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Methylation and Demethylation of Emerging Contaminants and Environmental Consequences

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ABSTRACT OF THE DISSERTATION

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by

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Contaminants of emerging concern (CECs) are ubiquitous in agroecosystems and aquatic environments. Transformations of CECs occur via biotic and abiotic pathways, resulting in the co-existence of CECs with many transformation products (TPs). Small changes in a chemical's structure, such as the addition or loss of a methyl group caused by methylation or demethylation, may bring significant alterations to its physicochemical properties, and further environmental behaviors. However, changes induced by methylation and demethylation in bioaccumulation, persistence in the environment, and toxicological effects of CECs are inadequately understood. Information about the occurrence of methylation and demethylation in higher plants and aquatic organisms is limited. In the first study of this dissertation research, the influence of methylation and demethylation on the uptake of four CECs (acetaminophen, diazepam, methylparaben and naproxen) and their methylated or demethylated TPs was characterized using two plant models - *A. thaliana* cell culture and hydroponically grown wheat seedlings. Results

showed that methylation generally increased a chemical's hydrophobicity, leading to increased uptake and accumulation in both plant models, as well as greater translocation in wheat seedlings. The second study considered the occurrence of methylation and demethylation in plants after uptake, and results showed that demethylation was generally more extensive than methylation. The rate of demethylation or methylation was dependent on the bond strength of R-CH_3, with demethylation of methylparaben and methylation of acetaminophen more pronounced than the other compounds. In the third study, changes in the bioaccumulation of and acute toxicity to an aquatic invertebrate, *Daphnia magna*, were further characterized as the result of methylation or demethylation. Methylation of CECs generally enhanced their acute toxicity, which was attributed to increased hydrophobicity. Greater bioaccumulation of methylated counterparts was concurrently observed to support this conclusion. Demethylation occurred in *D. magna* at different rates for different CECs and their TPs, indicating differences in the level of activity of the involved enzymes. Results from this dissertation research underline the environmental significance of simple, commonly occurring transformation reactions such as methylation and demethylation, and highlight the need to consider TPs for a more holistic understanding of the environmental fate and risks of CECs.

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Acronyms

List of acronyms used in this dissertation

BCF	Bioconcentration Factor
BPA	Bisphenol A
CEC	Contaminant of Emerging Concern
DM-diazepam	Demethylated Diazepam, i.e., Nordiazepam
DM-Methylparaben	Demethylated Methylparaben, i.e., 4-Hydroxybenozic Acid
DM-Naproxen	Demethylated Naproxen, i.e., 6-*O*-Desmethyl Naproxen
EDCs	Endocrine Disruption Compounds
EPA	Environmental Protection Agency
LOD	Limit of Quantification
M-Acetaminophen	Methylated Acetaminophen, i.e., p-acetanisidide
MS	Mass Spectrum
MTBE	Methyl Tert-Butyl Ether
MTL	Monitoring Trigger Level
PBDE	Polybrominated Diphenyl Ether
PCDE	Polychlorinated Diphenyl Ether
PPCP	Pharmaceuticals and Personal Care Product
QSAR	Quantitative Structure-Activity Relationship
RSD	Relative Standard Deviation
SD	Standard Deviation
TBBPA	Tetrabromobisphenol A
TBBPA DME	Tetrabromobisphenol A Di-methyl Ether
TBBPA MME	Tetrabromobisphenol A Mono-methyl Ether
TP	Transformation Product
TWW	Treated Wastewater
UPLC	Ultra Performance Liquid Chromography
WWTP	Wastewater Treatment Plant

Chapter 1 Introduction

1.1 Background of Resources Reuse

Climate change, exponential population growth, and inefficient water use are exacerbating water scarcity globally, leading to one of the biggest challenges for humankind to access adequate and safe freshwater resources.[1–4] Extreme weather events such as droughts occur with increasing magnitude, frequency, and duration around the world.[5] California, for instance, suffered through a multi-year drought from 2012 to 2016, and plunged into an even more severe and unprecedented drought in 2021-2022. Two consecutive emergency regulations have been enacted in California to limit urban water use at the beginning of 2022. These measures include restricting outdoor irrigation by homeowners to once or twice a week in Los Angeles County and prohibiting the irrigation of non-functional lawns in commercial, industrial, and institutional areas. Although most areas of California have been relieved from the long-lasting drought with plentiful precipitation in 2023, the central United States continues to suffer different degrees of dryness (Figure 1-1). Frequent severe droughts can lead to not only fluctuations in water availability, but also increased salinity in certain surface water systems, which can compromise water quality, posing a more significant challenge.[6]

Numerous water management strategies are being developed to meet the increasing water demands. The reuse of treated wastewater (TWW) is one of the most promising courses of action, as it can provide a reliable substitute for freshwater used in applications

such as irrigation, industrial processes, and drinking water supplies.[7] Water reuse can also support the sustainability of groundwater and surface water resources, which are often in overdraft status. Furthermore, it can improve the diversity of community water supplies, contributing to long-term water resource sustainability.[8]

Accounting for 92% of global water footprint in the period 1996-2005, agricultural production contributes the most to the total water footprint and suffers a direct impact from water shortages.[9] The utilization of TWW for irrigation purposes has been increasingly practiced all over the world, especially in arid and semi-arid regions.[2,10] For example, TWW makes up over 50% of total irrigation water in Israel,[11,12] and has long been used in China,[13] the Mediterranean basin[14] and some African countries.[15,16] However, only a small percentage of the total discharge water is reclaimed for further use in the United States based on current estimates, with less than 1% of water demands met through water reuse.[8] The U.S. Environmental Protection Agency (EPA) released the National Water Reuse Action Plan in February 2020, which urged the nation to collaborate on advancing water reuse to secure and support water resources.[8] The State Water Resource Control Board of California aims to increase the use of recycled water from 714,000 acre-feet per year (afy) in 2015 to 2,500,000 afy by 2030, to meet the increasing water demands of the growing population and minimize the impacts of decreasing flow and precipitation in the state.[17] The use of TWW for agricultural irrigation offers multiple benefits, including: 1) conserving freshwater resources; 2) providing a cost-effective alternative to freshwater sources; 3) providing additional

nutrients, such as nitrogen and phosphorus, which can enhance crop growth and help maintain soil fertility; 4) minimizing the direct discharge of TWW into the environment and the associated negative ecological effects; and 5) serving as a sustainable water source during droughts and other extreme conditions.[18]

Biosolids are a byproduct of wastewater treatment and are disposed of by land application, advanced treatment, landfill, and incineration.[19] The increasing scale of water treatment generates larger amounts of biosolids. In the U.S., approximately 47% of the produced biosolids are used for land application, of which about 60% are for agricultural fields; and this practice takes place in all 50 states.[20] Using biosolids as fertilizers in agriculture also provides many advantages, such as: 1) improving soil structure and increasing soil's ability to retain water and nutrients; 2) providing a cost-effective option for farmers compared to synthetic fertilizers; 3) contributing to the environmental sustainability by avoiding landfill and/or incineration; 4) increasing carbon sequestration and help mitigating climate change; and 5) reducing reliance on synthetic fertilizers that can have negative environmental impacts and are often energy-intensive to produce.[21,22]

1.2 CECs in TWW and Biosolids

Contaminants of emerging concern (CECs) are chemicals and other substances with no regulatory standards but have been recently detected in the environment and have the potential to cause adverse effects at environmentally relevant concentrations.[23] CECs consist of many different types of chemicals based on their purposes of use, including flame retardants, pharmaceuticals and personal care products (PPCPs), endocrine-

disrupting chemicals (EDCs), nanomaterials, among others. Flame retardants such as polybrominated diphenyl ethers (PBDEs) and tetrabromobisphenol A (TBBPA) are added to manufactured materials (i.e., plastics, textiles, surface coating) to prevent or slow the development of ignition. Prescribed pharmaceuticals like amoxicillin and over-the-counter drugs like acetaminophen are widely used by individuals for personal health. PPCPs also contain many types of preservatives and anti-bacterial substances, like triclosan. Antibiotics and veterinary medicines are widely applied to improve the production of livestock. For a long time, these substances were unknown, unidentified, unexpected, or unsuspected pollutants due to limitations in analytical methodologies.[24] It was also challenging to assess the impact of CECs on human health and the environment due to the lack of data or risk assessment tools.[20]

After emission from varied sources, including household sewers and industrial effluents, CECs are carried in contaminated wastewater to wastewater treatment plants (WWTPs).[25,26] The removal efficiency of CECs during treatments depends on the design and performance of individual WWTPs, as well as the physicochemical properties of CECs.[27–29] Many studies have shown that numerous CECs are present at trace levels in the treated effluent in the ng L^{-1} to µg L^{-1} range around the world, including Spain,[30] Germany,[31] the United States,[32,33] China,[34,35] and South Africa.[36] The concentrations of CECs are generally higher in biosolids because of the higher organic matter content, and are in the µg kg^{-1} to mg kg^{-1} range.[37–39] For example, triclosan and triclocarban were detected at 2715 and 1265 µg kg^{-1} respectively, in biosolids, in a study conducted in the

U.S.[38] The use of TWW and biosolids in agriculture, and/or their direct discharge into the environment, can introduce CECs to agricultural ecosystems and surface aquatic ecosystems, posing potential risks to ecosystems and human health.[27,40–43]

Many CECs contain active functional groups such as hydroxyl, carboxyl, and amide groups in their chemical structures, and are susceptible to many biotic and abiotic transformations in the environment and organisms.[34,44–47] Transformation products (TPs) of CECs can be directly introduced into WWTPs in municipal wastewater, leachates, and surface runoff. For instance, pharmaceuticals can be metabolized in the human body after consumption and are excreted in large portions as metabolites, particularly conjugates.[48–50] TPs can also be formed during the treatment processes in WWTPs via microbial transformations, photochemical transformations and oxidation and halogenation by disinfection processes.[41,44,51,52] These processes may also transform some TPs back to the parent CECs, such as hydrolysis/deconjugation of the conjugates of estrogens, leading to the "negative removal" for certain CECs in WWTPs.[53–55] Transformations of CECs may also take place in agroecosystems and aquatic environments after TWW and biosolids are discharged or applied. Soils,[56–58] plants,[59–62] terrestrial organisms,[63,64] algae,[65,66] aquatic organisms[67–69] and photochemical degradation[70,71] have all been reported to mediate CEC transformations. In some cases, TPs may pose higher ecological risks than their parent compounds, as they may have a greater bioaccumulation potential, increased toxicity to organisms, or longer persistence in the environment.[72–74]

Even though it is well known that TPs can co-exist with CECs in TWW and biosolids, information on their occurrence in TWW and biosolids, as well as their fate in agroecosystems and aquatic ecosystems, is still limited. The lack of experimental data hinders the development of solid risk assessments for CECs.

1.3 Fate of CECs in Plants

Through the irrigation of TWW and the land application of biosolids in agricultural fields, CECs present in these resources are introduced to agroecosystems and come into contact with plants (Figure 1-2). There are increasing studies showing that plants can take up, accumulate, translocate, and metabolize CECs, leading to human exposure via dietary consumption.

1.3.1 Plant Uptake of CECs

Assuming the majority of irrigated TWW and applied biosolids are received by soil, roots would serve as the major pathway for CEC uptake into plants.[75–77] Mechanistic understanding of CEC uptake remains rather limited. Based on the current knowledge, root uptake of CECs occurs primarily through passive diffusion,[27,75] although an energy-dependent active process mediated by transporters is likely for certain hormone-like compounds such as naproxen, clofibric acid, hydrocinnamic acid and perfluoroalkyl acids.[78–80] Translocation of CECs from roots to above-ground tissues, such as stems, leaves and fruits, has also been observed by previous studies, with concentrations of CECs generally being more substantial in roots.[40,76,77,81,82] Both biotic and abiotic factors

have been shown to affect the uptake, bioaccumulation and translocation of CECs by plants. These factors include plant physiology, soil pore water chemistry, the physicochemical properties of CECs, and the experimental conditions.[27,75,81,82]

Plant physiology plays an important role in plant uptake of CECs.[83] Plants exposed to stressors such as drought, salinity and high temperature can respond with various adaptive mechanisms such as heightened antioxidant defense, hormone regulation, and metabolic modifications.[84] The water and nutrient uptake and photosynthetic efficiency can decrease significantly in plants grown under stressed conditions.[85] Therefore, it may be assumed that non-stressed plants have greater potential for CEC uptake and accumulation. Other than plant physiology, plant species within the same genus, even varieties of the same plant species, have shown different patterns of CEC uptake. For example, different carrot genotypes displayed distinct uptake patterns for metformin, ciprofloxacin and narasin.[86] Based on the current knowledge, the ability of crop plants to uptake and accumulate CECs in the edible tissues decreases in the following order: leafy vegetables > root vegetables > cereals and fodder crops > fruit vegetables.[83]

The physicochemical properties of CECs, such as hydrophobicity and speciation, can strongly affect their uptake and translocation in plants.[27,76,83] Many CECs in TWW and biosolids are polar compounds with low volatility and contain ionizable functional groups, like hydroxyl, carboxyl and amide groups.[75] Only the dissolved CEC fraction in soil pore water would be considered available for root uptake.[27,75] For neutral CECs, root uptake usually involves two pathways: 1) equilibrium between the aqueous phase in plant

roots and the peripheral solution such as soil pore water; and 2) chemical sorption by the lipophilic root solids.[27,87] Ionized CECs, on the other hand, may undergo disassociation in soil pore water depending on the solution pH.[75,77,87] The electrical attraction or repulsion to the negatively charged root surface, along with the ion trap effects, which occur when CECs are neutral in the apoplast (pH 4~6) but ionized inside the cell (pH 7~7.5), can greatly influence their uptake and translocation in plants.[75] A linear relationship has been often observed between the hydrophobicity, e.g., log K_{ow}, and the bioaccumulation of neutral CECs in plants.[76,81,82] However, using log K_{ow} to estimate the bioaccumulation of ionizable CECs is not accurate, partly because lipid bilayers can more easily accommodate charged organic species than *n*-octanol.[75,81,82]

Different experimental settings, such as hydroponic cultivation, greenhouse soil cultivation and field experiments, have also exhibited great influence on the uptake and accumulation of CECs in plants. Hydroponic experiments provide simplified conditions,[27,39,88] while greenhouse soil cultivation and field experiments have more environmental relevance. The uptake of CECs by plants is usually evaluated by bioconcentration factor (BCF), which is calculated as the ratio of the concentration of CECs in plant tissues to that in soil pore water, or the growth media for hydroponic experiments. BCF values of CECs in roots can be high up to 840 L kg^{-1} in hydroponic settings,[81] while the values obtained from soil experiments may be much smaller,[89] suggesting the availability of CECs for plants decreased greatly in soil pore water during to phase partitioning.

1.3.2 Plant Metabolism of CECs

CECs with active functional groups, such as carboxyl, hydroxyl, and amide groups, are susceptible to metabolism in plants via various enzymatic activities after being taken up. This metabolic process is similar to the hepatic detoxification system and is known as the "green liver".[90] Three metabolic phases are usually involved in the metabolism of xenobiotics in plants: Phase I metabolism is an activation process that includes hydroxylation, dealkylation, oxidation and reduction, that are catalyzed by cytochrome P450s, esterase, peroxidase, or other enzymes to enhance reactivity and polarity of xenobiotics; Phase II metabolism is predominantly conjugation with polar biomolecules, such as amino acids, sugars and glutathione, to further increase the hydrophilicity and mobility of xenobiotics; Phase III metabolism refers to the sequestration of conjugated metabolites in plant cells, including the storage in vacuoles and the incorporation into cell walls.[27,75,90]

There have been only a small number of studies focusing on the metabolism of CECs in plants. Plant cell systems, such as *A. thaliana* cell culture,[88,91–95] carrot cell culture,[96] rice cell cultures[97,98] and horseradish hairy root cell culture,[62,99] have been used as a simple and fast approach for characterizing metabolites of various CECs. Whole plants, either hydroponically cultivated or grown in soil, have also been used to understand plant metabolism of CECs.[59,100,101] For example, phase I metabolites of carbamazepine, 10,11-epoxide-carbamazepine and 10,11-dihyro-10,11-dihydroxy-carbamazepine, were observed in the leaves and fruits of tomato and cucumber,[102] leaves

and roots of sweet potato and carrot,[103] and leaves of *Typha* spp. (a plant with potential use in phytoremediation).[104] Diclofenac was found to be hydroxylated to 4'-OH-diclofeanc in barley,[62] horseradish root cell culture[62] and bulrush.[105] Two single benzene-ring metabolites of TBBPA were identified in pumpkin plants and rice cell cultures.[98,106] Phase I metabolism was also reported for epimers of tetracycline in pinto bean leaves.[107] Phase II metabolism has been found to occur extensively for some CECs in plants. For example, conjugation with amino acids was reported for naproxen,[59] ibuprofen,[59] diclofenac,[92] and gemfibrozil[108] in *A. thaliana* cells and whole plants. Glycosylation was observed for diclofenac,[62] sulfamethoxazole[91,109] and di-n-butyl phthalate[101] in *A. thaliana*, triclosan, naproxen, diclofenac, ibuprofen and gemfibrozil in carrot cell cultures,[96] bisphenol A and carbamazepine in lettuce,[110] and TBBPA in pumpkin seedlings.[111] Acetaminophen and chlortetracycline were conjugated with glutathione in cucumber seedlings and maize seedlings, respectively.[107,112] Conjugation with other biomolecules, such as saccharides, malonic acid, and sulfate, was also occasionally reported for some CECs, such as triclosan in carrot cells and diclofenac in *A. thaliana* cells.[92,113] Other than conjugation, methylation is also a type of phase II metabolism and has been reported for TBBPA in pumpkin seedlings.[106] The investigation of phase III metabolism of CECs in plants is relatively limited, as quantitative evaluation of phase III products would require the use of isotope (e.g., ^{14}C) labeling to account for the non-extractable or bound residues, although phase III is expected to be dominant in determining the final destination of xenobiotics in plants. For instance, nearly all ^{14}C-

labeled naproxen, diclofenac, bisphenol A and nonylphenol were found in non-extractable bound residues in lettuce and collards.[114]

1.4 Fate of CECs in the Aquatic Environment

1.4.1 Occurrence of CECs in Aquatic Ecosystems

Numerous studies have documented the occurrence of CECs in aquatic environments in many countries and regions. CECs are introduced into aquatic environments via discharge of TWW from WWTPs, agricultural activity, landfill leachates, and surface runoff (Figure 1-3). Concentrations of CECs ranges from ng L^{-1} to μg L^{-1} in impacted surface water and from μg kg^{-1} to mg kg^{-1} in the sediment.

