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Uptake of tire-derived compounds in lettuce under realistic agricultural growing conditions

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Additionally, I want to bring awareness to the people of the Nir Oz kibbutz in Israel, since one of the soils used in this project was sampled in this kibbutz. During the October 7 attacks, 46 out of the 400 inhabitants of the village were killed and 70 people abducted, including the Bibas family and their 4-year- and 9-month-old children who remain abducted to this day.

ABSTRACT

Water scarcity exacerbated by climate change has led to an increased reliance on treated wastewater (TWW) for irrigation, introducing contaminants of emerging concern (CECs) into agricultural systems. This thesis investigates the behaviour and uptake of tire wear compounds (TWCs) in leafy crops grown under realistic agricultural conditions, focusing on their potential to enter the human food chain. A greenhouse experiment was conducted using lettuce plants grown in soils with varying clay content, irrigated with TWC-spiked water. Results show that soil properties influence TWC bioavailability and plant uptake, with higher clay content reducing accessibility. Additionally, a detection study of leafy greens from supermarkets and agricultural fields highlights the presence of TWCs in commercial produce. Findings demonstrate that plant uptake is governed by compound-specific properties and accessibility in soil. This research contributes to understanding the environmental fate of TWCs and informs strategies for safer reuse of treated wastewater.

ZUSAMMENFASSUNG

Der durch den Klimawandel verschärfte Wassermangel hat zu einer verstärkten Nutzung von behandeltem Abwasser (TWW) zur Bewässerung geführt, wodurch neuartige Schadstoffe (CECs) in landwirtschaftliche Systeme eingebracht werden. Diese Arbeit untersucht das Verhalten und die Aufnahme von Reifenabriebverbindungen (TWCs) in Pflanzen, die unter realistischen landwirtschaftlichen Bedingungen angebaut werden, mit besonderem Fokus auf deren potenziellen Eintritt in die menschliche Nahrungskette. In einem Gewächshausversuch wurden Salatpflanzen in Böden mit unterschiedlichem Lehmgehalt kultiviert und mit TWC-angereichertem Wasser bewässert. Die Ergebnisse zeigen, dass Bodeneigenschaften die Bioverfügbarkeit und Aufnahme von TWCs erheblich beeinflussen, wobei ein höherer Lehmgehalt die Verfügbarkeit verringert. Eine Untersuchung von Blattgemüse aus Supermärkten und landwirtschaftlichen Feldern belegt zudem die Präsenz von TWCs in erwerblichen Lebensmitteln. Die Ergebnisse zeigen, dass die Aufnahme von TWCs durch verbindungsspezifische Eigenschaften und Verfügbarkeit im Boden bestimmt wird. Diese Forschung trägt zum Verständnis des Umweltverhaltens von TWCs bei und liefert wichtige Erkenntnisse für eine sicherere Wiederverwendung von behandeltem Abwasser.

ABBREVIATIONS

TWC – tire wear derived compound

TWP – tire wear particle

CEC – contaminant of emerging concern

WWTP – wastewater treatment plant

NER – non-extractable residue

LC/MS – liquid chromatography / mass spectrometry

LOQ – limit of quantification

BCF – bioconcentration factor

TSCF – translation stream concentration factor

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1. INTRODUCTION

Water scarcity is projected to increase due to climate change, placing mounting pressure on agricultural systems to feed and sustain a growing human population.[1,2] In water-scarce regions, the demand for alternative irrigation sources like treated wastewater irrigation (TWW) is increasing to help to alleviate these shortages.[3] However, modern wastewater treatment plants often fail to fully remove organic micropollutants or chemicals of emerging concern (CECs), potentially introducing these chemicals into agricultural systems.[4] Once present in the agricultural environment, these compounds can be taken up by edible crops and transferred into the human food chain.[5]

Similarly, using composted wastewater treatment sludge as biosolids for fertilizer can introduce contaminants such as microplastics[6], per- and polyfluoroalkylated substances (PFAS)[7], pharmaceuticals and personal care products[8] into agricultural fields. While biosolid application aligns with circular economy principles, the contamination issues associated with sludge pose significant challenges to its applicability as a sustainable agricultural practice.[9,10]

This research focuses on tire wear derived chemicals (TWCs) and their behaviour in agricultural systems. TWCs are compounds added to tire rubber material during the manufacturing process to achieve desired mechanical and chemical properties.[11] Transformation products of tire additives are also classified as TWCs.[12] During the lifetime of the tire, tire wear particles (TWPs) are generated by abrasion of the rubber against the road surface, especially during braking and acceleration.[13] Once these TWPs reach the environment, TWCs can leach out of the rubber material, causing considerable ecotoxicological effects.[14] Tire wear particles accumulate on road surfaces and are washed into sewage systems[15] during rainfall events, from which they can enter agricultural areas. In Germany it is estimated that between 1400 – 2800 tons of tire wear particles enter agricultural areas yearly via biosolids application,[16] and TWCs have been detected in treated wastewater effluent. Although TWCs are well studied in aquatic environments[11], little is known about their fate and behaviour in soils, especially agricultural soils. In agricultural soils close to low traffic roads, TWPs have been detected[17]. TWCs have been shown to occur in agricultural runoff[18]. Previous work has shown that under hydroponic conditions, TWCs are taken up and metabolized by plants.[19] In a real agricultural setting however, additional factors such as soil type, temperature and irrigation influence the uptake of CECs.[20] Soil especially has a large influence on plant uptake, reducing bioaccessibility through sorption.[21]

Therefore, it is necessary to study the behaviour and fate of these compounds under realistic agricultural growing conditions and to investigate the occurrence of TWCs in commercially available produce. This research aims to fill this knowledge gap by investigating the uptake, translocation, and occurrence of TWCs in crops grown in soils irrigated with contaminated water, thereby contributing to the development of strategies for mitigating the risks associated with the use of treated wastewater in agriculture.

2. STATE OF KNOWLEDGE

2.1 Fate, behaviour and properties of tire wear compounds

Chemicals used as additives in the production of tire rubber are not defined by their chemical classes but rather through their functionality. Therefore, chemicals found in rubber comprise a heterogenous and diverse spectrum of different compounds. In this study, five prominent tire derived chemicals were selected and their most important chemical characteristics as well as factors determining their environmental fate will be subsequently discussed. A summary of the physicochemical properties of the compounds can be found in table 1.

Table 1: Physicochemical properties of selected TWCs

Compound	**HMMM**	**DPG**	**6PPD**	**6PPD-q**	**BTH**
M [g/mol]	390.22	211.11	268.20	298.17	135.01
pKa	7.01	10.12	6.7	N/A	7.8
log K_{ow}	1.61[45]	2.89[46]	4.84[32]	4.30[34]	2.01[47]

2.1.1 Hexa(methoxymethyl)melamine (HMMM)

HMMM is commonly used in the production of tire rubber as a crosslinking agent and is present throughout the water cycle, for example in WWTP effluent and influent[22] as well as natural surface waters[23]. The compound exhibits low sorption to pyrogenic carbonaceous materials[24] (log K_{ow} = 1.61) and soils[25] and is therefore expected to be readily available and mobile in a soil system. Biodegradation of HMMM occurs, with 50-60 % of the parent compound degraded in 15 days by white rot fungi[26]. Transformation products of HMMM can be more persistent then the parent compound and a number of these TPs have been detected in WWTPs, however full mineralization does not occur with ~50 % of HMMM removed in 18 days via activated sludge.[22]

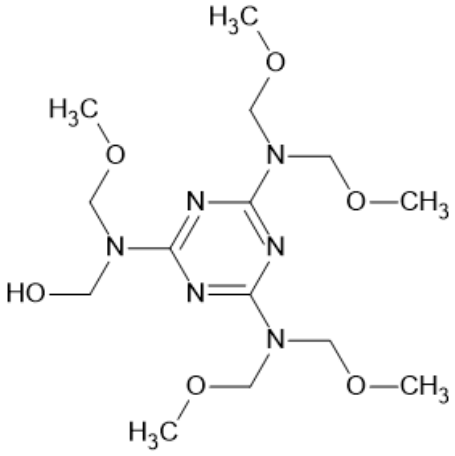

Figure 1: Structure of HMMM

2.1.2 1,3-Diphenylguanidine (DPG)

DPG is used as a vulcanization agent in rubber production and is widely distributed in the environment with detection in multiple compartments such as soils, WWTPs and surface waters.[15,27,28] It shows strong sorption to pyrogenic carbonaceous materials and soils.[24,25] Due to its pKa of 10.12, DPG is expected to be present in its cationic form under environmental conditions, indicating electrostatic interactions with negatively charged soil constituents such as clay minerals, potentially hindering its mobility in high clay content soil.[29,30] Regarding its stability, DPG is shown to be stable towards degradation by hydrolysis, biotransformation by activated sludge and white rot fungi in short term lab scale experiments.[25,31]

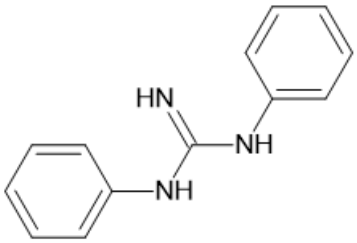

Figure 2: Structure of DPG

2.1.3 *N*-(1,3-dimethylbutyl)-*N*'-phenyl-p-phenylenediamine (6PPD)

6PPD is commonly used in rubber formulas as an anti-ozonation agent, protecting the rubber from damaging reactions with atmospheric ozone. With a pKa of 6.7, 6PPD is primarily present in both its cationic and neutral form at environmental pH values, indicating retention by clay minerals in soil. In snow samples, 6PPD is predominantly found in the particulate phase, indicating a tendency to sorb to organic material (log K_{ow} = 4.84). [32] As 6PPD is designed to react with ozone, its stability is low by design, resulting in a number of different oxidation transformation byproducts dominating its environmental fate.[33] No data on the stability of 6PPD in soil is available, however after 2 h in biologically active river water it was found that 57 % of 6PPD was degraded.[34]

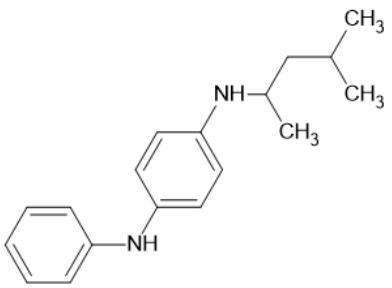

Figure 3: Structure of 6PPD

2.1.4 *N*-(1,3-dimethylbutyl)-*N*′-phenyl-*p*-phenylenediamine-quinone (6PPD-q)

6PPD-q is a prominent transformation product of 6PPD, generated through oxidation of 6PPD exhibiting high toxicity to some fish species.[14] As it shows strong sorption to rubberized asphalt mixtures[35] and rubber materials combined with its low aqueous solubility of 38 µg/L[36] and its prevalence in the particulate phase of snow[32] it is expected to be immobile in soil systems with high organic carbon levels. It has additionally been shown that bioretention cells provide an effective method of reducing 6PPD-q loads to freshwater through strong soil retention[37]. In simple deionized water systems, 6PPD-q has been shown to stable in short (14 d) time frames[36], however this stability is expected to rapidly decrease in more complex systems, as 6PPD-q can be further oxidized by reactive oxygen species[32,38] and is prone to photodegradation.[39]

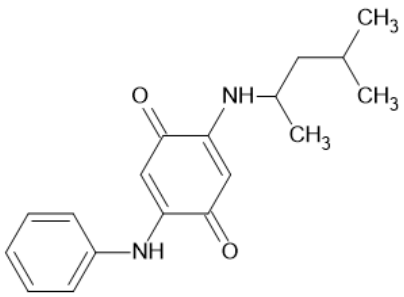

Figure 4: Structure of 6PPD-quinone

2.1.5 Benzothiazole (BTH)

BTH and its derivates such as 2-mercapto-BTH are used as vulcanization accelerators in rubber production and are commonly found in WWTP effluent and influent.[40–42] Benzothiazole derivatives are also used in corrosion inhibitors, antifreezes, biocides, photochemicals and pharmaceuticals.[43] Occurrence in other environmental compartments like soil, water and sediment and even human urine has also been documented.[43] BTH has been shown to reversibly sorb to sandy river sediment with sorption-desorption hysteresis occurring, pointing towards BTH being moderately mobile in soil.[44] Regarding its stability, BTH is susceptible to microbial biodegradation, however the rate of biodegradation fluctuates between hours and weeks depending on the microbial strain.[43] In WWTPs, BTH is not always fully removed, with a large variance in removal rates in conventional treatment facilities.[45,46]

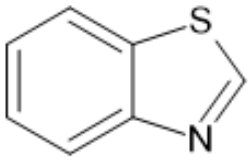

Figure 5: Structure of BTH

2.2 Plant interactions with xenobiotics

2.2.1 Bioavailability and bioaccessibility

The terms bioavailability and bioaccessibility are two separate, conflatable and closely related concepts of importance when dealing with organic contaminants in soil. Bioavailability refers to the amount of compound that is freely available to cross an organisms cellular membrane, while bioaccessibility refers to the amount of compound available to the organism in the environment including a fraction that can be available at a later point in time (i.e. through slow desorption).[47,48] Basically, the bioaccessable fraction includes the bioavailable fraction plus potentially bioavailable fractions.

2.2.2 Mobility of organic compounds in soil

The mobility of an organic compound refers to the ability of the compound to traverse the soil profile horizontally or vertically. This has a strong impact on the bioaccessibility of the compound as highly mobile compounds are more likely to reach the plant roots and be subsequently taken up. Compounds with low mobility will be retained stronger by the soil, leading to less uptake but potentially longer presence in the soils. The most important factors influencing the mobility of organic compounds in soils will be discussed here.

