Water is too precious to waste

Trade-offs of sewage effluent reuse in agricultural sub-surface irrigation

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Chapter 4
Aerobic and anaerobic biodegradation during sub-surface irrigation with sewage effluent

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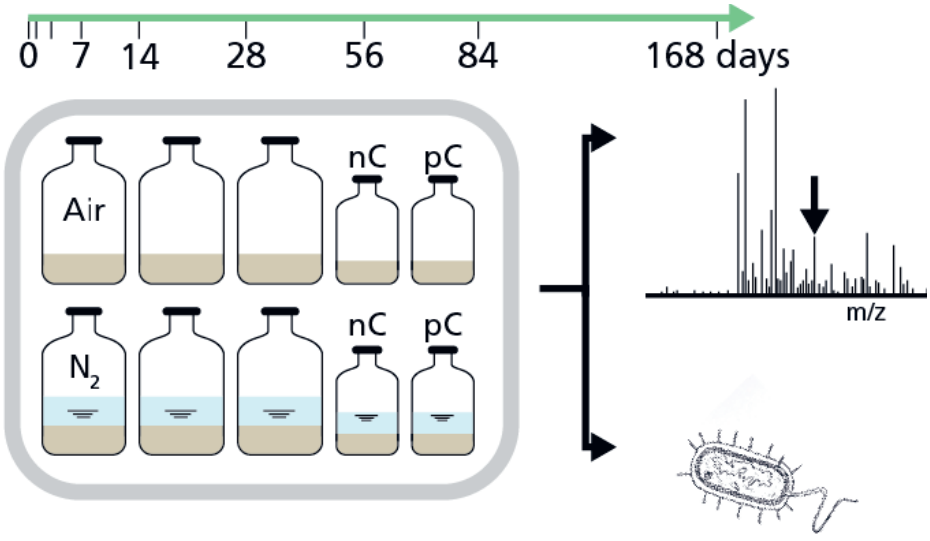

Abstract

The reuse of sewage treatment plant (STP) effluent for agricultural irrigation is increasingly considered in order to meet the current and future water demand. The presence of contaminants of emerging concern (CoECs) in the STP effluent might stimulate mechanisms of degradation in the soil microbiome. In this study a six-months batch experiment was conducted, under aerobic and anaerobic conditions, following the OECD 307 ready biodegradability protocol for the unexposed community and an enhanced version of the test for the exposed communities. STP effluent was used in the experimental replicates to facilitate realistic concentrations.

The biodegradation of 27 CoECs as well as microbial community changes were followed in this long-term batch experiment. As a negative control, to find out whether microbial community diversity and composition changes are permanent the shallow groundwater and the deeper groundwater of the SSI field were collected in the winter five months after the water supply for the SSI system was turned off. Overall, CoECs showed higher biodegradation rates with the pre-exposed community than the unexposed community. Temporal shifts were also observed in the anaerobic bottles for both the pre-exposed community and unexposed community. Hence, the presence of CoECs in STP effluent do stimulate mechanisms of degradation and adaptation in the soil microbiome. The magnitude and mechanisms of functional responses in ecosystems to such alterations from sewage effluent is still unknown and warrants further investigation.

4.1 Introduction

Globally, the reuse of sewage effluent for irrigation purposes is increasingly encouraged as one of the practical solutions against the mismatch between the demand for and availability of freshwater resources (Voulvoulis, 2018). However, sewage treatment plants (STPs) are not designed to remove contaminants of emerging concern (CoECs) - such as pharmaceuticals, biocides, personal care products, antibiotics and antibiotic resistant genes (Blum et al., 2018; Krzeminski et al., 2019). Thus, irrigation techniques with STP effluent may cause adverse health effects amongst farm workers and the consumers through direct exposure or via crop contamination (Kibuye et al., 2019; Picó et al., 2019). An innovative solution to avoid direct contact of effluent with crops and field workers is the use of STP effluent in sub-surface irrigation (SSI) systems (Narain-Ford et al., 2020; de Wit et al., 2022).

Additionally, when using SSI as a method of supply the quality of STP effluent may improve due to soil passage and related biodegradation processes of CoECs (Narain-ford et al., 2022; Narain-Ford et al., 2020). In SSI systems, as opposed to sprinkler and drip irrigation, CoECs are expected to primarily be removed via sorption and aerobic and anaerobic transformation processes. Anaerobic transformation of CoECs is generally less energetically favorable than transformation under aerobic conditions. However, some aerobically recalcitrant CoECs can be bio-transformed under strictly anaerobic conditions and little is known about the organisms and enzymatic processes involved in their transformation (Ghattas et al., 2017; Schulze et al., 2019; Wang et al., 2022). Thus, monitoring the changes in CoECs concentration in SSI systems is valuable as it provides information about how the SSI system functions and how the CoECs distribute within such systems. Not much information is available on CoECs biodegradation potential in soils exposed to effluents via SSI. Many processes can influence CoECs concentrations fluctuations in a SSI system besides biodegradation, such as leaching, sorption, and dilution among others. Hence, changes in concentration cannot solely be attributed to microbial biodegradation activity.

It is well known that microorganisms are able to develop metabolic degradation mechanisms to novel environmental pollutants, in which heterotrophic bacteria can utilize organic micro-pollutants as their growth substrates (Poursat et al., 2019; Wang et al., 2022). Furthermore,

co-metabolism results in the transformation of organic micro-pollutants by nonspecific enzymes produced by microorganisms using a different substrate for their primary metabolism (Benner et al., 2015). This may lead to enhanced CoECs removal rates in irrigation reuse systems, benefiting the receiving waters (Albergamo et al., 2019a). Even though currently platforms are available for analytical transformation product screening via high-resolution mass spectrometry (Helmus et al., 2022), transformation products might be present at trace concentrations, making detection and quantification challenging even with modern techniques. Therefore, in addition to analytical tools, complementary molecular tools are needed for monitoring CoECs biodegradation.

The biodegradation rate of CoECs is a key parameters for environmental risk assessment (Bertelkamp et al., 2014; Hale et al., 2020). Under the European REACH regulation, biodegradability and persistency are determined by a range of laboratory-based methods, developed by the Organization for Economic Co-operation and Development (OECD, 2002). The results of these tests allow for fast categorization of the tested compounds from readily biodegradable to persistent in the environment (ECHA, 2017). The commonly used ready biodegradability test for soil (OECD 307 for aerobic and anaerobic transformation in soil) suffer from several well-known problems that can affect their outcome such as: 1) spiking the test substances at relatively high concentrations in the range of 2 to 100 mg/L, as sole carbon and energy source (Greskowiak et al., 2017); 2) variability of the lag phase time required for a microbial community to start degrading a compound combined with relatively short test duration (Dalmijn et al., 2021) and 3) neglecting adaption of the microbial community (Driver et al., 2017; Poursat et al., 2019). Hence, these OECD tests do not reflect realistic environmental conditions and cannot predict degradation constants under relevant conditions, i.e. with realistic concentrations, a variety of carbon sources, and after the community has been pre-exposed to the compounds or mixture of compounds of interest. A better understanding of biodegradation processes in different environments can revolutionize the science of CoECs remediation. Along these lines the aim of this study is to investigate the influence of long-term exposure of soil to environmentally relevant concentrations of organic pollutants on biodegradation half-lives and adaptive responses of the bacterial community.

To this end, a batch experiment was performed with soil taken

from a full-scale cropland with SSI fed by STP effluent (Narain-ford et al., 2022). Water and soil of each batch were analyzed for 89 CoECs relevant for effluents, as based upon an earlier full field study (Narain-ford et al., 2022). 16S amplicon sequencing was used to gain insight into the microbial community dynamics at the start at end of the batch experiment. Moreover, to see how the microbial communities adapt to weather influences and drainage after the growing season is finished, also groundwater and effluent samples were taken at the field site. Such an extensive analysis with incubation material from a full-scale field under real farming conditions is novel and ensures that environmental fate processes such as (an)aerobic degradation of organic pollutants and microbial community adaptation responses occur under realistic conditions.

4.2 Materials and methods

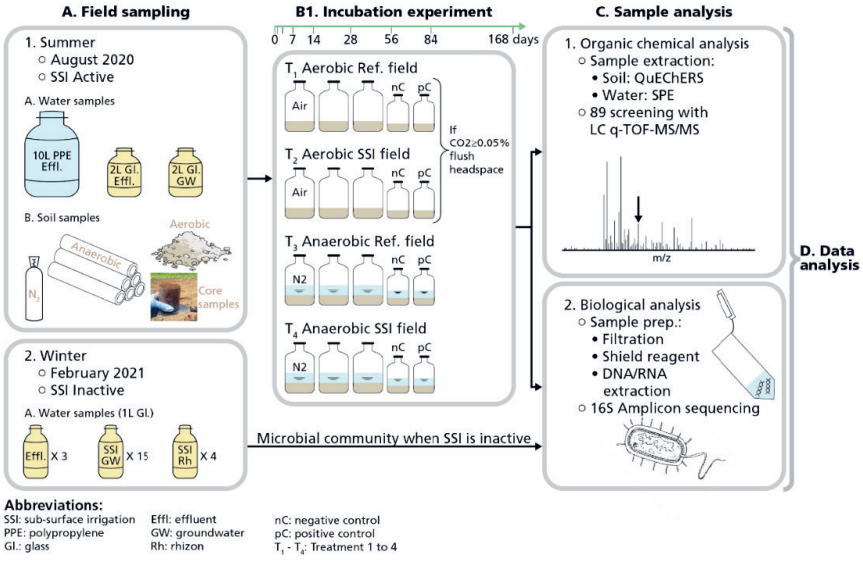

Figure 4.1 Schematic representation of the experimental set-up, including study area and field sampling (4.2.1), incubation experiments, chemical (4.2.3) and microbiological analysis (4.2.4) and data analysis (4.2.5).

4.2.1 Study area and field sampling

4.2.1.1 Study area

Field samples were taken from a cropland operated with SSI fed by STP effluent in Haaksbergen, the Netherlands (52°10'42.9"N 6°42'43.8"E) (Narain-ford et al., 2022). Infiltration of the effluent during the growing season (April-September) occurs via a series of parallel pipes at a depth of ca. 1.20m. Since the start of SSI in 2015 no other direct irrigation has taken place at this site. As reference location without SSI, the cropland at the other side of the surface water with the same farming conditions was selected. Field sampling at our study area (Narain-ford et al., 2022) took place during two rounds: in the summer of August 2020 and in the winter of February 2021. A geographical representation of the study area and locations of the sampling sites are presented in Narain-ford et al. (2022).

4.2.1.2 Summer sampling

On the 20th of August 2020, while the SSI system was active, aerobic and anaerobic soil was collected from three locations in the middle of the SSI field right next to an infiltration pipe, and also from three locations in the middle of the reference location (Figure 4.1 Section A-1). As required by OECD 307 (OECD, 2002) aerobic soil was collected from the top 20 cm layer in polypropylene bags and loosely-tied during transportation. Also anaerobic soil, from both the SSI and reference field, that is the soil below the phreatic groundwater, was collected in PVC pipes (n = 6, width: 10cm, length: 1m) under a gentle nitrogen flow. At the SSI field site the phreatic groundwater was 1m below soil surface and at our reference location it was 1.5 m below soil surface. The headspaces of the PVC pipes used to collect the soil samples were filled with nitrogen, immediately sealed with duct tape on both ends and stored in plastics bags filled with nitrogen. The plastic bags were immediately sealed and kept cool during transportation to the laboratory.

A previous study done at this site (Narain-ford et al., 2022) shows that next to an infiltration pipe in a SSI system concentrations of CoECs can be equal or even higher than their retrospective concentration in STP effluent. Therefore, elucidating the maximum impact of STP effluent, the incubation experiments were conducted with 72-H composite STP effluent collected via an automatized sampler in a plastic container

(V=10L) and a 2L autoclaved pre-cleaned glass bottle (V sampled = 2L). All effluent samples were stored separately from the soil samples and kept cool during transportation to the laboratory. To pre-condition soil also shallow groundwater from the reference field was collected, stored separately and kept cool until arrival in the laboratory. Parallel to the soil samples appropriated for the batch experiments, undisturbed core samples were taken in replicate of the aerobic and anaerobic soil of both the SSI field and the reference location using the soil sampling ring kit - model A53 (Eijkelkamp) according to manufacturer protocol. The soil properties are presented in SI-4.2.

4.2.1.3 Winter sampling

To study the soil microbiome in winter, when SSI is inactive, water samples were collected in February 2021 from the rhizosphere, the shallow groundwater and the deeper groundwater of the SSI field and reference location in 1L autoclaved glass bottles (Figure 4.1 Section A-2). To sample the shallow- and deeper groundwater a peristaltic pump was used. To sample pore water pre-installed rhizons were used as previously described elsewhere (Narain-ford et al., 2022). Field triplicates were taken to increase the precision of the measurements. 24-H composite effluent and 24-H composite influent were also sampled during this period (Table 4.1). All water samples were stored separately based on their origin (effluent, groundwater, pore water), kept cool during transportation and stored at 4°C upon arrival to the laboratory until filtration the next morning. All winter field samples (n=28) were filtered within 48 hours after arrival to the laboratory.

Table 4.1 Selected locations during winter water sampling

Location	Measuring point	Specifics	Groundwater Level[1] (m-soil surface)	Sampling Depth[1] (m-soil surface)	Volume of water sampled
SSI field	CloseNext	Close to STP, next to pipe	1.03	0.6	600 mL
				1.0	3× 1 L
	MidNext	Middle of field, next to pipe	0.51	0.6	450 mL
				1.0	3× 1 L
	MidNext_D	Middle of field, next to pipe deep well		9.6	
	MidBet	Middle of field, in between pipes	0.57	0.6	100 mL
				1.0	3× 1 L
Reference field	Ref. field	Reference field - Shallow groundwater	0.62	grab sample from phreatic groundwater	3× 1 L
Sewage Treatment Plant (STP)	24-H STP influent	Standardized program at STP for 24 hour composite sample			3× 1 L
	24-H STP effluent	Standardized program at STP for 24 hour composite sample			3× 1 L
	Effl_discharge	Grab sample from effluent discharge into surface water			3× 1 L

[1]Soil surface (m + mean sea level): 20.322 - 20.995.