The concentration of CECs in surface water is largely influenced by the population density, environmental conditions (e.g., precipitation and temperature) and terrigenous supply, displaying temporal and spatial variations.[26,115–117] Studies conducted in the U.S. in recent decades have shown the occurrence of hundreds of CECs in various watersheds, with the maximum concentration at 35 μg L^{-1} for sucralose in water.[26,115,118] A comprehensive review of the occurrence of CECs in Latin America, including studies performed in 11 different countries between 1999 and 2018, has shown the common detection of 17β-estradiol, bisphenol A and estrone. The highest concentration of CECs detected reached 1100 μg L^{-1} for clindamycin in Costa Rica.[119] Many survey studies have also been reported for countries in Europe, such as the Sava River in Slovenian and Croatian,[120] rivers receiving TWW in Ireland,[43] and impacted rivers and lakes in

Sweden.[121] An EU-wide survey of CECs in European river waters indicated that benzotriazole, caffeine, carbamazepine, tolyltriazole, and nonylphenoxy acetic acid were among the most frequently detected and/or at the highest concentrations.[122] In addition, levels of CECs in some developing countries may be higher than those in developed countries, likely due to less rigorous treatment at WWTPs. For example, naproxen as high as 140 μg L^{-1} was reported in a study originated in India and up to 167 μg L^{-1} for lamivudine in Kenya.[123]

Research focusing on the occurrence of CECs in sediment is less prevalent and typically involves fewer CECs. Furthermore, the concentration of CECs in sediments usually exhibits less seasonal variations, which suggests that sediment samples may serve as a more stable marker for CEC monitoring in aquatic environments.[115,124–126] High detection frequencies and concentrations of estrone and 17β-estradiol have been reported in sediments from the mouth of the Manokin River in the U.S., with the highest concentration at 58.4 μg kg^{-1} and 11.5 μg kg^{-1}, respectively.[126] In the Southern California Bight, triclosan, 4-nonylphenol and bis(2-ethylhexylphthalate) have been detected in all sediments at median (maximum) concentrations of 5.1 (8.6), 30 (380), and 121 (470) μg kg^{-1}, respectively.[127] The highest total concentration of antibiotics in sediments from the intertidal zones of the Yellow River Delta, China was measured to be 178.77 μg kg^{-1}.[124] Studies conducted in African countries such as Morocco showed even greater CEC concentrations in the sediment (e.g., up to 5.1 mg kg^{-1} for bisphenol A).[128] Interestingly, several surveys have shown the presence of some hydrophilic CECs in sediments, such as

acetaminophen and caffeine, which were previously thought to have limited sorption to solids due to their low hydrophobicity.[129–131]

Aquatic organisms living in impacted aquatic environments have been sampled for detection of CECs. CECs have been found in the tissues of fish, mussels and oysters collected from the impacted water systems in the U.S., with the maximum concentration detected at 3000 ng g^{-1} (dry weight) for 4-nonylphenol in mussels, suggesting potential bioaccumulation of CECs in aquatic organisms.[126,132–134] Diazepam was detected in all collected flatfish liver samples in Southern California but was infrequently detected in sediments, highlighting the biomagnification potential of certain CECs.[127] The accumulation of PBDEs in fish livers was comparable to that of legacy organochlorines.[127] Water snakes and small common carps living in an e-waste-contaminated water pond were reported to accumulate plasticizers and organophosphorus flame retardants in their tissues.[135] Therefore, aquatic organisms may be exposed to low levels of CEC mixtures in the environment, and bioaccumulation in aquatic organisms is possible for some CECs.

1.4.2 Toxicity of CECs on Aquatic Organisms

As CECs are continuously introduced into aquatic environments via various pathways, they may be considered as pseudo-persistent contaminants, causing long-term, mixed, low-dose exposure to aquatic organisms. A comprehensive review of the ecotoxicity of human pharmaceuticals concluded that for all human medicines tested, acute effects to aquatic organisms were unlikely, unless spill incidents occurred, due to

their trace level occurrence in aquatic environment.[136] The chronic lowest observed effect concentrations of most tested pharmaceuticals in standard laboratory organisms are about two orders of magnitude higher than what detected in the effluents.[136] However, recent studies showed that some CECs may exert unintended adverse effects to organisms, such as endocrine disruption and developmental toxicity, at environmentally relevant levels.[137–140] These chemicals are known to have adverse effects on non-target aquatic organisms. The investigation on toxic effects of CECs on aquatic organisms includes two main types of exposure: direct exposure to the real environment, such as TWW and impacted water, and exposure to water spiked with CECs under controlled conditions.

Chronic effects, including sublethal effects, have been often observed in aquatic organisms exposed to affected water bodies or TWW. For example, fathead minnows and freshwater mussels were caged for 4 weeks upstream and downstream of the discharge from WWTPs, and were found to develop multiple biomarker responses, such as oxidative stress, enzyme induction, shifts in gene expression and alteration of immune functions.[141] The growth and yield of green algae and reproduction of daphnia were inhibited by TWW and exhibited dose-response effects.[142] Juvenile rainbow trout exposed to TWW showed significantly different plasma cortisol and glucose response to the secondary stressor.[143] However, it is often difficult to interpret the impact of CECs in this type of experiments, as various other stressors, such as water temperature and bacteria in the real environment, may also induce such biomarker responses.[141] The low concentrations of CECs in TWW also could not explain the sublethal effects observed on

algae and daphnia.[142] In addition, interactions of compounds in CEC mixtures should be further considered.

The exposure of aquatic organisms to artificially spiked CECs, on the other hand, provides comparable toxicological data under controlled conditions. Most research has been devoted to the toxic effects of CECs at the individual level, while in realistic situations, CECs are always present as a mixture. Exposure to CECs at environmental relevant levels cause multiple adverse effects. For example, marine mussels exposed to atorvastatin at around 1.2 μg L^{-1} exhibited key fatty acid metabolism disruption and suppression of xenobiotics efflux through P-glycoprotein and membrane diffusion.[144] Gemfibrozil was shown to reduce plasma androgens in goldfish (*Carssius auaratus*) after exposure to 1.5 μg L^{-1} for 4 and 14 days;[145] while the concentration of gemfibrozil in WWTP effluent was found to be in the range of 10-3830 ng L^{-1}.[146] The adverse effects of CECs have also been shown at the population level. For example, a 7-year, whole lake experiment conducted in northwestern Ontario, Canada, showed that chronic exposure of fathead minnow (*Pimephales promelas*) to 5-6 ng L^{-1} of the synthetic estrogen, 17 α-ethynylestradiol, led to the near extinction of this species.[140] Aquatic invertebrates have been widely adopted to derive acute toxicity end-points, e.g., LC_{50} values, for target CECs. The acute toxicity of CECs varied greatly, even for compounds belonging to the same chemical class and displayed species-specific effects. For example, the EC_{50} and LC_{50} values varied largely among the 12 tested polychlorinated diphenyl ethers (PCDEs, used as hydraulic oil, electrical insulators, lubricants, flame retardants, and plasticizers)

for *S. obliquus*, *D. magna*, and *D. rerio*, respectively.[147] Exposure to 17α-ethinylestradiol, acetylsalicylic acid, and bisphenol A significantly affected the embryonic development of sea urchins, with different LC_{50} values for *Mysidopsis juniae* and *Artemia sp*.[148] The concentrations that induced 50% growth inhibition in algae of metolachlor, erythromycin, and triclosan also showed multiple-fold differences between freshwater and marine algae, reflecting the species-specific sensitivity.[149] Mixed exposure of silver nanoparticles, polystyrene nanoplastics and 5-fluorouracil displayed interaction toxicity to marine mussels, with exponentially increased oxidative damage compared to individual contaminants,[150] highlighting the importance to consider chemical interactions when investigating the toxic effects of CECs in the real environment.

Some government agencies in the U.S., such as EPA and California State Water Resources Control Board, have tried to put some regulations to control CECs in aquatic environments. For example, the Science Advisory Panel for CECs in California's aquatic ecosystems has developed strategies to identify the monitoring trigger levels (MTLs) of CECs in aquatic environments based on their lowest effect values available from established databases, such as the Computational Toxicology (CompTox) database (https://comptox.epa.gov/dashboard/) and the NORMAN database (https://www.norman-network.com/nds/), for aquatic organisms.[151] However, for the TPs of CECs, such data are usually not experimentally available. Due to the huge and continually increasing number of CECs in aquatic environments, it is unrealistic to examine the toxicity effects of all CECs. Several studies have attempted to develop a prioritization process to select

CECs that require the most attention for aquatic organisms based on their monitoring data, production volume, persistence and prevalence in the environment, bioaccumulation potential, and biological effects.[152–155] Several modeling tools, like machine learning and quantitative structure-activity relationships (QSARs), have also been developed to predict the bioaccumulation, biotransformation and toxicological effects of CECs.[154,156–160] For example, Sequence Alignment to Predict Across Species Susceptibility (https://seqapass.epa.gov/seqapass/) was adopted by the Science Advisory Panel for CECs in California's aquatic ecosystems to predict the behaviors of CECs across species without available toxicological data from the existing database.[151] The incorporation of such tools is of vital importance to improve risk assessment of CECs due to the limited experimental resources.

1.4.3 Biotransformation of CECs in Aquatic Organisms

Studies have often revealed that TPs of CECs occur simultaneously in the tissues of aquatic organisms with their parent compounds, sometimes at even higher concentrations. For instance, metabolites of organophosphorus flame retardants were found in the same order of magnitude as their parent compounds in water snake and small common carps collected from an e-waste-affected site.[135] Norsertraline, the demethylated TP of sertraline, was found to be bioaccumulated at a greater degree than sertraline in the liver of rudd collected from the TWW-impacted Niagara River.[161] Nordiazepam, the demethylated TP of diazepam, was also frequently detected in aquatic organisms along with diazepam.[127] Therefore, TPs of CECs in aquatic organisms could originate from two

sources – uptake from the ambient environment, and transformation taking place in the organism upon the uptake of the parent compound.

Research focusing on the biotransformation of CECs in aquatic organisms, including aquatic plants such as algae, invertebrates such as daphnia, and vertebrates such as fish, is limited. However, the identification of CEC metabolites in aquatic organisms is crucial for evaluating the ecological risks of CECs. Prevalent phase I and phase II enzyme activities were frequently induced in aquatic organisms after CEC exposure, such as cytochrome P450 enzymes and glutathione transferases.[69,72,162] Although some common metabolites might be expected, the pattern of CEC metabolism could also vary between different species. For example, three phase I metabolites and 10 phase II metabolites were identified in marine mussels exposed to diclofenac,[163] while 7 phase I metabolites and 3 phase II metabolites were found in *H. Azteca* and *G. pulex*.[72] Significant differences in biotransformation rates were observed for different species or between opposite sexes of fishes exposed to CECs.[157] Certain aquatic species, such as glass eels, displayed low metabolic activity, with few metabolites detected after CEC exposure,[155] while the absence of biomagnification effects of PFRs in water snakes was attributed to the active biotransformation.[135] Biotransformation of CECs in algae shared some similar pathways as that in terrestrial plants, as in the case of hydroxylation, demethylation and glycosylation of bisphenols.[65]

1.5 Methylation and Demethylation of CECs

Methylation and demethylation are common transformation pathways for chemicals in the environment, especially for compounds with -OCH_3, -NCH_3-, -SCH_3, and/or the corresponding -OH, -NH-, and -SH groups in their chemical structures. Demethylation is a common phase I metabolism typically catalyzed by cytochrome P450 enzymes that are ubiquitous in humans, terrestrial organisms and aquatic organisms.[50,69,164–168] Sometimes demethylation can also occur via the catalysis of esterase or non-enzymatic hydrolysis for -$COOCH_3$ and result in the formation of carboxyl groups.[169] Methylation of CECs, on the other hand, is a phase II metabolism typically catalyzed by methyltransferases.[170–173] Various substrates are susceptible to the activity of methyltransferases, such as nucleic acids, lipids and many xenobiotics.[170,174,175] Unlike other phase II metabolism, methylation usually leads to increased hydrophobicity, but it is considered a detoxification pathway in most cases.[64]

Methylation and demethylation are among the most common transformations for CECs in the environment. For example, triclosan was methylated during the WWTP treatment, and the derived methyl triclosan was frequently detected in TWW along with triclosan, sometimes at even higher concentrations.[117,176,177] Acetaminophen was reported to methylate during microbial degradation in soil.[178] TBBPA monomethyl ether (TBBPA MME) and dimethyl ether (TBBPA DME) were frequently detected in the environment along with TBBPA, sometimes at higher concentrations.[68,179] TBBPA MME and TBBPA DME were also formed through abiotic methylation in the natural presence of methyl

iodide in aquatic environments.[180] Biotic methylation of TBBPA was also observed to occur through biologically mediated transformations in sediments,[174] earthworms (*Metaphire guillelmi* and *Eisenia fetida*),[64] and plants. Methylation of BPA was promoted by Mycobacterium strains like PYR-1 and PCP1.[175] Methylation of diclofenac was observed in aquatic invertebrates.[72] Demethylation of common CECs has been previously reported as well, such as the *O*-demethylation of naproxen in humans, terrestrial plants, microbes and soils,[59,165,181] and the *N*-demethylation of diazepam in humans, terrestrial plants and aquatic organisms.[88] Several studies have also shown the demethylation of methylated CECs back to the parent compound. For example, methyl triclosan was back converted to triclosan in *A. thaliana* and fish.[61,182] Demethylation of TBBPA DME and TBBPA MME back to TBBPA was observed in pumpkin plants.[106] The back conversion of diclofenac methyl ether in aquatic invertebrates was also reported.[72]

As an important type of TPs, methylated CECs are usually more hydrophobic (higher log K_{ow}) than their corresponding parent compounds, and therefore may pose increased ecological risks. For example, consistently higher concentrations and BCFs of methyl triclosan, as compared with triclosan, were observed in fish, snails and algae collected from TWW-impacted streams.[176,183–185] BPA mono- and dimethyl ether were more toxic to the development of zebrafish embryos than BPA itself.[175] Diclofenac methyl ether showed greater bioaccumulation and further higher acute toxicity to *H. azteca* and *G. pulex* than diclofenac.[72] However, exceptions exist to this general rule. For instance, the methyl ethers of TBBPA were less toxic to zebrafish development[137] and

earthworms (*Metaphire guillelmi* and *Eisenia fetida*) in terms of acute exposure.[64] Lower bioaccumulation factors of methyl triclosan than triclosan was reported in algae (*Cladophora spp.*).[184] These studies suggested that changes in environmental behaviors of CECs induced by methylation were molecule-specific.

The investigation of back-and-forth conversion between methylation and demethylation of CECs is important to obtain a more complete understanding of the environmental cycling of CECs. This previously neglected conversion circle implies prolonged persistence of such contaminants in the environment. This transformation circle needs to be further investigated for more comprehensive and accurate risk assessment for CECs that are susceptible to such reactions. In addition, research is needed to quantitatively evaluate differences in non-target toxicity between CECs and their methylated or demethylated derivatives.

1.6 Knowledge Gaps and Problem Statement

Although methylation and demethylation of several CECs in agroecosystems and aquatic invertebrates were studied previously,[67,72,106,174,175,180] our overall knowledge of the transformation potential, fate and ecological risks of these methylated or demethylated TPs is limited.

Many CECs contain active functional groups such as hydroxyl, methoxyl, carboxyl, ester and amide groups in their chemical structures, making them susceptible to methylation or demethylation under biotic and abiotic conditions. However, the specific molecular properties that promote methylation or demethylation need to be better

understood. For example, methyltransferases in plants are involved in many important metabolic activities; [170,171,173] therefore, the similarity in chemical structures to the endogenous biomolecular substrates may influence the potential for methylation of xenobiotics in plants. The bond strength of the chemical bond connecting the methyl group and the major fragment (R-CH_3) may also affect the potential for demethylation of CECs. Furthermore, methylated or demethylated TPs may be demethylated or methylated back to the parent CECs, respectively.[61,106] This previously ignored metabolic circle may effectively prolong the persistence of CECs in the environment and lead to unrecognized environmental risks.

Changes in environmental behaviors induced by methylation or demethylation are poorly understood with few experimental observations. Methylation of CECs can lead to increases in bioaccumulation, and acute and developmental toxicity in organisms,[72,175,184,185] while exceptions also exist.[64] Demethylation of CECs also does not necessarily lead to lower bioaccumulation and toxicity.[74] Methylation and demethylation may also affect the transport, translocation and persistence of CECs by inducing changes in their hydrophobicity, solubility and pK_a. The fate of demethylated and methylated TPs of CECs in agroecosystems and aquatic environments warrants a more systematic evaluation.

Given the large and ever-increasing number of CECs in the environment, it is unrealistic to experimentally investigate all CECs, let alone their methylated and/or demethylated TPs.[83,154] The incorporation of modeling tools, such as QSARs and models

based on the use of molecular descriptors, can help predict environmental behaviors and provide an alternative way to assessing the risks of CECs and their TPs. Such modeling approaches need to be validated and refined using experimental data.

In conclusion, changes in the environmental behaviors of CECs induced by simple transformation reactions such as methylation and demethylation need to be systematically explored through rigorously designed experiments. The experimental data should be further incorporated into existing models to help validate the utility of models and also allow the prediction for a wide range of CECs for which experimental data may never be available.

1.7 Research Objectives

The overall objective of this dissertation project is to elucidate changes in the environmental behaviors of CECs as induced by common transformations, such as methylation and demethylation, in plants and aquatic organisms. This information could contribute to a more holistic and accurate understanding of risks of CECs associated with the beneficial reuse of TWW and biosolids in agriculture, as well as in ecosystems impacted by these waste sources. The specific objectives are:

1) To investigate the changes induced by methylation and demethylation in the bioaccumulation of CECs in higher plants;

The uptake of four CECs, including acetaminophen, diazepam, methylparaben, and naproxen, and their corresponding methylated or demethylated derivatives, by two plant models, i.e., *A. thaliana* cells and wheat seedlings, will be investigated. The translocation

of these CECs and TPs in wheat seedlings and their persistence in culture media will also be simultaneously characterized and compared.