Sorption is the key process determining mobility. Compounds can sorb onto soil constituents such as clay or soil organic matter, lowering their mobility. Absorption to soil organic matter is the predominant sorption mechanism for hydrophobic, neutral compounds. Their sorption behaviour is assessed via the octanol-water partitioning coefficient K_{OW} serving as an estimation of the more specific but sometimes unavailable water-organic carbon partitioning coefficient K_{OC}. For charged or ionizable compounds however, sorption behaviour is more complex as it strongly depends on environmental parameters such as pH or mineral composition of the soil.[29] In this study, the investigated compounds include hydrophobic, neutral compounds such as 6PPD-q as well as charged compounds such as DPG, which is cationic at the pH of the employed soils.

The soils used in this study vary in their composition, the Sa'ad soil especially is composed to a large percentage of clay minerals. As clay minerals present in soil typically exhibit a negative surface charge at soil pHs, cationic compounds can bind to negatively charged mineral surface functional groups via cation exchange interactions. This process depends on the minerals cation exchange capacity (CEC), type of exchangeable cations and surface charge distribution.[49,50]

Sorption leads to the formation of non-extractable residues (NER). Non-extractable residues are defined as the fraction of an organic compound and its transformation products immobilized on solid surfaces.[51] NERs are differentiated into three different types: Type I NERs are formed by the sequestered fraction, i.e. compounds immobilized via adsorption, electrostatic interactions or physical entrapment in micropores. This process is considered reversible, albeit at a potentially slow rate. Type II NERs consist of the compound fraction covalently bound to the solid surface in an irreversible fashion unless soil is dramatically altered. Type III NERs involve the incorporation and assimilation of the parent compound or its transformation products into biomass.[52] The presence of plants in the soil system can influence the formation and release of NERs. Plants may reduce the formation of NERs and facilitate the release of type I NERs, thereby increasing their bioavailability.[53]

2.2.3 Uptake of xenobiotics into plants

The uptake of xenobiotics into plants can be divided into two main parts: The uptake of the compound into the plant system through the roots and the subsequent translocation of the compound into the aerial plant tissue.

In a simple hydroponic system, the dissolved fraction of the compound is taken up through the roots by passive diffusion via the root membrane. To achieve this, the compound must move through the rhizosphere through the root epidermis into the root endosphere. Once in the root endosphere, there are two available pathways for the compound to move through root tissue towards the xylem: the apoplastic and symplastic pathways. In the apoplastic pathway the compound travels through the intercellular space towards the casparian strip while in the symplastic pathway the compound enters the interior of the root cells and migrates to the casparian strip through cytoplasm via the plasmodesmata. Uptake of xenobiotics into the plant system is traditionally considered to be a passive process, however protein-mediated active uptake mechanisms have been demonstrated for some pesticides[54] and are hypothesized for the pharmaceutical Metformin.[55]

2.2.3 Translocation of xenobiotics in plants

To enter the plants vascular system and further transport to the aerial tissues, the compound must cross the casparian strip into the xylem. In mammals, the blood brain barrier can be seen as an analogous system to the plants casparian strip as a biological barrier regulating the passage of solutes into crucial biological systems. From this functional similarity, rules that govern the passage of a xenobiotic compound through the blood brain barrier can be transposed onto the ability of a compound to cross the casparian strip.[56] One such rule derived from mammalian pharmacokinetics is Lipinski's rule of five, predicting the passage of a compound through the blood brain barrier according to five molecular characteristics: molecular weight <500 Da, <5 H-bond donors, log K_{ow} <5 and <10 H-bond acceptors.[57] A study investigating a large dataset of transpiration stream concentration factors confirmed the general transferability of Lipinski's rule of five to plants, however more restrictive cutoffs for the molecular characteristics were found: molecular weight <350 Da, <4 H-bond donors, log K_{ow} 1-3 and <7 H-bond acceptors.[58] A compound that matches these criteria is considered likely to permeate the blood brain barrier and therefore also likely to permeate the casparian strip and enter the plants vascular system.[58]

Of these criteria, hydrophobicity is considered to be the most impactful, with moderately hydrophobic compounds with a log K_{ow} of 1.8 deemed as ideal for plant uptake.[59] Once the compound crosses the casparian strip, it is transported passively with the transpiration flow towards the aerial tissues.

Studies on plant uptake and translocation are usually performed in a hydroponic system, providing maximum bioavailability and minimal interfering parameters. In more realistic soil systems however, the bioavailability of a compound is further influenced by its stability, mobility and formation of non-extractable residues in the system, generally lowering bioavailability.

2.3. Occurrence of contaminants of emerging concern in produce

The occurrence of CECs in produce is an issue potentially affecting consumer health. Studies monitoring the occurrence of these contaminants have mostly focused on pharmaceuticals and personal care products as these compounds are ubiquitously found in WWTPs, leading to their presence on agricultural fields via treated wastewater irrigation. A large scale survey of pharmaceuticals in Israel in agricultural soil, irrigation water and commercial produce found 18 detectable CECs in leafy greens varying in concentration from 0.1 ng/g to 2470 ng/g (for the persistent anticonvulsant carbamazepine).[60] Leafy greens are considered to be the produce class with the highest potential risk as the entire plant is consumed unlike plants where only the fruits or tubers are consumed. Leafy greens also show the highest potential for CEC uptake.[61] The endocrine disrupting chemical bisphenol A was detected in commercially available lettuce at 2.0 ng/g.[62] In lettuce harvested from fields in Spain and irrigated with reclaimed wastewater, seven antibiotics were detected at frequencies ranging between 29 and 100 % and concentrations ranging between 0.12 – 1.19 ng/g.[63] Commercially available produce can serve as a potential exposure route for CECs to consumers, therefore the investigation of TWCs in produce can provide important data on potential consumer risks.

3. AIMS AND HYPOTHESES

As plant uptake is a potential route for TWCs to enter the human food chain via TWW irrigation or biosolid application, the aim of this thesis is to:

1. Investigate TWC uptake in edible crops under realistic agricultural circumstances.
2. Assess the concentrations of TWCs in commercially available leafy greens and produce from agricultural fields.

Previous studies in controlled hydroponic systems have demonstrated that TWCs can be taken up by plants. However, as soil has a strong influence on bioaccessibility of organic compounds, the need for investigation under realistic agricultural circumstances arises.

To achieve objective 1, a greenhouse experiment was conducted in which lettuce plants are cultivated in agricultural soils irrigated with TWC-spiked water. At maturity, the lettuce plants were harvested, and TWC concentrations in plant tissue, soil and pore water were analyzed. To achieve objective 2, the concentrations of TWCs in lettuce from supermarkets and leafy greens from agricultural fields were analyzed.

Based on literature research, previous experiments, and the study design, the following hypotheses are proposed:

Hypothesis 1: Soil clay content influences TWC uptake. Higher soil clay content is expected to reduce TWC bioaccessibility due to charge-based interactions with cationic compounds. Leaf TWC concentrations are hypothesized to decrease with increasing soil clay content, being lowest in Sa'ad soil, followed by Ein Hashlosha soil, and highest in Nir Oz soil.

Hypothesis 2: TWC concentrations in lettuce follow the order HMMM > BTH > DPG > 6PPD-q > 6PPD. HMMM is hypothesized to exhibit the highest uptake due to its stability, and moderate hydrophobicity. BTH is hypothesized to show intermediate uptake, due to its hydrophobicity and mobility but limited by susceptibility to degradation. DPG uptake is expected to strongly depend on clay content, with its cationic nature (pKa 10.12) reducing bioaccessibility through interactions with clay minerals. 6PPD-quinone is hypothesized to be taken up more than 6PPD but less than HMMM, DPG, and BTH, due to its higher stability and mobility in low organic carbon soils. 6PPD is hypothesized to show the lowest uptake due to its hydrophobicity, limited stability, and partial ionization at soil pH.

Hypothesis 3: TWCs are hypothesized to be detected in produce, with BTH and HMMM appearing more frequently and at higher concentrations due to their uptake potential and widespread occurrence in wastewater.

4. MATERIALS AND METHODS

4.1 Materials

Acetonitrile (LC/MS grade) was purchased from Sigma-Aldrich. d5-6PPD-quinone was purchased from HPC Standards. HMMM was purchase from TCI Europe. DPG, BTH and 6PPD were purchased from Sigma Aldrich. d4-BTH was purchased from LGC standards.

Lettuce (*Lactuca sativa*) seedlings were obtained from the Hishtil nursery in Ashkelon, IL. The arable soils used in the greenhouse experiment were previously collected from lysimeter stations in the Israeli Negev region: Sa'ad (31.470185, 34.534356), Nir Oz (31.309697, 34.402075) and Ein Hashlosha (31.351722, 34.403471). Selected properties of the studied soils are found in table 2.

Table 2: Properties of soils used in the greenhouse experiment[64]

Property	Sa'ad	Nir Oz	Ein Hashlosha
Clay (%)	27	4	13
Silt (%)	48	14	40
Sand (%)	25	82	47.5
Organic matter (%)	2.27 ± 0.07	1.04 ± 0.05	2.64 ± 0.01
$CaCO_3$ (%)	16	4	8
Cation exchange capacity (meq/100g)	21	11	16
pH	7.6	7.5	7.5

4.2 Methods

4.2.1 Greenhouse experiment

To investigate the potential plant uptake of TWCs, a greenhouse experiment was conducted. Lettuce plants (*Lactuca sativa*) were grown in three different soils collected from the Israeli Negev region (table 2*)*. Initially, three seedlings were planted in each pot and thinned out to one seedling over the course of two weeks. The plants were irrigated three times daily using an automated drip irrigation system.

After this initial acclimatization period, the irrigation water was spiked with a mixture of BTH, 6PPD, d5-6PPDq, HMMM and DPG at a concentration of 1000 ng/mL by adding 1 mL of a previously prepared stock mixture (100 µg/L) in ACN to 100 ml of water and irrigating manually while skipping automated dripped irrigation. Spiked solution was freshly prepared prior to each irrigation event, to minimize degradation of unstable compounds. To differentiate between 6PPD-quinone formed via oxidative transformation of 6PPD, the deuterated form of 6PPD-quinone, d5-6PPD-quinone was used. Negative controls were analogously irrigated with 100 mL of water with 1 mL of ACN added to it. Spiking occurred at regular intervals (3x weekly) during the growth period for a total of 1 mg of each compound added to the pots (fig A1*).*

During the growth period, pot location was randomized and adjusted regularly to minimize potential localized differences in humidity and temperature. The irrigation water volume was increased as the water demand of the plants increased. Temperature and humidity in the greenhouse were monitored continuously.

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Figure 6: Greenhouse setup

After 38 days of growth, the plants were determined to have reached a commercial size and were harvested. During harvest, the plants were partitioned in outer and inner leaves by separating the 10 outermost leaves from the rest of the plant. Roots were removed from the soil and washed with water. The soil in the pots was separated in a top (10 cm) and bottom section. Plant and soil samples were lyophilized and homogenized. Lyophilized samples were stored frozen until extraction.

4.2.1.1 Pore water sampling

To investigate the evolution of the concentrations of TWCs in the soil pore water, Rhizon samplers were utilized to extract pore water from the pots through suction by a syringe through the hydrophilic porous polymer membrane of the Rhizon sampler. In the beginning of the experiment, pore water was sampled immediately after spiking, 6 h and 24 h after spiking. As some of the compounds exhibit limited stability in water, samples were immediately extracted into acetonitrile following the protocol detailed in section 4.2.3.4 and extracts were stored frozen until analysis. Originally it was planned to perform a weekly pore water sampling, however, due to increased water demand of the plants, the soil in the root zone proved too dry to extract adequate pore water volumes as plant growth increased, limiting further sample collection after the first week of spiking.

4.2.2 Environmental Samples

For the monitoring study, fifteen lettuce heads (in triplicate) were sampled from Swiss supermarkets by the consumer magazine K-TIPP. The lettuces were grown in Switzerland, Spain and Italy and were purchased between February and April of 2023.

Additionally, thirteen different leafy green samples from Israel collected in a previous sampling campaign were used. These samples were collected in 2017 as part of a large survey investigating the occurrence of pharmaceuticals in produce.[60] Each of the samples represents a different agricultural field. For each sample, 10-15 plants were harvested, lyophilized and homogenized and treated as a composite sample.

4.2.3 Sample preparation and Extraction

4.2.3.1 Environmental Samples

The lettuce samples collected from Swiss supermarkets were frozen, shipped to Vienna and lyophilized. Israeli leafy green samples were already homogenized and lyophilized from a previous sampling campaign and were shipped to Vienna. As only trace concentrations of TWCs were expected in these samples an extraction protocol with pre-concentration was employed: Lyophilized lettuce heads were homogenized in a blender and three 1.5 g subsamples taken from each sample, extracted separately and combined before pre-concentration for a total mass of 4.5 g of each sample. Subsamples were transferred into 50 ml centrifuge tubes and 0.67 µg of ISTD (d5-6PPD-quinone & d4-BTH) was added along with 4 g of stainless-steel beads and 30 ml of LC/MS grade acetonitrile. The samples were subsequently subjected to bead-beating at 3000 rpm before centrifugation at 17000 g for 20 min at 20 °C. After centrifugation, 20 ml of supernatant was extracted into a separate vessel. 4 ml of ACN was added to the samples and extraction was repeated twice for a total extraction volume of 28 ml per subsample. Subsamples were combined and vacuum filtered through a 0.2 µm nylon filter before concentrating them to ~ 5 ml in a rotary evaporator (226 mbar, 60 °C). Concentrated samples were syringe filtered through a 0.2 µm filter into LC/MS vials before analysis.

4.2.3.2 Biomass

After harvest, the lettuce plants obtained in the greenhouse experiment were partitioned into three sections: the inner leaves, outer leaves (10 outermost leaves) and roots (washed with water). The samples were lyophilized and the dried biomass homogenized using a blender. For the leaf samples a subsample of 1 g was taken and weighed into a 15 mL centrifuge tube, for the roots the entire sample was used. 4 g of stainless steel beads, internal standard (500 ng d4-Benzothiazole) and 4 mL of Acetonitrile were added. Samples were then subjected to 3 min of beadbeating at 3000 RPM, followed by centrifugation at 5000 rpm for 5 min and recovery of 1 mL of supernatant. The extraction was repeated twice with 2 mL acetonitrile added each time and 2 mL supernatant were recovered in the subsequent extractions. The extracts were combined for a total extract volume of 5 mL, mixed and filtered through a 0.22 µm filter into a LCMS vial.