4.2.2 Batch experiment setup

4.2.2.1 Batch soil preparation

All material collected in August 2020 was used to conduct a six-month batch incubation experiment. Upon arrival the anaerobic soil from both the SSI field and reference field were immediately transferred under a gentle flow of nitrogen to a nitrogen filled glovebox (O_2 < 0.05%, T = 20±2°C) and dried for four days with silica containers. The aerobic soils were then placed in polypropylene containers, dried in the oven at 30°C for two days, dry-sieved (>2 mm) and placed in polypropylene containers in a climate control room of 20±2°C until further processing.

4.2.2.2 Batch set-up and operation

A six month batch incubation experiment was performed following the OECD 307 ready biodegradability protocol (OECD, 2002), with four treatments existing of soil collected from the reference field or soil collected from the SSI field and the composite effluent, under

aerobic or anaerobic conditions. Figure 4.1, section B shows the four treatments. Each experimental replicate consisted of a 250 mL glass vacuum bottle filled with 80 grams of soil, whereas the positive and negative control consisted of 100 mL glass bottle filled with 40 grams of soil. The volume of the bottle of the negative and positive control was reduced so that the bottle could fit into the holding system of the gas chromatograph for $CO_{2\,(g)}$ analysis. Before the soil was placed inside the glass bottles the moisture content was measured in triplicate for every type of soil (n=12).

Soil was preconditioned for a week with shallow groundwater from the reference location in a dark climate controlled room at 20±2°C to prevent algae growth and/or CoECs loss due to photolysis. The bottles under aerobic conditions were operated for 84 days, while the bottles under anaerobic conditions were operated for 168 days (Table 4.2). Further following OECD 307, soil water capacity, soil type and microbial activities of all aerobic soil samples were analyzed within 48hours after their collection from the field by Wageningen University Laboratory via standard methods conforming to (inter)national standards.

Table 4.2 Batch set-up and operations

Condition		Experimental replicate	Weight soil[2]	Volume of reference field groundwater[1] for pre-conditioning soil (mL)	Test compound	Volume of water added (mL)
Aerobic	SSI field (adapted community)	1	80	10	STP effluent	10
		2				
		3				
		Negative control	40	5	Ref field GW[1]	5
		Positive control			1.5 mg/L Benzoic acid (ultrapure water)	5
	Reference field	1	80	10	STP effluent	10
		2				
		3				
		Negative control	40	5	Ref field GW[1]	5
		Positive control			1.5 mg/L Benzoic acid (ultrapure water)	5
Anaerobic	SSI field (adapted community)	1	80	30	STP effluent	30
		2				
		3				
		Negative control	40	15	Ref field GW[1]	15
		Positive control			1.5 mg/L Benzoic acid (ultrapure water)	15
	Reference field	1	80	30	STP effluent	30
		2				
		3				
		Negative control	40	15	Ref field GW[1]	15
		Positive control			1.5 mg/L Benzoic acid (ultrapure water)	15

[1] Shallow groundwater from reference field
[2] The exact weight of soil added to each bottle is available from https://data.mendeley.com/datasets/htdgbzhr3p

The aerobic bottles were flushed weekly with synthetic air composed of 80% N_2 and 20% O_2, whereas the anaerobic bottles were not flushed in accordance to OECD 307. The CO_2 (g) concentration was measured right before the bottles were de-capped. To stop the degradation process soil samples were frozen at -20°C.

4.2.3 Organic chemical analysis

4.2.3.1 Standards and reagents

All chemicals used were of analytical grade. More details are provided elsewhere (Narain-ford et al., 2022).

4.2.3.2 Sample preparation

Soil samples were extracted using an in-house created and validated Quechers method. 30 g freeze-dried soil was added to 50 mL sterile polypropylene (PP) tubes filled with 6 g anhydrous magnesium sulphate ($MgSO_4$) and 1.5 g sodium acetate (NaAc) and spiked with 50 µL of a 200 µg/L stock internal standards solution. 30 mL of acetonitrile was added, mixed for one hour and subsequently centrifuged for 15 minutes. The supernatant was then transferred to clean 50 mL sterile PP tubes and mixed for 15 minutes with the addition of 300 mg magnesium sulphate ($MgSO_4$), 100 mg primary secondary amine (PSA) and 100 mg octadecyl (C18), whereafter the sample was evaporated to 0.5 mL under a gentle nitrogen flow. The water portion of the anaerobic samples was extracted directly after opening the vacuum glass bottles using a generic solid-phase extraction method described elsewhere (Narain-ford et al., 2022). The water portion of the aerobic bottles was not extracted, the soil samples were analyzed as a whole.

4.2.3.3 HPLC-MS/MS and GC-MS/MS analysis

All water and soil samples were analysed with a maXis 4G LC high-resolution q-TOF-MS/MS upgraded with a HD collision cell and equipped with an ESI source (Bruker Daltonics, Wormer, the Netherlands) according to (Narain-ford et al., 2022), with one slight modification: we selected a 1.8µm column instead of a 2.6µm to optimize the separation of the CoECs (Figure 4.1, section C1). The samples were screened for 89 CoECs that were previously detected at our field site (Narain-ford et al., 2022). https://data.mendeley.com/datasets/htdgbzhr3p presents the target list. The CO_2 concentration in the headspace of the positive and negative control incubation bottles was measured with a TRACE GC Ultra gas chromatograph (Thermo Fisher, Breda, The Netherlands), equipped with a FID and a Hayesep Q 1/8" 80–100 2 m + Hayesep N 1/8" 80–100 0.73 m column, further details of the procedure can be found in Dalmijn (2021).

4.2.4 Molecular analysis

4.2.4.1 Sample preparation

All water samples were filtered over 0.22-µm-pore-size polycarbonate track-etch filter membranes (Sartorius) to collect bacterial biomass (Figure 4.1, section C2). The filters were stored in autoclaved Eppendorf tubes at -20 °C in 200 µL DNA / RNA Shield reagent (Zymo Research, Irvine, CA, USA) to prevent biological degradation from freeze-thaw cycling and unexpected freezer failures until further analysis. DNA was extracted from the filters and soil samples (200 mg) using the DNeasy PowerSoil Kit (QIAGEN Benelux B.V) according to the manufacturer's protocol (Qiagen). DNA extracts (100 µl) were stored at -20 °C until further analysis. The quantity and quality of DNA was measured with the Nanodrop (NanoPhotometer N60/N50, Implen GmbH, München, Germany). The quantity of DNA was also measured with the Qubit 2.0 fluorometer (Life Technologies) for higher accuracy according to the manufacturer protocol. The DNA concentrations ranged from 0.273 to 71.5 ng/µL. RNA was extracted from the filters and soil (200 mg) using the RNeasy PowerSoil Total RNA kit, according to the manufacturer's protocol (QIAGEN Benelux B.V), with one exception: During the final steps of the extraction, 1 ml of 100% (v/v) isopropanol was added for nucleic acid precipitation to the eluted RNA, and samples were incubated overnight at - 20 °C. The RNA extracts were stored at - 80 °C. To measure the quantity of RNA a Qubit RNA HS (high sensitivity, 5 to 500 ng) and RNA BR (broad range, 20 to 1000 ng) Assay Kits was used for higher accuracy. After RNA extraction, DNA was removed using the TURBO DNA-free kit from Invitrogen (Carlsbad, CA), based on the routine DNase treatment protocol. Hereafter, the quantity of RNA was measured with the Nanodrop (NanoPhotometer N60/N50, Implen GmbH, München, Germany).

4.2.4.2 16S Amplicon sequencing

DNA extracts were amplified in a T100™ Thermal Cycler (Bio-Rad, Germany). The V4–V5 hypervariable region of the bacterial 16S ribosomal RNA (rRNA) gene was targeted using the following primer set: 518F (5'-GTGYCAGCMGCCGCGGTAA-3') and 926R (5'-CCGYCAATTYMTTTRAGTTT-3') (Huse et al., 2014), which amplify a 411-bp long fragment of the 16S rRNA genes of Archaea and

Bacteria. The thermal cycling conditions are presented in Table SI-4.1. In total a PCR product was retrieved out of 75 samples. The PCR products were confirmed in a 2% (w/v) agarose gel electrophoresis and stored at - 20 °C until further analysis. The PCR products were purified using ExoSAP-IT™ PCR Product Cleanup Reagent (Applied Biosystems), confirmed using gel electrophoresis (DNA fragment of 411 bp) and then shipped (n = 73, V = 20 µL) to company MR DNA (Molecular Research LP, Shallowater, USA) for 16S rRNA gene Illumina sequencing. As positive controls two purified PCR products (20 µl) containing genomic DNA from bacterial mock communities MSA-1000™ 10 Strain Even Mix Genomic (ATCC, Manassas, VA, USA) as a template were also sent for sequencing.

4.2.5 Data analysis

Statistical analysis for all measurements was performed with the R statistical environment (R Core Team, 2021) version 4.1.2 (1 November 2021). All data were first tested for normality by Shapiro-Wilk tests. When possible, models were used to determine the time necessary to reach 50% of degradation. The DRC R package (Ritz et al., 2015) was used to fit a three - parameters log-logistic models to the degradation data. In the model the upper limit (indicated by parameter d) was set to 1. Student's test (t-test) was used to compare the half-lives of each compound for the reference field and the SSI field, specified per aerobic and anaerobic condition.

Bacterial community analysis of the 16S rRNA gene sequences were also performed within the R statistical environment (R Core Team, 2021). For phylogenetic indices, alpha diversity (at the family level) was evaluated based on non-parametric Shannon's diversity index, and Chao richness using the Vegan package in R. The observed richness represents the number of unique species observed in our samples and is dependent on the sequencing depth, while the Chao1 index estimates the total richness. The Shannon diversity index (H) combines both richness and the evenness of the communities.

Differences in the community composition between samples were evaluated with beta diversity. 'Bray-Curtis dissimilarity' was used, which uses abundance data and calculates differences in feature abundance. The beta-diversity are visualized using PCoA. Permutational Multivariate Analysis of Variance (PERMANOVA) were performed using the Adonis function from the R package Vegan.

4.3 Results and discussion

4.3.1 Pre-exposure enhances biodegradation

Overall, the SSI field showed shorter half-lives than the reference field. For aerobic conditions 4 CoECs, for aneaerobic water 11 and for anaerobic soil 9 CoECs have significantly shorter half-live in the SSI field as compared to the reference field (Table 4.3). When datapoints are missing in the beginning phase of the experiment, as for triethyl phosphate under aerobic conditions, the model cannot accurately predict the half-lives and the t-test is not significant. However, we can clearly see from Table 4.3 that in this situation the SSI field has a better degradation potential than the reference field. Yet, pre-exposure prior to any standard ready biodegradability test is not allowed under the current ECHA guidelines. A substantial body of literature confirms that pre-exposure can affect the result of biodegradation test and supports the inclusion of such pre-exposed community in these test (Poursat et al., 2019). In a literature compilation on the degradation of chlorinated hydrocarbons, Driver et al. (2017) found that rate constants derived from column experiments were considerably higher than in batch experiments. They attributed this behavior to (i) better microbial adaptation as a result of the development of steady-state conditions within the column with a constant supply of substrate, as well as (ii) the fact that the flow through the column prevents the accumulation of waste products of microbial metabolism, which could decrease biodegradation performance. In addition Dechesne et al. (2014) shows that degradation processes vary greatly for the same CoECs between different soil types emphasizing the diversity of microbial metabolism in heterogeneous systems. Since during sub-surface irrigation the point of entry is the shallow groundwater (de Wit et al., 2022) where during the irrigation period the goal is to raise groundwater to provide water to the crops, the STP effluent will more or less stay in the system until drainage flushes it out. Therefore we selected a batch experiment to represent the relative steady-state situation of the groundwater. From the 89 CoECs in our target list, 47 are retrieved in our batch samples. These 47 CoECs have all previously been detected in the SSI field (Narain-ford et al., 2022). Of the 47 CoECs, 17 are below the limit of quantification. These 30 CoECs can be classified as 9 persistent mobile compounds (PM), 5 persistent hydrophobic compounds (Pm), 9 non-persistent mobile compounds (pM) and 4 low persistent and low

mobile compounds (pm), following Narain-ford et al. (2022) cut-off value of 57-day half-life in soil and a log *D* of 1.49. Table 4.3 reveals the degradation half-lives of the 23 CoECs with high persistency and/or high mobility, thereby excluding the four low persistent and low mobility compounds and the three transformation products BAM, 2-Hydroxyquinoline and 4-Hydroxyquinoline. The degradation plots of the 23 CoECs are presented in SI-4.3, while further information about the targeted transformation products is given in https://data.mendeley.com/datasets/htdgbzhr3p.

Table 4.3 Half-lifes of CoECs (in days), in soils from the SSI and reference field under aerobic and anaerobic conditions. In green if the half-life in the Reference field is significantly higher, in blue if it is significantly lower.