2) To characterize the interconversion between CECs and their methylated or demethylated TPs in higher plants.

The interconversion between selected CECs and their corresponding methylated or demethylated TPs in two plant models, i.e., *A. thaliana* cells and wheat plants, will be experimentally evaluated investigated. The potential for methylation or demethylation will further predicted from the bond strength ($R\text{-}CH_3$) and other molecular descriptor via a computational chemistry model, and the predictions will be evaluated against the experimentally derived data.

3) To assess the effect of methylation and demethylation on bioaccumulation and acute toxicity, and the interconversion between CECs and their TPs in aquatic invertebrates.

The acute toxicity and bioaccumulation of the selected CECs and their methylated or demethylated TPs will be determined using standard protocols with *D. magna* as the model organism. The interconversion between CECs and their TPs in *D. magna* will be simultaneously characterized. The *in vivo* experimental data will be further compared to *in silico* data generated with QSAR models to allow more general predictions of the effect of demethylation/ methylation on the environmental behaviors of CECs in aquatic organisms.

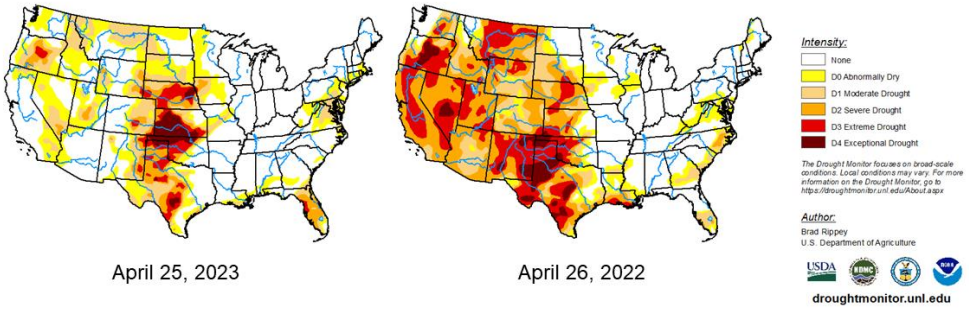

Figure 1-1 Drought map of United States (accessed on 04/25/2023).

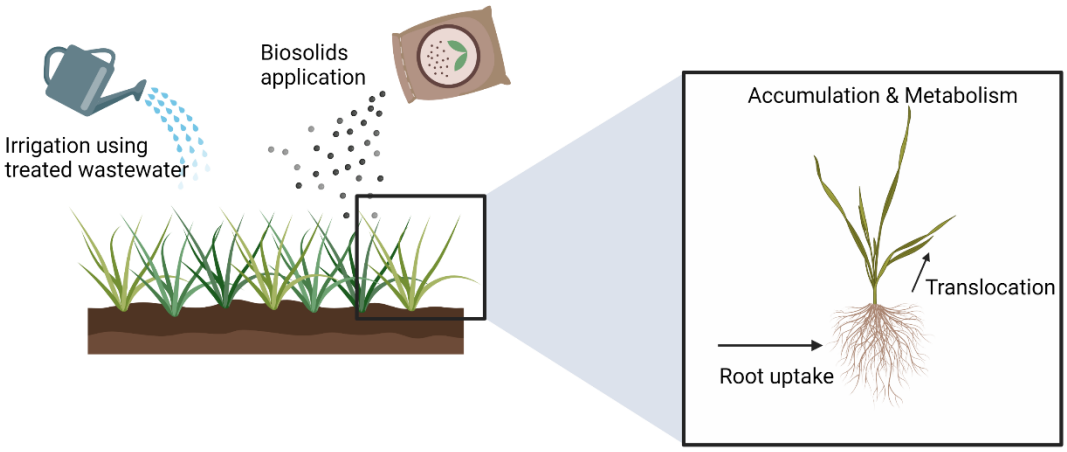

Figure 1-2 Fate of CECs in plants: introducing, uptake and metabolism (Created with BioRender.com).

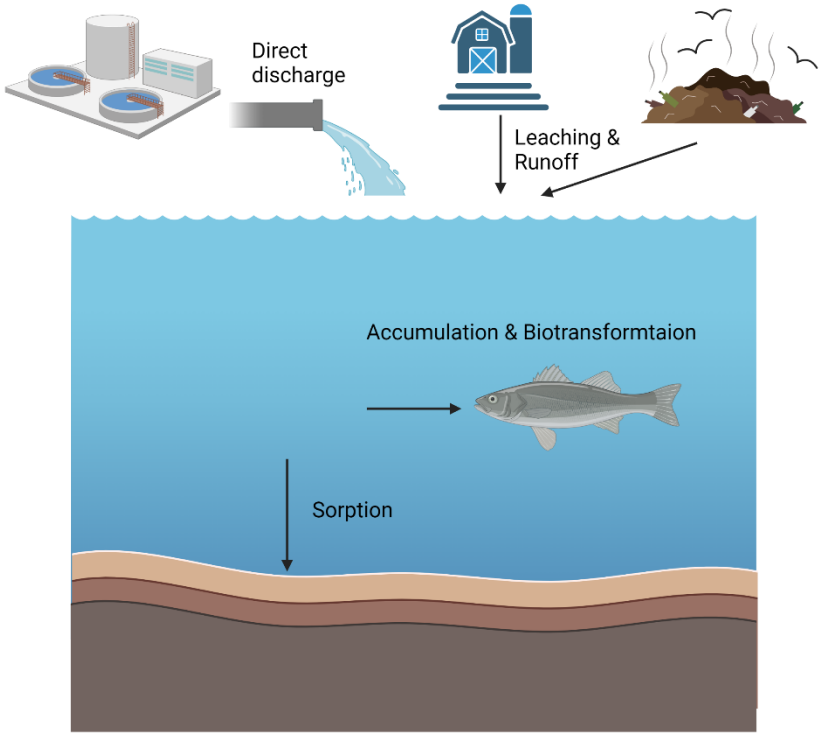

Figure 1-3 Fate of CECs in aquatic environments (Created with BioRender.com).

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Chapter 2 Influence of Methylation and Demethylation on Plant Uptake of Emerging Contaminants

Abstract

Contaminants of emerging concern (CECs) as well as their transformation products (TPs) are often found in treated wastewater and biosolids, raising concerns about their environmental risks. Small changes in chemical structure, such as the addition or loss of a methyl group, as the result of methylation or demethylation reaction, may significantly alter a chemical's physicochemical properties. In this study, we evaluated the difference in accumulation and translocation between four CECs and their respective methylated or demethylated derivatives in plant models. Suspended *Arabidopsis thaliana* cell culture and wheat seedlings were cultivated in nutrient solutions containing individual compounds at 1 mg/L. The methylated counterpart was generally more hydrophobic and showed comparative or greater accumulation in both plant models. For example, after 1 h incubation, methylparaben was found in *A. thaliana* cells at levels two orders of magnitude greater than demethylated methylparaben. In contrast, demethylated counterparts, especially those with the addition of a hydroxyl group after demethylation, showed decreased plant uptake and limited translocation. For example, acetaminophen and demethylated naproxen were not detected in the shoots of wheat seedlings after hydroponic exposure. Results from this study suggest that common transformations such as methylation and demethylation may affect the environmental fate of CECs, and should

be considered to obtain a more comprehensive understanding of risks of CECs in the environment.

2.1 Introduction

Contaminants of emerging concern (CECs) refer to contaminants that are recently discovered in the environment and may pose potential adverse effects, such as developmental toxicity and endocrine disruption, to non-target organisms and human health at environment-relevant concentrations [1–3]. Because of their widespread use, CECs are ubiquitously present at trace levels in treated wastewater and biosolids. [3–8]. Many CECs contain reactive functional groups such as hydroxyl, carboxyl, and amide, making them susceptible to biotic and abiotic transformations during treatment at wastewater treatment plants (WWTPs) [6,9,10]. Therefore, in addition to the parent form of CECs, transformation products (TPs) are also often present in treated wastewater and biosolids, sometimes at even higher concentrations [11]. Treated wastewater and biosolids have been increasingly applied to agricultural lands in recent years in beneficial reuse practices, which serves as a conduit for plants to be contaminated with CECs and their TPs, posing potential human health and ecological risks [12–15].

Methylation and demethylation are among the most common transformation reactions for many CECs. Biotic demethylation is a phase I metabolism process facilitated mainly by cytochrome P450 enzymes that are ubiquitous in organisms [16–19]. For example, as a common pharmaceutical itself, nordiazepam (demethylated diazepam or DM-diazepam) is also a demethylated metabolite of diazepam excreted after oral

administration in humans [20] (Figure 1). Likewise, demethylation can convert naproxen to *O*-desmethyl naproxen (DM-naproxen), and methylparaben to 4-hydroxybenzoic acid (DM-methylparaben) through microbially mediated phase I metabolism [21,22] (Figure 1). Abiotic demethylation of herbicides and some CECs was also observed after advanced oxidation processes during wastewater treatment [23]. Therefore, demethylated counterparts are among the most commonly observed TPs of CECs. Biotic methylation is a phase II metabolism mediated by methyltransferases [24]. Methylated acetaminophen, i.e., p-acetanisidide (M-acetaminophen) (Figure 1), is a major metabolite of acetaminophen in soil [25]. Methyl triclosan is the primary TP of the antimicrobial triclosan after WWTP treatment [26]. Tetrabromobisphenol A (TBBPA), a brominated flame retardant, was found to be *O*-methylated by microbes, as well as in pumpkin plants and earthworms [27–29]. Naturally occurring methyl iodide can also cause the abiotic methylation of phenolic contaminants [30].

The addition or loss of a methyl group during transformations alters a compound's physicochemical properties [31], which may subsequently affect its fate and risk in the environment. As uptake of CECs into plants is known to depend closely on a chemical's physicochemical properties, such as lipophilicity (i.e., K_{ow}) [32–34], it may be hypothesized that methylation or demethylation changes a chemical's behavior and fate in the soil-plant continuum. Despite their frequent occurrence, the environmental significance of simple transformation reactions such as methylation and demethylation is often overlooked. In this study, we compared plant accumulation and translocation of four pairs

of compounds differing only in a methyl group in their structures using two plant models, *Arabidopsis thaliana* cells and wheat seedlings. Four CECs (acetaminophen, diazepam, methylparaben, and naproxen) and their respective methylated or demethylated counterparts (M-acetaminophen, DM-diazepam, DM-methylparaben, and DM-naproxen) were chosen as the test compounds because of their widespread use and occurrence in the environment [35–37]. Of these compounds, DM-diazepam is not only a TP of diazepam, but also a pharmaceutical itself, and DM-methylparaben is not just a TP of methylparaben, but also an industrial raw material [36,38]. Results from this study contribute to a better understanding of the implications of simple transformation reactions such as methylation and demethylation on the environmental behavior and potential risks of CECs.

2.2 Materials and Methods

2.2.1 Analytes, Surrogates, and Solvents

All analytical standards were purchased with reported purities ≥ 98%. Acetaminophen, diazepam, DM-diazepam, d_5-diazepam (used as surrogate for diazepam and DM-diazepam), naproxen, DM-methylparaben and methylparaben were purchased from Sigma-Aldrich (St. Louis, MO). M-Acetaminophen was purchased from Santa Cruz (Dallas, TX). DM-naproxen and d_4-methylparaben (used as surrogate for DM-methylparaben and methylparaben) were purchased from Toronto Research Chemicals (Toronto, Ontario, Canada). d_4-Acetaminophen (used as surrogate for acetaminophen and M-acetaminophen) and d_3-naproxen (used as surrogate for naproxen and DM-naproxen)

were purchased from C/D/N Isotopes (Pointe-Claire, Quebec, Canada). HPLC-grade methanol, acetonitrile, methyl tert-butyl ether (MTBE) and acetone were purchased from Fisher Scientific (Fair Lawn, NJ). Ultrapure water was generated by an in-house Milli-Q water purification system (Millipore, Carrigtwohill, Cork, Ireland). Radioisotope labeled compounds were not used in this study, and therefore the uptake efficiency or mass balance of the target compounds in plants could not be derived.

2.2.2 Uptake in *Arabidopsis thaliana* Cells

The *A. thaliana* cell suspension (cell line T87, CCL84839) was obtained from the Arabidopsis Biological Resource Center at Ohio State University (Columbus, OH) and was maintained in the laboratory at 24 °C and 130 rpm in NT-1 media with constant light (Text S1). An aliquot (5 mL) of the *A. thaliana* cell culture was added to fresh, autoclaved NT-1 media (25 mL), and incubated for 3 d, after which each cell suspension was spiked with individual compounds to arrive at an initial concentration of 1 mg/L. Control treatments included positive and negative control groups containing CECs spiked in nonviable cells (autoclaved at 121 °C for 45 min), CECs in blank culture solution, or viable cell culture solution without CECs. Each treatment was prepared in triplicate, and was sampled at 1, 3, 6, 11, 24, 48, and 96 h. Entire samples were transferred to polypropylene centrifuge tubes (50 mL) and were immediately centrifugated at 3500 rpm for 30 min. The cell matter was stored at -80 ˚C until further analysis, and the supernatant was transferred into a 40 mL glass bottle and stored at -20 ˚C until further analysis.

2.2.3 Uptake in Wheat Seedlings

Wheat seedlings used in this study were germinated from seeds to avoid potential background contamination. Sterilized seeds were germinated on a moist filter paper on a tray in the dark at room temperature. The tray was then transferred into a growth chamber (24 ℃, 16:8 h light:dark ratio) after 2 d for seedlings to grow. When the seedlings grew to about 5 cm in height, they were transplanted into a 50-mL polypropylene centrifuge tube wrapped in aluminum foil (to prevent light exposure to the roots) and then cultivated in the growth chamber. Initially filled with water, the solution in the tubes was replaced to 1/4 strength and then 1/2 strength Hoagland® nutrient solution at 2-day intervals to allow wheat seedlings gradually acclimating to the nutrient media. Once acclimated, the media were replaced with 30 mL fresh 1/2 strength Hoagland® nutrient solution spiked with a single compound of interest from individual stocks (1000 mg/L) to reach a nominal chemical concentration of 1 mg/L. Water was added to each tube every other day to make up the lost water throughout the incubation experiment.

Triplicate containers were sacrificed at 0, 3, 6, 12, 24, 48, 96, 168 and 240 h after the treatment. Plants were rinsed with deionized water, dried with paper towels, and separated into roots and shoots. The nutrient solutions remained in the centrifuge tubes and separated plant tissues were stored at -80 ℃ until further analysis. Transpiration stream concentration factors of target compounds were not measured in wheat seedlings in this study, due to challenges in collecting adequate amount of xylem sap for analysis.

2.2.4 Sample Preparation

Deuterated compounds were used as surrogates during extraction for QA/QC. Extraction of nutrient solutions from *A. thaliana* cells and wheat seedlings was carried out using a similar method to a previous study [39], with minor modifications. Briefly, 50 μL of the surrogate solution (10 mg/L) was added to a 5 mL aliquot of nutrient solution. The nutrient solution samples were then extracted by HLB cartridges (6 mL, 150 mg). Methanol, and then water, 7 mL each, were added to each HLB cartridge for precondition, followed by the addition of the sample and then 5 mL of 5% methanol in water for clean-up. For elution and collection of the target analytes, a final pass-through of 15 mL methanol was performed. The resulting methanol eluent was collected in a glass tube, dried using a nitrogen evaporator, reconstituted in 1 mL methanol-water mixture (1:1, v/v), and filtered through a 2 mm PTFE filter into a 1.5 mL HPLC vial for instrumental analysis.

Plant cell matter and wheat tissues were freeze dried at -50 ˚C for at least 72 h to remove moisture and weighed. Before extraction, 50 μL of a depurated compound (10 mg/L) was added to each sample as the recovery surrogate. Samples were firstly extracted with 10 mL MTBE via sonication for 30 min. The sonication process was then repeated with 10 mL fresh MTBE one additional time and 10 mL acetonitrile twice. Extracts from the extraction were combined and dried by a nitrogen evaporator, followed by reconstitution in 1 mL methanol and dilution with 20 mL water. The resulting liquids were cleaned up with HLB cartridges using a similar protocol as described above. The

final extracts were dried under a gentle nitrogen gas flow, reconstituted in 1 mL methanol: water (1:1, v/v), and filtered through a 2 mm PTFE filter before instrument analysis.

2.2.5 UPLC-QqQ-MS/MS Analysis

Analytical methods for all compounds were established on a Waters ACQUITY ultra-performance liquid chromatography (UPLC) with a Waters Triple Quadrupole mass spectrometer (QqQ-MS/MS) (Waters, Milford, MA). An ACQUITY BEH C18 column (100 × 2.1 mm i.d., 1.7 μm; Waters, Milford, MA) in a 40 ℃ column compartment was used for chromatographic separation. The mobile phases (A and B) were 0.01% formic acid in water and methanol, respectively. Extracts were injected (5 μL) and separated along the following solvent gradient: 0-1 min, 5% to 40% B; 1-2 min, 40% to 90% B; 2-4 min, 90% to 95% B; and 4-6 min, re-equilibration with 5% B. The flow rate was 0.3 mL/min. The MRM transitions of all target compounds were optimized and are provided in Table S1. Data were processed by TargetLynx XS software (Waters, Milford, MA).

2.2.6 Quality Assurance and Quality Control

The recoveries and limits of quantification (LOD) for the target compounds are provided in Table S2. Method blanks and matrix blanks were included to check for possible contamination and carryover. One solvent blank and one check standard (100 μg/mL) were injected after every 10 samples. No target analytes were detected in the solvent blanks. Data were calculated as mean ± standard deviation (SD). Systematic

differences were evaluated at a significance level of 0.05 and the data were processed and graphed using SPSS Statistics 27 (IBM Corp, Armonk, NY) and Prism 9 (GraphPad, La Jolla, CA), respectively.

2.3 Results and Discussion

2.3.1 Accumulation in *A. thaliana* Cells

No significant difference was found in the biomass between the treated groups and control groups for experiments using *A. thaliana* cells. No target analytes were detected in the method blanks. The concentrations of target analytes in control groups with no cells, or with nonviable cells, varied in the range of 93.4-116.0% of the spiked concentration at the end of exposure as compared to the initial concentrations, suggesting stability of these compounds under abiotic conditions.