Due to an error during shipping and extraction of the root samples, it was not possible to use the data obtained from the roots and they will be omitted from further discussion.

4.2.3.3 Soil

The soil body of the pots was partitioned into the top 10 cm and the bottom part. Visible roots were removed from the soil. Soils were homogenized and from each section a combined sample was taken and lyophilized. Dried samples were ground using a mortar and pestle and a 2.5 g subsample was weighed into 15 mL centrifuge tubes. Internal standard (2.6 µg d4-Benzothiazole) and 4 g of stainless steel beads were added. 2 mL of 150 mg/L EDTA solution was added and the mixture was agitated on the bead beater at 4800 RPM for 3 min. 5 mL of acetonitrile was added and beadbeating was repeated, followed by the addition of 2 g Na_2SO_4 and 1 g NaCl and subsequent beadbeating, followed by centrifugation at 5000 RPM for 30 min. 4 mL supernatant was transferred into a clean glass vial, followed by the addition of 2 mL acetonitrile to the soil. After beadbeating and centrifugation, 2 mL of supernatant was combined with the first extract. Extracts were vortexed and filtered through a 0.22 µm nylon syringe filter into LCMS vials.

4.2.3.4 Pore Water

Aqueous pore water samples were extracted using a liquid-liquid extraction protocol: Internal standard (1.6 µg d4-Benzothiazole) was added into 3 mL of water sample, followed by the addition of 2 mL ACN and 300 mg NaCl. The mixture was shaken for 5 min at 400 RPM and the organic phase was transferred into a clean glass vial. 2 mL acetonitrile were added to the aqueous phase, followed by shaking. The organic phase was combined with the first extract, mixed and filtered through a 0.22 µm nylon syringe filter into a LCMS vial.

4.2.4.1 QA/QC

For the greenhouse experiment, triplicate lettuce plants were grown in each soil and irrigated with water without TWC spiking to serve as a negative control group.

During extractions, method blanks consisting of UPLC-grade acetonitrile were extracted at set intervals during the extraction procedure to identify and control potential contamination issues. For the leaf samples, after every seven extracted samples, a method blank was extracted. For the soil samples, after every nine extracted samples, a method blank was extracted.

Instrumental solvent blanks were used during measurement to identify potential carry over effects during the measurement.

A surrogate standard was employed to account for inconsistencies during extraction. To quantify the samples obtained from the greenhouse experiment, d4-Benzothiazole was used as a surrogate standard, the samples from the detection study were quantified using both b4-Benzothiazole and d5-6PPD-quinone as surrogate standards. Since d5-6PPD-quinone was added to the greenhouse system as a target compound, it was omitted from the standard mix for this set of samples.

For quantification of the samples obtained from the greenhouse experiment, a ten-point (0.1 – 100 ng/ml) matrix matched calibration curve (table A3) was prepared by spiking negative control biomass or soil with a defined amount of the compounds, then extracting them analogous to real samples to account for potential matrix effects on the measurement and to account for compound recovery. Negative control biomass and soil were analyzed and confirmed to not contain any analytes before preparing a matrix matched calibration curve.

For each compound, the limit of quantification (LOQ) was determined following the formula:

$$LOQ = mean\ (blank) + 3\ x\ \sigma\ (blank)$$

For compounds, for which no analyte signal was detected in the blanks, the lowest point on the calibration curve was set as LOQ. Values below LOQ were removed from the dataset.

4.2.4.2 Instrumental

Samples were analyzed via ultra-performance liquid chromatography – triple quadrupole mass spectrometry (Agilent 1290 Infinity II – Agilent 6470) using a C18 column (Acquity HSS T3, 1.8 µm, Waters, 50 mm) in positive ionization mode (capillary voltage: 2500 V) via multiple reaction monitoring (MRM). Flow rate of the LC was 0.6 mL/min at a column temperature of 40 °C. Injection volume was 5 µL. Mobile phase was made up of ultrapure water (Phase A) and acetonitrile (Phase B) containing 0.1 % formic acid each. The eluent gradient was held at 95 % Phase A for 1 min, then decreased to 5 % Phase B over 9 min, held at 5 % for 2 min and increased to 95 % for 1 min, followed by column re-equilibration at 95 % Phase A for 1 min. Electrospray ionization (ESI) was performed at a gas temperature of 240 °C and a gas flow rate of 5 L/min. The nebulizer was set to 30 psi with a sheath gas temperature of 250 °C and a gas flow rate of 11 L/min. Transitions from precursor ions to product ions can be found in table A4.

4.2.4.3 Data analysis

Raw data from LC/MS-MS measurement was processed using Agilent MassHunter Quantitative Analysis software. Further data processing was performed in R (Version 4.2.2).[65]

5. RESULTS AND DISCUSSION

5.1 QA/QC

In the method blanks used in the extraction no TWCs were detected. In the negative control biomass samples obtained from the greenhouse, concentrations were below LOQ for all compounds in all sample except for one sample of the outer leaves grown in the Sa'ad soil showing a concentration of 6PPD-quinone at 0.5 ng/g dw.

In the soil negative control samples, DPG was detected consistently at trace levels in the soil negative control samples in the Sa'ad and Ein Hashlosha soils as well as in one sample of the Nir Oz soil. As the soils used in the study are agricultural soils that have been irrigated with freshwater for two decades and DPG is detected in surface waters, this might account for the presence of DPG in the soils. In all other soil negative control samples, no TWCs are detected

LOQs of all compounds in each matrix are presented in table A4.

Due to the high LOQ (plant = 10 ng/µl; soil = 7.5 ng/µl) of benzothiazole in soil and plant biomass obtained from the greenhouse experiment all results concerning benzothiazole in the greenhouse experiment were omitted from the subsequent results.

5.2 Detection study

In this section the levels of tire wear compounds detected in leafy green samples collected from various fields in Israel and supermarket samples from Switzerland will be presented. Further detailed discussion, statistical analysis and estimation of human daily intake is published in Sherman et al[66]. Since the work presented here focused on the concentrations obtained, further discussion of the results is omitted from this thesis and referred to Sherman et al.

In Fig 7, the concentrations and detection frequencies of TWCs in the analyzed samples are presented. Sample information can be found in table A1. 71 % of samples contained at least one TWC. Six of the sixteen studied TWCs were detected in at least one plant sample. Benzothiazole shows the highest detection frequency (42.9 %) and the largest mean concentration (ranging from 8.3 – 238 ng/g dw). 2-hydroxybenzothiazole was present in one sample at a concentration of 665 ng/g dw. This sample also contained the highest measured concentration of BTH. Three substituted *p*-phenylenediamines were detected in the samples: 6PPD (detection frequency of 25 % and maximum concentration 0.4 ng/g dw), IPPD (detection frequency of 10.7 % and maximum concentration 0.1 ng/g dw) and CPPD (detection frequency of 3.6 % and maximum concentration 0.3 ng/g dw). DPG was present in 21.4 % of the samples at concentrations ranging from 0.3 – 2.1 ng/g dw. 5-methyl-1H-benzotriazole, HMMM, aniline, DPPD and all substituted *p*-phenylenediamine quinone derivatives were below limit of quantification in all samples.

	DPG	2OHBTZ	BTZ	6PPD	IPPD	CPPD
IL-01	<LOQ	665	238	<LOQ	<LOQ	<LOQ
CH-04	2.1	<LOQ	14.7	0.4	0.1	<LOQ
IT-04	0.4	<LOQ	28.8	0.2[b]	0.1[b]	<LOQ
IT-02	<LOQ	<LOQ	<LOQ	<LOQ	0.1	<LOQ
CH-02	<LOQ	<LOQ	<LOQ	0.3	<LOQ	<LOQ
CH-05	<LOQ	<LOQ	18.2[b]	0.3	<LOQ	<LOQ
CH-03	<LOQ	<LOQ	<LOQ	0.2	<LOQ	<LOQ
IL-12	<LOQ	<LOQ	<LOQ	0.2[a]	<LOQ	<LOQ
IL-02	<LOQ	<LOQ	101	<LOQ	<LOQ	<LOQ
IL-03	<LOQ	<LOQ	42.7	<LOQ	<LOQ	<LOQ
IL-04	<LOQ	<LOQ	52.2	<LOQ	<LOQ	<LOQ
IT-01	0.6	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
CH-01	0.4	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
ES-01	0.4	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
ES-02	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
ES-05	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
IT-05	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
IT-03	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
IL-05	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
IL-08	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
IL-06	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
IL-07	<LOQ	<LOQ	<LOQ[a]	<LOQ	<LOQ	<LOQ
ES-03	<LOQ	<LOQ	10.1	<LOQ	<LOQ	<LOQ
ES-04	<LOQ	<LOQ	9.1[b]	<LOQ	<LOQ	<LOQ
IL-09	<LOQ	<LOQ	8.3	<LOQ	<LOQ	<LOQ
IL-10	<LOQ	<LOQ	14.7	<LOQ[a]	<LOQ	<LOQ
IL-11	<LOQ	<LOQ	18.4[b]	<LOQ	<LOQ	<LOQ
IL-13	0.3	<LOQ	<LOQ	0.2[a]	<LOQ	0.3[a]

Figure 7: Concentrations of TWCs in leafy green samples. Units are ng/g dw. Superscript a indicates that one measurement duplicate was below LOQ. Superscript B indicates that variance between measurement duplicates was <30 % (adapted from Sherman et al, 2024[66])

5.2 Plant uptake

5.2.1 Soil

In Fig 8, the concentrations of TWCs in the top (10 cm) and bottom soil at the time of harvest are presented. There is a strong variation between the concentrations of the compounds which can be partially attributed to the differences in the extraction recoveries ranging from 7 % to 107 % (table 3). Depending on soil type and compound, the formation of non-extractable residues can strongly influence the recovery. As only the recoverable fraction of the compound is deemed accessible for plant uptake, no correction for recovery was performed. Additionally, stability and mobility of the compounds in soil influence the soil concentrations.

6PPD is present at low concentrations (maximum concentration of 1.8 ng/g dw) in the soil. The limited stability of 6PPD likely caused it to be degraded quickly in the soil system. Formation of non-extractable residues could also contribute to the low concentrations 6PPD, as it showed low soil recoveries with a maximum of 43 % (table 3), however as the no soil control showed a recovery of 63 %, degradation is the most likely cause.

6PPD-quinone exhibits the largest soil concentrations of all compounds in all soils at a maximum concentration of 69.1 ng/g dw. Recoveries in all three soils were between 83-97 %. The high soil concentrations combined with the recoveries indicate that 6PPD-quinone is readily accessible in the soil. The low organic carbon content of the studied arid soils (table 2) likely enhanced the accessibility of 6PPD-quinone in this experiment. Due to its hydrophobic nature, 6PPD-quinone strongly sorbs to rubber materials[35] and is retained by soils with high organic carbon content.[37]

DPG was found in the soil at a maximum concentration of 23.01 ng/g dw in one sample of the Sa'ad soil. This sample however is a possible outlier as the concentration is five times higher than in the other two samples. Surprisingly, concentrations of DPG are similar in the top and bottom soil. Compared to the other compounds, this could indicate some vertical mobility of DPG in the soil column. However, DPG is cationic at soil pH and therefore expected to exhibit charge-based interactions with negatively charged soil constituents, hindering its mobility. As DPG is consistently present in the negative control soil samples, the detection of DPG in bottom soil is more likely to be attributed to the background concentrations of DPG in the system than to vertical mobility.