			Aerobic			Anaerobic water			Anaerobic soil		
Class[1]	Analyte Name	Type	Ref. field	SSI field	t-test p-value	Ref. field	SSI field	t-test p-value	Ref. field	SSI field	t-test p-value
pM	Caffeine	IV	2.21 ± 0.17*	1.40 ± 0.12*	0.002	6.90 ± 1.01*	3.91 ± 0.76*	N.S.	3.25 ± 0.29*	0.80 ± 0.46	0.000
	Diclofenac	III	< LoQ	< LoQ	-	42.13 ± 9.64*	2.77 ± 0.54*	0.002	155.83 ± 32.96*	26.38 ± 4.04*	0.000
	Diglyme	I	2.86 ± 0.65*	2.62 ± 0.59*	N.S.	< LoQ	< LoQ	-	31.70 ± 3.61*	21.70 ± 3.05*	N.S.
	MCPP-p	II	< LoQ	<LoQ	-	17.80 ± 7.31*	3.75 ± 0.79*	N.S.	< LoQ	< LoQ	-
	Paracetamol	III	< LoQ	<LoQ	-	4.39 ± 0.62*	3.44 ± 0.38*	N.S.	< LoQ	< LoQ	-
	Saccharin	V	< LoQ	<LoQ	-	20.43 ± 1.69*	2.14 ± 0.61*	0.005	< LoQ	< LoQ	-
	Sotalol	III	< LoQ	<LoQ	-	27.33 ± 1.60*	3.40 ± 0.56*	0.000	1.19 ± 0.26*	2.27 ± 0.15*	0.04
	Triethyl phosphate	I	7.50 ± 0.97*	0.14 ± 0.13	N.S.	67.07 ± 12.80*	6.45 ± 1.51*	0.003	7.15 ± 0.54*	1.73 ± 0.19*	0.000
	N-Phenyl urea	I	1.9e+38*	5.83*	0.000	15.93 ± 10.22	3.15 ± 0.79*	N.S.	26.76 ± 3.13*	17.47 ± 2.99*	0.01
Pm	1H-benzotriazole	I	3.73 ± 0.32*	1.37 ± 0.23*	0.001	22.88 ± 1.42*	1.87 ± 0.56*	0.003	21.98 ± 1.46*	0.08 ± 0.05	N.S.
	DEET	III	5.55 ± 1.04*	3.11 ± 0.85*	N.S.	23.78 ± 1.86*	4.22 ± 0.70 *	0.000	1.88 ± 0.25*	0.62 ± 0.23*	0.000
	Dimethanamid-p	III	34.13 ± 3.68*	5.72 ± 0.69*	0.000	*No fit*	*No fit*		*No fit*	*No fit*	
	Fipronil	II	< LoQ	< LoQ	-	49.61 ± 9.75*	4.49 ± 1.27*	0.01	< LoQ	< LoQ	-
	Metolachlor-s	II	< LoQ	< LoQ	-	< LoQ	< LoQ	-	7.03 ± 0.74*	16.02 ± 0.82*	0.000
PM	4,6-Dinitro-o-cresol	II	128 ± 362	6.77 ± 1.17*	N.S.	< LoQ	< LoQ	-	2510 ± 5730	60.75 ± 15.03*	N.S.
	Atenolol	III	< LoQ	< LoQ	-	14.89 ± 2*	2.32 ± 0.64*	0.009	< LoQ	< LoQ	-
	Carbamazepine	III	< LoQ	< LoQ	-	*No fit*	*No fit*	-	*No fit*	*No fit*	-
	Diuron	II	< LoQ	< LoQ	-	22.75 ± 40.43	5.4 ± 9.73	N.S.	< LoQ	< LoQ	-
	Furosemide	III	< LoQ	< LoQ	-	45.28 ± 3.15*	1.9 ± 0.74*	0.02	< LoQ	< LoQ	-
	Metoprolol	III	< LoQ	< LoQ	-	21.43 ± 1.31*	2.91 ± 0.63*	0.000	8.56 ± 0.82*	2.84 ± 0.3*	0.000
	Sulfamethoxazole	III	*No fit*	*No fit*	-	40.87 ± 4.99*	3.66 ± 1.1*	0.005	25.43 ± 2.59*	0.35 ± 0.2	0.000
	Tramadol	III	353.1 ± 1982	1.30 ± 0.12*	N.S.	4.85 ± 1.08*	2.79 ± 0.83*	N.S.	55.73 ± 1.93*	33.36 ± 7.31*	0.003
	Venlafaxine	III	185.41 ± 94.25	1.47 ± 0.24*	N.S.	*No fit*	*No fit*		*No fit*	*No fit*	

[1]pM = non-persistent, mobile, Pm = persistent, hydrophobic, PM = persistent, mobile.
[2]I = industrial chemicals, II = biocide, III = pharmaceutical , IV = stimulent, V = artifical sweeteners
*indicates that the model predictions on the half-life are statistically significant (p-value <0.05)

4.3.2 Bacterial community changes due to long-term STP effluent exposure

Soil microorganisms, especially bacteria, which represent the most abundant group, play a central role in ecosystems, including the carbon, nitrogen, and metals cycling as well as the biodegradation of environmental contaminants. Soil microbial abundance and diversity are typically the highest in the top 10 cm and decline with depth (Aislabie and Julie R. Deslippe, 2018). The microbial activity of the aerobic soil (rhizosphere, top 20 cm) from the SSI field in summer was almost a factor of three higher than the reference location (Figure 4.2). This confirms that SSI with STP effluent leads to enrichment of the soil microbes, even while the rhizosphere does not come in direct contact with the STP effluent (Narain-ford et al., 2022).

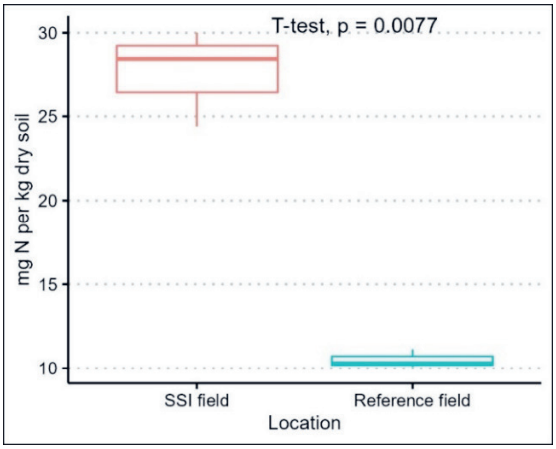

Figure 4.2 Microbial activity mg N/kg dry soil for the rhizosphere in the SSI and reference field

In the batch samples no significant difference was seen between the microbial community composition of the SSI field and reference field at the start of the experiment (0 days), which is to be expected as both bottles were fed with the same 72H-effluent at T0(0days). For the anaerobic bottles the differences between the microbial communities in time do become significant ($p = 0.044$). Heatmaps representing the 40 most abundant bacterial families for the batch samples (≥ 4% of relative abundance) and winter samples (≥ 2% of relative abundance) for the average ($n = 3$) of all summer and winter replicates can be founded in the Figure 4.2. The most distinctive families (at a cut-off of more than 5% occurrence when looking at the top 40 most abundant bacterial

families) for the batch samples are the *peptococcaceae,* methylophilaceae and *holophagaceae*. The *peptococcaceae* family was only detected in our anaerobic bottles and not our aerobic bottles, which is in agreement with previous studies (Zaan et al., 2012). The Methylophilaceae family is known for its use in biofilters for denitrification (Wang et al., 2022). The *holophagaceae* family is also commonly found in treated wastewater and is rarely if ever detected in agricultural soil that is not exposed to sewage effluent (Becerra-Castro et al., 2015).

When looking at the top 40 most abundant bacterial families the batch samples and winter samples have 15 families in common, of which the *oxalobacteraceae* (12,95%), *chitinophagaceae (*10,28%), *comamonadaceae* (7,42%) and *burkholderiaceae* (5,21%) are the most prevalent across all samples within this study (at a cut-off of more than 5% occurrence). In the winter samples (Figure 4.3B) the family *oxalobacteraceae* has the highest relative abundance in effluent samples, but not in the influent or reference samples. The general metabolism of oxalobacteraceae families is mostly aerobic/microaerobic to facultative anaerobic in some species; only members of the genus *Oxalobacter* have a strictly anaerobic lifestyle. The Oxalobacter formigenes plays a role in the destruction of oxalate in the gastrointestinal tract, where it decarboxylates oxalate, generating formate and CO_2 (Oropeza et al., 2012). The *Chitinophagaceae* family which has the second highest relative abundance across all our samples is mainly prevalent during the batch experiment, where the effluent was collected during the summer. They were reported by Wu et al., (2019) as dominant nitrifiers in a bench-scale SBR treating anaerobic digestate for total nitrogen removal. Members of the *comamonadaceae* family occur highly frequently in a wide range of full-scale STPs. They were found to be more specialized to the degradation of non-aromatic plant exudates, such as amino acids (Oropeza et al., 2012). Indeed Lünsmann et al., (2016) found that they were involved to a lesser extend in the degradation of toluene throughout a 5 years constructed wetland model system, whereas the majority of the toluene degradation enzymes matched the family of the *burkholderiaceae*. The main pathway of toluene degradation occurred via two subsequent monooxygenations of the aromatic ring (Lünsmann et al., 2016). In our study this family was found in a lesser extent in the rhizosphere in the winter study and also to a lesser extent in the batch aerobic bottles, but dominated in the anaerobic bottles.

As can be seen in Figure 4.3 and Figure 4.4 the sampling time 0 days seems to be most related to 168 days, while the samples taken at 84 days seem to be the most distinctive. This could imply that the shifts observed in microbial communities are temporal and maybe even recoverable, which is in line with previous research (Price et al., 2018). Mansfeldt et al. (2020) also observed that the effluent significantly alters bacterial communities when added to natural systems, but could not relate such alterations to functional effects.

It is important to highlight that not only the conditions influenced the results of this location, but also the location at which we took samples to conduct the batch experiment. The Bray–Curtis dissimilarity analysis revealed significant differences in the microbial structure amongst the 8 locations ($F_{7,15} = 2.61$; $R^2 = 0.7$; $p < 0.001$) and 6 depths ($F_{5,15} = 3.05$; $R^2 = 0.6$; $p < 0.001$). Hence, as expected the microbial structure of our study area is very heterogeneous.

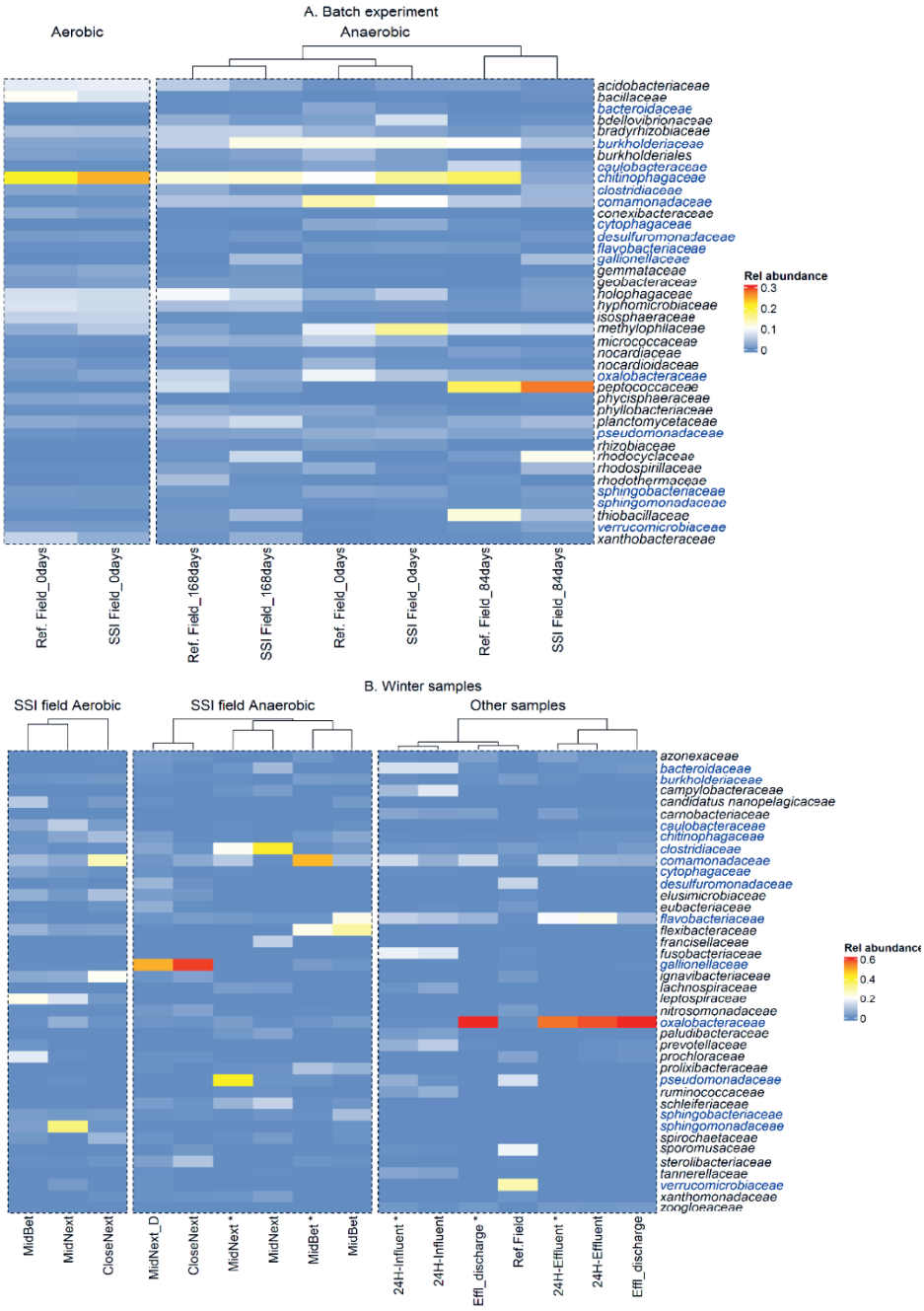

Figure 4.3 Heatmap of the 40 most abundant bacterial families for the batch (summer) and winter samples. Similar bacterial families between A. Batch experiment and B. Winter samples are displayed in blue.