The individual compounds were found to be taken up by live *A. thaliana* cells. The levels in the plant cell matter reached maxima within 3 h for all compounds except acetaminophen, which exhibited the highest accumulation at 6 h into the incubation (Figure 2). The level of chemicals in the cell matter decreased quickly thereafter. At the end of 96-h cultivation, the level in the cell matter was < 0.3 μg/g (dry weight, d. w.) for most compounds, suggesting rapid metabolism in viable plant cells and likely excretion into the aqueous medium. Among the different compounds, DM-diazepam and diazepam appeared to be accumulated to higher levels than the other compounds and were also more recalcitrant to metabolism. After 96 h of incubation, 4.78 ± 0.90 μg/g of DM-

diazepam or 3.63 ± 1.74 μg/g of diazepam still remained in the *A. thaliana* cells. In previous studies, CECs including acetaminophen, diazepam and naproxen were found to be readily metabolized in different plant species [40–42].

The CECs and their methylated/demethylated derivatives showed different accumulation potentials in *A. thaliana* cells. For example, acetaminophen was detected in the *A. thaliana* cells at significantly higher concentrations than M-acetaminophen at any given sampling time point ($p < 0.05$). After 6 h of incubation, 6.10 ± 1.57 μg/g of acetaminophen was found in the *A. thaliana* cells, while the level was only 2.52 ± 0.57 μg/g for M-acetaminophen (Figure 2a). In comparison, methylparaben was found to accumulate more than DM-methylparaben at all sampling time points. For example, at 1 h, methylparaben was found at 11.6 ± 2.81 μg/g in the cell matter, while DM-methylparaben at only 0.06 ± 0.00 μg/g. The difference in accumulation by *A. thaliana* cells between DM-methylparaben and methylparaben may be partly attributed to the fact that DM-methylparaben was present mostly in an ionized form in the nutrient media (Table 1). Negatively charged chemicals are known to not easily cross the negatively charged cell walls and membranes and are limited in their plant uptake [43,44]. Like methylparaben, higher concentrations of naproxen than DM-naproxen were also detected in the cell matter throughout the exposure time (Figure 2d). After 1 h of incubation, 12.31 ± 2.46 μg/g of naproxen was found to be in the cell matter, while the level was only 2.10 ± 0.40 μg/g for DM-naproxen. However, no statistically significant difference was observed between diazepam and DM-diazepam in their levels in *A. thaliana* cells during

the exposure experiment. This may be attributed to the fact that log K_{ow} of DM-diazepam is similar to that of diazepam (Table 1).

As the test chemicals were taken up by *A. thaliana* cells, the levels of CECs and their methylated or demethylated derivatives in the culture media concurrently decreased. In the culture media, the concentration of DM-methylparaben and methylparaben, and DM-naproxen and naproxen all decreased rapidly, and their level fell below the detection limit after just a few hours into the incubation (Figure S1). In comparison, the decrease of acetaminophen and M-acetaminophen, and DM-diazepam and diazepam was relatively slower, with 0.12-0.40 mg/L, or 12-40% still remaining in the cell culture media after 48 h of exposure. The dissipation of CECs and their methylated or demethylated derivatives in the culture media was further fitted to the first-order decay model, and the fit was generally good, with $R^2 > 0.63$. The half-life $T_{1/2}$ was then calculated from the first-order rate constant (Table 2). The estimated $T_{1/2}$ values were very small for methylparaben, DM-methylparaben, and naproxen. The dissipation of DM-naproxen was so rapid that $T_{1/2}$ could not be derived. Methylation appeared to increase $T_{1/2}$ for acetaminophen and DM-diazepam, with statistically significant difference ($p < 0.05$ between acetaminophen and M-acetaminophen, and $p < 0.001$ between DM-diazepam and diazepam).

A mass balance approach was not followed in this study, as subsequent transformation products in the *A. thaliana* cells were not characterized. Given that the compounds considered in this study were stable under abiotic conditions, the rapid dissipation in the culture media and limited accumulation in the *A. thaliana* cells

suggested that the CECs and their methylated or demethylated counterparts underwent rapid metabolism in the *A. thaliana* cells. In the case of acetaminophen, methylparaben, and naproxen, demethylation introduced a hydroxyl or carboxyl group into the molecule. As shown in previous studies, compounds with a hydroxyl or carboxyl functional group can undergo rapid conjugation with various biomolecules in plants [42,45,46]. The conjugated intermediates are substantially larger in molecular size and may become "immobilized" once formed in the *A. thaliana* cells [47–49]. Future research should consider the formation of conjugates for demethylated compounds and understand the fate and risks of such plant-origin conjugates.

2.3.2 Accumulation and Translocation in Wheat Seedlings

Uptake and translocation of the paired compounds were further measured in wheat plants grown hydroponically in nutrient solutions. Roots and shoots of wheat seedlings were collected and analyzed separately to understand the in-plant translocation. Target CECs and their methylated or demethylated counterparts showed great stability in hydroponic solution without wheat seedlings, with recoveries ranging from 99.1-125.7% of the initial spiked concentration after 240 h incubation. No compounds of interest were detected in the untreated hydroponic solution or wheat seedlings.

In general, the level of chemicals in the plant tissues first increased and then decreased, suggesting uptake into the roots from the hydroponic media, followed by translocation from roots into shoots and/or metabolism in the plant. All CECs and their methylated or demethylated TPs were detected in wheat roots, and the concentrations

were much higher than those in shoots, indicating generally limited translocation (Figure 3). Among the different CECs, acetaminophen and DM-naproxen were not detected in wheat shoots, while DM-methylparaben was only found occasionally at trace levels. The accumulation of acetaminophen was also limited in the roots, which may explain its absence in the shoots. From a previous study [42], after formation from naproxen through demethylation, DM-naproxen was found to metabolize readily through phase II and phase III pathways in *A. thaliana* cells. The rapid metabolism of DM-naproxen in plants may have contributed to its absence in the shoots.

Higher concentrations were consistently detected for M-acetaminophen than acetaminophen in both wheat roots and shoots (Figure 3a). In wheat shoots, only M-acetaminophen was detected, suggesting that methylation rendered acetaminophen more mobile and a greater potential to translocate from roots to shoots. In general, DM-methylparaben was found to be taken up more rapidly than methylparaben into wheat roots and reached 20.66 ± 2.78 μg/g (d.w.) at 6 h after the treatment (Figure 3c). In comparison, the highest level of methylparaben in roots was observed at 12.34 ± 1.33 μg/g after 96 h of exposure. However, methylparaben consistently exhibited much higher concentrations than DM-methylparaben in the shoots, suggesting a greater potential for translocation for methylparaben (Figure 3c). Both compounds were found to undergo rapid metabolism, and their levels after 10 d of incubation were considerably lower than at earlier time points in the roots, while essentially no DM-methylparaben was found in the shoots. As the demethylated derivative of naproxen, although DM-naproxen was

taken up quickly and reached 33.32 ± 8.41 μg/g in wheat roots after 24 h, it appeared to be rapidly metabolized (Figure 3d), as only 0.33 ± 0.02 μg/g DM-naproxen was detected in the roots after 10 d. In comparison, naproxen was accumulated in both roots and shoots at consistently higher concentrations than DM-naproxen throughout the experiment (Figure 3d). In wheat shoots, DM-naproxen was consistently below the detection limit, suggesting limited translocation, and/or rapid transformations in the roots via pathways such as conjugation.

Both DM-diazepam and diazepam showed significant accumulation in wheat plant (Figure 3b). At the end of 10-d exposure, 32.74 ± 0.64 μg/g and 13.12 ± 2.79 μg/g of diazepam were detected in roots and shoots, respectively, while the corresponding values were 15.36 ± 1.51 μg/g and 11.81 ± 0.40 μg/g for DM-diazepam, suggesting active translocation after entry in the roots. Among the four pairs of compounds considered in this study, diazepam and DM-diazepam have the largest log K_{ow} (Table 1). Between diazepam and DM-diazepam, the root accumulation of DM-diazepam was greater than diazepam during the first few sampling time points; however, an opposite trend was observed after 48 h of incubation, where the level of diazepam appeared to be significantly greater than DM-diazepam (Figure 3b). Levels of both diazepam and DM-diazepam in the shoots increased over time, and there was no statistically significant difference between their concentrations at the same time points. It must be noted that unlike the other compounds considered in this study, demethylation of diazepam does not introduce a hydroxyl group into the structure and therefore, the almost identical

accumulation of diazepam and DM-diazepam may be attributed to their similar physicochemical properties (Table 1).

As CECs and their methylated or demethylated derivatives were taken up by wheat seedlings, their levels in the nutrient solution decreased (Figure S2). The rate of dissipation was similar between acetaminophen and M-acetaminophen, and between methylparaben and DM-methylparaben. However, diazepam and naproxen appeared to decline at a slower rate than their demethylated counterparts (Figure S2). Consequently, the estimated $T_{1/2}$ values were also significantly longer for diazepam and naproxen than their demethylated derivatives (Table 2). The prolonged availability of diazepam and naproxen in the nutrient solution may have contributed to their relatively high accumulation in wheat seedlings (Figure 3b and 3d).

Translocation factor (TF) in the whole wheat plant was calculated as the ratio of the concentration in shoots to that in roots (C_{shoot}/C_{root}) at the end of exposure [32,50–52]:

$$\text{Translocation Factor (TF)} = \frac{\text{Concentration in shoots}}{\text{Concentration in roots}} \qquad (1)$$

The derived TF values of the four pairs of compounds are shown in Figure 4. The TF values for all target compounds in this study were less than 1, reflecting generally limited mobility from roots to shoots. With the exception of diazepam and DM-diazepam, TF values were generally greater for the methylated compound than the demethylated counterpart, although the difference was statistically significant only between

acetaminophen and M-acetaminophen. For example, the derived TF was 0.30 ± 0.01 for M-acetaminophen, while the TF for acetaminophen was 0 as acetaminophen was not found in the shoots (Figure 3a). However, diazepam and DM-diazepam showed an opposite trend, where DM-diazepam exhibited a greater TF (0.77 ± 0.10) than its methylated counterpart diazepam (0.40 ± 0.09), and the difference was statistically significant (p <0.01, Figure 4). As noted above, demethylation of diazepam does not lead to significant changes in physicochemical properties, and in fact, log K_{ow} 2.93 of DM-diazepam was slightly greater than that for diazepam (log K_{ow} 2.82) (Table 1). These results suggest that the effect of methylation or demethylation on the translocation of CECs in whole plants is specific to the molecular structure of individual compounds, and the changes that the reaction brings to the compound's properties, such as hydrophobicity. When demethylation results in increased polarity (or decreased hydrophobicity), reduced plant uptake and translocation may be expected. In contrast, methylation generally leads to increased hydrophobicity and may be expected to contribute to enhanced plant uptake and translocation.

2.3.3 Correlation Between Accumulation and Physicochemical Properties

Physicochemical properties of organic compounds, such as hydrophobicity (indicated by K_{ow}, partition coefficient between octanol and water) and ionization, are known to greatly influence their accumulation in organisms [13,14,53]. Several previous studies reported a positive linear relationship between log K_{ow} and root accumulation in various plant species for neutral xenobiotics [13,32,52,54]. For ionic xenobiotics, the situation

is more complicated. On the one hand, charged compounds are generally less accumulative than neutral species, especially anions, because the cell membranes are negatively charged. On the other hand, ionic species may interact with cell walls and membranes, involving in processes such as “ion trap”, which may contribute to more accumulation in plants [43,54,55]. For ionizable xenobiotics, it is important to determine their fraction of neutral species (f_n) in order to predict their bioaccumulation potential in plants, since neutral molecules are usually considered to be taken up more readily by plants than their ionized forms [32,50]. Therefore, pK_a and the ambient pH are important factors regulating the plant uptake of ionizable compounds. In this study, pH of the plant cell culture solution and the whole wheat hydroponic culture solution were measured to be 5.80 and 5.10, respectively. The fraction of the neutral species (f_n) for the compounds in the culture media was calculated using [32,55]:

$$f_n = \frac{1}{1 + 10^{i(pH - pK_a)}} \qquad (2)$$

where *i* is 1 for acids and -1 for bases.

By considering K_{ow} for the neutral species and the dissociation rate of ionizable compounds, the pH-adjusted octanol-water partition coefficient log D_{ow} was estimated as (Table 1):

$$logD_{ow} = logK_{ow} + logf_n \qquad (3)$$

As log D_{ow} discounts for the ionized fraction, it is expected to correlate more closely with bioaccumulation than log K_{ow} for ionizable compounds.

Among the four pairs of compounds considered here, methylation and demethylation had varied effects on the accumulation in *A. thaliana* cells for the different CECs, and the effect was molecule-specific. In wheat seedlings, the demethylated derivative in each pair, when demethylation causes the introduction of a polar functional group (e.g., hydroxyl group), was accumulated at a comparatively reduced level and also exhibited more limited translocation. The limited accumulation of demethylated derivatives, especially in the shoots, may be partly attributable to their rapid subsequent metabolism, such as conjugation. Demethylation of M-acetaminophen, methylparaben and naproxen led to the introduction of a hydroxyl or carboxyl group into the molecule, resulting in lower log K_{ow} and log D_{ow} values than those for their parent form (Table 1). As demonstrated in previous studies, CECs with functional groups such as hydroxyl group are highly susceptible to conjugation with biomolecules in plants, including amino acids, sugars, and sulfate [42,56–58]. In contrast, the *N*-demethylated diazepam derivative, DM-diazepam, has a slightly greater log K_{ow} (2.93) than diazepam (2.82) (Table 1). The high similarity in log K_{ow} and log D_{ow} between diazepam and DM-diazepam may explain their almost identical accumulation in *A. thaliana* as well as in wheat seedlings. Therefore, when demethylation leads to a decreased log K_{ow} or log D_{ow} value for the compound (as observed for *O*-demethylation in this study), it may generally result in

reduced plant accumulation. The reduced accumulation may be caused by a decrease in uptake into the root because of the increased polarity, and/or rapid Phase II transformations such as conjugation with endogenous biomolecules in plants. Conversely, when a compound becomes methylated and its log K_{ow} or log D_{ow} increased, as in the case of conversion of acetaminophen to M-acetaminophen (i.e., p-acetanisidine), DM-naproxen to naproxen, and DM-methylparaben to methylparaben, plant uptake and translocation are likely enhanced.

To better understand the relationships between physicochemical properties of CECs and their accumulation in plants, the bioconcentration factor (BCF), calculated as the ratio of chemical concentration in *A. thaliana* cells, wheat roots or shoots at the end of exposure, to the initially spiked concentration, and log TF in wheat seedlings for all target compounds are plotted against their log D_{ow} values (Figure 5). Positive linear relationships were observed between log BCF and log D_{ow} for the different treatments in this study (Figure 5a, b, and c, $p < 0.05$), indicating that the bioaccumulation of methylated or demethylated CEC derivatives in plants was closely related to the pH adjusted hydrophobicity parameter log D_{ow}. This linear relationship between log D_{ow} and accumulation was also observed for vegetables grown hydroponically in previous studies [32,54,59]. Therefore, differences in accumulation by *A. thaliana* cells or wheat seedlings caused by methylation or demethylation may be largely explained by the change imparted on K_{ow} or D_{ow}.

No significant correlation was found between log TF and log D_{ow} in this study (Figure 5d, $p > 0.05$), likely due to the limited number of compounds considered in this study. Wu et al. (2013) observed a generally negative correlation for pharmaceuticals and personal care products in lettuce, spinach, cucumber and pepper. Another study conducted by Li et al. (2018) did not show any significant correlation between log TF and log K_{ow} for neonicotinoids in Japanese mustard. Different treatments, plant species and compounds were used in those studies, suggesting that the translocation of xenobiotics in plants may be affected by not only the physicochemical properties of the xenobiotics, but also the inherent characteristics of plants. In addition, plants have a cascade of enzymes that are capable of facilitating metabolic transformations, and metabolism affects TF, as rapid metabolism in the root would translate into a diminished TF. Also, weak acidic CECs dissociated in the cytosol could be repelled by the negatively charged cell membranes, and therefore, become "trapped" in root cells, which may also limit their translocation [51]. Active metabolism, such as conjugation with endogenous plant biomolecules, and the possible "ion trap" in root cells, likely contributed to the lack of apparent translocation for acetaminophen, DM-naproxen and DM-methylparaben in this study.

While physicochemical parameters such as pK_a and K_{ow} are available for many man-made compounds, they are often unknown for TPs. In the absence of experimentally measured values, models based on molecular descriptors may be used for obtaining approximate physicochemical properties for TPs. For example, ChemAxon provides a

calculator for predicting log K_{ow}, pK_a, and log D_{ow} of organic compounds. In the case of bisphenol A (BPA), methylation may be predicted to increase its log D_{ow} (pH at 5) from 4.04 to 4.19 for BPA monomethyl ether and further to 4.34 for BPA dimethyl ether. Likewise, while TBBPA has a log D_{ow} (pH at 5) of 7.11, it increases to 7.41 for TBBPA dimethyl ether. Methylation of diclofenac to diclofenac-methyl ether increases its log D_{ow} (pH at 5) from 3.21 to 4.4; log D_{ow} of methyl triclosan (5.13) is also greater than triclosan (4.98). However, methylation of compounds does not always result in increased log D_{ow} values. For example, caffeine (-0.55) would show a smaller log D_{ow} than some of its demethylated products, i.e., paraxanthine (0.24) and 7-methylxanthine (0.02). Hence, methylation and demethylation change the physicochemical properties of CECs, and the change induced is highly molecule-specific. Tools like ChemAxon could help predict basic properties of organic compounds, including TPs that do not always have experimentally derived values. It is feasible to incorporate changes in physicochemical properties, using either experimentally derived or estimated values, into well-established empirical relationships to evaluate the potential influence of common transformation reactions such as methylation and demethylation on plant uptake for a large range of CECs in the scenarios of beneficial reuse of treated wastewater effluent and biosolids.