HMMM exhibits a maximum soil concentration of 0.89 ng/g dw. Compared to the other compounds, soil concentrations of HMMM are rather low. As a moderately stable compound, it is unlikely that this low concentration is caused by full degradation of the compound, partial microbial degradation and abiotic hydrolysis are however expected to occur. In all three studied soils, HMMM has the lowest recoveries of all compounds (table 3), pointing towards the formation of non-extractable residues in soil. Low soil recoveries of HMM have been reported in literature.[25]

Table 3: Soil recoveries of analyzed TWCs

	DPG	BTZ	HMMM	6PPD	d5-6PPD-quinone
No soil	94 ± 9 %	99 ± 3 %	98 ± 2 %	61 ± 3 %	97 ± 2 %
NO	107 ± 0 %	37 ± 3 %	7 ± 1 %	43 ± 1 %	96 ± 0 %
SA	99 ± 2 %	56 ± 10 %	17 ± 2 %	24 ± 3 %	83 ± 2 %
EH	99 ± 5 %	45 ± 2 %	20 ± 10 %	23 ± 1 %	87 ± 5 %

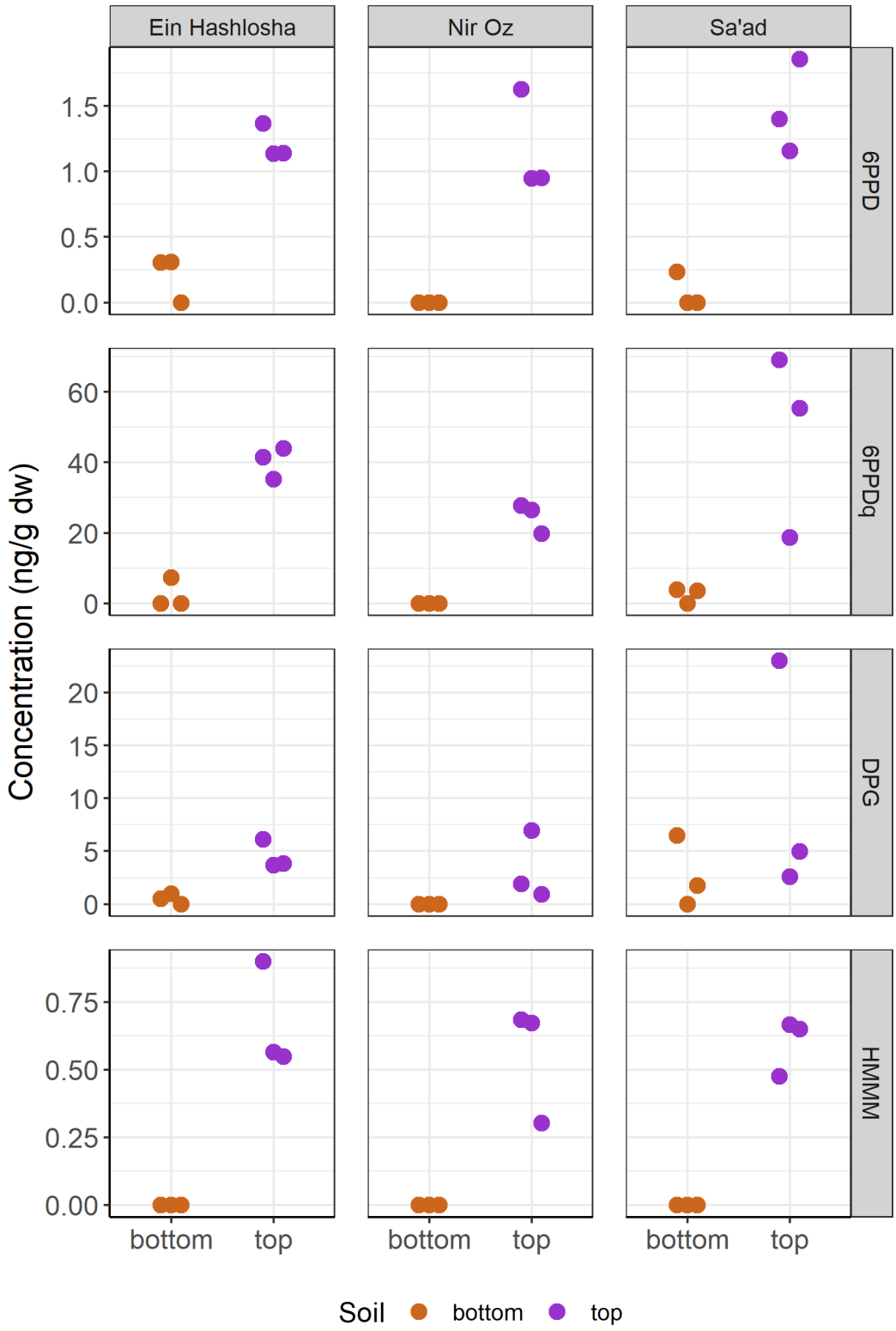

Figure 8: Soil concentrations of TWCs in top and bottom soil

In Fig 9, the concentrations of TWCs in soil with a plant compared to the bare soil without plants are displayed. 6PPD shows lower concentrations in the soils with a plant, indicating depletion by the plants. In the Nir Oz and Ein Hashlosha soils, concentrations of DPG are lower in the soils with a plant, in Sa'ad soil they are equal, indicating stronger retention by the soil and lower depletion by the plants. HMMM shows no difference in the concentration between bare soil and soil with plant, however only trace concentrations of HMMM can be found in both, likely due to the formation of non-extractable residues. For 6PPD-quinone, depletion with respect to the bare soil can only be observed in the Nir Oz soil, possibly connected to the largest uptake of 6PPD-quinone in the Nir Oz soil.

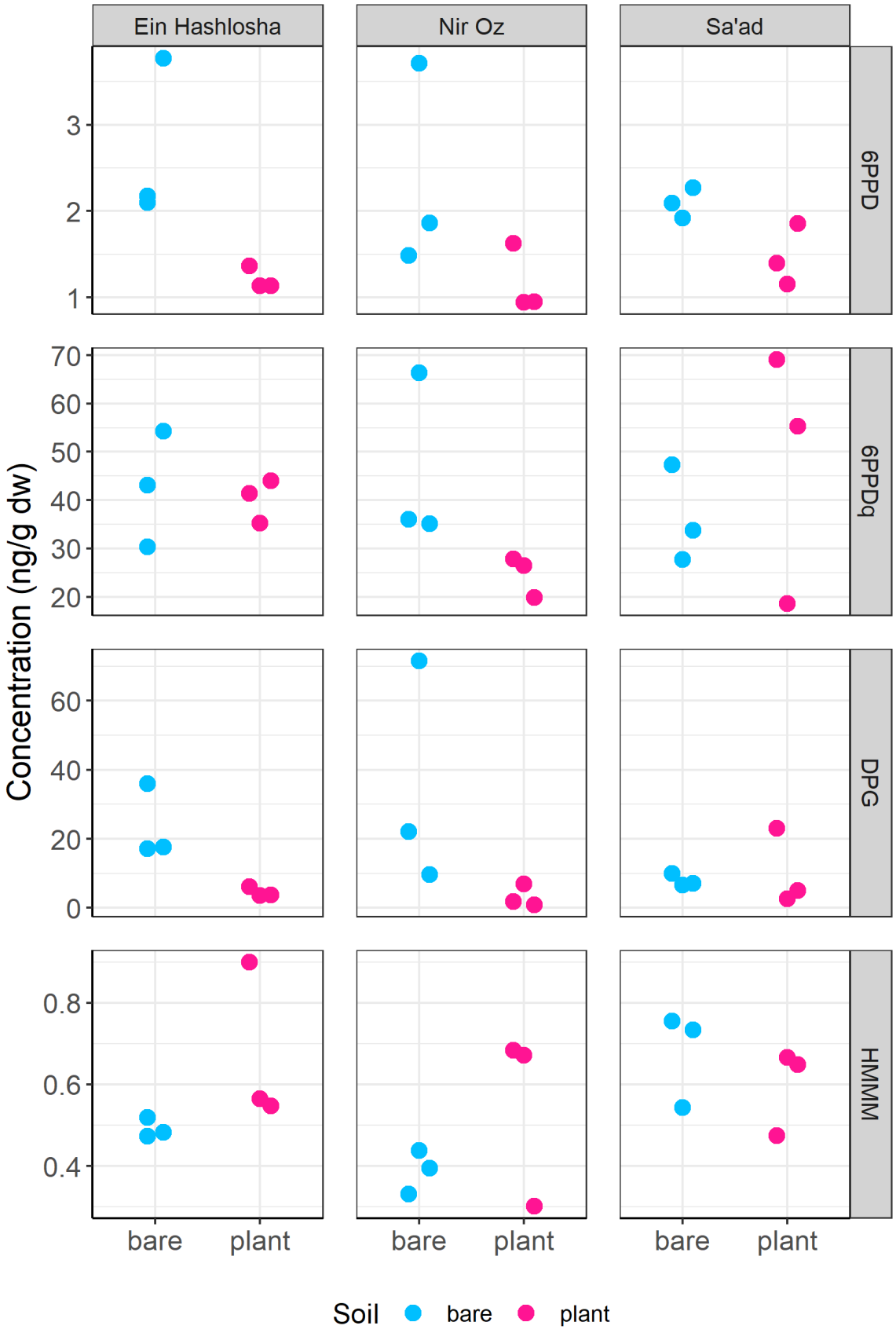

Figure 9: Soil concentration of TWCs in pots with plant and without

5.2.2 Leaves

In Figure 10 the concentration of TWCs in the inner and outer (outermost 10 leaves) leaves grown in the three different soils is presented. Generally, all compounds were taken up by the plants, with the lowest uptake occurring in the plants grown in the Sa'ad soil. This soil has the highest clay content (table 2) of the tested soils, potentially leading to higher retention of the compounds in the soil by sorption to the clay mineral surfaces. This effect of the clay soil was observed for all tested compounds.

Generally, the levels of TWCs in the outer leaves are higher than in the inner leaves. Due to the older age of the outer leaves, their larger size and their larger exposure to light, evapotranspiration is higher in the outer leaves than in the inner leaves. Assuming a mostly passive transpiration stream uptake effect, as the compounds are transported through the plant system with the flow of water, higher evapotranspiration leads to a larger accumulation of the compounds in the outer leaves compared to the inner leaves[67]. The same trend has been observed in studies for carbamazepine[8].

6PPD shows the lowest leaf concentration in all soils with a maximum concentration of 3.75 ng/g dw. The hydrophobicity of 6PPD (log K_{ow} = 4.77) is far from the optimal value for plant uptake. Additionally, 6PPD is susceptible to biotic and abiotic degradation and shows limited stability in complex aqueous systems[68]. As the concentration of 6PPD was low in the bare soil (fig 9), 6PPD likely degraded in the system.

6PPD-quinone was taken up by the plants with a maximum concentration of 94.45 ng/g dw at similar levels to DPG and HMMM even though as a rather hydrophobic (log K_{ow} = 4.30) compound it is not ideal for plant uptake. 6PPD-quinone is shown to be rather stable in simple aqueous systems[36] however this stability likely decreases with increased solution complexity.

DPG has a maximum leaf concentration of 66.04 ng/g dw. DPG is a moderately hydrophobic compound and is readily taken up by plants. It shows the lowest concentrations in plants grown in the Sa'ad soil (highest clay content). As DPG is cationic at the pH of the studied soils, retention by sorption to clay minerals is expected. DPG also shows the highest concentrations in plants grown in the Nir Oz soil which contains the least clay of all studied soils.

HMMM has a maximum leaf concentration of 32.51 ng/g dw; it is moderately hydrophobic and readily taken up by plants. In a previous hydroponic study using the same compounds, concentrations of HMMM exceeded concentrations of DPG by one order of magnitude. Combined with the low soil concentrations of HMMM, this points towards the formation of non-extractable residues hindering the uptake of HMMM in a soil system.

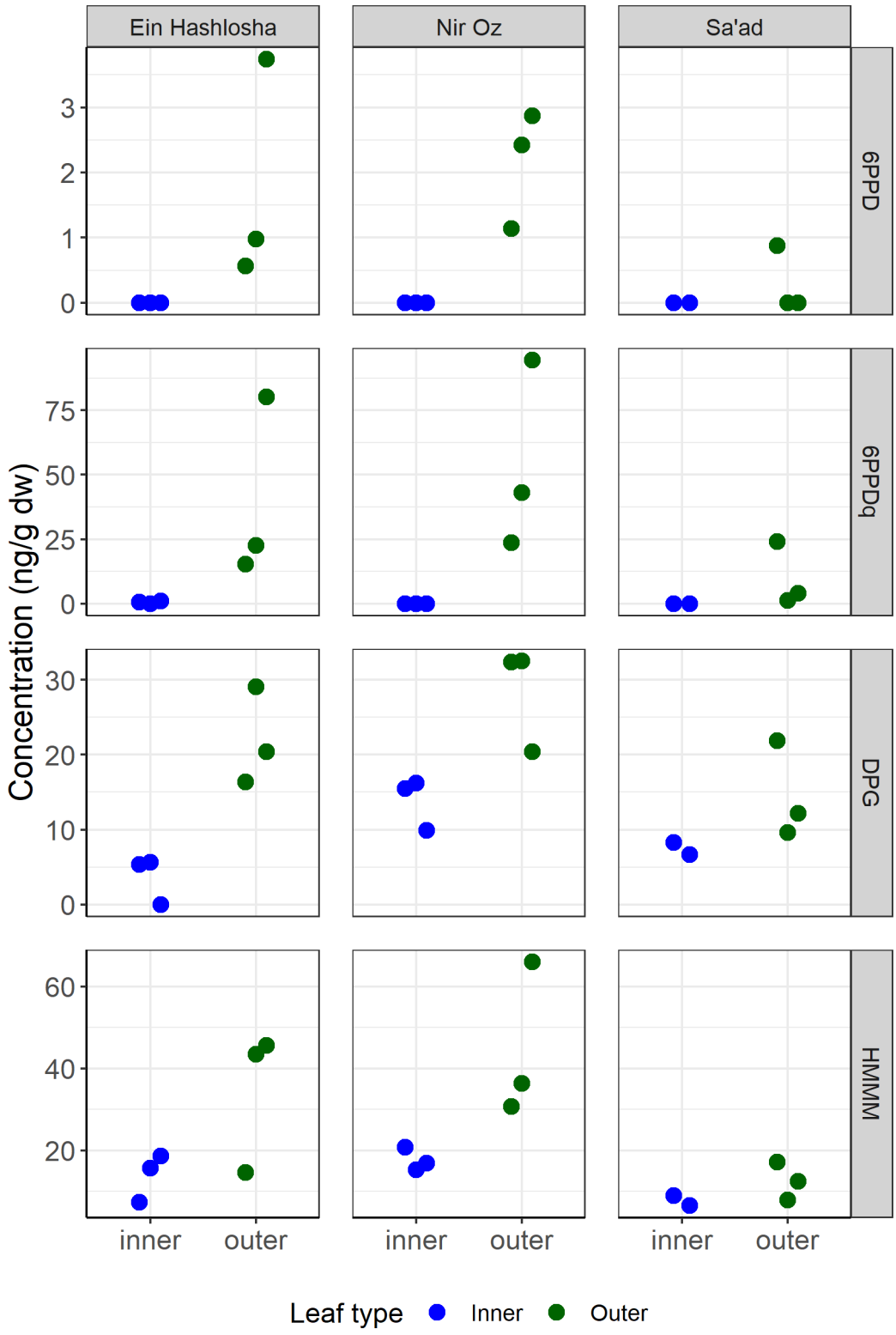

Figure 10: Leaf concentration of TWCs after harvest in inner and outer leaves

Generally, leaf concentrations of TWCs follow the order Nir Oz > Ein Hashlosha > Sa'ad for each of the compounds, with lower clay content of the soil (table 2) leading to higher concentrations of TWCs in the plant biomass. Nir Oz (1.04 %) soil also contains lower organic carbon and clay than the Sa'ad (2.27 %) and Ein Hashlosha soils (1.04 %), increasing the bioaccessibility of the compounds.