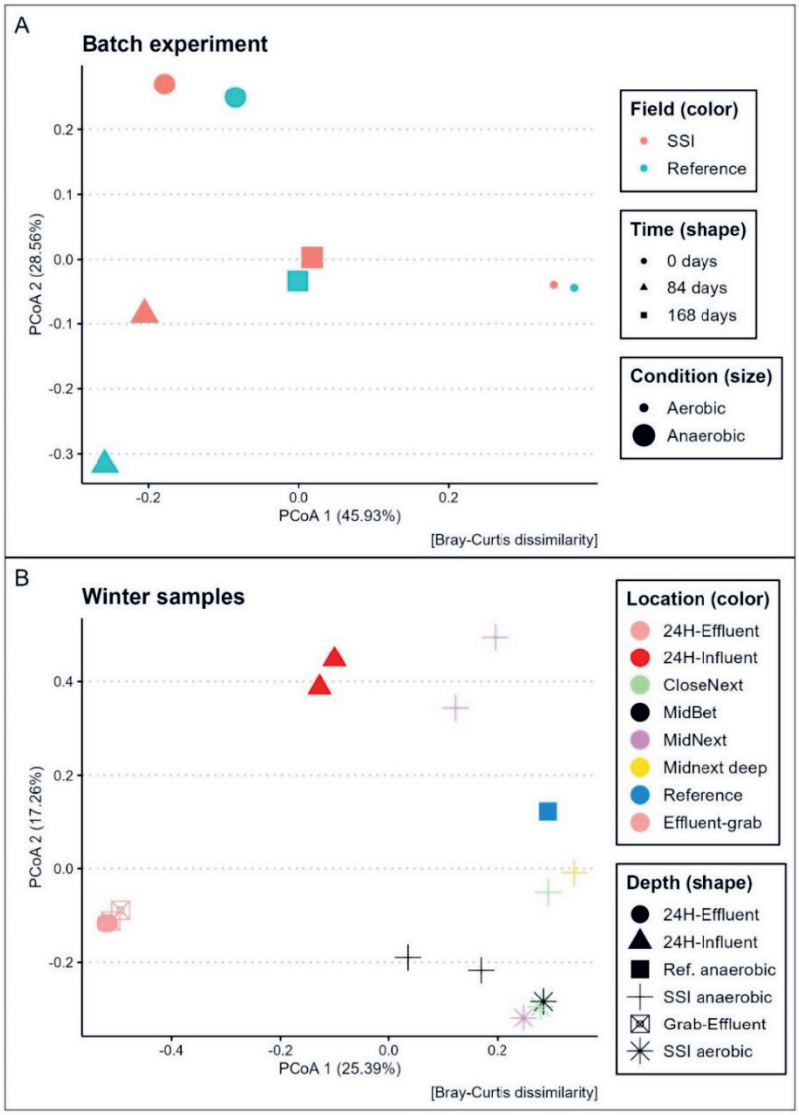

Figure 4.4 Beta diversity analysis by Principal Coordinates Analysis (PCoA).

Comparison of the alpha diversity indices (Figure 4.5) reveal subtle significant differences between the aerobic and anaerobic conditions. The observed species richness has between 125 and 175 OTUs. Thus, Chao1 is higher in its richness, which suggests that the sequencing depth was not enough to catch all the diversity present in the harvested environment. No significant shifts in microbial richness and composition are observed when looking at the alpha diversity

analysis (Figure 4.5). In addition, as the anaerobic condition fosters through time with effluent from the STP it seems that the abundance of the families become similar between the reference field (not pre-exposed) and SSI field (pre-exposed) community.

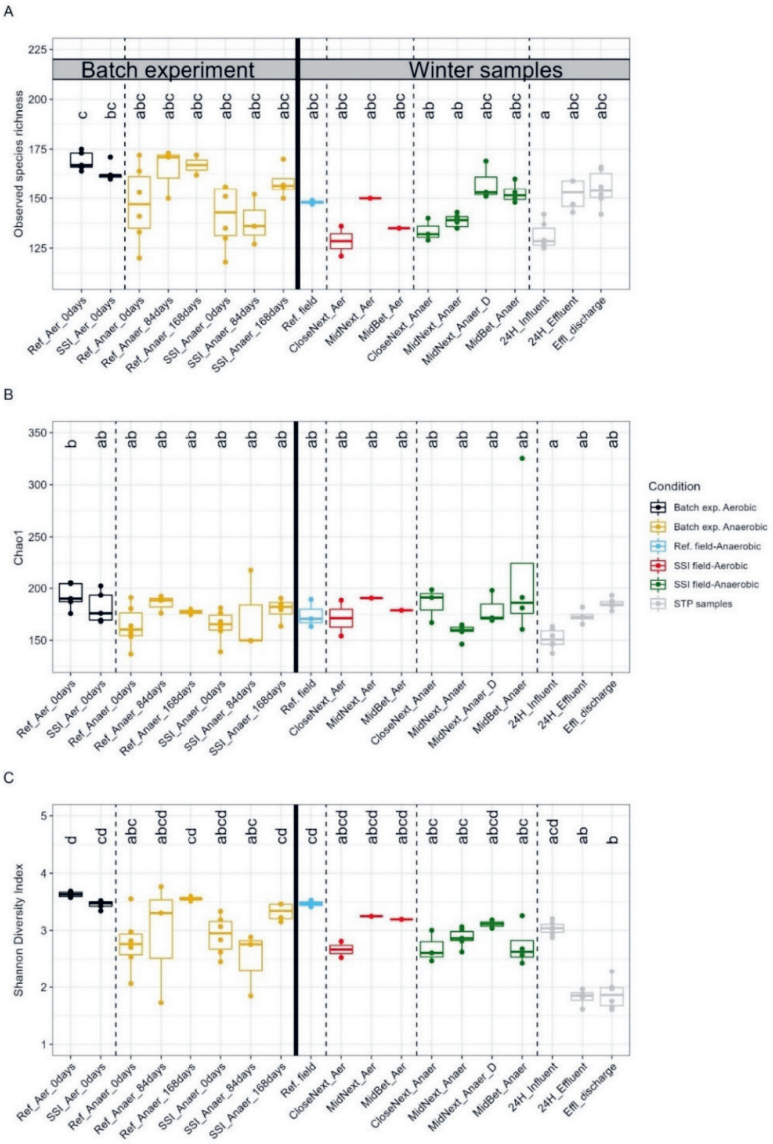

Figure 4.5 Alpha diversity analysis of each sample: A. Observed species richness, B.Chao1 and C. Shannon Diversity index. For location abbreviations, see Table 4.1. Each colour represents a condition. Samples found to differ significantly ($p<0.05$ based on the Benjamin-Hochberg method) from other samples are indicated with different letters.

4.3.3 Perspectives of CoECs biodegradation in field condition

In this study STP effluent was used in the experimental replicates to facilitate realistic concentrations, which according to (Christou et al., 2017a) is limited. Overall, CoECs showed higher biodegradation rates with the pre-exposed community than the unexposed community. We therefore suggest pre-exposure as a strategy to improve the quality of obtained biodegradation half-lives, in line with previous research (Poursat et al., 2019). One of the bottlenecks with testing with realistic concentrations is that the CoECs concentrations might be under the limit of detection once the test progresses, thus the formed transformation products might be under the limit of detection. Thus, in this study non-targeted screening of transformation products were not considered. Dealing with low levels and measuring transformation products requires methods that can go to picogram/liter. In addition semi-automated methods for screening transformation products are currently still under development (Helmus et al., 2022). Thus, future studies should examine also the transformation products formed during batch incubation studies with SSI relevant concentrations of CoECs and inoculum.

4.4 Conclusion

Overall, CoECs in the bottles with the pre-exposed community from the SSI field showed significantly shorter biodegradation half-lives than the unexposed community from the reference field. The microbial community composition also shifted based on the created conditions. The composition of effluent bacteria showed significant differences as the anaerobic conditions fostered for the anaerobic bottles. However, the functional effects of such alterations observed here is yet unknown. Nevertheless, the present study showed that the presence of CoECs in STP effluent can stimulate mechanisms of degradation and adaptation in the soil microbiome, which may lead to better water quality.

Data availability

Supplementary data to this article are available from the datadata repository (Narain-Ford et al., 2022): https://data.mendeley.com/datasets/htdgbzhr3p

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

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Annex B - Supplementary information chapter 4

SI-4.1.1 PCR thermal cycling conditions

Initial denaturation at 95 °C for 5 minutes (1 cycle), 30 cycles of 95 °C for 30 seconds, 55 °C for 30 seconds, and 72 °C for 60 seconds, followed by a final extension at 72 °C for 5 minutes. Each 25 µl reaction mixture included 5 µl of extracted DNA (approximately 5.5 ng), 12.5 µl of Taq PCR Master Mix (Qiagen), 2.5 µl of each primer (5 µM) and 2.5 µl of nuclease-free water (ThermoFisher Scientific). Negative controls, no template DNA added, were included in every PCR.

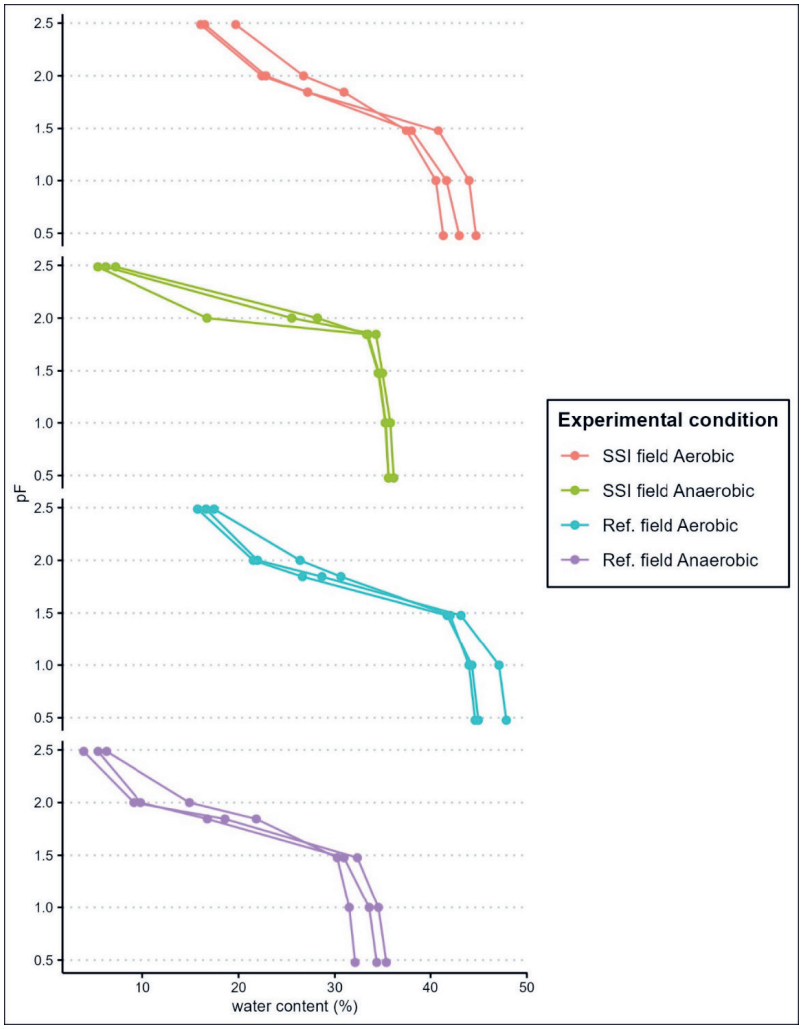
After cDNA synthesis, all samples were amplified in a 25 µl PCR reaction using the same primer set (515F and 926R) and adding ~ 8 ng of cDNA as template. The PCR protocol and the cycling program that were followed were the same as previously described. Negative control with nuclease-free water and positive control with a known DNA sample as a template were included in the PCR. Then 5 µl of each amplified were visualized in a 2% (w/v) agarose gel electrophoresis. For this purpose, 0.8 g of agarose 2% (w/v) (Hispanagar; Sphaero Q, Leiden, The Netherlands) was dissolved in 80 ml of 1X TAE Buffer (Bio-Rad) with 0.25 ml of 50X Midori Green Advance DNA Stain (Nippon Genetics Europe GmbH). The DNA fragments were separated by loading 5 µl of each PCR product with 1 µl of 6X orange loading dye to the agarose gel. A DNA molecular weight marker GeneRuler DNA Ladder Mix (100 – 10.000 bp) (ThermoFisher Scientific) was included in the outer slots of each gel, as standard for the calculation of the fragments. To improve the separation of the fragments, gel electrophoresis was performed with a voltage of 100 V at 400 mA for 60 minutes. Then the DNA bands were visualized under an ultraviolet (UV) transilluminator (Syngene Chemi-Genius Q Bio-imaging system) and identified using the GeneSnap™ software.

SI-4.1.2 PCR product purification

The PCR products (20 µL) were purified with the ExoSAP-IT™ PCR Product Cleanup Reagent (Applied Biosystems) by incubating 20µl of PCR product and 8 µl of 1:5 ExoSAP-IT™ for 30min at 37 °C to degrade primers and nucleotides and then incubated at 80 °C for 15 minutes to inactivate the ExoSAP - enzymes.

SI-4.2 Soil properties and batch incubation conditions.