2.4 Environmental Implications

Simple reactions such as methylation and demethylation are common abiotic and biotic transformations, which contribute to the co-occurrence of many TPs of man-made chemicals in the environment. As demonstrated in this study, methylation and

demethylation could result in changes in a chemical's physicochemical properties, and the magnitude of change is specific to the molecule and the types of functional groups undergoing the conversion. The changes in a chemical's physicochemical properties could subsequently lead to different environmental behaviors and risks, such as accumulation and translocation in higher plants. Moreover, a methylated or demethylated derivative may have increased or decreased biological activity as compared to the parent compound. Given that there are numerous CECs in sources such as treated wastewater and biosolids, the co-existence of additional TPs presents another layer of challenge to the risk assessment of man-made chemicals. Although not explored in this study, differences may be similarly expected in microbial degradation and hence persistence of TPs, and further, bioaccumulation and toxicity to non-target organisms, such as aquatic and terrestrial invertebrates. For instance, methylated diclofenac showed a 430-fold increase in acute toxicity to *Hyalella azteca* than diclofenac [61]. BPA mono- and dimethyl ethers were also found to result in enhanced mortality and developmental toxicity in zebrafish embryos than BPA [62]. Methyl triclosan was shown to exhibit greater bioaccumulation in snails but reduced bioaccumulation in algae compared to triclosan [63,64]. The potential influence of methylation and demethylation on the phytotoxicity of CECs was not explored in this study; further research should consider this aspect by evaluating changes in enzyme activities and photosynthetic efficiency, among other endpoints [41].

It must be noted that the experiments in this study were conducted under simplistic conditions. More processes and variables are involved in the soil-plant system under field conditions and their interactions likely determine the ultimate fate and risks of a chemical. For instance, methylation or demethylation may alter a compound's stability in the rhizosphere as well as its adsorption to the soil solid phase, which in turn influence the chemical's availability for plant uptake. As a chemical's log K_{ow} increases, its adsorption to soil increases while its presence in the soil porewater decreases, leading to a reduced availability for uptake into plant roots. The interactions of these fate and transport processes in the soil-plant system may therefore amplify or diminish the effects brought upon by the transformations and should be further studied under field-relevant conditions.

A significant bottleneck to the holistic assessment of environmental risks is the sheer number of CECs and the fact that experimentally determined physicochemical properties are often not available for their transformation intermediates. It is likely that for many CECs, transformation reactions consistently lead to reduced biological availability and lower non-target toxicity. In this case, only certain transformation reactions for a subset of CECs may pose an increased risk. Predicting essential physicochemical parameters such as pK_a and log D_{ow} using well-established chemical calculation tools may generate the first line of information for identifying TPs with enhanced potential for bioaccumulation or non-target toxicity. This approach may be used to effectively direct

future research efforts to better understand the environmental significance of common transformation reactions for CECs.

Tables

Table 2-1. Physicochemical properties of selected CECs and their methylated or demethylated counterparts.

Compound	logK_{ow}[a]	pK_a[c]	pH 5.8		pH 5.1	
			f_n	logD_{ow}[d]	f_n	logD_{ow}[d]
Acetaminophen	0.46	9.38	1.00	0.46	1.00	0.46
M-Acetaminophen	1.03	1.5[b]	1.00	1.03	1.00	1.03
DM-Diazepam	2.93	2.85[b]	1.00	2.93	0.99	2.93
Diazepam	2.82	3.40	1.00	2.82	0.98	2.81
DM-Methylparaben	1.58	4.54	0.05	0.30	0.22	0.91
Methylparaben	1.96	8.34	1.00	1.96	1.00	1.96
DM-Naproxen	2.84[b]	4.34[b]	0.03	1.37	0.15	2.01
Naproxen	3.18	4.18	0.02	1.55	0.11	2.21

[a]Measured values collected from PubChem: https://pubchem.ncbi.nlm.nih.gov/.

[b]Predicted by ChemAxon and collected from The Human Metabolome Database: https://hmdb.ca/.

[c]Measured value from CompTox Chemicals Dashboard: https://comptox.epa.gov/dashboard/.

[d]Calculated logD_{ow} values crosschecked with the logD_{ow} values predicted by ChemAxon: https://disco.chemaxon.com/calculators/demo/plugins/logd/.

Table 2-2. The dissipation half-life of test compounds in *A. thaliana* cell culture media and wheat seedling hydroponic solution.

Compound	Dissipation half-life ($T_{1/2}$, h)	
	A. thaliana cell media	Hydroponic solution
Acetaminophen	20.4 (14.0-30.3)[a]	11.6 (7.2-19.7)
M-Acetaminophen	34.0 (23.69-50.0)	13.7 (6.8-30.2)
DM-Diazepam	49.7 (23.1-146.0)	36.2 (9.0 -109.9)
Diazepam	106.2 (46.2-988.1)	245.5 (194.5-323.8)
DM-Methylparaben	0.69 (0.68-0.70)	8.2 (5.3-13.2)
Methylparaben	1.05 (0.42-1.58)	12.2 (6.3-26.9)
DM-Naproxen	N.A.[b]	8.3 (6.0-11.7)
Naproxen	0.9 (0.7-1.0)	67.4 (25.1-174.7)

[a]Values expressed as "the best fit value (95% CI)".
[b]N.A. - not available due to extremely rapid dissipation.

Figures

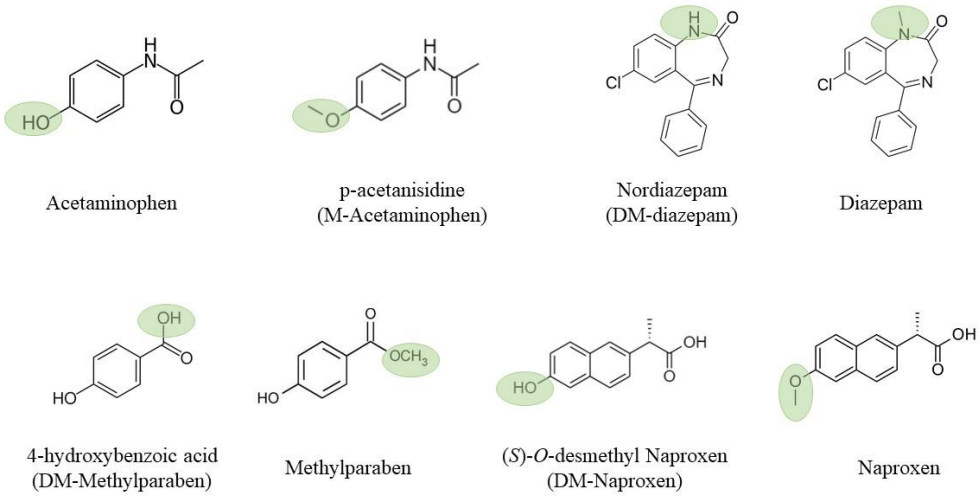

Figure 2-1. Chemical structures of the target compounds considered in this study; methylated or demethylated part indicated with a green circle.

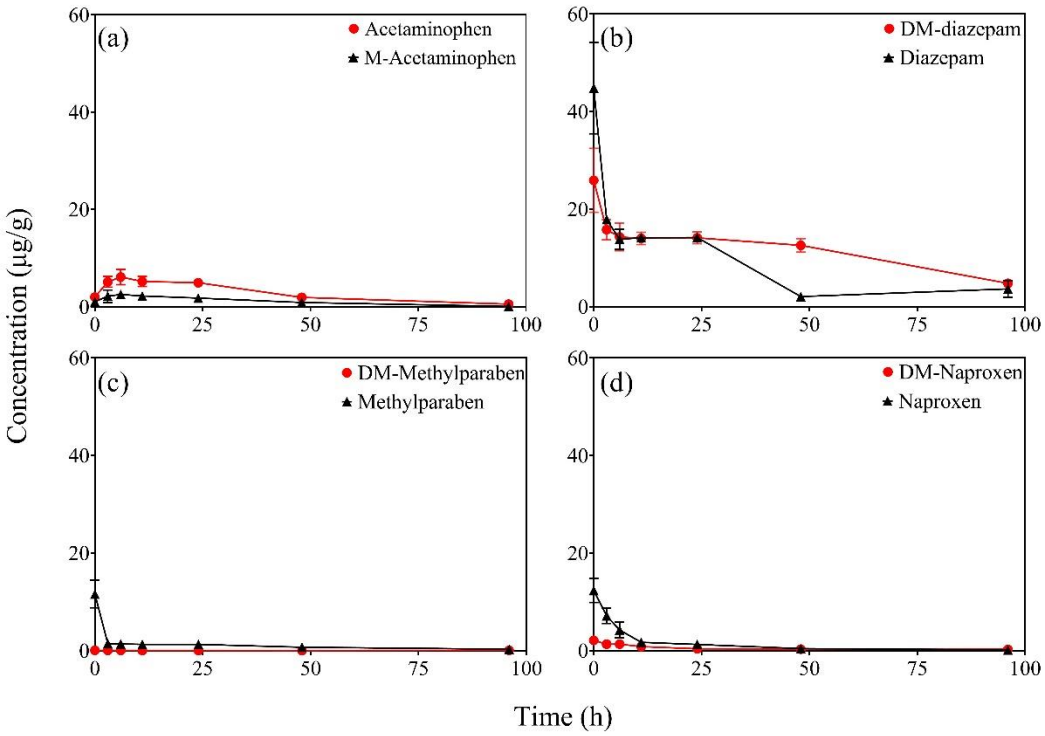

Figure 2-2. Accumulation of CECs and their methylated or demethylated transformation products in *A. thaliana* cells.

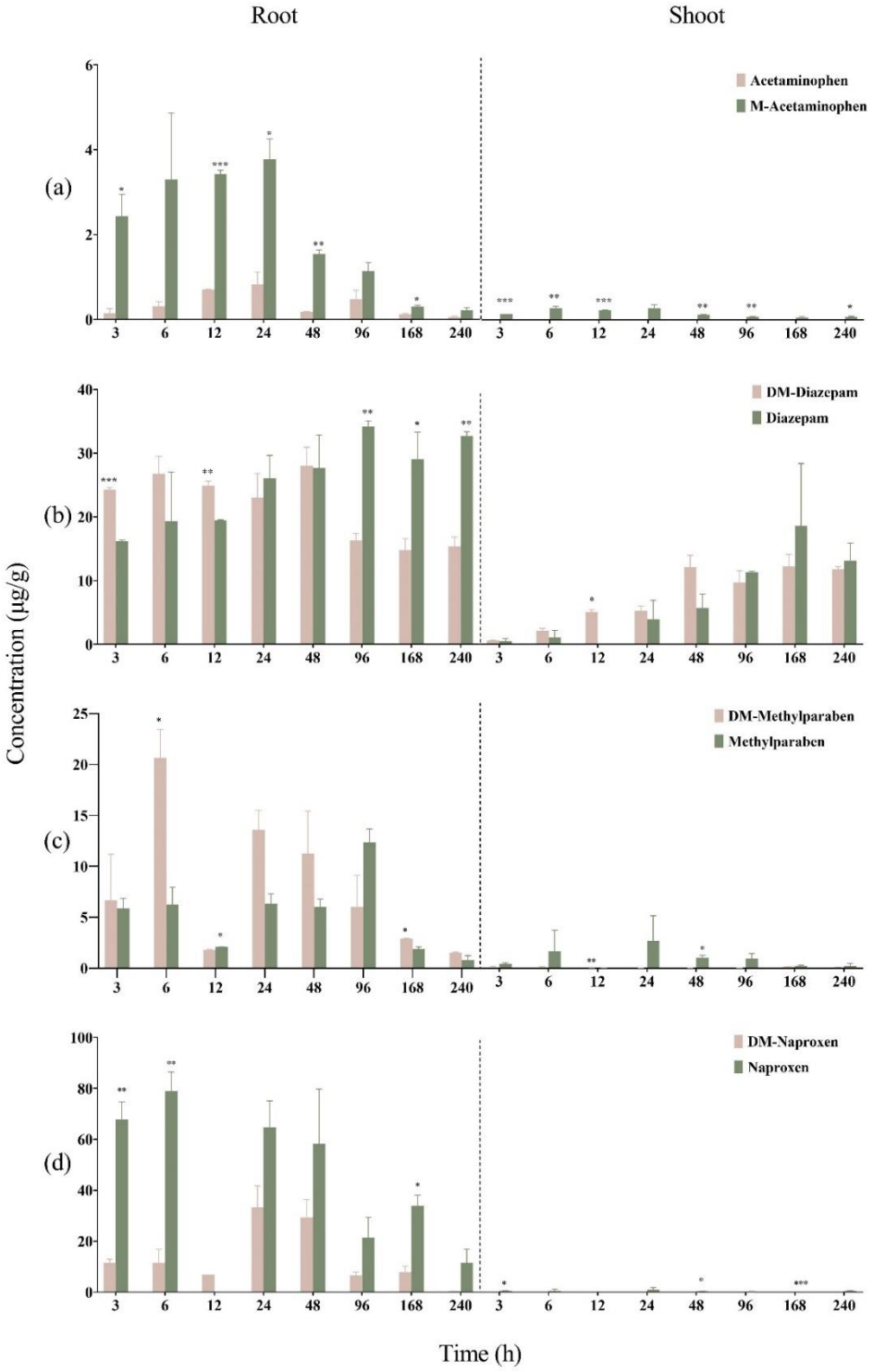
Root
Shoot
(a)
Acetaminophen
M-Acetaminophen
(b)
DM-Diazepam
Diazepam
(c)
DM-Methylparaben
Methylparaben
(d)
DM-Naproxen
Naproxen
Concentration (μg/g)
Time (h)
3
6
12
24
48
96
168
240

Figure 2-3. Accumulation of CECs and their methylated or demethylated counterparts in wheat roots and shoots.

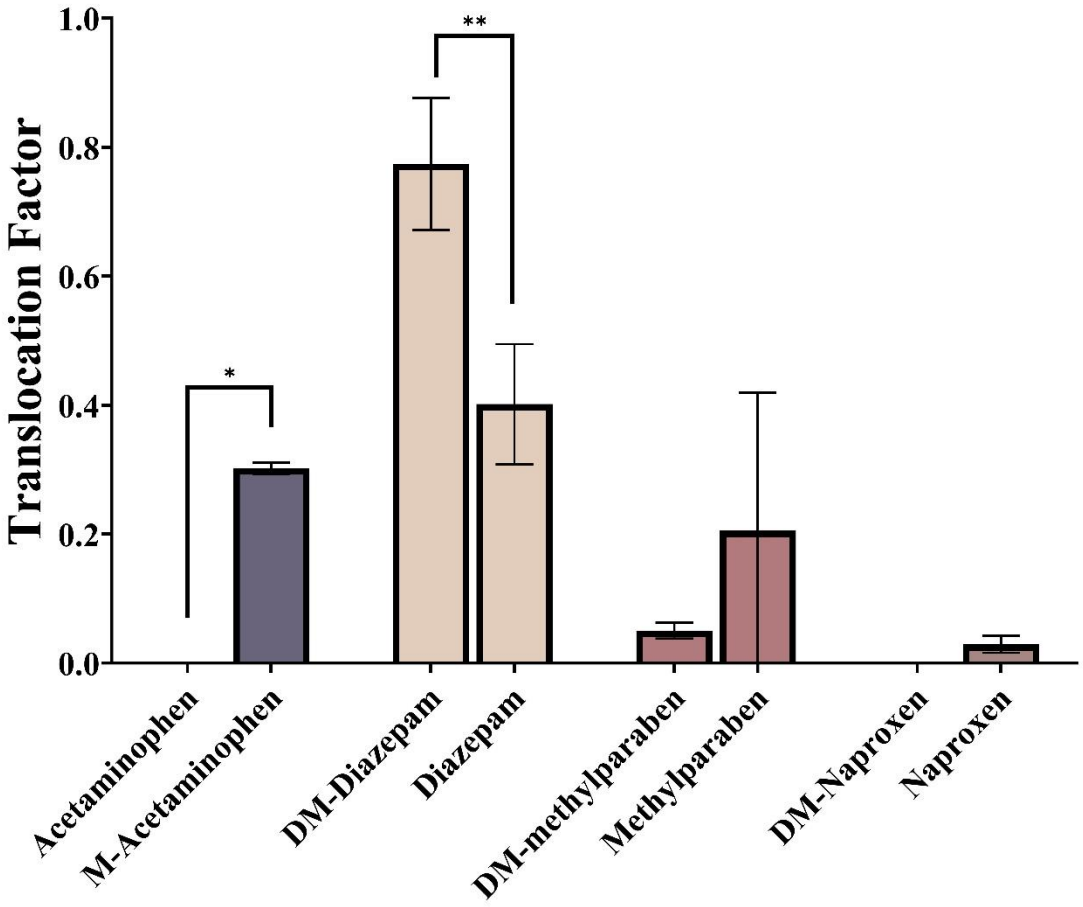

Figure 2-4. Translocation factor (TF) of test compounds and their methylated or demethylated counterparts in wheat seedlings at 10 d.

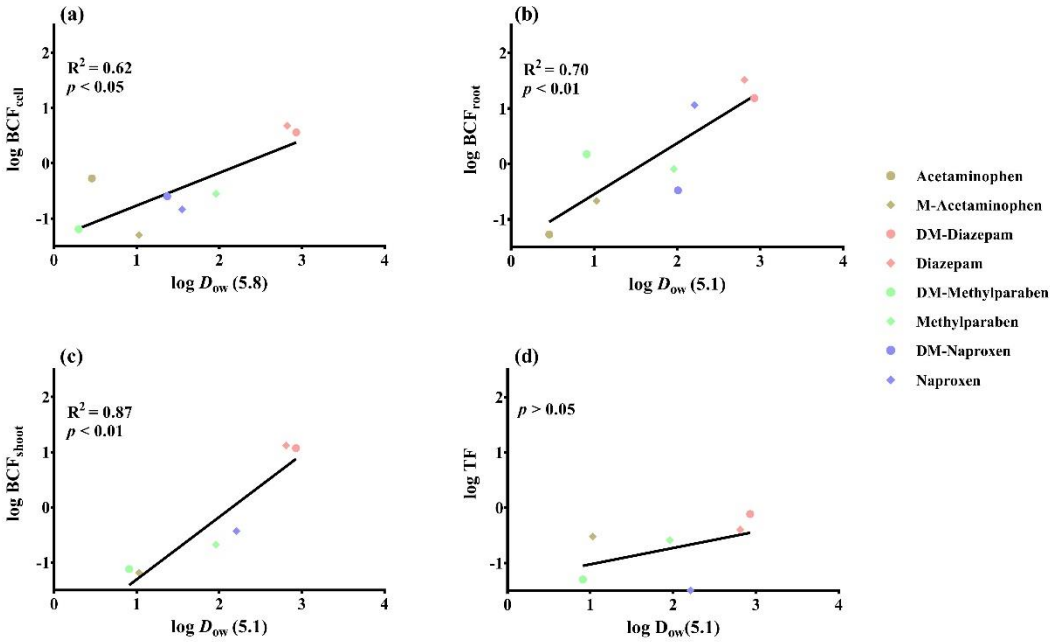

Figure 2-5. Correlations between log D_{ow} and (a) bioconcentration factor in *A. thaliana* cells, (b) bioconcentration factor in wheat root, (c) bioconcentration factor in wheat shoot, and (d) translocation factor in wheat seedlings.