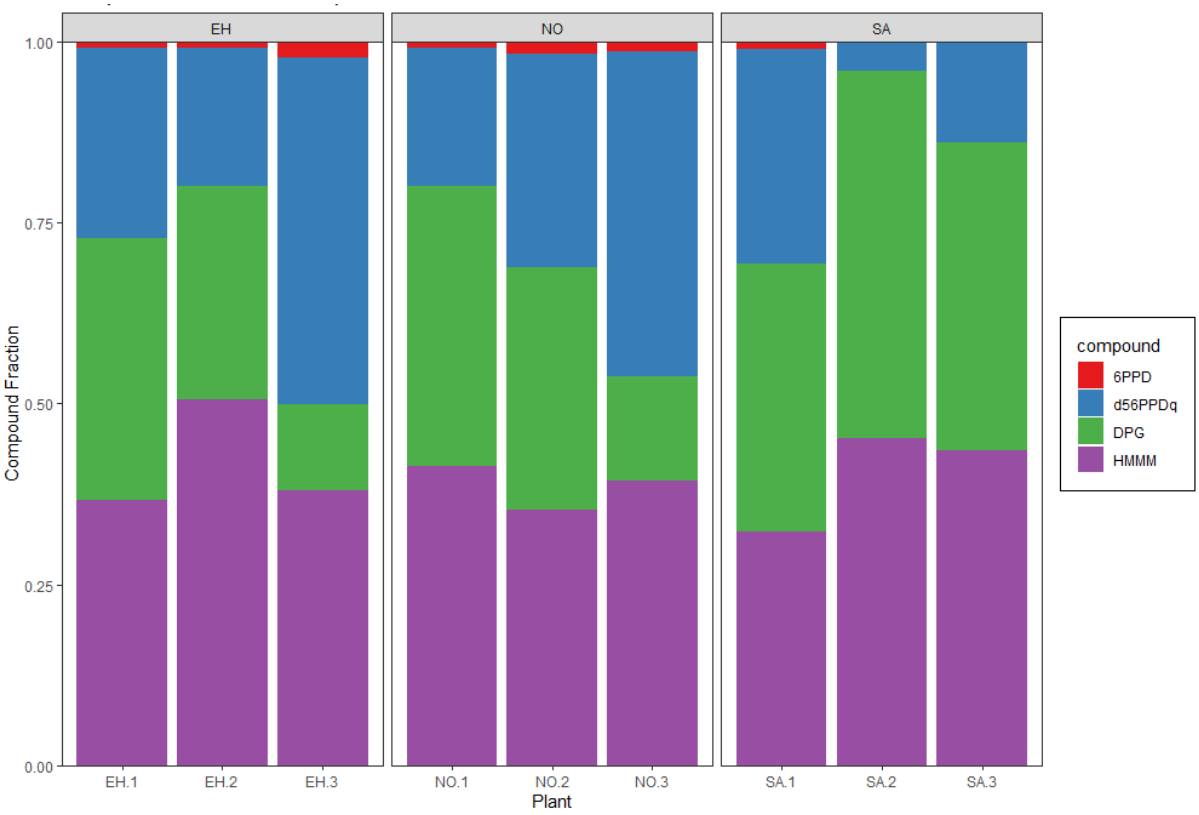

Figure 11: Compound profile of each singular plant after harvest

Soil type does not appear to have a large impact on the compound profile in the plants (fig 11). All compounds were affected equally by the soil type, even though their properties would imply different accessibility in different soil types. For example, DPG is present in its cationic form which leads to stronger retention by negatively charged clay minerals, so a lower fraction of extractable DPG was expected in the Sa'ad soil. Although the soils differ in their organic carbon content (table 2), as arid soils they are generally low in organic carbon, having a negligible effect on sorption of hydrophobic compounds. Compound properties appear to play a limited role in controlling the variation between soils.

5.2.3 Bioconcentration factors

Combining the concentrations of TWCs in soil and leaf leads to the bioconcentration factor defined as

$$BCF_{soil} = \frac{C_{leaf}}{C_{soil}}$$

The bioconcentration factor yields a measure to which degree the compounds are concentrated in the leaves relative to the available concentration in the soil. In fig 12, the bioconcentration factors based on the soil concentrations are displayed.

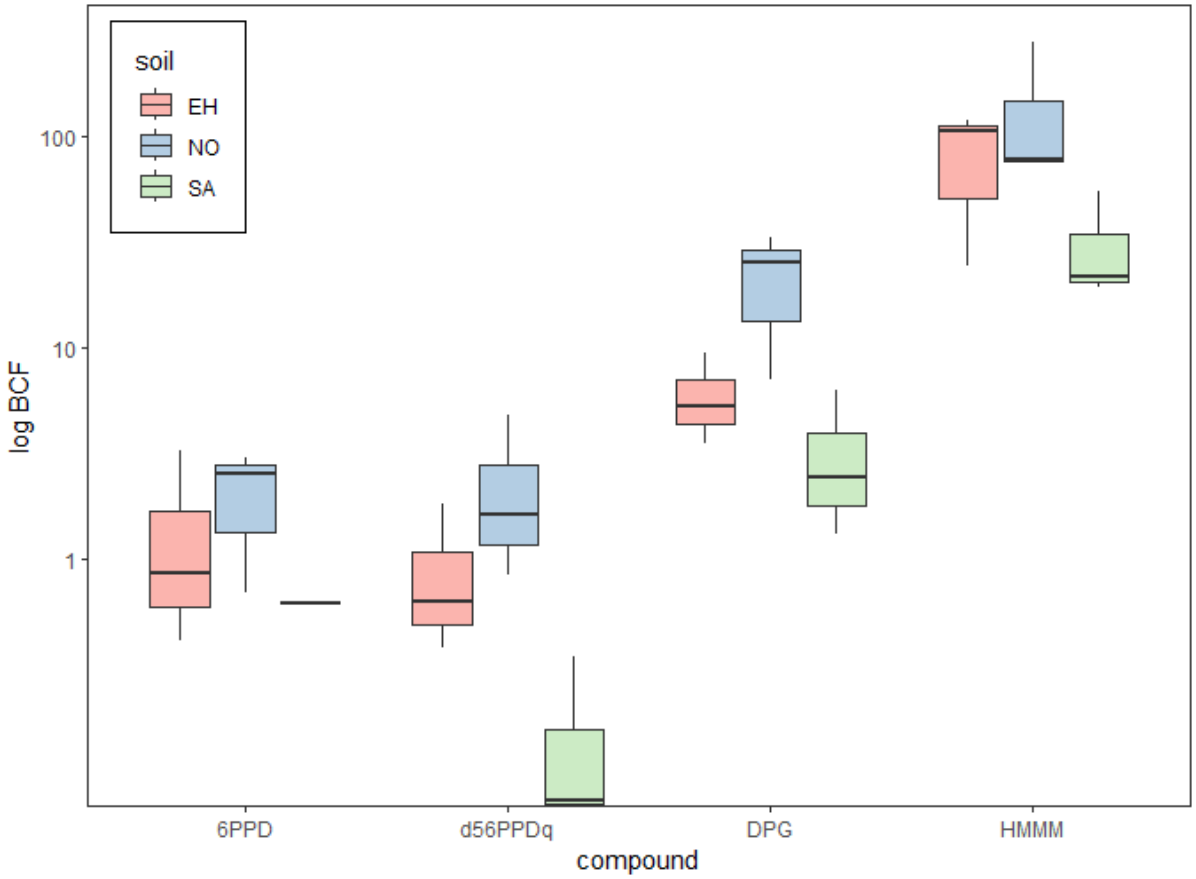

Figure 12: Logarithmic bioconcentration factors of TWCs

HMMM and DPG exhibit the largest bioconcentration factors of the tested compounds, as they are readily taken up by the plants if available. Even though soil concentrations of HMMM and DPG are rather low (fig 8), they show high concentrations in the plant leaves. As moderately hydrophobic compounds (table 1), HMMM and DPG are readily taken up by the plants.

Although 6PPD-quinone was present in the leaves at surprisingly high amounts, it was also present in the soils at a high level. As the physicochemical properties of 6PPD-quinone (table 1) are outside of the ideal range of lipophilicity for plant uptake, the high concentrations in the leaves were surprising.

This can be explained by its low soil bioconcentration factor: 6PPD-quinone was taken up to a large degree because it was readily accessible in the soil, not due to a high uptake.

6PPD exhibits a low bioconcentration factor and is also present in the leaves at low levels. Compared to 6PPD-quinone which has a similar hydrophobicity, 6PPD was taken up less due to its limited stability in the soil.

Comparing these soil bioconcentration factors to the translocation factors (C_{leaf} / C_{root}) obtained from previous hydroponic experiments[19], the same trend is observed as the factors follow an order of HMMM >> DPG > 6PPDq ≈ 6PPD. Uptake and translocation mechanisms do not differ between hydroponically grown plants and plants grown in soil. The difference in leaf concentrations between the hydroponic and the soil experiments points towards the importance of the bioaccessible compound fraction in determining plant uptake as a controlling factor in the uptake of these compounds under realistic growing circumstances as translocation trends are preserved and independent of the growing matrix. Even though a compound is outside the optimal range of hydrophobicity for plant uptake, levels in the plants can be similar to a less accessible compound with a more optimal hydrophobicity for plant uptake.

5.2.4 Pore water

In Fig 13, the pore water concentration for DPG recovered through the Rhizon samplers in the 24 h after the initial spiking for the plants grown in each respective soil are displayed, comparing replicates with a plant to replicates without plant. Dashed lines show the concentration of DPG in the pore water at the end of the experiment.

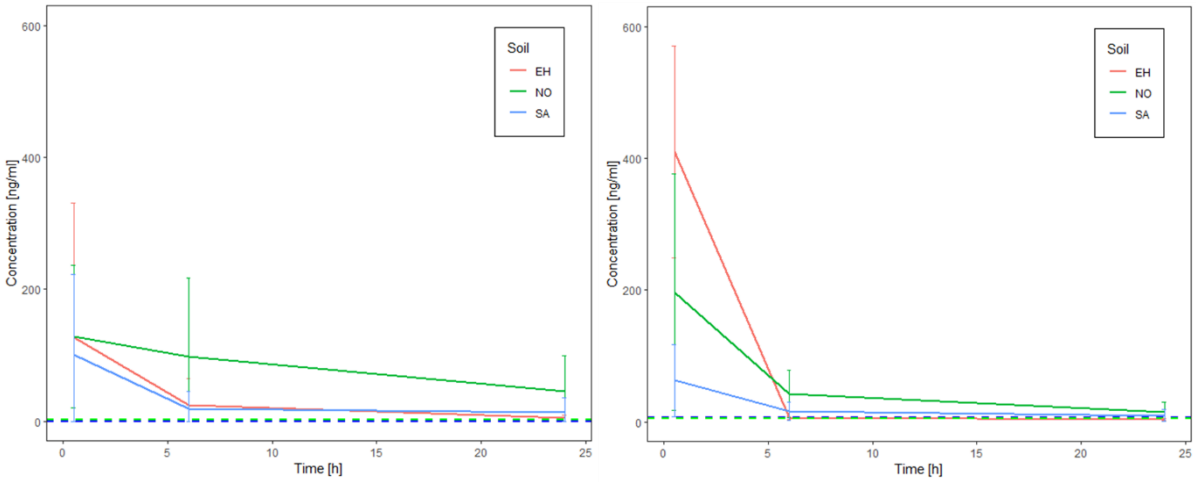

Figure 13: Pore water concentrations of DPG (left without plants; right with plants)

In Fig 14, the pore water concentration for HMMM recovered through the Rhizon samplers in the 24 h after the initial spiking for the sample grown in each respective soil are displayed, comparing replicates with plant to replicates without plant. Dashed lines show the concentration of HMMM in the pore water at the end of the experiment.

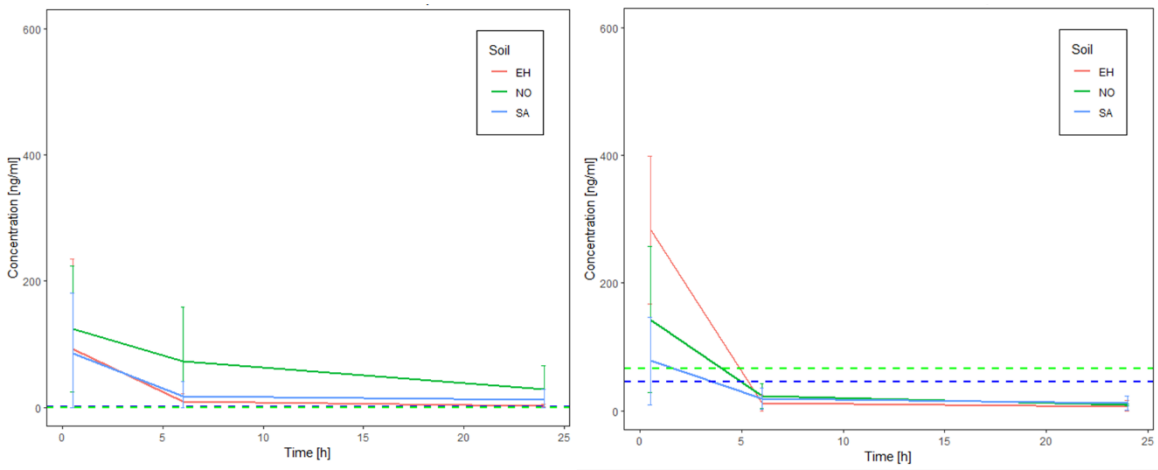

Figure 14: Pore water concentrations of HMMM (left without plants; right with plants)

Pore water sampling was met with some issues, as pre-experiments showed that 6PPD and 6PPD-quinone could not be recovered through the rhizon samplers, possibly due to sorption of these rather hydrophobic compounds to some material in the rhizon samplers, as 6PPD-quinone has been shown to sorb strongly to plastic materials[36]. In table 4, the recoveries of the individual compounds through the rhizon samples from an aqueous test solution are presented.