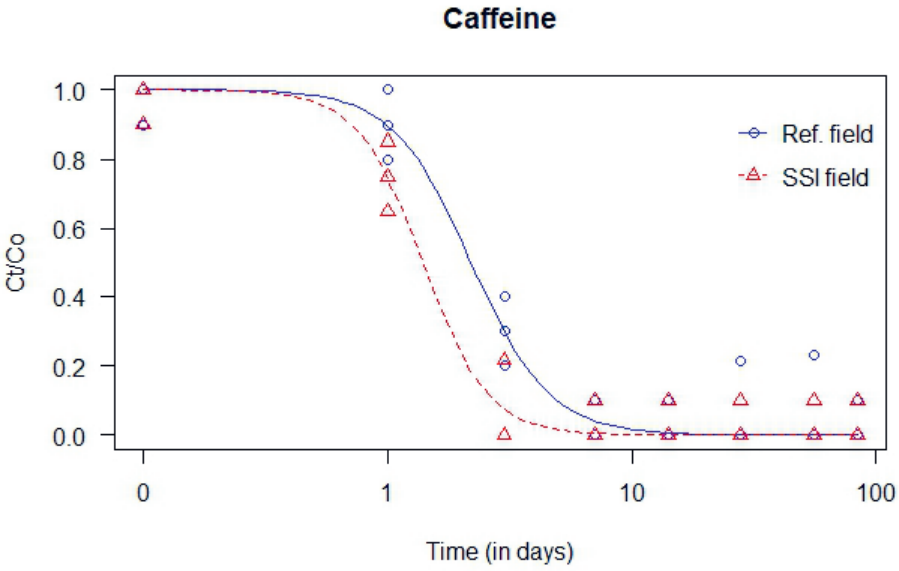
The pF curves confirm that aerobic soil has a better potential to retain moisture than anaerobic soil, which is expected since organic matter is enriched thereby increasing its potential to retain water (Kopittke et al., 2019). $CO_{2\ (g)}$ levels in all aerobic batch replicates were below 0.05%, in accordance with OECD guideline 307 (https://data.mendeley.com/datasets/htdgbzhr3p). The Chloride bromide ratio, a tracer of effluent, confirmed that during winter 2021 most effluent was flushed out of the system compared to when it was active in 2020 (https://data.mendeley.com/datasets/htdgbzhr3p). At this field site we observed that precipitation surplus and drainage during normal years removes CoECs from the SSI system (Narain-ford et al., 2022).

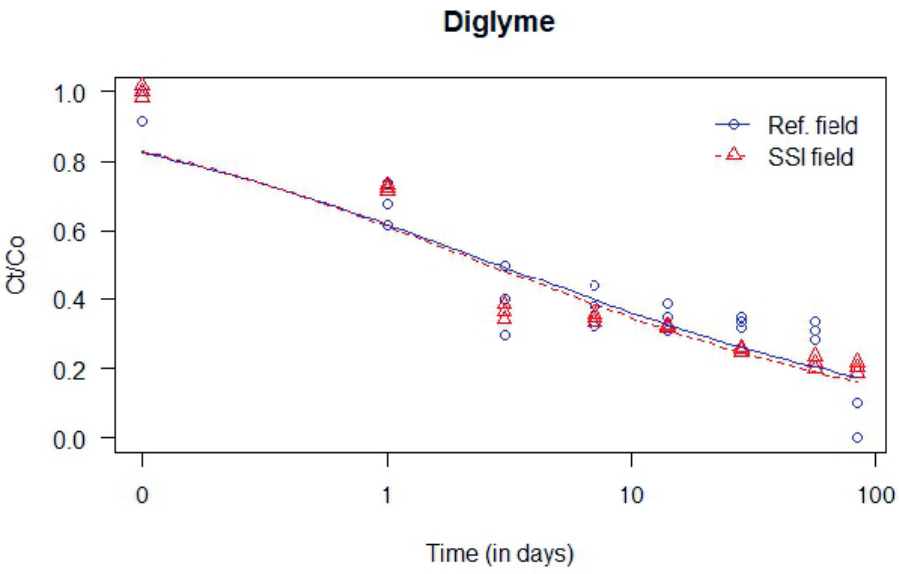


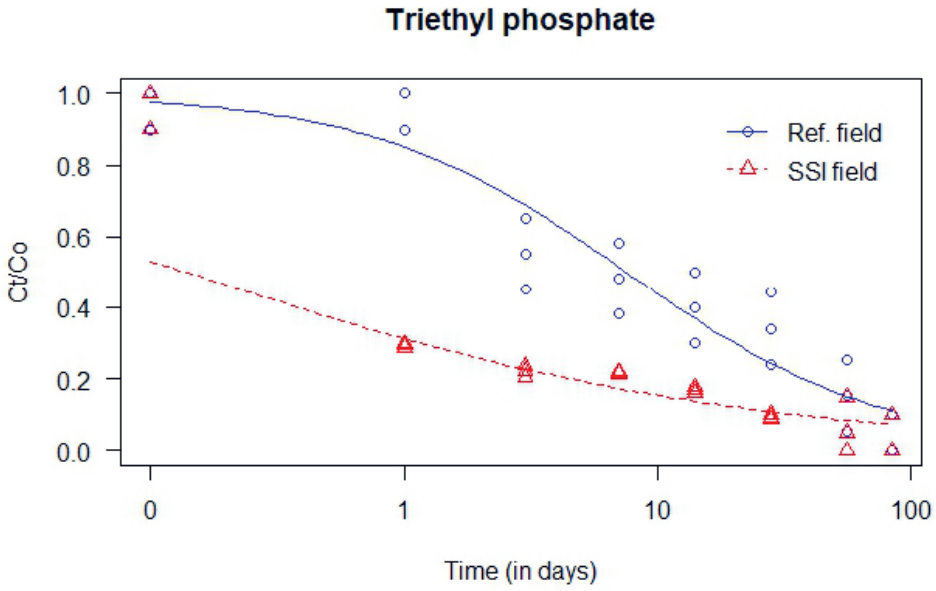
SI-4.3 Degradation plots

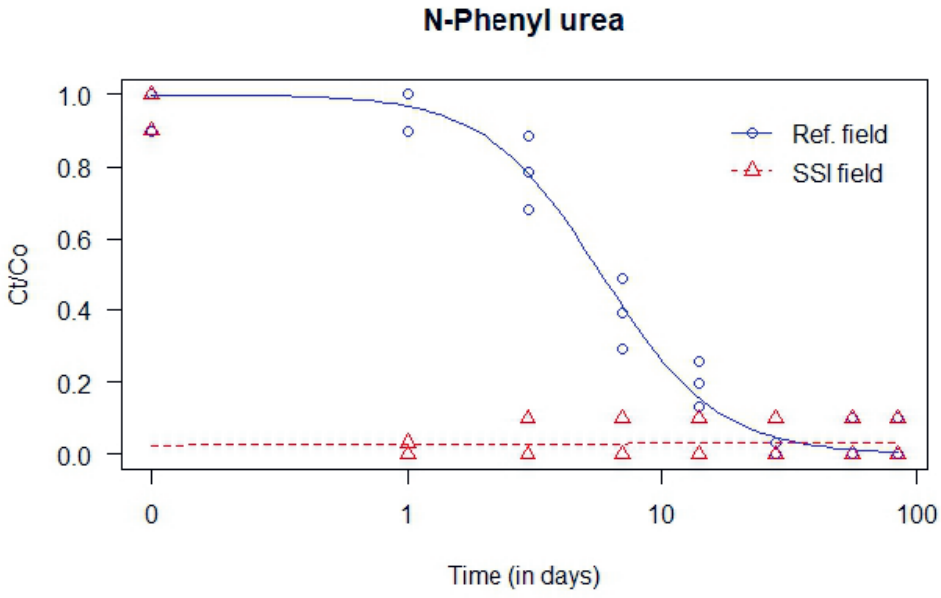
The DRC R package (Ritz et al., 2015) was used to fit a three - parameters log-logistic models to the degradation data based on the best fit.

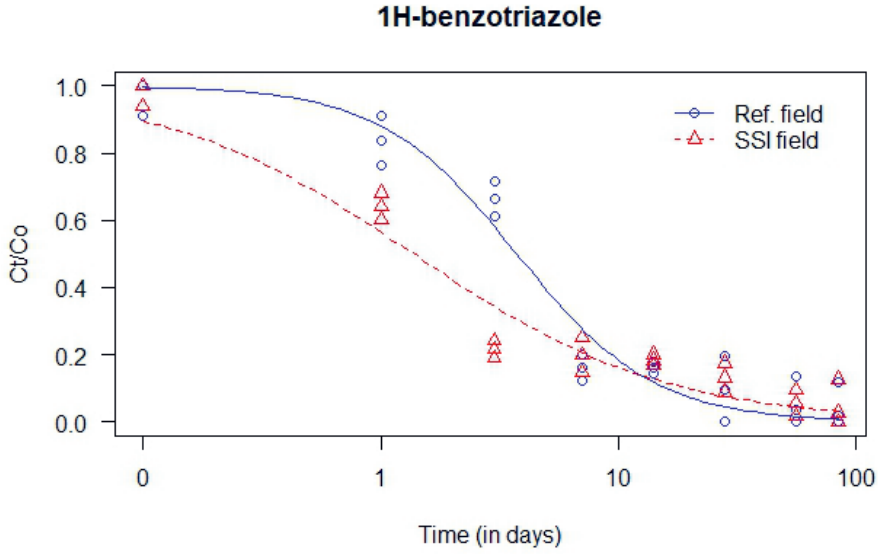
SI-4.3.1 Aerobic degradation plots






Triethyl phosphate
Ct/Co
1.0
0.8
0.6
0.4
0.2
0.0
0
1
10
100
Time (in days)
Ref. field
SSI field


N-Phenyl urea
Ct/Co
1.0
0.8
0.6
0.4
0.2
0.0
0
1
10
100
Time (in days)
Ref. field
SSI field


1H-benzotriazole
Ref. field
SSI field
Ct/Co
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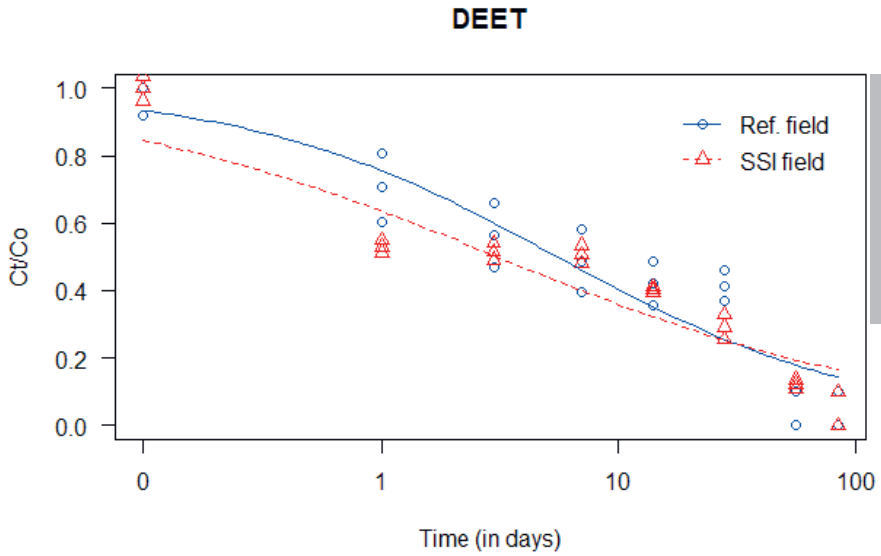
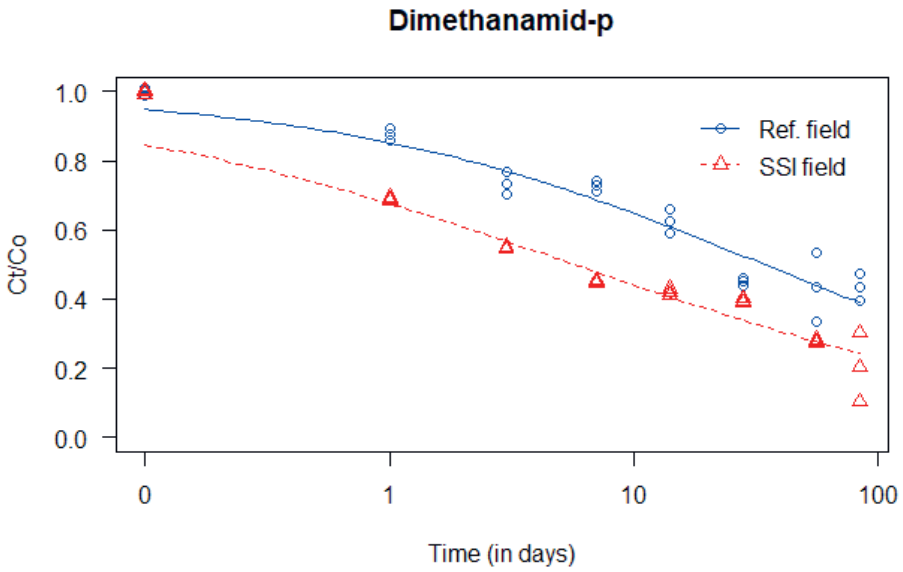
0.8
0.6
0.4
0.2
0.0
0
1
10
100
Time (in days)


DEET
Ref. field
SSI field
Ct/Co
1.0
0.8
0.6
0.4
0.2
0.0
0
1
10
100
Time (in days)