Supplementary Information

Text S2-1. NT-1 media

The *A. thaliana* suspension cell culture solution was prepared with the following ingredients: 4.3 g Murashige and Skoog basal salt mixture (MS), 30 g sucrose, 0.18 g KH_2PO_4, 100 mg myo-Inositol, 220 µL of 2 mg/mL 2,4-D stock solution and 100 µL of 10 mg/mL Thiamine stock solution. These components were dissolved in 800 mL deionized water. Then the pH of the solution was adjusted to 5.8 with 5 M NaOH solution. Finally, the volume of the solution was adjusted to 1 L. An aliquot (75 mL) of the final solution was added to a 250 mL glass flask, and autoclaved for 20 mins. After it was cooled down to room temperature, 15 mL of *A. thaliana* suspended cells was added into flasks, and then maintained at 24 °C and 130 rpm with constant light for 7 days before it can be sub-cultured.

Table S2-1. MRM transitions of target compounds on UPLC-MS/MS

Compound	MRM (m/z)			
	Quantification	CV/CE*	Qualification	CV/CE
ESI+				
Acetaminophen	151.97 > 109.99	38/22		
M-Acetaminophen	166.03 > 124.07	38/22	166.03 > 92.74	38/24
d4-Acetaminophen	156.03 > 113.99	40/12	156.03 > 96.75	40/22
DM-diazepam	271.03 > 139.99	56/28	271.03 > 165.03	56/28
Diazepam	285.03 > 154.02	56/26	285.03 > 193.09	56/32
d5-Diazepam	290.10 > 198.07	54/34	290. 10 > 154.11	54/26
ESI-				
DM-Methylparaben	137.09 > 93.08	34/15		
Methylparaben	151.05 > 92.03	38/20	151.05 > 136.00	38/14
d4-Methylparaben	155.05 > 96.05	36/20	155.05 > 140.01	36/14
DM-Naproxen	215.15 > 171.15	21/6	215.15 > 169.15	21/28
Naproxen	229.15 > 185.15	17/8	229.15 > 170.15	17/16
d3-Naproxen	232.18 > 188.10	14/5	232.18 > 173.14	14/18

*CV-cone voltage (kV), CE-collision energy (eV).

Table S2-2. Detection limits and recoveries of target compounds

Compound	LOQ* ng/mL	Recovery (%)				
		A. thaliana cells	Wheat roots	Wheat shoots	Cell culture media	Wheat hydroponic solution
Acetaminophen	0.5	93.3 ± 7.7	83.2 ± 1.0	78.1 ± 0.7	112.0 ± 6.1	113.4 ± 5.7
M-Acetaminophen	0.2	63.4 ± 7.1	63.8 ± 13.3	63.2 ± 1.1	96.0 ± 2.7	98.8 ± 4.0
DM-Diazepam	0.2	82.5 ± 1.7	70.7 ± 3.8	60.0 ± 3.7	75.1 ± 2.3	77.3 ± 1.9
Diazepam	0.25	95.9 ± 8.9	83.3 ± 10.2	69.5 ± 11.5	114.9 ± 6.7	85.0 ± 13.0
DM-Methylparaben	3.0	80.8 ± 6.7	42.4 ± 10.1	54.8 ± 4.7	101.0 ± 5.8	100.8 ± 1.5
Methylparaben	1.5	64.3 ± 6.9	100.7 ± 2.8	97.4 ± 1.7	126.2 ± 3.2	97.3 ± 0.7
DM-Naproxen	3.0	65.0 ± 5.2	39.9 ± 7.3	42.6 ± 6.3	90.3 ± 2.5	90.3 ± 9.6
Naproxen	2.0	115.8 ± 3.0	89.1 ± 2.8	84.5 ± 3.6	80.3 ± 6.6	100.5 ± 1.3

*LOQ, limit of quantification.

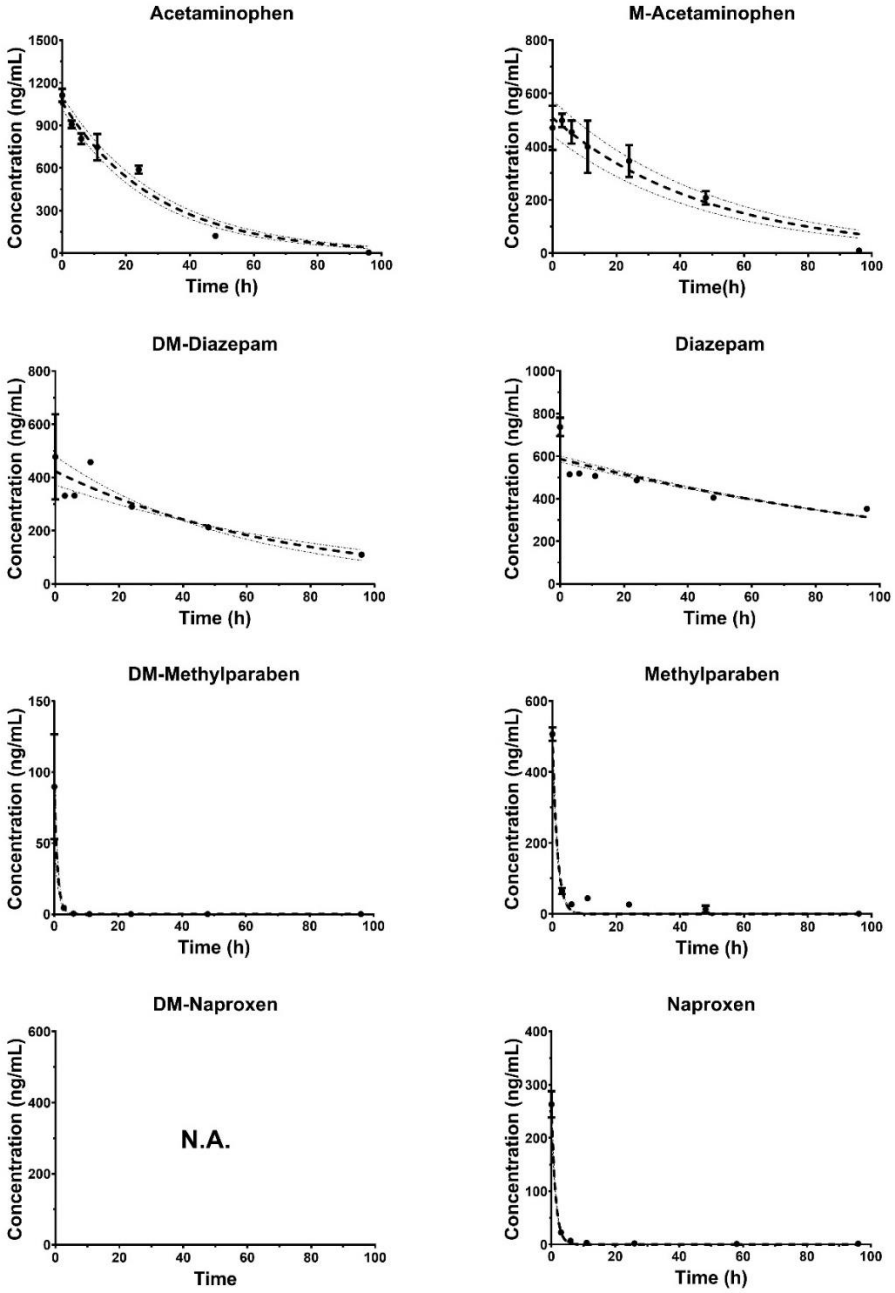

Figure S2-1. The first-order dissipation kinetics of target compounds in plant cell culture media.

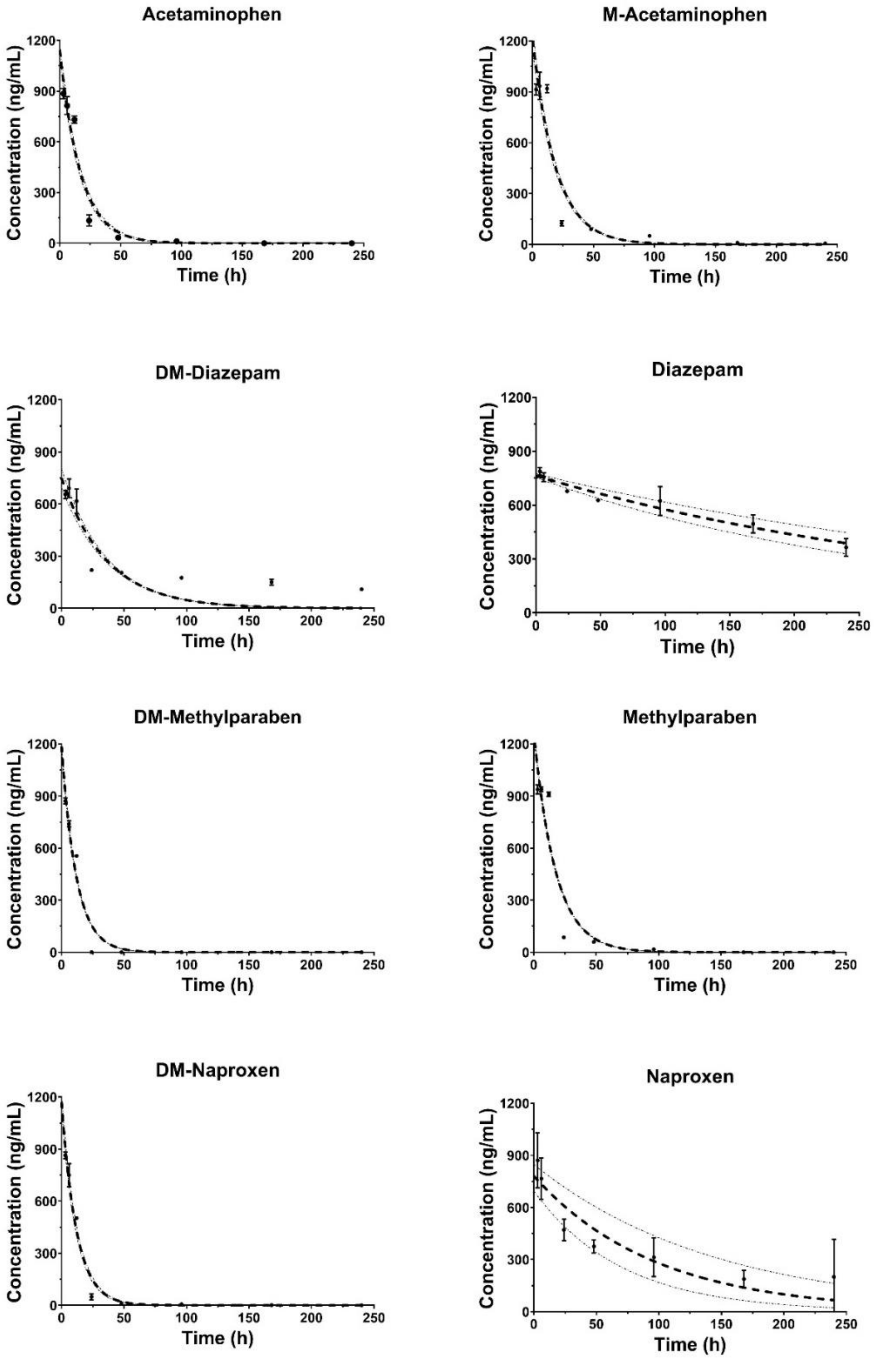

Figure S2-2. The first-order dissipation kinetics of target compounds in wheat hydroponic solutions.

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Chapter 3 Methylation and Demethylation of Emerging Contaminants in Plants

Abstract

Many contaminants of emerging concern (CECs) have reactive functional groups and may readily undergo biotransformations including methylation and demethylation. Such transformations have been reported to occur during human metabolism and wastewater treatment, leading to the propagation of the number of CECs. When treated wastewater and biosolids are used in agriculture, CECs and their transformation products (TPs) are introduced into soil-plant systems. However, little is known if transformation cycles, such as methylation and demethylation, take place in higher plants and hence affect the fate of CECs in terrestrial ecosystems. In this study, we explored the interconversion between four common CECs (acetaminophen, diazepam, methylparaben and naproxen) and their methylated or demethylated TPs in *A. thaliana* cells and whole wheat seedlings. The methylation-demethylation cycle occurred in both plant models, with demethylation generally taking place at a greater degree than methylation. The rate of demethylation or methylation was dependent on the bond strength of R-CH_3, with demethylation of methylparaben or methylation of acetaminophen being more pronounced. Although not explored in this study, these interconversions may exert influences to the behavior and biological activity of CECs, particularly in terrestrial ecosystems. The study findings highlight the prevalence of biologically mediated transformations along the human-wastewater-soil-plant continuum and the need to

consider these circular transformations to obtain a more accurate understanding of the environmental fate and risks of CECs.

3.1 Introduction

The use of treated wastewater and biosolids promotes environmental and agricultural sustainability and is increasingly practiced around the world.[1–3] However, numerous contaminants of emerging concern (CECs) are present in the wastewater treatment plant (WWTP) effluent and biosolids.[3–6] Reuse of these resources introduces CECs into agroecosystems, where some CECs may be taken up by plants and enter terrestrial food chains.[3,4,7] Even though the transfer of CECs from WWTP effluent or biosolids to higher plants has been increasingly reported, in most cases only the parent form of CECs is considered.[8–10] Many CECs, unlike legacy contaminants such as PCBs and organochlorine pesticides, possess reactive functional groups like hydroxyl and carboxyl groups, making them more susceptible to abiotic and biotic transformations. Such transformations have been reported for CECs after human consumption,[11,12] during treatment at WWTPs,[13,14] and in other biologically-mediated processes.[15–18] For instance, methyl triclosan was often detected alongside triclosan in WWTP effluent and biosolids, sometimes at even higher levels.[19,20] Acetaminophen can be methylated by microorganisms in soil.[21] Therefore, with the use of treated wastewater and biosolids, CECs are often introduced into the agroecosystems together with their transformation products (TPs), such as methylated or demethylated TPs, before they come into contact with plants.[4,22–24]

Plants also have a cascade of enzymes capable of many biotransformation reactions.[25,26] For example, demethylation is a common transformation for xenobiotics as phase I metabolism catalyzed by cytochrome P450 enzymes, and esterase catalyzed or nonenzymatic hydrolysis for esters.[27–30] Diazepam was previously found to be demethylated to nordiazepam (DM-diazepam) in *Arabidopsis thaliana* cell cultures, cucumber and radish seedlings.[31,32] Naproxen was demethylated to 6-*O*-desmethylnaproxen (DM-naproxen) in *A. thaliana* cells.[33] Methylation, as a phase II metabolism catalyzed by methyltransferases,[34,35] was also reported for a broad spectrum of substrates ranging from nucleic acids, lipids to xenobiotics.[34,36] For example, tetrabromobisphenol A (TBBPA) was converted to TBBPA *mono*- and *di*-methyl ethers in pumpkin seedlings.[37] Therefore, in-plant transformations such as methylation or demethylation may occur after CECs or their TPs are taken up into plants, influencing the environmental cycling of CECs. It is further plausible that methylation and demethylation happen simultaneously and form a metabolic cycle within plants. For example, exposure of pumpkin plants to TBBPA dimethyl ether showed that the methylated TBBPA metabolite was demethylated back to TBBPA.[37] This interconversion between CECs and their methylated or demethylated TPs may effectively prolong the environmental persistence of CECs, leading to uncertainties in our understanding of their ecological and human exposure and risks.

To date, there has been little research on interconversions of CECs in plants, even though plants play a critical role in terrestrial ecosystems including agricultural systems.

Here we examined the interconversion between four pairs of CECs, i.e., acetaminophen, diazepam, methylparaben and naproxen, and their methylated or demethylated counterparts in *A. thaliana* cells and wheat seedlings. These compounds were selected because of their ubiquitous occurrence in the environment.[23,31,38] Additionally, TPs of these CECs are known to possess biological activity. For instance, DM-diazepam is not only a TP resulting from demethylation of diazepam but also a pharmaceutical in its own right.[39] Similarly, DM-methylparaben also serves as a raw material in various industrial applications.[40] *A. thaliana* cell suspensions were selected in this study due to the easiness for cultivation, high metabolic activity, and their common use as a fast-screening tool for evaluating plant metabolism.[41,42] Whole plants, on the other hand, are more complex in structures as they have differentiated organs as well as associated microorganisms.[43,44] Whole plants are therefore complementary to cell models as they provide more environmental relevance.[45] Results from this comparative evaluation provide knowledge on the occurrence of such plant-mediated interconversions and the potential significance of this process to the environmental fate and risks of CECs.

3.2 Materials and Methods

3.2.1 Chemicals and Materials

Analytical standards (purity > 98%), including acetaminophen, diazepam, naproxen and methylparaben, and their methylated or demethylated counterparts, i.e., *O*-methylated

acetaminophen (M-acetaminophen), DM-diazepam, DM-naproxen and 4-hydroxybenzoic acid (*O*-demethylated methylparaben, DM-methylparaben), were purchased from Sigma-Aldrich (St. Louis, MO), Santa Cruz Biotechnology (Dallas, TX) or Toronto Research Chemicals (Toronto, Ontario, Canada). The deuterated standards d_5-diazepam (Sigma-Aldrich), d_4-methylparaben (Toronto Research Chemicals), d_4-acetaminophen and d_3-naproxen (C/D/N isotopes, Pointe-Claire, Quebec, Canada) were used as internal standards. HPLC grade solvents, including methanol, acetonitrile and methyl tert-butyl ether (MTBE), were purchased from Fisher Scientific (Fair Lawn, NJ). Ultrapure water was made using an in-house Milli-Q water purification system (Millipore, Carrigtwohill, Cork, Ireland).