Table 4: Recovery percentages of TWCs through rhizon samplers

Compound	DPG	BTH	6PPD	6PPD-q	HMMM
Recovery %	100.4	95.1	1.3	8.5	100.8

Originally, it was planned to conduct pore water sampling weekly throughout the growing period of the lettuce to observe changes in the pore water concentrations as the amount of TWCs added to the pot system increased through continued spiking of the irrigation water. However, as the plants increased in size and their growth accelerated, the water demand of the plants increased, lowering the water content in the root zone of the pots. This reduced water content in the root zone proved to be too dry for adequate pore water recovery through the rhizon samplers as they were placed in the root zone. Increasing the irrigation volume to provide adequate water content for the rhizon samplers was deemed to be disadvantageous for plant health, so the pore water sampling was omitted in favour of healthy plants.

From the limited pore water data available however, some information can be gathered:

In the 24 h after the initial spiking, the concentration of TWCs in the pore water (which is the fraction available for plant uptake[69]), decreases rapidly, as the water is distributed throughout the soil system, sorption to soil components occurs and the plants are taking up some of the compound. For HMMM in the replicates with plant (fig 14), the concentration at the end of the experiment was higher than initial concentrations, and higher than the concentration in the replicates without plant at the same timepoint, indicating the possibility of a solubilization process potentially caused by root exudates of the plant. However, as the final concentration is based on only one obtained sample, this may not be a significant difference. In addition, as the water content of the soil decreased with increased plant transpiration and the TWC concentration increased with ongoing spiked irrigation this effect could be caused by a concentrating effect.

Although the rhizon samplers are a practical way of sampling pore water *in situ* they apparently require almost complete water saturation of the soil, making it difficult to extract pore water under optimal plant growth conditions for lettuce plants, especially as the water content in the root zone is depleted through plant transpiration. This could potentially be overcome by increasing the suction on these rhizon samplers. In addition, recovering the more hydrophobic of the compounds (6PPD & 6PPD-quinone) was not possible. For future experiments, the addition of multiple rhizon samplers per pot for more robust replicates could also be considered, although this would drastically increase the number of samples needing to be processed. Investigating the dissolved fraction in plant uptake experiments has the chance to provide valuable data as the fraction of the compounds available to the plants is the dissolved fraction rather than the total soil concentration.

6. CONCLUSIONS & OUTLOOK

6.1 Conclusions

In the following paragraphs conclusions regarding the hypotheses presented in section 3 are presemted:

Hypothesis 1: The hypothesis that soil clay content influences TWC uptake was confirmed. Leaf concentrations followed the predicted trend, decreasing with increasing clay content. The lowest levels were observed in Sa'ad soil (highest clay content), intermediate levels in Ein Hashlosha soil, and the highest levels in Nir Oz soil. This trend was observed for all tested compounds.

Hypothesis 2: The hypothesis that TWC concentrations in lettuce follow the order HMMM > BTH > DPG > 6PPD-q > 6PPD was rejected. Instead, compounds were taken up in the order 6PPDq ≈ HMMM ≈ DPG >> 6PPD.

HMMM was taken up, however at levels similar to DPG and 6PPD-quinone. In soil, HMMM bioaccessibility was strongly limited by the formation of non-extractable residues, evidenced by its low soil recovery percentages and low concentrations in soil. However, HMMM exhibited the highest BCF of the studied compounds, confirming its tendency for plant uptake and limitation by accessibility. BTH was not quantifiable due to its high LOQ. DPG was taken up at similar levels to 6PPD-quinone and HMMM. Surprisingly, the compound profile was unaffected by soil type, so DPG was not specifically affected stronger by increasing clay content, even though it is fully cationic at soil pH. 6PPD was taken up the least as hypothesized. Its limited stability and hydrophobicity rendered it both unfavourable for plant uptake and not accessible to the plants. 6PPD-quinone was taken up at levels similar to DPG and HMMM which was surprising, considering its hydrophobicity and assumed limited stability. However, it proved to be readily accessible in soil, leading to high uptake even though its hydrophobicity is outside the ideal range for plant uptake. This is supported by its low BCF and high concentration in soil, indicating that the high uptake was caused by its accessibility.

Hypothesis 3: TWCs were detected in produce. BTH was detected frequently and at high concentrations, owing to its bioaccessibility and ubiquitous occurrence in wastewater. Surprisingly, HMMM was not detected in any of the samples. However, in the greenhouse experiment, HMMM was found to be strongly limited by its bioaccessibility through the formation of non-extractable residues. This could be the cause for HMMM being <LOQ in the produce samples. In realistic scenarios, the bioaccessibility of HMMM limits its uptake by plants. 6PPD and other substituted *p*-phenylenediamines were detected occasionally at low concentrations, which is surprising, considering 6PPDs low uptake in the greenhouse experiment.

In the following paragraphs some additional conclusions obtained from this work are presented.

Low vertical mobility of the compounds in soil was observed. In an agricultural scenario in these specific soils, this indicates their accumulation in the upper soil horizon, where they are potentially accessible to plant roots. If environmental conditions change, compounds retained as type I NERs could potentially be remobilized and be available for plant uptake in the future.

The available concentration in the pore water decreases quickly after irrigation with spiked water. This points towards the increased potential for plant uptake for regular irrigation with contaminated water. A single irrigation event with highly concentrated TWC-contaminated water (for example a flooding scenario) poses less potential for plant uptake than prolonged irrigation with lower concentrations

As concentrations of TWCs are larger in outer leaves compared to inner leaves, a predominantly passive transpiration stream uptake mechanism can be assumed. Outer, older leaves are larger and subjected to higher temperature and more sunlight and therefore show increased transpiration.

In conclusion, plant uptake is a potential route for TWCs to enter the human food chain. Compounds are taken up under realistic agricultural conditions and are detected in produce. Uptake is controlled by a combination of two main factors: Accessibility in soil and compound-specific properties. Studies assessing the potential health risks of these compounds to consumers should take this into account.

6.1 Consideration for future research

Future research should more closely examine the formation of non-extractable residues in soil as these have a large effect on bioaccessibility. Especially for a compound like HMMM, where the formation of NERs appears to have a large impact on its bioaccessibility, a closer investigation of the involved mechanisms and elucidation of the different types of NERs formed could provide valuable insights. This could be studied by utilizing ^{14}C-labelled compounds and adapting a procedure similar to Wu et al.[53]

Optimizing pore water sampling will also provide a clearer insight into the bioaccessible fraction. Investigation and refinement of different sampling techniques like centrifugation (as described in Carter et al[70]), lysimeters or vacuum extraction could provide more robust pore water data which is crucial in understanding bioaccessibility.

Additionally, as soil clay content has a strong influence on uptake of TWCs, a detailed investigation of the sorption mechanisms on the individual soil constituents could provide fruitful data. Specifically, an investigation on effects of different clay mineral types such as kaolinite (1:1, uncharged layers) or montmorillonite (2:1, charged layers) on the sorption behaviour of cationic compounds like DPG could yield a deeper understanding of the behaviour of these compounds in soil systems.

Applying a mathematical model for plant uptake of ionizable chemicals as published by Trapp et al[71] for the diverse and heterogenous family of TWCs could provide a list of potentially understudied compounds of interest, which could then be further investigated in a similar greenhouse experiment.

However, as these models depend on empirical physico-chemical data, which is scarce and often not standardized, a robust experimental determination of basic physico-chemical parameters of common TWCs such as hydrophobicity, pKa, stability and biodegradation would provide a valuable and important dataset, enabling researchers to base models and risk assessments on sound, high quality data.

Further investigation into the entrance pathways for TWCs into agricultural fields, by elucidating TWC levels in treated wastewater and biosolids will provide a deeper insight into the scope of potential risks associated with TWCs and agriculture. Differentiating the entrance pathways of TWCs by atmospheric deposition, biosolid fertilization and TWW irrigation through a systematic study investigating these compartments for the occurrence and fluxes of TWWs could provide a more complete picture of the fate of these compounds in agricultural systems.

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I. APPENDIX

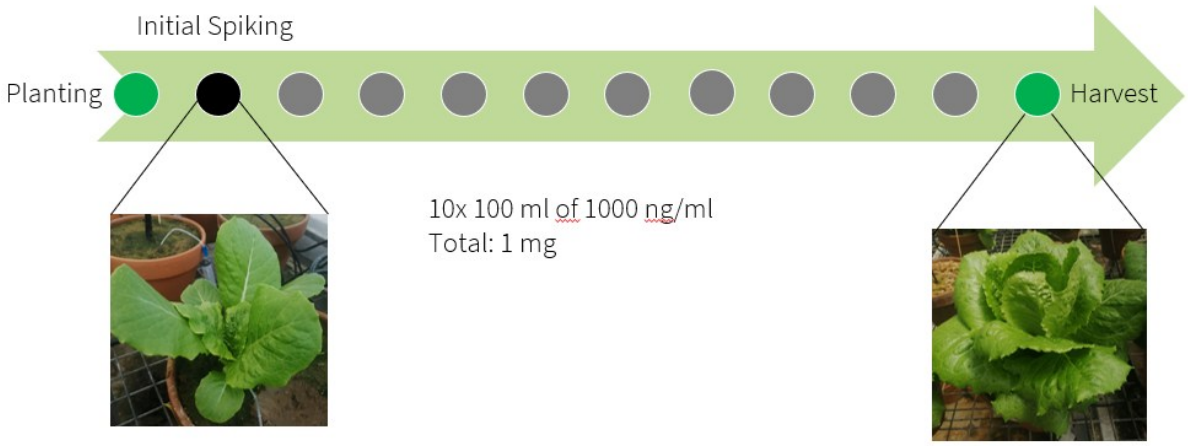

Figure A1: Experimental timeline of spiking events

Table A1: Sample details

Sample	Crop	Country of origin
IL-01	Lettuce	Israel
IL-02	Parsley	Israel
IL-03	Mint	Israel
IL-04	Mint	Israel
IL-05	Parsley	Israel
IL-06	Coriander	Israel
IL-07	Parsley	Israel
IL-08	Parsley	Israel
IL-09	Celery	Israel
IL-10	Dill	Israel
IL-11	Parsley	Israel
IL-12	Beetroot leaf	Israel
IL-13	Beetroot leaf	Israel
CH-01	Chicory	Switzerland
CH-02	Corn salad	Switzerland
CH-03	Corn salad	Switzerland
CH-04	Lettuce	Switzerland
CH-05	Corn salad	Switzerland
IT-01	Lettuce	Italy

IT-02	Lettuce	Italy
IT-03	Lettuce	Italy
IT-04	Spinach	Italy
IT-05	Arugula	Italy
ES-01	Lettuce	Spain
ES-02	Lettuce	Spain
ES-03	Lettuce	Spain
ES-04	Lettuce	Spain
ES-05	Lettuce	Spain

QA/QC

Table A2: Mass of TWC spiked on biomass for matrix matched calibration

Calibration level in ng/ml	**Spiked mass of TWC in ng**
0.1	0.5
1	5
2.5	12.5
5	25
7.5	37.5
10	50
25	125
50	250
75	375
100	500

Limits of quantification for TWCs in the greenhouse experiment:

Table A3: LOQs of TWCs

Compound	**LOQ plant ng/µl**	**LOQ soil ng/µl**	**LOQ pore water ng/µl**
DPG	1	0.1	0.1
BTH	10	7.5	2.5
6PPD	0.1	0.1	0.1
d5-6PPD-q	0.1	0.1	0.1
HMMM	0.1	0.1	0.1

Table A4: Transitions from precursor ions to product ions for all analyzed TWCs, quantifier ions in **bold**

Compound name	Surrogate standard	Precursor ion m/z	Product ion m/z	Fragmentor voltage (V)	Collision energy (V)	Cell accelerator voltage (V)	t_R (min)
2-aminobenzothiazole	**d4-BTZ**	**151**	**109**	**150**	**30**	**5**	**1.7**
2-aminobenzothiazole		151	65	150	38	5	1.7
2-hydroxybenzothiazole	**d4-BTZ**	**152**	**124**	**140**	**22**	**4**	**3.6**
2-hydroxybenzothiazole		152	92	140	26	4	3.6
2-mercaptobenzothiazole	**d4-BTZ**	**168**	**135**	**135**	**28**	**5**	**4.2**
2-mercaptobenzothiazole		168	124	135	24	5	4.2
2-mercaptobenzothiazole		168	109	135	28	5	4.2
6PPD	**d5-6PPD-q**	**269**	**184**	**150**	**45**	**5**	**5.0**
6PPD		269	107	150	45	5	5.0
6PPD		269	93	150	45	5	5.0
6PPD-quinone		299	241	150	53	5	7.1
6PPD-quinone		299	215	150	30	5	7.1
6PPD-quinone	**d5-6PPD-q**	**299**	**187**	**150**	**30**	**5**	**7.1**
Aniline	**d4-BTZ**	**94**	**77**	**100**	**22**	**4**	**2.8**
Aniline		94	51	100	23	4	2.8
Aniline		94	50	100	41	4	2.8
Benzothiazole	**d4-BTZ**	**136**	**109**	**150**	**31**	**5**	**3.9**
Benzothiazole		136	77	150	27	5	3.9
Benzothiazole		136	65	150	38	5	3.9
Benzothiazole-d4		140	113	150	31	5	3.8
Benzothiazole-d4		140	81	150	19	5	3.8
Benzothiazole-d4		**140**	**69**	**150**	**36**	**5**	**3.8**
CPPD	**d5-6PPD-q**	**267.2**	**184**	**100**	**30**	**5**	**4.7**
CPPD		267.2	107	100	50	5	4.7
CPPD		267.2	93.1	100	46	5	4.7
CPPD-quinone		297.1	215	130	18	5	6.8