Dimethanamid-p
Ct/Co
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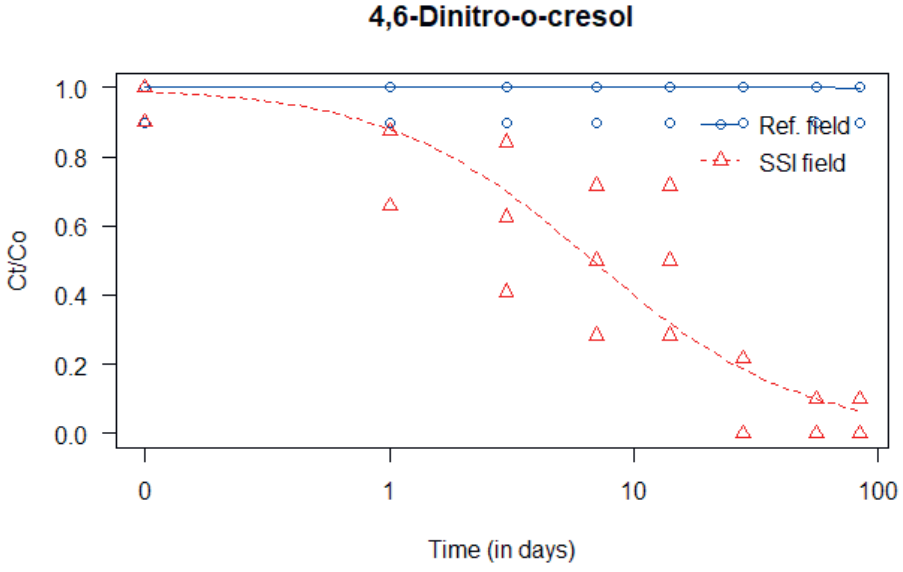
0.6
0.4
0.2
0.0
0
1
10
100
Time (in days)
Ref. field
SSI field


4,6-Dinitro-o-cresol
Ct/Co
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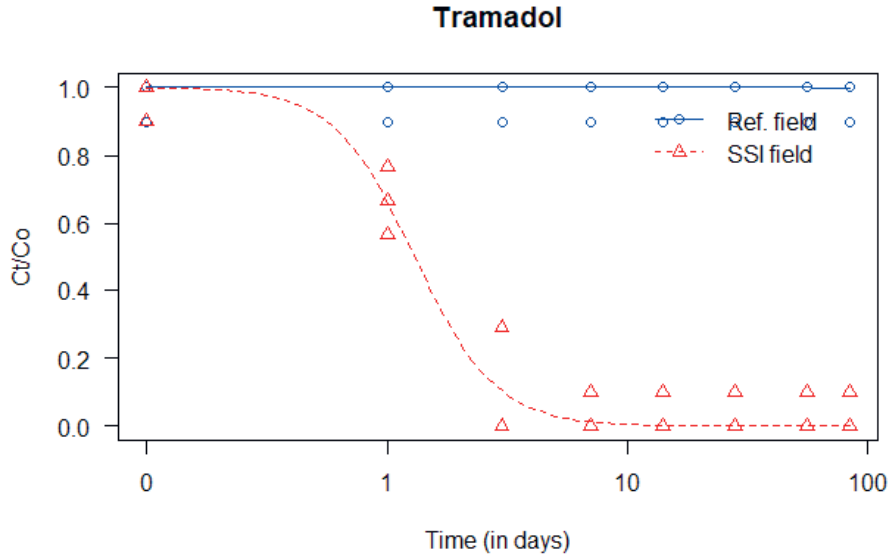
0.6
0.4
0.2
0.0
0
1
10
100
Time (in days)
Ref. field
SSI field


Tramadol
1.0
0.8
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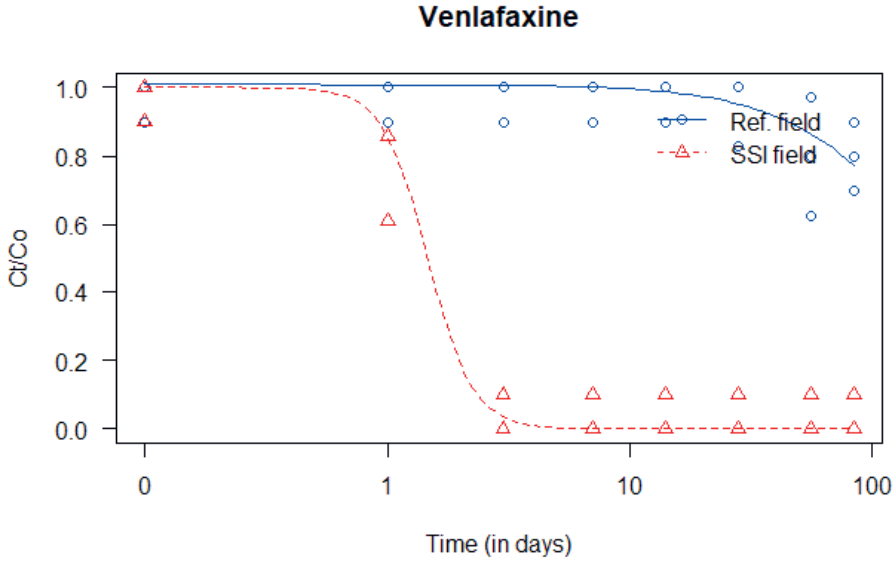
0.4
0.2
0.0
Ct/Co
Ref. field
SSI field
0
1
10
100
Time (in days)


Venlafaxine
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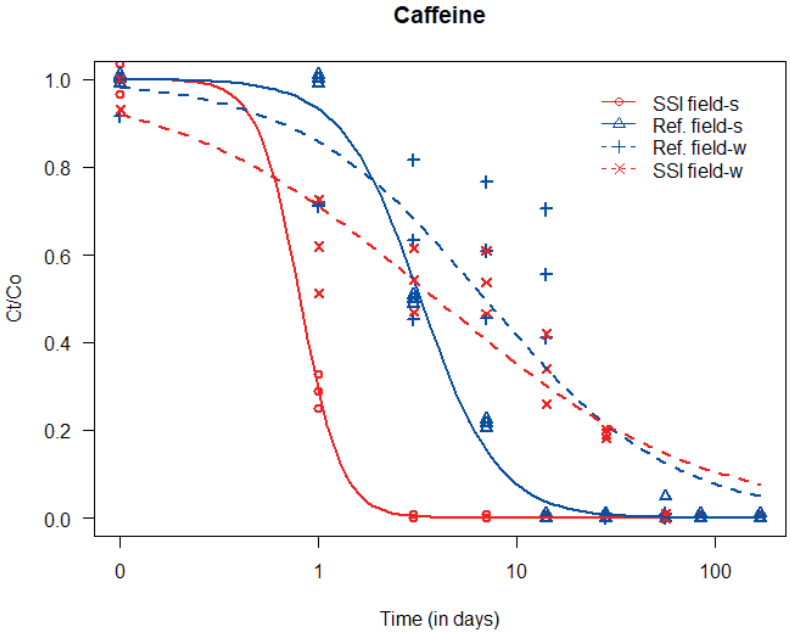
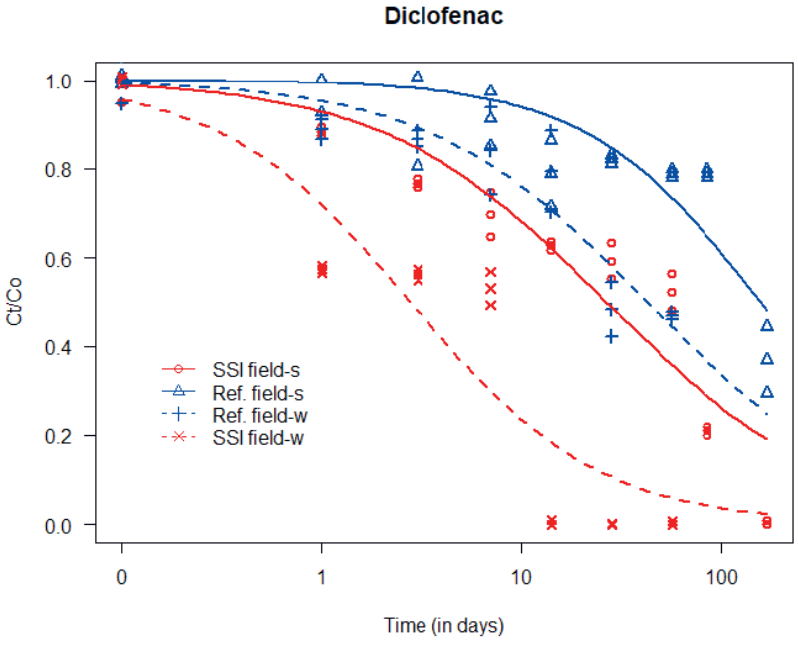
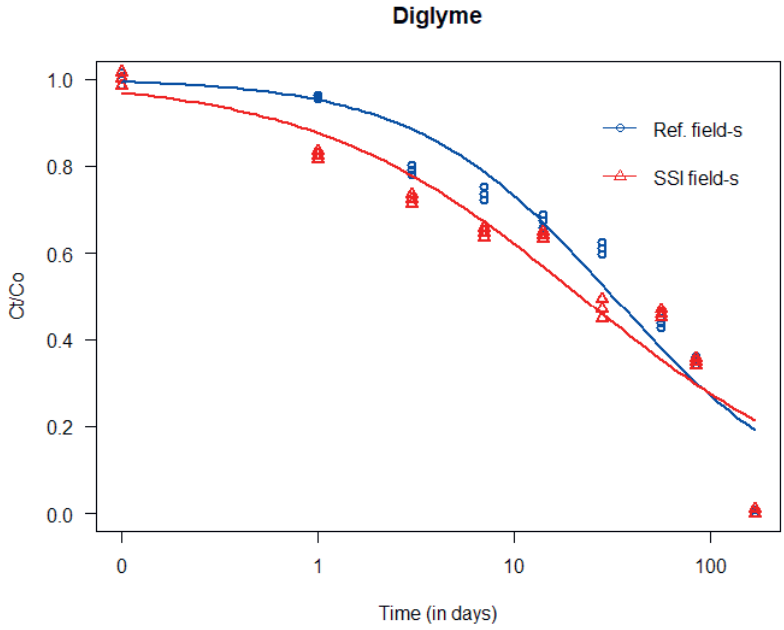
0.8
0.6
0.4
0.2
0.0
Ct/Co
Ref. field
SSI field
0
1
10
100
Time (in days)

SI-4.3.2 Anaerobic degradation plots






Diglyme
Ref. field-s
SSI field-s
Ct/Co
Time (in days)
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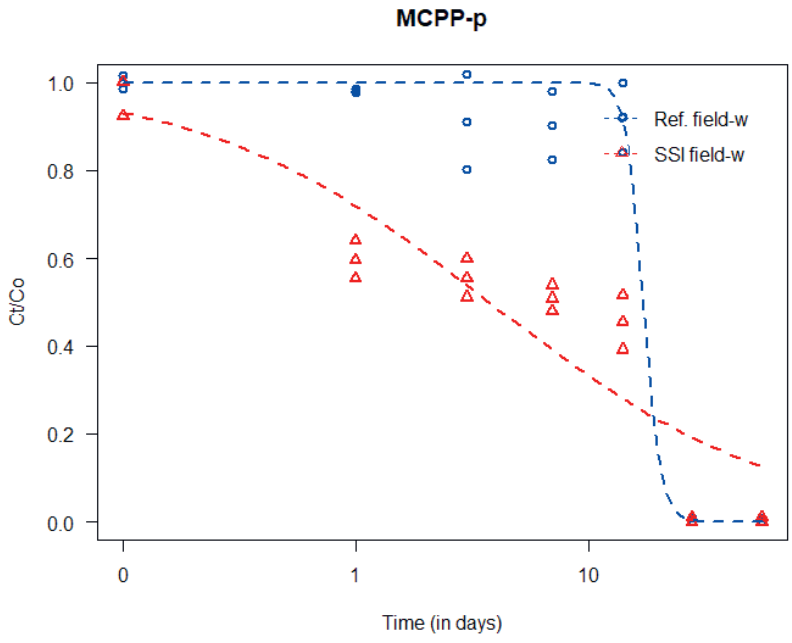
0.4
0.6
0.8
1.0
0
1
10
100


MCPP-p
Ref. field-w
SSI field-w
Ct/Co
Time (in days)
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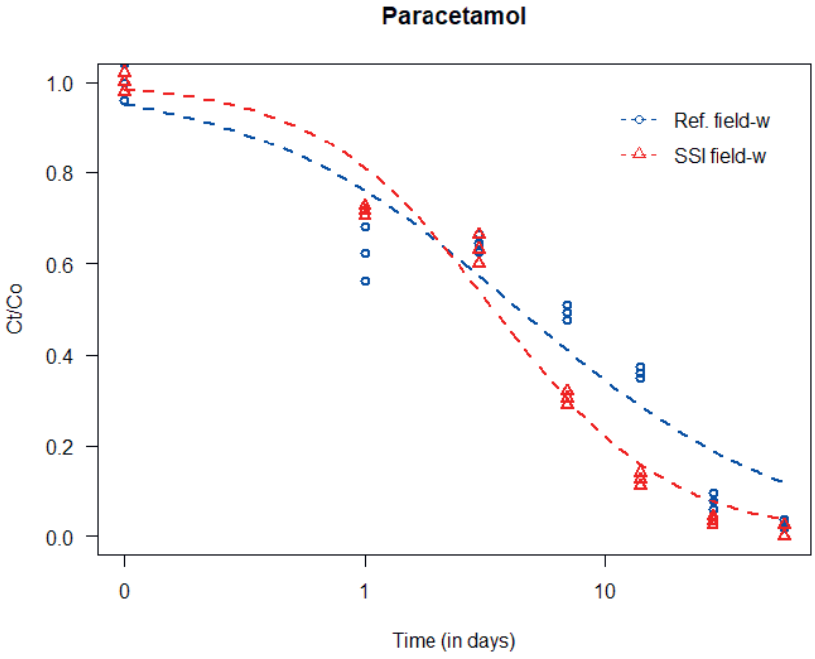
0.6
0.8
1.0
0
1
10