3.2.2 Treatment and Incubation of *Arabidopsis thaliana* Cells

A schematic description of experimental design used in this study is given in the Supporting Information (SI, Figure S1). *A. thaliana* cell suspension (cell line T87, CCL84839) was purchased from the Arabidopsis Biological Resource Center (Columbus, OH). The suspension was cultured and maintained at 24 °C and 130 rpm in NT-1 media with constant lighting.[23] An aliquot of 5 mL of *A. thaliana* cell culture was transferred to a glass flask containing 25 mL fresh, autoclaved (121°C, 45 min) NT-1 media every week. After 3 d incubation, the cell culture was ready for use in the experiments. The experimental design was similar to a previous study where the effect of methylation or demethylation on the bioaccumulation of CECs was examined in plant models.[23] Briefly,

to determine the interconversion between the selected CECs and their demethylated or methylated TPs in plants, the above target compounds were spiked individually into *A. thaliana* cell cultures at 1 mg/L. Control treatments including groups containing CECs with nonviable *A. thaliana* cells (autoclaved at 121°C for 45 min), CECs in blank culture solution, and viable cell suspension without CECs, were used for quality control purposes. After 0, 3, 6, 11, 24, 48 and 96 h of incubation, triplicate flasks from each treatment group were sacrificed, with the control treatment groups sampled only at 96 h. The cell suspension in each flask was transferred in its entirety to a 50 mL polypropylene centrifuge tube and immediately centrifugated at 3500 rpm for 30 min. The supernatants were gently pooled into another 50 mL polypropylene centrifuge tube and stored at -20 ˚C until analysis. The cell matter residues precipitated at the bottom of centrifuge tubes were then cleaned with 30 mL Milli-Q water and centrifugated at 3500 rpm for another 30 min. Biological activity of *A. thaliana* cells was not terminated during these steps. The resulting supernatant was discarded and the remaining cell matter was stored at -80 ˚C before analysis.

3.2.3 Treatment and Cultivation of Wheat Seedlings

Wheat seedlings were germinated from seeds in a seed germination tray kit in the dark at room temperature. When the seedlings grew to around 5 cm in height, five seedlings were transplanted into a 50-mL polypropylene centrifuge tube (foil-wrapped to prevent light exposure to the roots) containing 30 mL Milli-Q water. The wheat seedlings

were cultivated in a growth chamber at 24 ℃ with a 16:8 h light:dark schedule. The water solution in the tubes was replaced in 2-d intervals to 1/4 strength and then 1/2 strength Hoagland® nutrient solution to allow gradual acclimation for the seedlings. After seedlings were acclimated for another 2 d in the 1/2 strength Hoagland® nutrient solution, the hydroponic solution was replaced with 30 mL fresh 1/2 strength Hoagland® nutrient solution spiked with individual compounds at an initial concentration of 1 mg/L. Fresh Milli-Q water was added to each tube to replenish the water lost through evapotranspiration every other day. Control groups, including wheat seedlings growing in clean culture solution, and spiked culture solution in tubes without wheat seedlings, were used for quality control and assurance.

Triplicate containers were sacrificed after 0, 3, 6, 12, 24, 48, 96, 168 and 240 h of cultivation for each of the treatment groups. Control groups were sampled only after 240 h. Seedlings were rinsed, dried and separated into roots and shoots. The nutrient solution and the plant tissues were stored at -80 ℃ until analysis.

3.2.4 Sample Preparation and Chemical Analysis

Extraction methods were adopted from previous studies, with minor modifications.[23,46] *A. thaliana* cell culture media and wheat seedling hydroponic culture solution were processed using solid phase extraction (SPE). Prior to the extraction, deuterated compounds (50 μL, 10 mg/L in methanol) were added to 5 mL nutrient solution as recovery surrogates. HLB cartridges (150 mg, 6 cc) purchased from Waters (Milford, MA) were preconditioned with 7 mL methanol and 7 mL water in sequence.

The aqueous sample was passed through the preconditioned SPE cartridge, followed by the addition of 5 mL 5% methanol in water (v/v) for clean-up. A final elution using 15 mL methanol was carried out and the resulting eluent was collected in a glass vial, dried under a gentle nitrogen gas flow, and then reconstituted with 1 mL 1:1 (v/v) methanol:water. The final extracts were filtered through 0.2-μm PTFE filters into 1.5 mL glass vials for instrument analysis.

Plant tissues, including *A. thaliana* cell matter, wheat seedling roots and shoots, were freeze-dried at -50 ℃ for 3 d to remove moisture. Wheat roots and shoots were then cut into small pieces. Before extraction, a 50-μL aliquot of depurated compounds (10 mg/L in methanol) was added to the tissue samples as recovery surrogates. The samples were extracted with 15 mL MTBE in a sonication water bath for 30 min. The sonication extraction process was repeated with 15 mL fresh MTBE once and then 15 mL fresh acetonitrile twice. Extracts from all steps were combined and dried on a nitrogen evaporator, and then recovered using 1 mL methanol. The resulting samples were diluted with 20 mL Milli-Q water and then passed through HLB cartridges following a similar process to that given above for the aqueous samples. The eluent was then dried under a gentle stream of nitrogen gas, reconstituted in 1 mL 1:1 (v/v) methanol:water, and filtered through a 0.2-μm PTFE filter before instrument analysis.

Quantitative analysis of all target compounds in this study was conducted on a Waters ACQUITY TQD tandem quadrupole UPLC-MS/MS (Waters, Milford, MA). Chromatographic separation was performed using a Waters ACQUITY BEH C18 column

(100 × 2.1 mm i.d., 1.7 μm) at 40 ℃. The mobile phase A and B were 0.01 % formic acid in LC-grade water and pure optimal-grade methanol, respectively, with a flow rate of 0.3 mL/min. The flowing gradient was set as: 0-1 min, 5% to 40% B; 1-2 min, 40% to 90% B; 2-4 min, 90% to 95% B; and 4-6 min, re-equilibrate with 5% B. The injection volume was 5 μL. The MRM transitions of all target compounds were optimized and summarized in the Supporting Information (Table S1). Quantification was completed using the TargetLynx XS software (Waters, Milford, MA).

3.2.5 Computation of Bond Strength

To better understand the effect of molecular structures on methylation or demethylation transformations of CECs in plants, the strength of the chemical bond between the major fragment and the methyl group (i.e., R-CH_3, Figure S2) in the methylated compounds was estimated. Although indirect methods like bond-dissociation energies are frequently used to characterize the bond strength by considering enthalpy change when the bond is cleaved, they often do not accurately describe the intrinsic strength of a particular bond.[47,48] The calculation of compliance constants offers an alternative way to directly determine bond strength without referring to arbitrary or poorly defined states, therefore leading to more reliable results.[47,48] Compliance constants (cm/N) address the question of which displacement is caused by a given force on a single coordinate, while all other forces thereby introduced are allowed to relax. The relaxed force constants (N/cm), as the reciprocal of individual compliance constants, measure the

force required to distort a coordinate by a unit amount while allowing all other coordinates to relax. The relaxed force constants of R-CH_3 in the methylated TPs were computed using the software Compliance (version 3.0.2),[48,49] and the configurations of target compounds were optimized with density functional theory (DFT) calculations at B3LYP/6-31G* level by the software Gaussian 16 (Gaussian, Wallingford, CT) prior to computation. A larger value of relaxed force constant would indicate a stronger chemical bond strength of R-CH_3.

3.2.6 Quality Assurance and Quality Control

Recoveries of all target compounds for extraction efficiency and limits of quantification are given in SI (Table S2). Method blanks and matrix blanks were included during extraction to check for possible contamination. One solvent blank and one check standard (100 μg/L) were injected after every 10 samples to check cross-contamination and for continued calibration during analysis (RSD < 20%). No target analytes were detected in the method blanks, matrix blanks, or solvent blanks, indicating no carry-over contamination during extraction or instrument analysis. Data in this study were calculated as mean ± standard deviation (SD). The data were analyzed using SPSS Statistics 27 (IBM Corp, Armonk, NY) and graphed by Prism 9 (GraphPad, La Jolla, CA).

3.3 Results and Discussion

3.3.1 Interconversion in *A. thaliana* Cells

To explore the potential interconversion of selected CECs and their methylated or demethylated TPs in *A. thaliana* cells, we exposed *A. thaliana* cells to acetaminophen, M-acetaminophen, DM-diazepam, diazepam, DM-methylparaben, methylparaben, DM-naproxen and naproxen, individually. The changes in the level of the parent compound, the formed TP, and the unidentified portion were considered in estimating the mass balance (Figures S5 and S6). No methylated or demethylated products were found in the non-viable *A. thaliana* cell culture control groups spiked with their corresponding counterparts, suggesting that when methylation or demethylation was observed, it was due to biologically mediated transformations in live *A. thaliana* cells.

Shortly exposure to the methylated compounds M-acetaminophen, diazepam, methylparaben or naproxen, their demethylated counterparts started to appear in the *A. thaliana* cells (Figure 1). After reaching a peak level, the concentration of the demethylated products generally decreased as the incubation time further increased, likely due to subsequent metabolism of the demethylated intermediates in *A. thaliana* cells. After 11 h of cultivation, *A. thaliana* cells demethylated a small fraction of M-acetaminophen to acetaminophen, with 14.9 ± 1.9 ng/g (dry weight, d.w.) of acetaminophen found in *A. thaliana* cell matter, and the concentration further increased to 39.4 ± 27.0 ng/g at 48 h. The molar equivalent of acetaminophen to M-acetaminophen was approximately 0.05 in *A. thaliana* cells at 48 h. Demethylation of diazepam,

methylparaben or naproxen in *A. thaliana* cells appeared to take place immediately after the treatment. The concentration of DM-diazepam in *A. thaliana* cells spiked with diazepam increased to 394.5 ± 59.8 ng/g during the first 24 h and then decreased slightly. At the end of 96-h cultivation, the level of DM-diazepam in the cells was still at 320.5 ± 199.1 ng/g. Demethylation of methylparaben was found to occur extensively in this study. Noticeably, at 0 h, 7195.6 ± 434.9 ng/g of DM-methylparaben was found in *A. thaliana* cell matter. This was likely caused by the conversion during the sample preparation process after chemical spiking, including centrifugation, which lasted for about 1 h. When calculated as molar equivalent, it was approximately 0.68 for DM-methylparaben to methylparaben in the cell matter at 0 h. The level of DM-methylparaben in *A. thaliana* cells decreased thereafter, likely due to the rapid metabolism of DM-methylparaben.[23] At the end of exposure, only 49.7 ± 42.1 ng/g of DM-methylparaben remained in the cell matter.

In contrast, demethylation of naproxen was found to be less substantial under similar conditions, with the highest concentration (29.6 ± 19.2 ng/g) of DM-naproxen detected at 0 h. At the end of the 96-h cultivation, 10.8 ± 5.5 ng/g of DM-naproxen was found in the *A. thaliana* cell matter. This observation was in agreement with previous studies where DM-naproxen was found at lower levels than other metabolites in *A. thaliana* cells exposed to naproxen, or was not detected in Garden cress *Lepidium sativum* exposed to naproxen.[33,50] Naproxen conjugates in plants were reported in previous studies, suggesting that DM-naproxen is an intermediate metabolite of naproxen that can be

further transformed through phase II pathways such as conjugation.[33,50] Similarly, acetaminophen and DM-methylparaben were also found to be conjugated with biomolecules in plants.[51,52] The common occurrence of conjugation implies that the actual degree of demethylation of the CECs may be substantially greater than what was experimentally measured in this study.

Methylation of the demethylated compounds in *A. thaliana* cells was concurrently evaluated under similar conditions (Figure 2). Generally, methylation was less extensive as compared to the corresponding demethylation. For example, during the first 11 h of incubation, methylation of acetaminophen was limited, and M-acetaminophen was below detection. At 24 h into the incubation, 27.4 ± 3.7 ng/g M-acetaminophen was detected in the cell matter, which further increased to 38.0 ± 10.1 ng/g at 48 h. The methylated acetaminophen was then metabolized and was not detectable at the end of 96-h cultivation. Therefore, methylation of acetaminophen in *A. thaliana* cells was mostly negligible under the experimental conditions. Methylation of DM-diazepam to diazepam was not observed in *A. thaliana* cells throughout the 96-h incubation duration. Similarly, methylation of DM-methylparaben was also found at much slower rates than demethylation of methylparaben. Methylparaben was found at 68.0 ± 45.0 ng/g in *A. thaliana* cells at 48 h in the DM-methylparaben treatment. Naproxen was detected at 29.8 ± 23.3 ng/g at 0 h in *A. thaliana* cells treated with DM-naproxen and decreased thereafter. Overall, for the four demethylated CECs considered in this study, their methylation was more limited in relation to the demethylation of their counterparts.

Outside of the *A. thaliana* cells, trace levels of the methylated or demethylated TPs were occasionally found in the aqueous culture media (Figure S3). Among the different CECs, DM-naproxen was below the detection limit in the culture media spiked with naproxen. The absence of DM-naproxen in the culture media was consistent with the limited formation of DM-naproxen in *A. thaliana* cells. A similar pattern was also observed for acetaminophen and DM-diazepam, where their methylated products were not found in the cell culture media. The demethylated products of methylparaben and M-acetaminophen, and the methylated TP of DM-naproxen, were found in the range of 0-3.0 μg/L in the cell media. In contrast, the demethylated product of diazepam, i.e., DM-diazepam, was found at relatively high levels in the cell media. After 48 h of incubation, DM-diazepam reached 40.1 ± 0.3 ng/mL in the cell media treated with diazepam. The accumulation of DM-diazepam in the cell media may be attributed to the persistence of DM-diazepam in *A. thaliana* cell cultures,[23] and was in agreement with the finding that DM-diazepam was readily formed in *A. thaliana* cells.

3.3.2 Interconversion in Wheat Seedlings

The changes in the level of the parent compound, the formed TP, and the unidentified portion in hydroponic wheat seedling systems were considered to estimate the mass balance (Figures S7 and S8). The interconversion between CECs and their methylated or demethylated counterparts displayed different patterns in wheat roots and shoots (Figures 3 and 4). Results showed that demethylation of methylparaben and naproxen, and methylation of acetaminophen, DM-diazepam and DM-methylparaben

were significantly more extensive in roots than in shoots ($P < 0.05$). In addition, demethylation took place at a significantly greater extent as compared to the corresponding methylation in both wheat roots and shoots ($P < 0.05$), except for the demethylation of acetaminophen and methylparaben in wheat roots ($P > 0.05$), which aligns with the results in *A. thaliana* cells.

3.3.2.1 Wheat Roots

Demethylation and methylation of the test CECs in the wheat roots exhibited molecular specificity (Figure 3a and Figure 4a, respectively). Demethylation of naproxen in wheat roots was more pronounced (Figure 3a), with 18744.8 ± 2869.2 ng/g (d.w.) of DM-naproxen detected in wheat roots at 48 h in the naproxen-treated system. The level of DM-naproxen decreased with time but remained at 6697.0 ± 4404.7 ng/g at 240 h. In contrast, methylation of DM-naproxen was not detected in wheat roots (Figure 4a). These results suggested that naproxen in wheat roots was rapidly metabolized and/or translocated, and its demethylation to DM-naproxen was a substantial metabolism pathway in wheat roots. This was consistent with previous studies where demethylation of naproxen was found to take place in *A. thaliana* cells and seedlings, followed by subsequent conjugation reactions.[33,38] The limited methylation of DM-naproxen may be partly attributed to further metabolism and potential translocation of the derived naproxen, rendering it non-detectable. The demethylation product of diazepam, DM-diazepam, was also observed in wheat roots and the level of DM-diazepam reached

2707.7 ± 826.0 ng/g at the end of the 10-d exposure (Figure 3a). In contrast, methylation of DM-diazepam was relatively negligible, and diazepam was detected at 43.1 ± 30.5 ng/g at 12 h in the roots treated with DM-diazepam and became non-detectable thereafter (Figure 4a). This pattern was similar to that in *A. thaliana* cells, suggesting again that demethylation was substantially more active than methylation for the diazepam and DM-diazepam pair in plants.

In wheat roots exposed to 1 mg/L methylparaben, DM-methylparaben was found at trace levels for the first 168 h but increased thereafter, reaching 321.9 ± 16.5 ng/g at the end of experiment (Figure 3a). Demethylation of methylparaben in the roots was found to be more limited than that in *A. thaliana* cells. This may be attributed to the more rapid dissipation in wheat roots caused by active metabolism, translocation out of the roots, and/or microbial degradation in the rhizosphere. The concentration of methylparaben, on the other hand, increased to 589.7 ± 20.9 ng/g at 48 h in wheat roots grown in the DM-methylparaben spiked hydroponic solution, and then decreased to 36.5 ± 13.9 ng/g at the end of experiment (Figure 4a). No appreciable demethylation of M-acetaminophen was observed in the roots exposed to M-acetaminophen (Figure 3a). In comparison, methylation of acetaminophen to M-acetaminophen was more substantial (Figure 4a), with M-acetaminophen detected at 316.5 ± 20.8 ng/g after 12 h and 112.4 ± 25.8 ng/g at the end of experiment.

Among the four pairs of CECs and their corresponding methylated or demethylated TPs, naproxen and diazepam showed a greater degree of demethylation, while their

demethylated products showed little back conversion (i.e., methylation) in wheat roots. In contrast, acetaminophen and DM-methylparaben exhibited notable methylation, while demethylation of M-acetaminophen and methylparaben appeared to be limited. This observation indicates that even though methylation and demethylation could take place simultaneously in plants, the interconversion may be somewhat directional for individual CECs, with one transformation favored over the back transformation.