CPPD-quinone	**d5-6PPD-q**	**297.1**	**187**	**130**	**34**	**5**	**6.8**
CPPD-quinone		297.1	55.2	130	50	5	6.8
d5-6PPD-quinone		**304.2**	**246.1**	**110**	**36**	**4**	**7.0**
d5-6PPD-quinone		304.2	220.1	110	36	5	7.0
d5-6PPD-quinone		304.2	192.1	110	36	5	7.0
DPG		212	195	150	20	5	2.8
DPG	**d5-6PPD-q**	**212**	**119**	**150**	**20**	**5**	**2.8**
DPG		212	94	150	20	5	2.8
DPPD	**d5-6PPD-q**	**260.1**	**183**	**130**	**38**	**5**	**7.3**
DPPD		260.1	167	130	46	5	7.3
DPPD		260.1	156	130	46	5	7.3
DPPD-quinone	**d5-6PPD-q**	**291.1**	**263**	**150**	**22**	**5**	**6.4**
DPPD-quinone		291.1	235.2	150	34	5	6.4
DPPD-quinone		291.1	144	150	38	5	6.4
HMMM		391	283	150	15	5	4.7
HMMM		391	253	150	23	5	4.7
HMMM		391	207	150	19	5	4.7
HMMM	**d5-6PPD-q**	**391**	**177**	**150**	**35**	**5**	**4.7**
IPPD	**d5-6PPD-q**	**227.2**	**184**	**100**	**18**	**5**	**3.8**
IPPD		227.2	118	100	46	5	3.8
IPPD		227.2	107	100	46	5	3.8
IPPD-quinone		257.2	216	110	30	5	5.6
IPPD-quinone	**d5-6PPD-q**	**257.2**	**187**	**110**	**30**	**5**	**5.6**
IPPD-quinone		257.2	170	110	34	5	5.6
5M-1H-BTR	**d4-BTZ**	**134.1**	**77.1**	**108**	**31**	**5**	**3.2**
5M-1H-BTR		134.1	79.1	108	23	5	3.2
5M-1H-BTR		134.1	51.2	108	45	5	3.2

Plants:

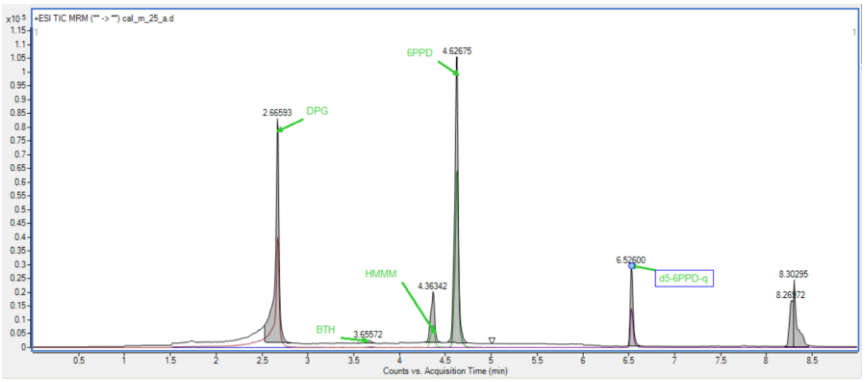

Figure A2: Chromatogram of TWCs in plant samples

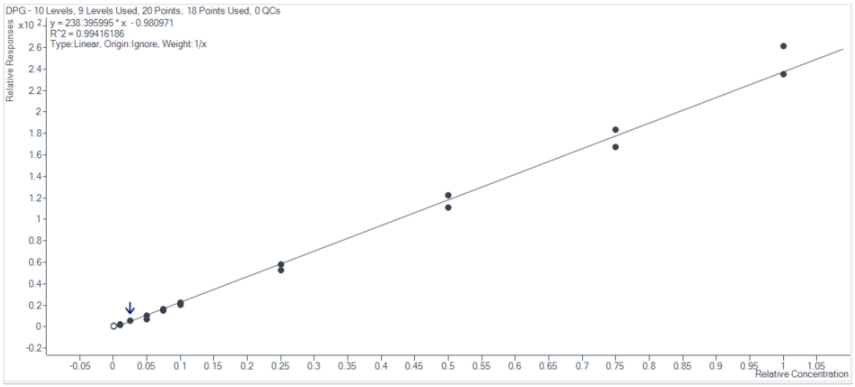

Figure A3: Calibration curve of DPG in plants

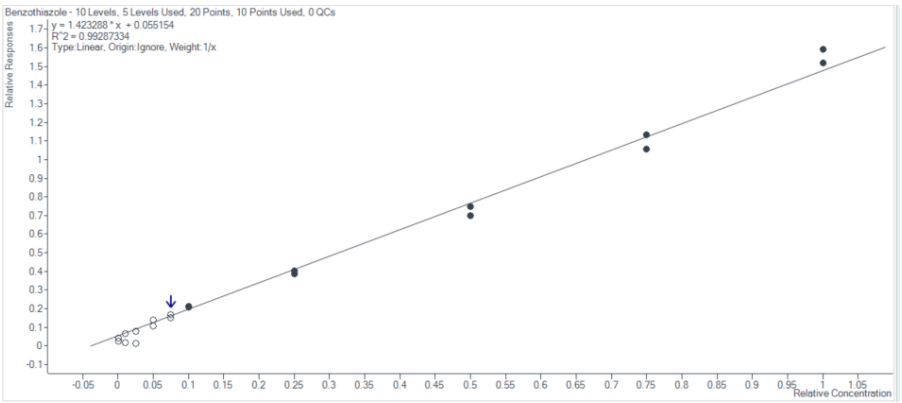

Figure A 4: Calibration curve of BTH in plants

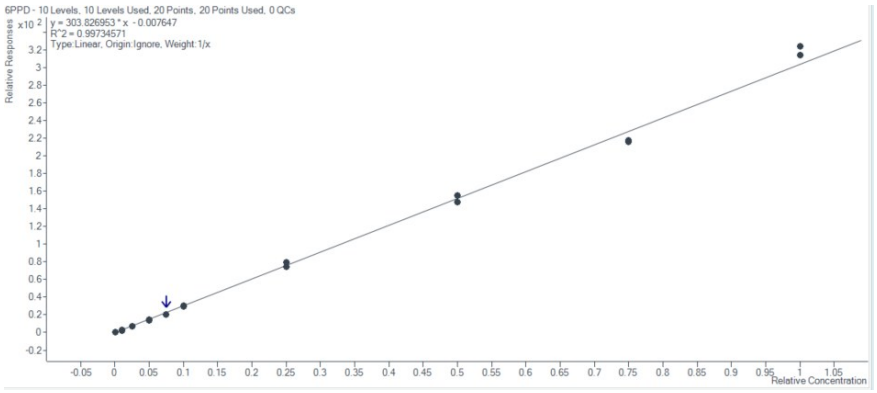

Figure A5: Calibration curve of 6PPD in plants

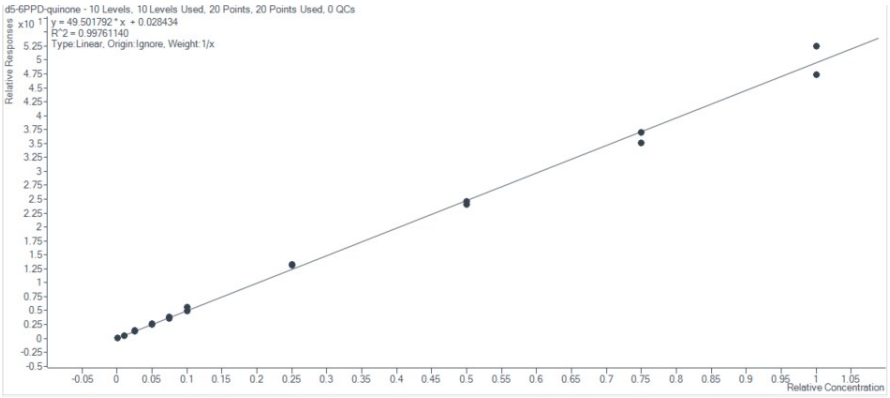

Figure A6: Calibration curve of d5-6PPD-q in plants

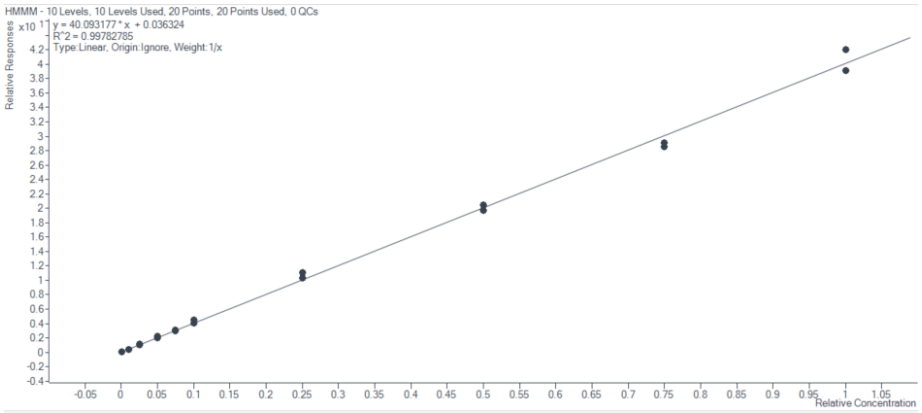

Figure A7: Calibration curve of HMMM in plants

Soil:

