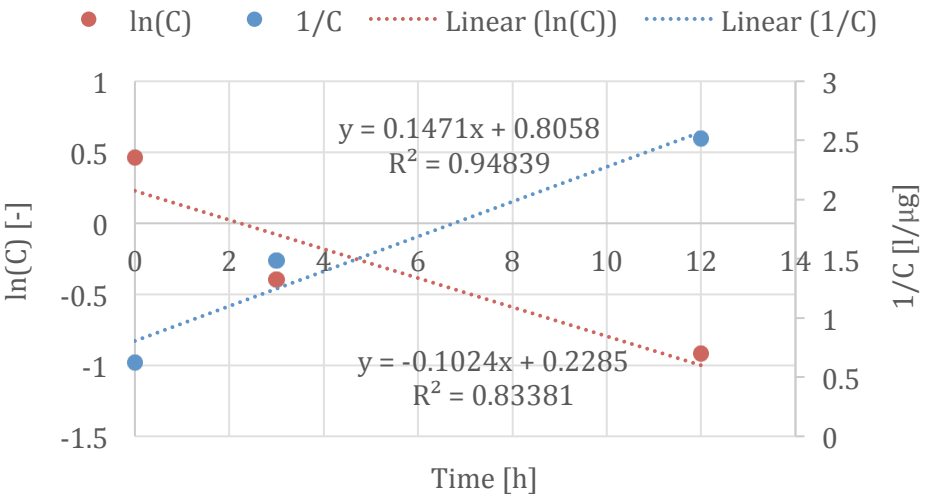
Linear regression of MBT media concentration data; C_0=36 µg/L MBT. Note that the data shown are the raw data (not corrected for MDL).



Linear regression of MBT media concentration data; C_0=1973 µg/L MBT.



Linear regression of MBT media concentration data; C_0=147 µg/L MBT.



Linear regression of MBT media concentration data; C_0=1.59 µg/L MBT. Note that only the first three sampling points are shown (because two of the samples were below the MDL).