Paracetamol
Ref. field-w
SSI field-w
Ct/Co
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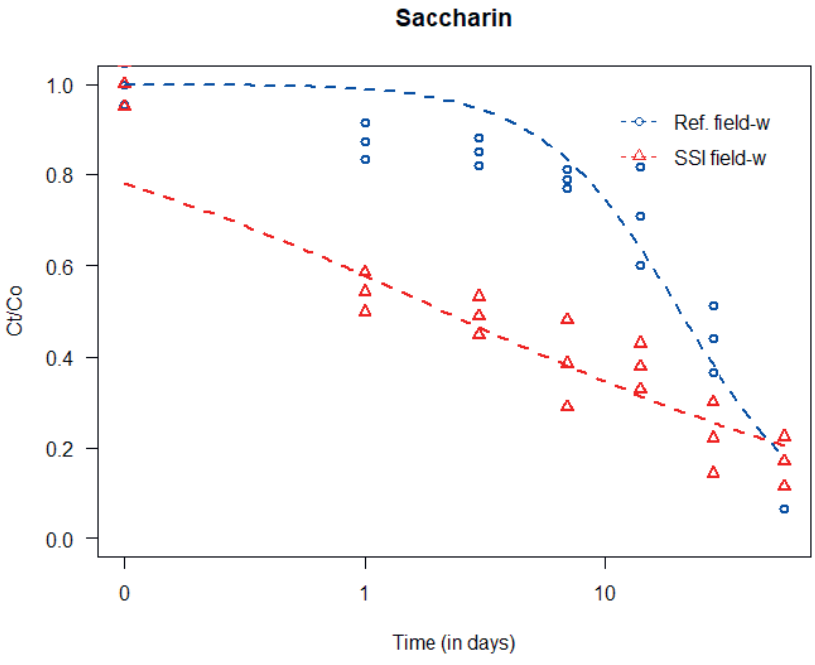
0.6
0.4
0.2
0.0
0
1
10
Time (in days)


Saccharin
Ref. field-w
SSI field-w
Ct/Co
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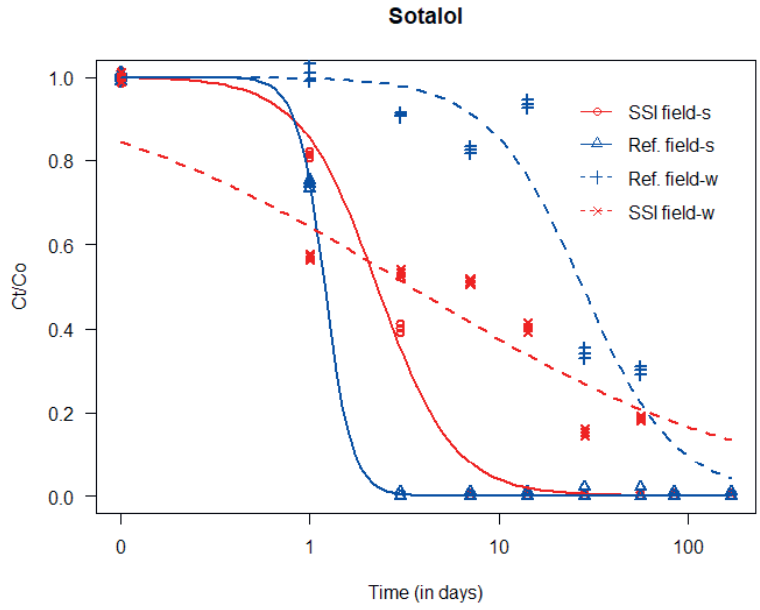
0.4
0.2
0.0
0
1
10
Time (in days)


Sotalol
SSI field-s
Ref. field-s
Ref. field-w
SSI field-w
Ct/Co
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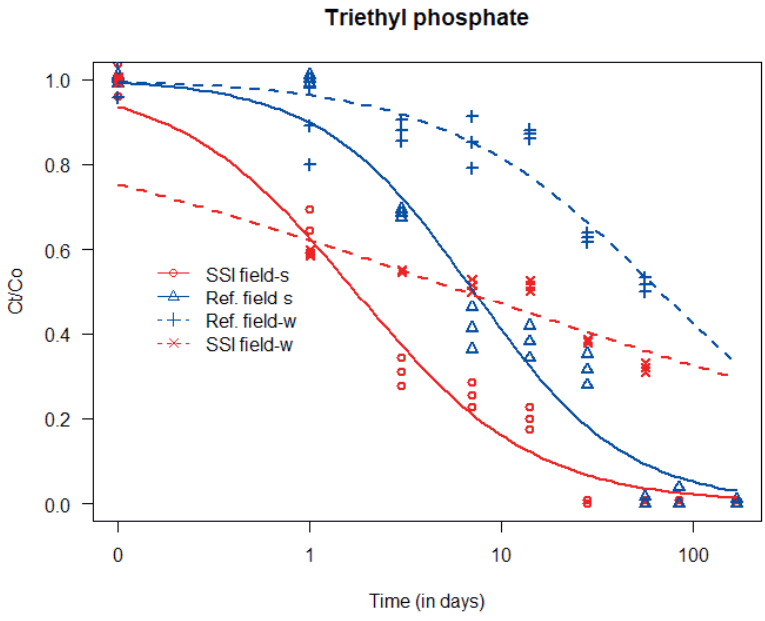
0.4
0.2
0.0
0
1
10
100
Time (in days)


Triethyl phosphate
SSI field-s
Ref. field s
Ref. field-w
SSI field-w
Ct/Co
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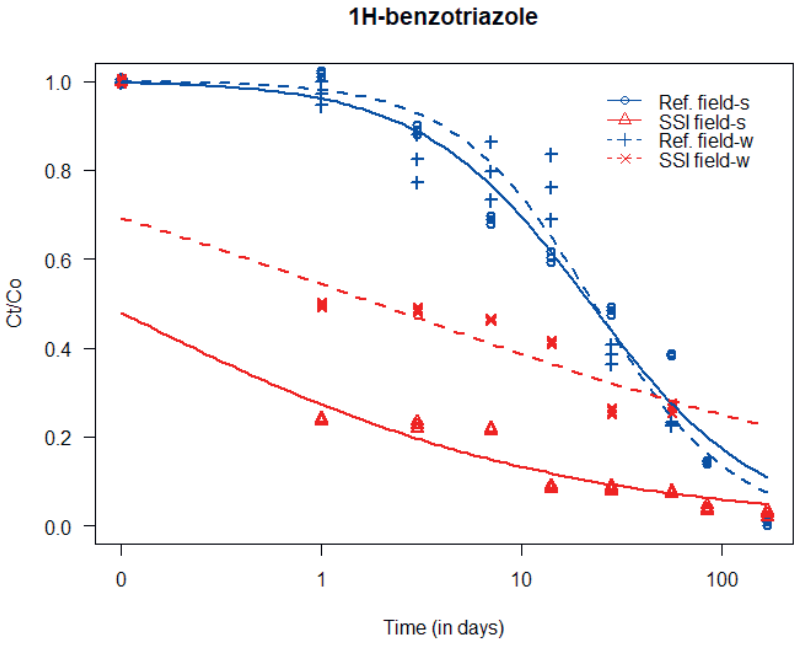
0.6
0.4
0.2
0.0
0
1
10
100
Time (in days)


1H-benzotriazole
Ref. field-s
SSI field-s
Ref. field-w
SSI field-w
Ct/Co
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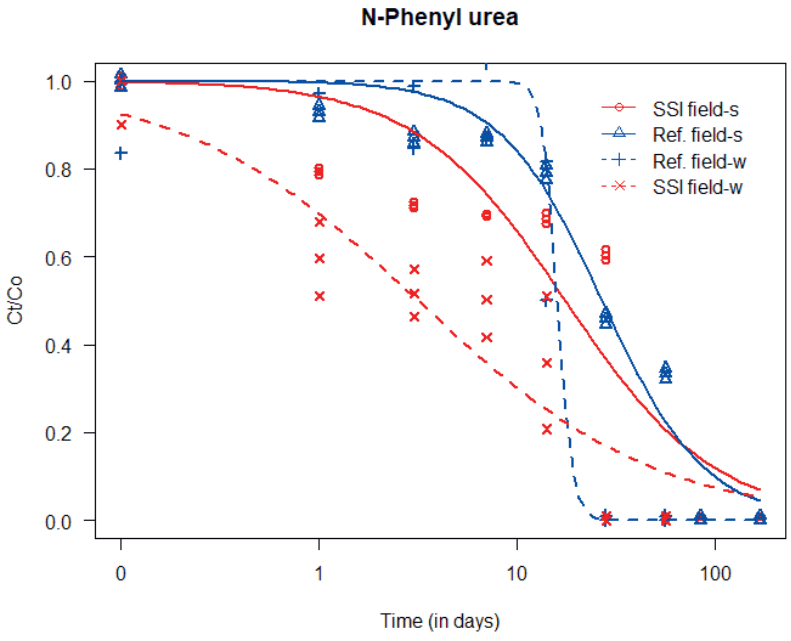
0.2
0.0
0
1
10
100
Time (in days)


N-Phenyl urea
SSI field-s
Ref. field-s
Ref. field-w
SSI field-w
Ct/Co
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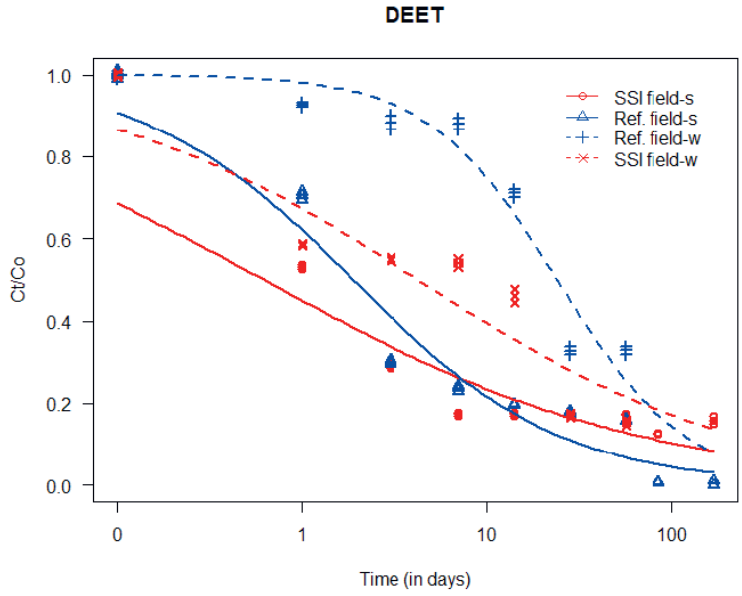
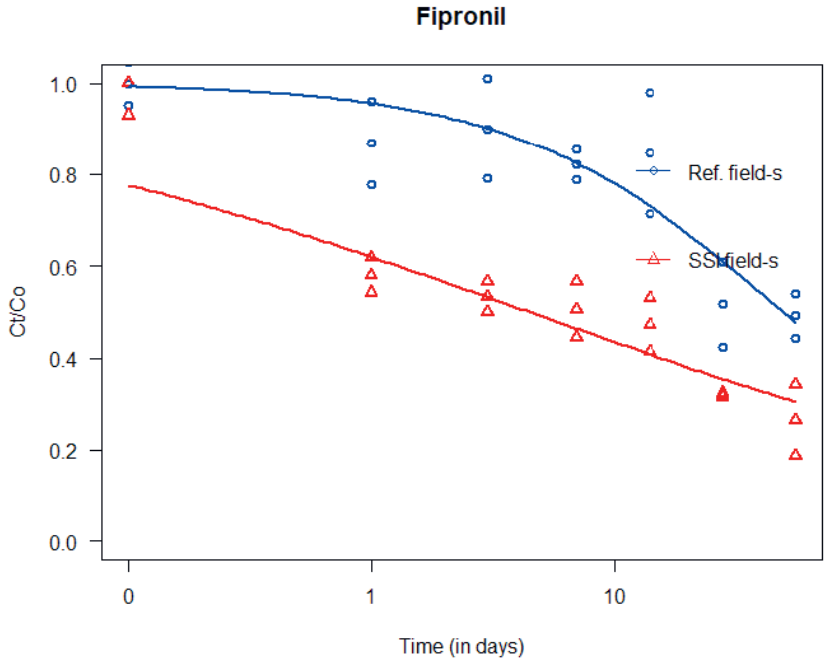
0.8
0.6
0.4
0.2
0.0
0
1
10
100
Time (in days)


DEET
SSI field-s
Ref. field-s
Ref. field-w
SSI field-w
Ct/Co
1.0
0.8
0.6
0.4
0.2
0.0
0
1
10
100
Time (in days)


Fipronil
Ref. field-s
SSIfield-s
Ct/Co
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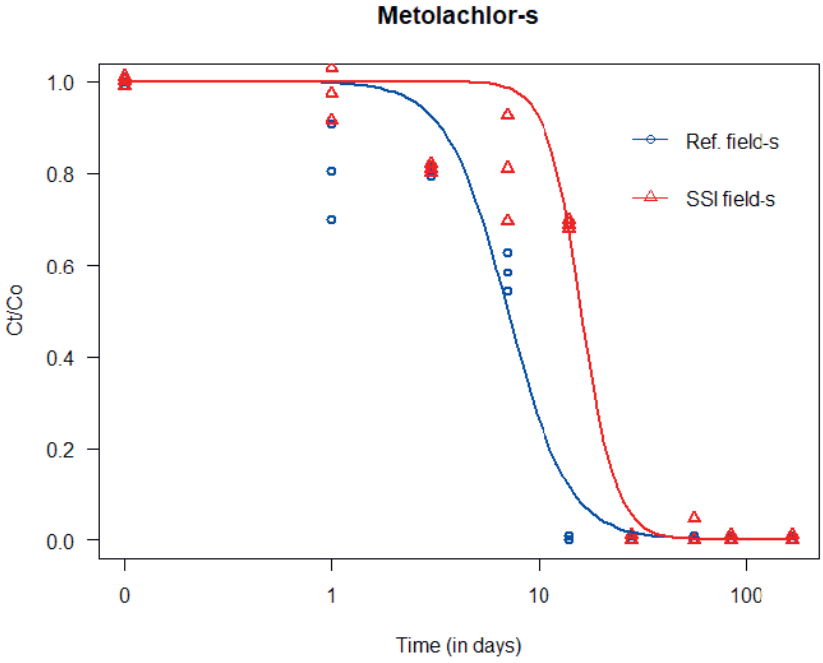
0.8
0.6
0.4
0.2
0.0
0
1
10
Time (in days)