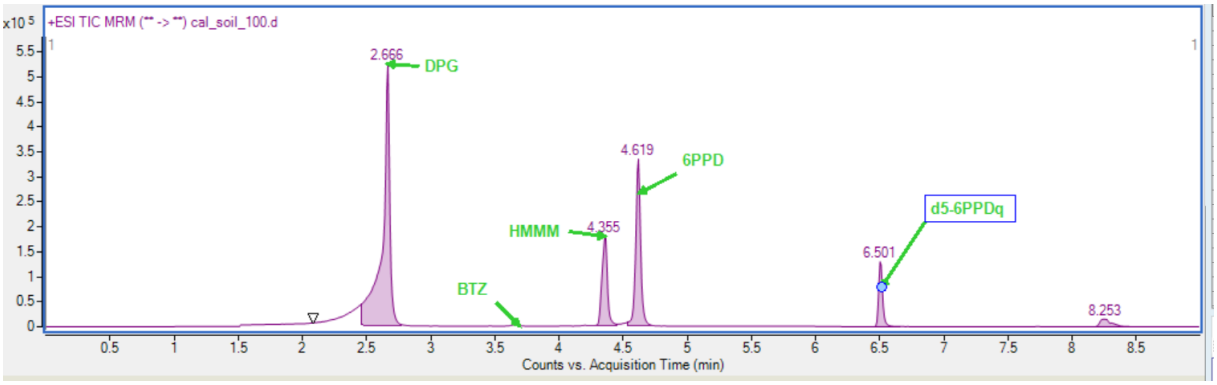

Figure A8: Chromatogram of TWCs in soil

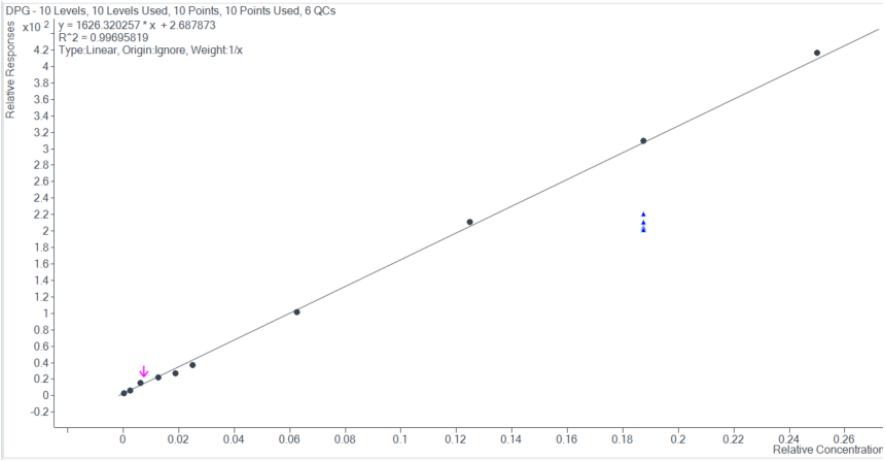

Figure A9: Calibration curve of DPG in soil

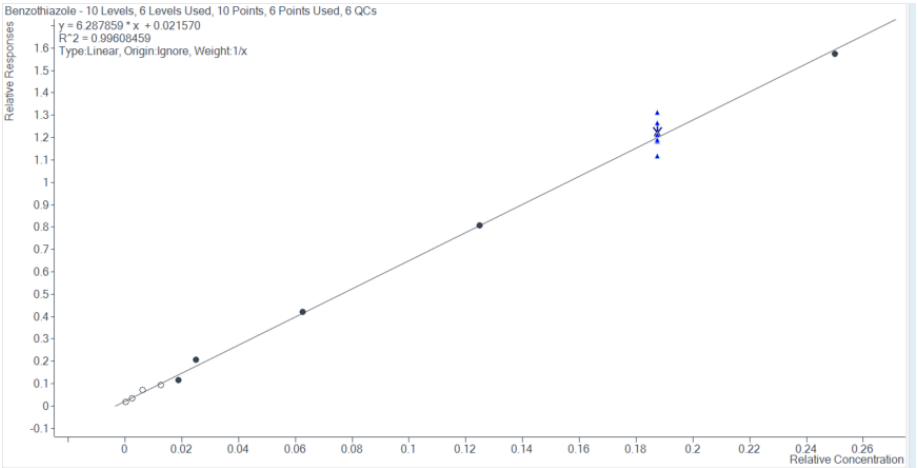

Figure A10: Calibration curve of BTH in soil

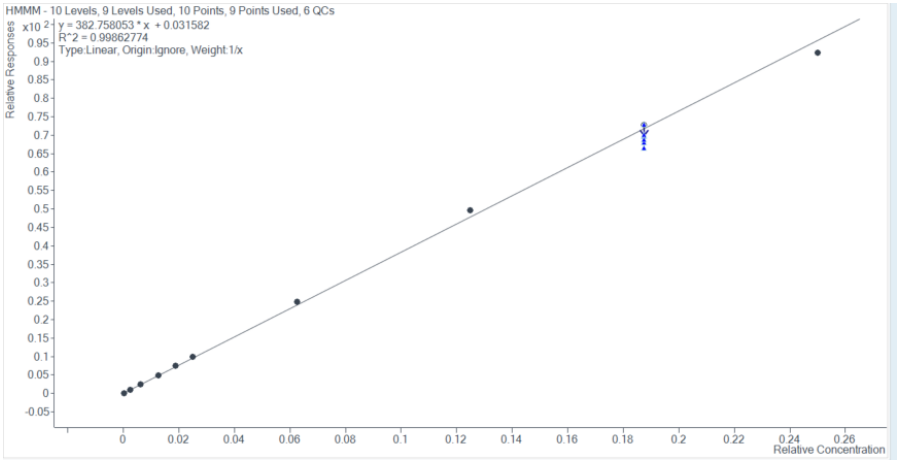

Figure A11: Calibration curve of HMMM in soil

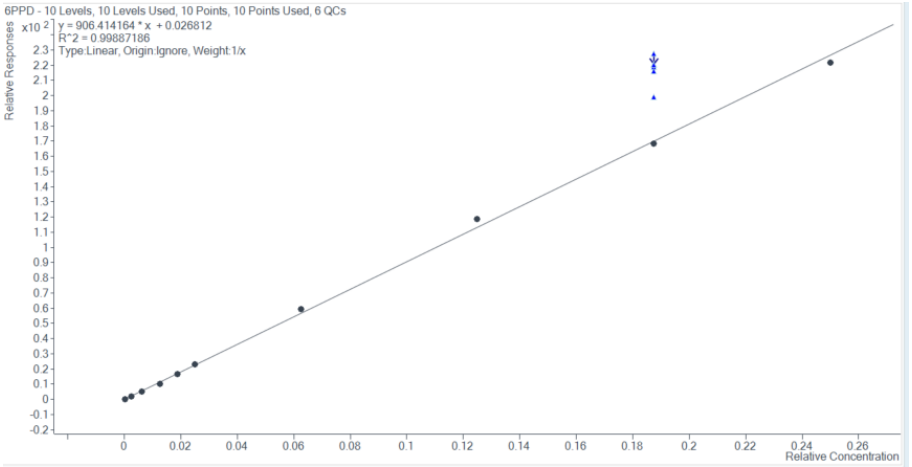

Figure A12: Calibration curve of 6PPD in soil

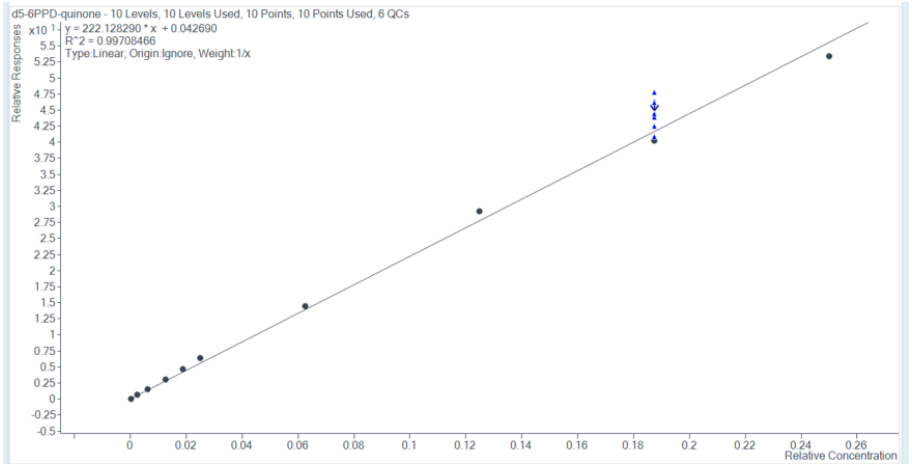

Figure A13: Calibration curve of d5-6PPD-q in soil

Pore water:

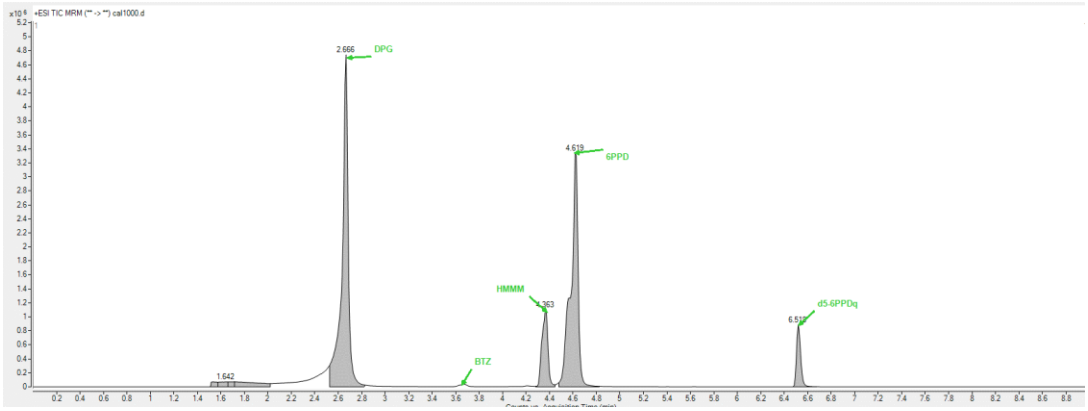

Figure A14: Chromatogram of TWCs in pore water

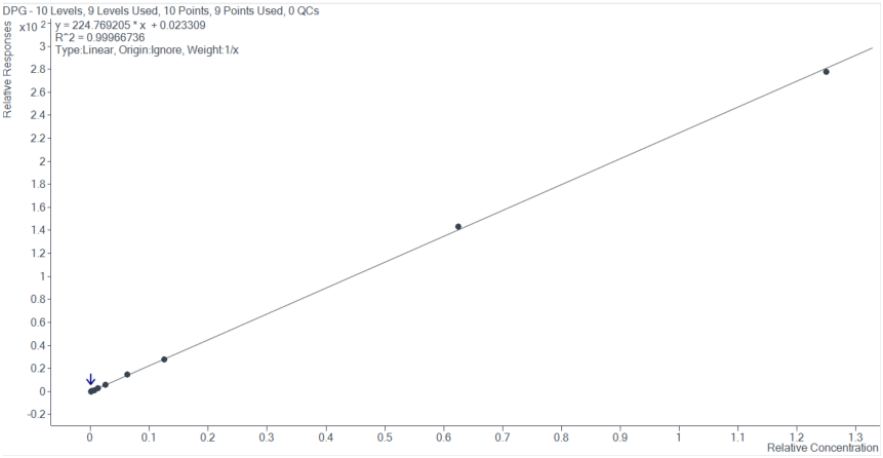

Figure A15: Calibration curve of DPG in pore water

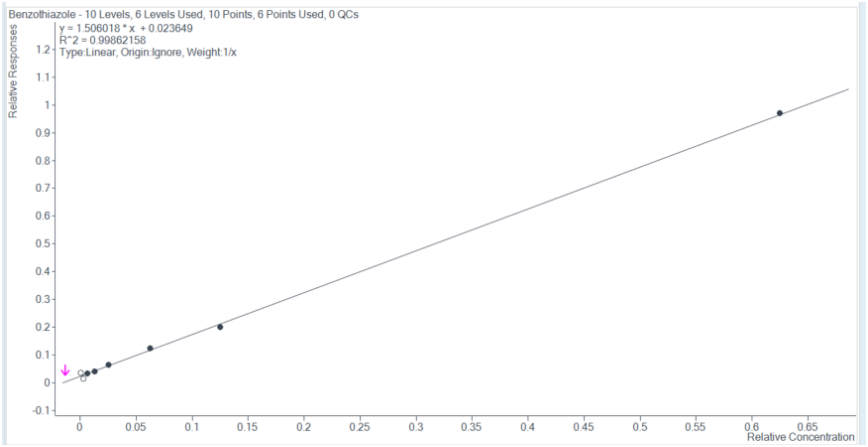

Figure A16: Calibration curve of BTH in pore water

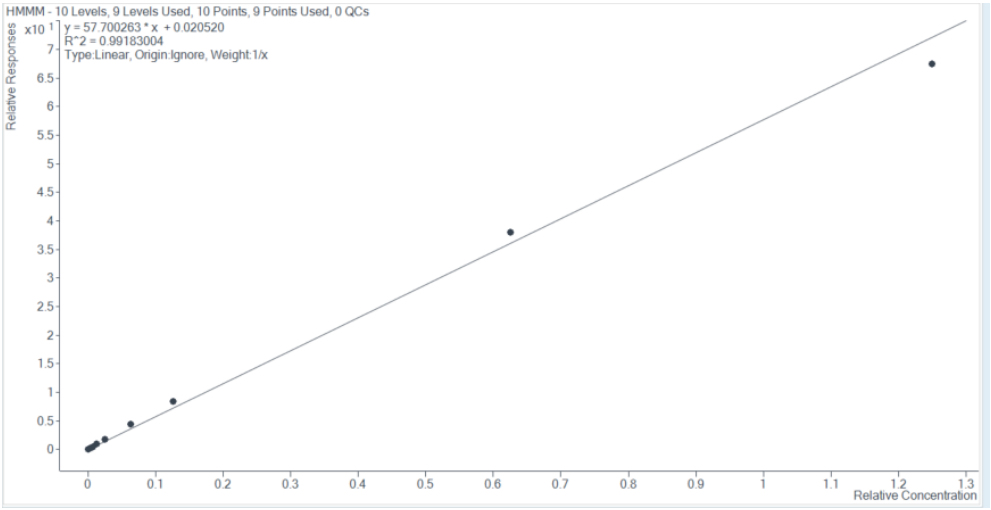

Figure A17: Calibration curve of HMMM in pore water

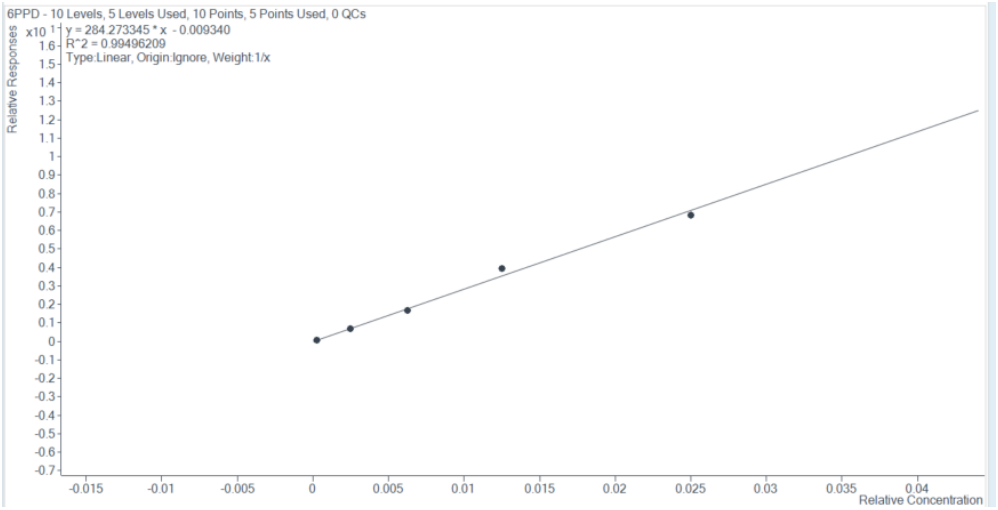

Figure A18: Calibration curve of 6PPD in pore water

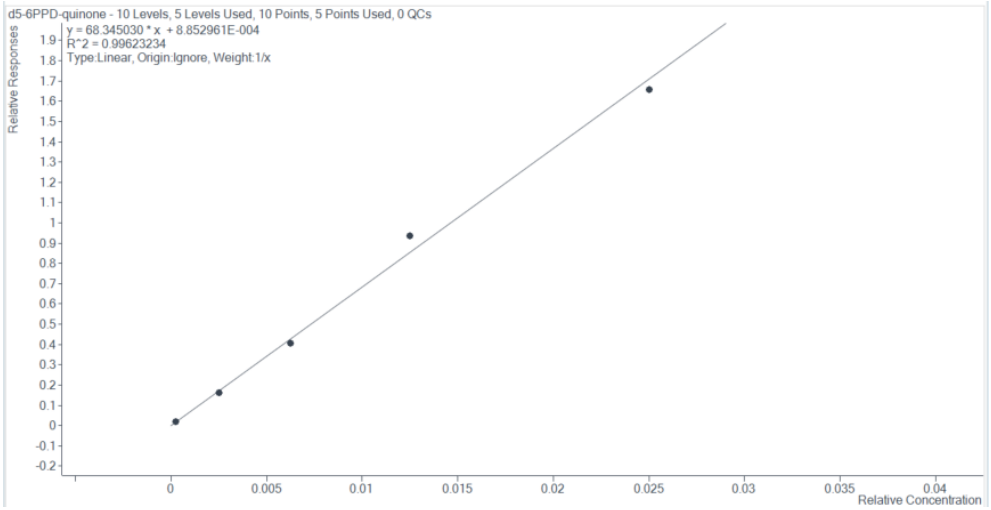

Figure A19: Calibration curve of d5-6PPD-q in pore water