Metolachlor-s
Ref. field-s
SSI field-s
Ct/Co
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0.6
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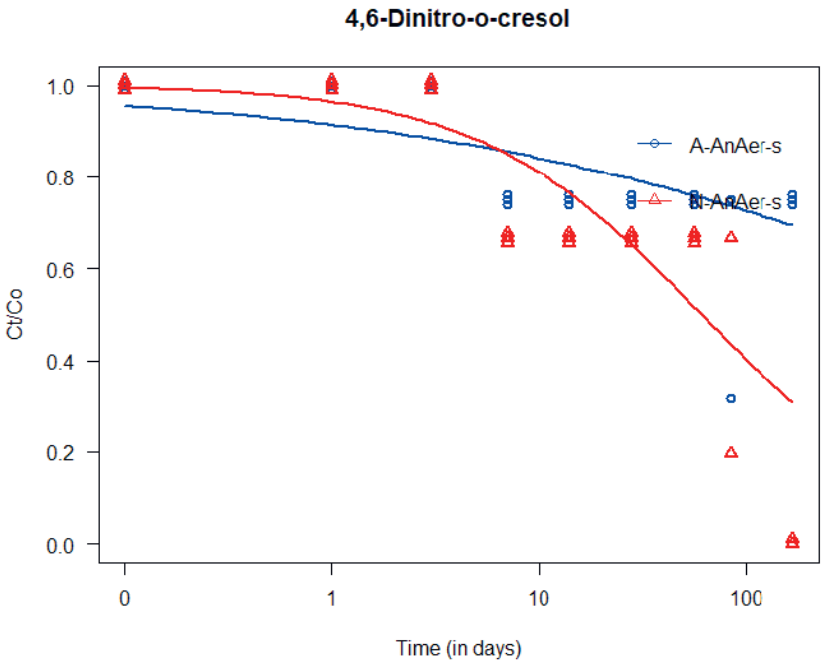
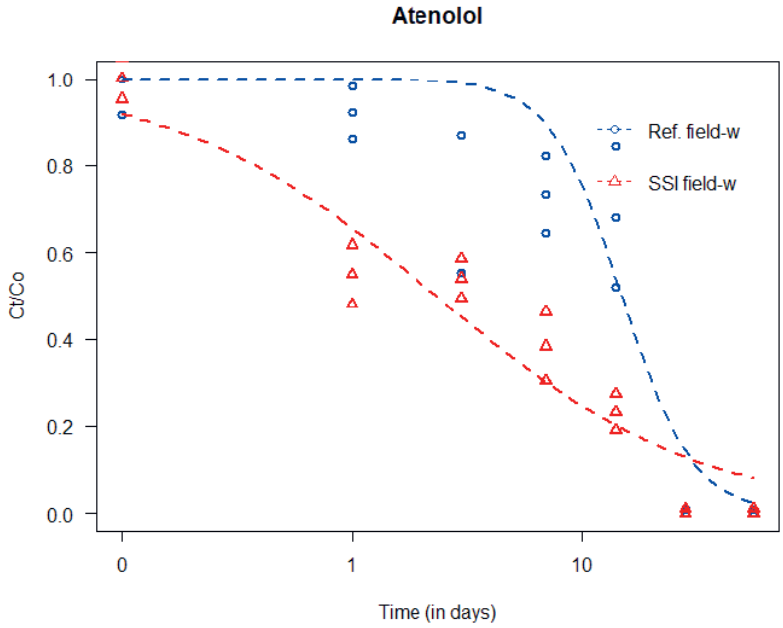
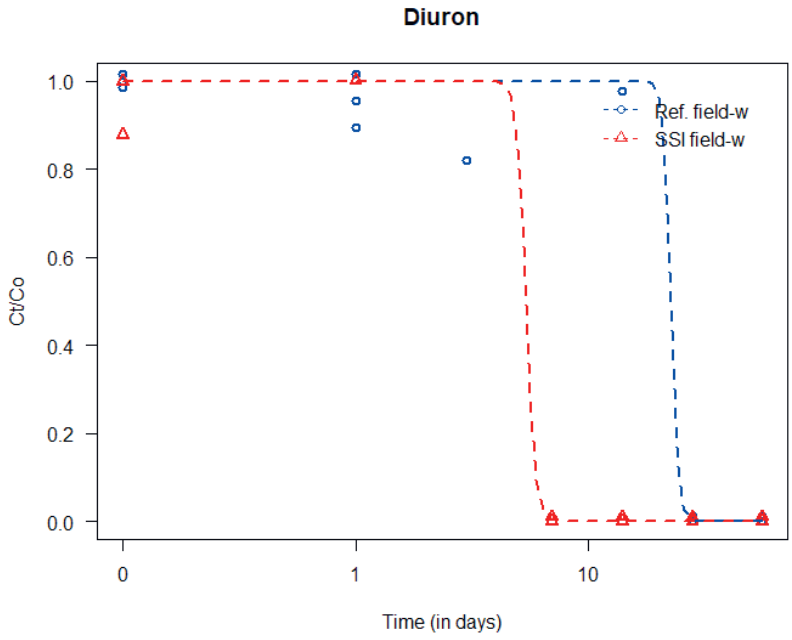
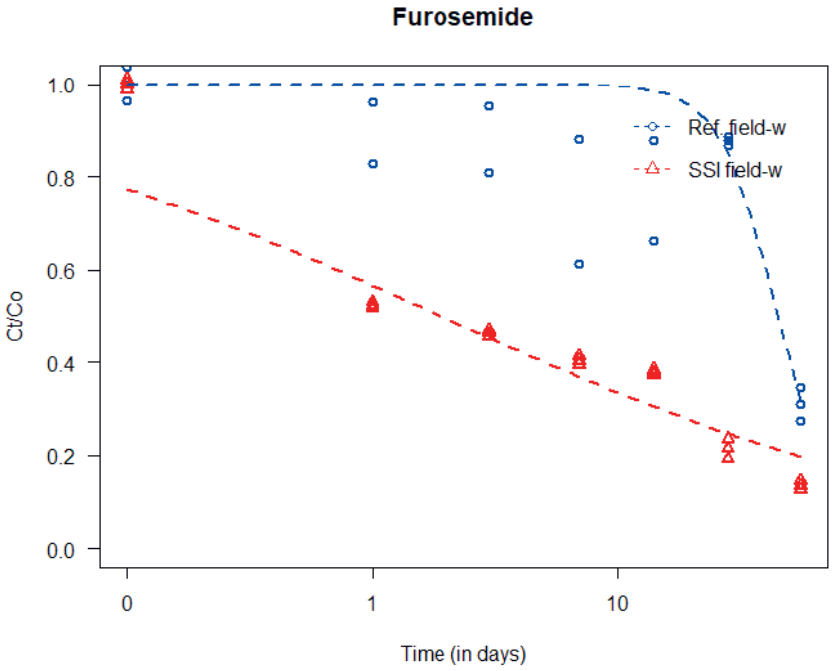
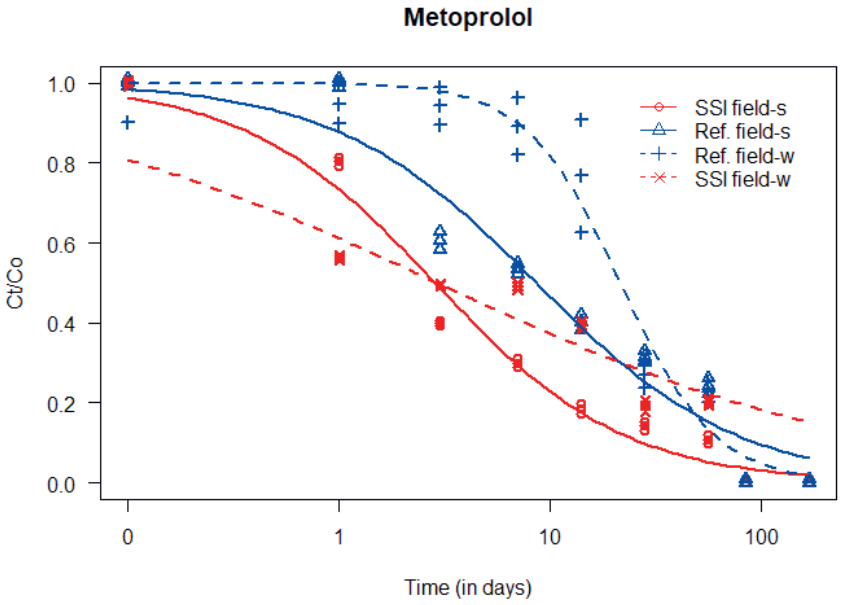
0.0
0
1
10
100
Time (in days)

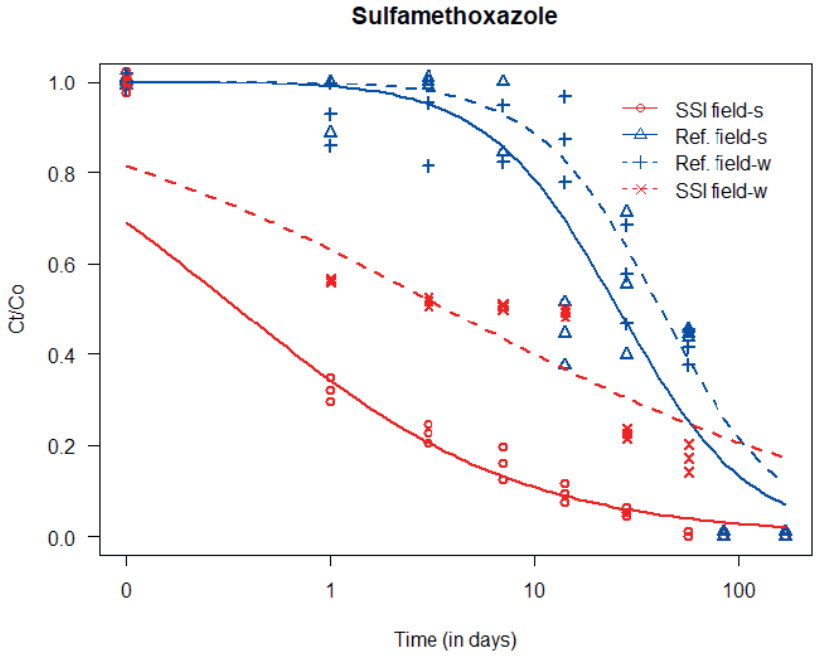

4,6-Dinitro-o-cresol
A-AnAer-s
N-AnAer-s
Ct/Co
1.0
0.8
0.6
0.4
0.2
0.0
0
1
10
100
Time (in days)

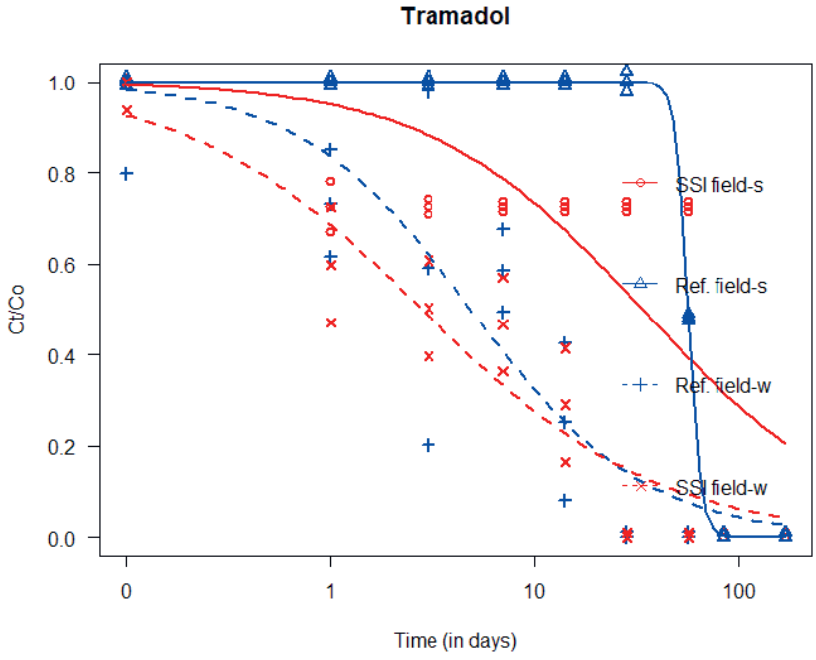

Atenolol
Ct/Co
1.0
0.8
0.6
0.4
0.2
0.0
0
1
10
Time (in days)
Ref. field-w
SSI field-w


Diuron
Ct/Co
1.0
0.8
0.6
0.4
0.2
0.0
0
1
10
Time (in days)
Ref. field-w
SSI field-w


Furosemide
Ct/Co
1.0
0.8
0.6
0.4
0.2
0.0
0
1
10
Time (in days)
Ref. field-w
SSI field-w


Metoprolol
Ct/Co
1.0
0.8
0.6
0.4
0.2
0.0
0
1
10
100
Time (in days)
SSI field-s
Ref. field-s
Ref. field-w
SSI field-w


Sulfamethoxazole
Ct/Co
1.0
0.8
0.6
0.4
0.2
0.0
0
1
10
100
Time (in days)
SSI field-s
Ref. field-s
Ref. field-w
SSI field-w


Tramadol
Ct/Co
1.0
0.8
0.6
0.4
0.2
0.0
0
1
10
100
Time (in days)
SSI field-s
Ref. field-s
Ref. field-w
SSI field-w