3.3.2.2 Wheat Shoots

The methylated or demethylated TPs of CECs found in wheat shoots could potentially have two sources, i.e., *in situ* transformation from the parent CEC in the shoots, and translocation of the TP from the roots. The interconversion between M-acetaminophen and acetaminophen took place at similar levels in wheat shoots (Figures 3b and 4b). After 48 h of incubation in hydroponic solution spiked with 1 mg/L M-acetaminophen, acetaminophen was detected at 161.1 ± 74.0 ng/g, which decreased to 51.8 ± 25.7 ng/g at the end of exposure (Figure 3b). It should be noted that acetaminophen was not found in wheat roots exposed to M-acetaminophen, and previous studies showed that the translocation of acetaminophen was negligible in wheat and cucumber seedlings.[23,51] Therefore, it is likely that the occurrence of acetaminophen in wheat shoots grown in the hydroponic solution spiked with M-acetaminophen was a result of demethylation taking place in the shoots, rather than translocation from the

roots. After 12 h of incubation in media spiked with 1 mg/L acetaminophen, 107.2 ± 0.2 ng/g of M-acetaminophen was found in wheat shoots (Figure 4b). Similar to wheat roots, demethylation of methylparaben in wheat shoots was relatively limited as compared to the other CECs, with DM-methylparaben detected at 166.7 ± 28.7 ng/g in the shoots at the end of experiment (Figure 3b). Methylation of DM-methylparaben was negligible, with methylparaben found at only 8.3 ± 3.5 ng/g in the shoots at 6 h (Figure 4b). The accumulation of DM-methylparaben was previously found to be very limited in wheat shoots,[23] which may explain the absence of its methylation in the shoots.

Demethylation of naproxen in wheat shoots was found at lower levels compared to the roots (Figure 3), which may be attributed to the limited translocation and/or rapid metabolism of DM-naproxen in wheat shoots.[23] Like in the roots, methylation of DM-naproxen was not observed in wheat shoots. Since DM-naproxen was not detected in the shoots of wheat seedlings exposed to DM-naproxen,[23] the absence of naproxen in wheat shoots exposed to DM-naproxen may be mainly due to limited plant uptake and translocation of DM-naproxen.

In contrast, the formation of DM-diazepam was substantial in the shoots of wheat seedlings exposed to diazepam, with the level increasing quickly and reaching 8839.0 ± 2275.1 ng/g at the end of exposure (Figure 3b). The levels in the shoots were higher than even those in the roots at the same time points. In a previous study, DM-diazepam was found to be metabolized faster in wheat roots than diazepam, while their levels in shoots were similar.[23] Therefore, after demethylation, the formed DM-diazepam may undergo

further metabolism, especially in roots. When considered in molar equivalents, about 8.0% of the total spiked diazepam was demethylated to DM-diazepam in wheat shoots, which was substantial given that some of the formed DM-diazepam was likely not extractable by solvent and/or had undergone further metabolism. By comparison, methylation of DM-diazepam was not significant in the shoots, with the highest level at only 46.0 ± 32.5 ng/g at 48 h and non-detectable at the later time points (Figure 4b).

3.3.2.3 Rhizosphere

The rhizosphere is usually considered an important player in the overall metabolism of xenobiotics by whole plants, as root exudates generally enhance the richness of microbial communities in the root zone, leading to a greater microbial abundance and accelerated microbial degradation.[45,53,54] Although the rhizosphere in a hydroponic system may differ greatly from that in soil in terms of microbial community abundance, it was likely that some of the transformations of the target CECs or their TPs occurred in the solution due to rhizosphere-mediated microbial degradation.[53,54] This could result in the occurrence of methylated or demethylated metabolites in the hydroponic solution and their subsequent uptake into the plant. In addition, previous studies also showed that some xenobiotics may be excreted from plant roots into their bathing solution.[4,18,54,55] Analysis for the target CECs in the nutrient solution in this study, however, generally showed an absence of the corresponding methylation or demethylation products in the nutrient solution, except for acetaminophen and M-acetaminophen (Figure S4).

Acetaminophen, as the demethylation product of M-acetaminophen, was detected in the hydroponic solution at 978.7 ± 102.0 ng/mL after 6 h of cultivation, although it was not found in the roots and at only 87.6 ± 1.3 ng/g in the shoots. M-acetaminophen, as the methylated TP for acetaminophen, was found in hydroponic solution at 341.2 ± 9.5 ng/mL at 3 h of cultivation, which was also higher than its concentration in the roots (279.5 ± 43.5 ng/g) or shoots (94.0 ± 6.2 ng/g). Therefore, the interconversion between acetaminophen and M-acetaminophen likely took place in the nutrient solution outside the plant, which may have contributed to their accumulation in the wheat seedlings.

3.3.3 Relationship With Bond Strengths

The calculated compliance constants of the methylated CECs are summarized in Table 1, along with the calculated R-CH_3 relaxed force constants. A stronger chemical bond is harder to break as it requires more energy, while it is easier to form as more energy may be released. The computation results of the relaxed force constants showed that the chemical bond strength between the methyl group and the major molecular fragment in the methylated CECs followed a general order of methylparaben < diazepam < naproxen < M-acetaminophen. Therefore, demethylation may be expected to occur more readily for methylparaben, but more slowly for M-acetaminophen. Conversely, methylation of DM-methylparaben may be expected to be the hardest, while it is relatively easy for acetaminophen. The trends observed for the four pairs of CECs in *A. thaliana* cells generally followed the prediction from the bond strengths. For example, the

demethylation of methylparaben in *A. thaliana* cells was the most extensive among the test compounds, followed by diazepam. In contrast, demethylation of M-acetaminophen or naproxen was negligible under the same conditions. Methylation from acetaminophen to M-acetaminophen was found to proceed more readily than the conversion from DM-naproxen to naproxen, while methylation of DM-diazepam was not observed. Due to the limited number of compounds considered in this study, a quantitative correlation between the calculated bond strength and transformation rates was not carried out. However, future studies may consider ascertaining such a relationship, with information from more compounds, in order to better understand the impacts of molecular structures on biotransformation in plants.

The demethylation and methylation processes involve distinct subfamilies of CYP450s, esterases and methyltransferases, [29,34,36,56] which may depend on plant species-specific enzyme activities, as well as the chemical structure of xenobiotics. The generally good agreement between the experimental results and bond strength-based predictions in this study suggests that evaluation of chemical characteristics such as the bond strength of R-CH_3 may be used to identify CECs with a high tendency for specific transformation reactions. Given the large number of CECs, such a first-cut screening approach may be invaluable for developing a priority list of CECs that may undergo such conversions. The usefulness of such predictions may be further improved by considering more compounds and different plant species, and by developing and refining quantitative structural-activity relationships.

3.3.4 Limitations and Environmental Implications

To ensure confident identification and quantitative measurement of CECs and their TPs, an artificially high concentration (1 mg/L) was used in the growth media for *A. thaliana* and wheat seedlings. This concentration was likely orders of magnitude higher than the environmentally relevant levels. In addition, hydroponic cultivation was a simplified system, and the absence of soil should impart significant influences on the adsorption and hence the availability of CECs for plant uptake. Microorganisms in rhizosphere soil under field conditions likely play a great role in facilitating transformations of CECs, and therefore, the interconversion of CECs and their TPs in the soil-plant continuum may exhibit patterns different from observations from this study. Nevertheless, results from the controlled experiments in this study clearly showed that plants can mediate transformations of CECs such as methylation and demethylation. In some cases, demethylated products were found at relatively high levels under experimental conditions. Given that a large fraction of TPs was likely non-extractable or conjugated, the actual occurrence of such transformations in plants may be much more pronounced than that detected in this study. Conjugated metabolites may become deconjugated upon ingestion, for example, by enzymes in the gastrointestinal tract, releasing bioactive molecules.[9,57,58] The methylated or demethylated TPs likely retain or have even increased biological activity. For example, DM-diazepam (i.e., nordiazepam), although a demethylated TP of diazepam, is itself a drug for treating anxiety. The

addition or loss of a methyl group alters the physicochemical properties of a compound, leading to different environmental behaviors such as bioaccumulation, metabolism, and toxicity. For example, diclofenac methyl ether showed greater acute toxicity to aquatic invertebrates (*Gammarus pulex* and *Hyalella azteca*) than diclofenac.[59] Bisphenol A *mono*- and *di*-methyl ether also displayed greater developmental toxicity to zebrafish embryos than bisphenol A.[17] Therefore, when considering the whole life cycle of CECs, e.g., along the entire human-wastewater-soil-plant-human continuum, such circular interconversions may effectively prolong the persistence of CECs and contribute to enhanced human and ecotoxicological risks, underscoring an urgent need to consider such interconversions for more comprehensive risk assessment.

For the four pairs of CECs considered in this study, demethylation appeared to proceed more readily than methylation, and there were also differences among different compounds. A preliminary analysis showed a dependence of the methylation or demethylation rate on the bond strength of R-CH_3 of the compounds. As CYP450s, esterases and methyltransferases are involved in the metabolism of many xenobiotics, CECs with similar functional groups like -OH, -OCH_3, -NH-, and -NCH_3- may also undergo the methylation and demethylation cycle. With more experimental observations, it is feasible to predict the likelihood of such transformations using basic chemical structures and molecular descriptors. This is particularly valuable given that CECs and their TPs are numerous in numbers and identifying compounds or structural features

conducive to interconversions constitutes an important first step to better understand the significance of this phenomenon for the overall environmental fate and risks of CECs.

Tables

Table 3-1 Molecular descriptors for bond strength of target compounds (the bond between the major molecular fraction and methyl group).

Compounds	Compliance constant (cm/N)	Relaxed force constant (N/cm)
M-acetaminophen	0.214	4.67
Diazepam	0.224	4.46
Methylparaben	0.233	4.29
Naproxen	0.215	4.65

Figures

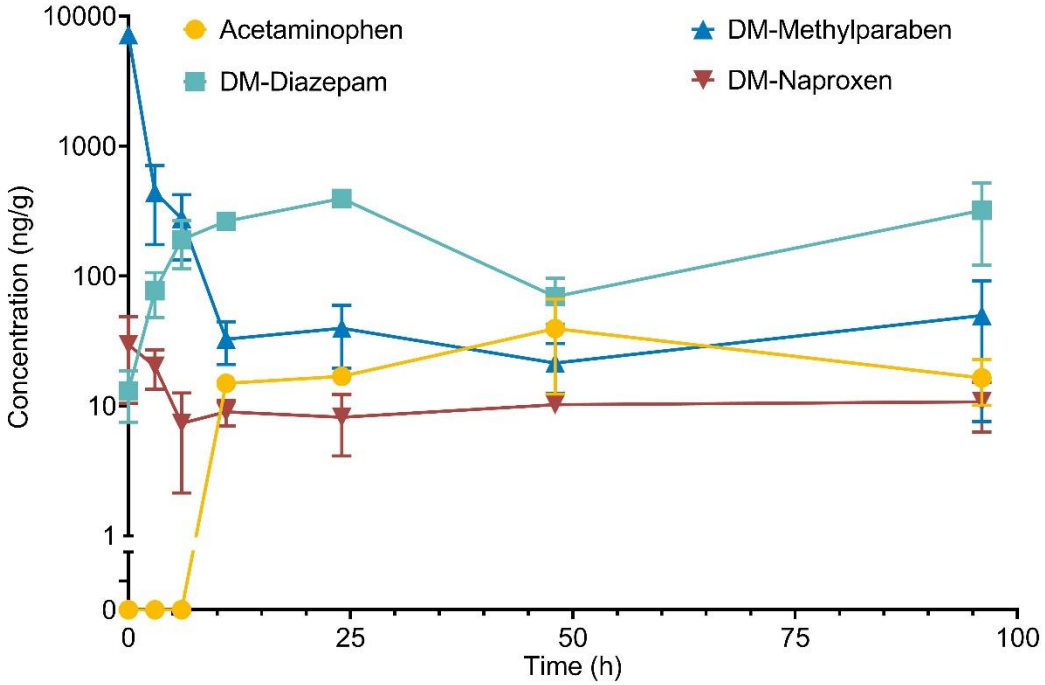

Figure 3-1 Formation of demethylated TPs in A. thaliana cells spiked with methylated compounds (data presented as mean ± SD, n = 3).

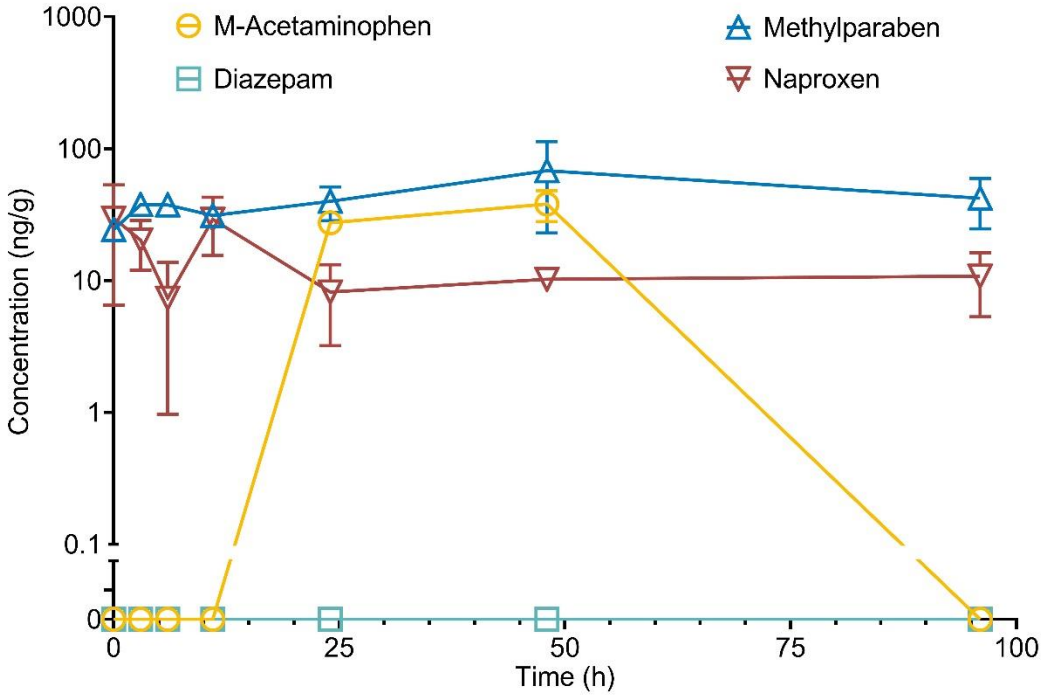

Figure 3-2 Formation of methylated TPs in A. thaliana cells spiked with demethylated compounds (data presented as mean ± SD, n = 3).

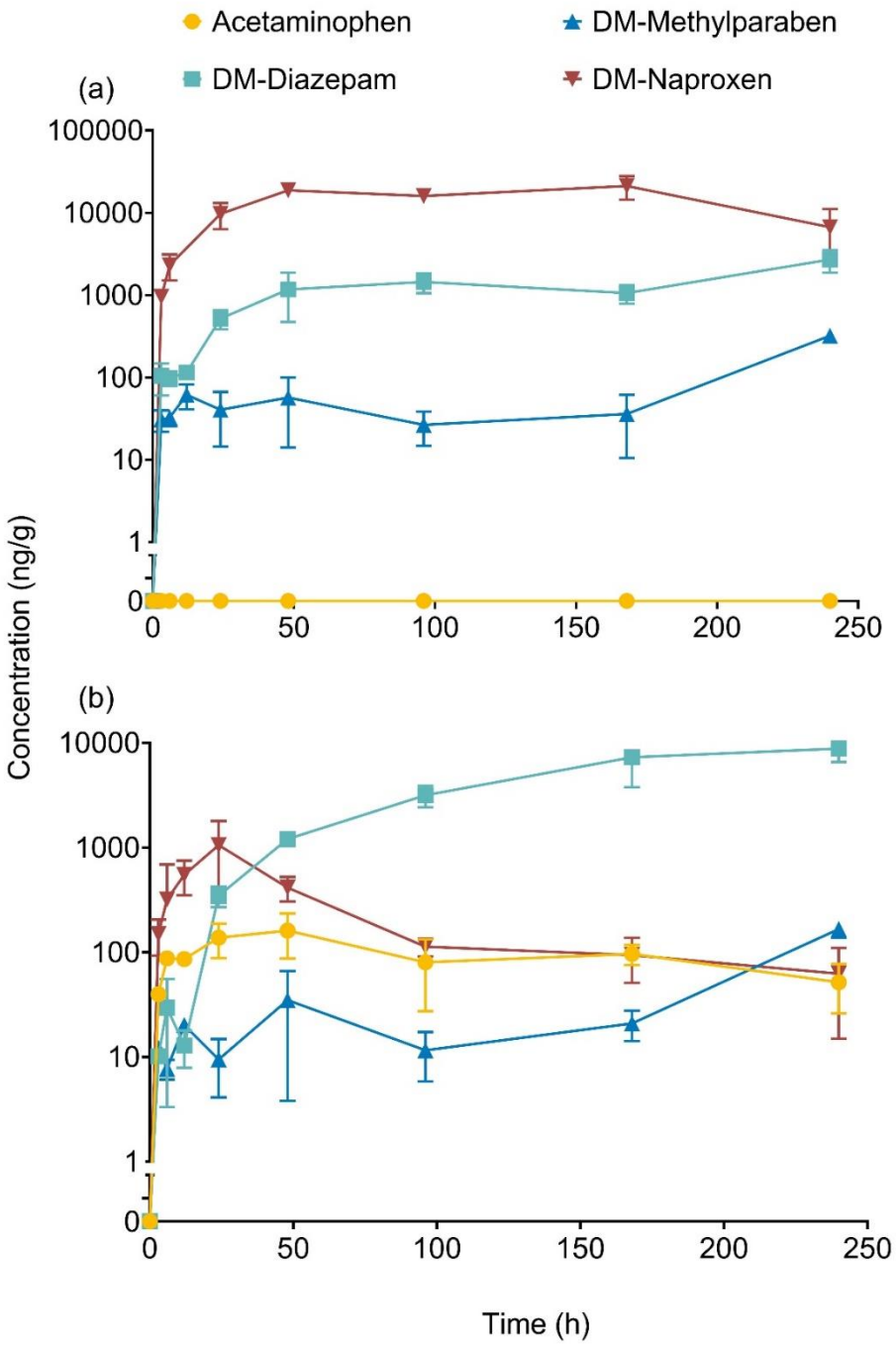

Figure 3-3 Formation of demethylated TPs in wheat seedlings exposed to methylated compounds: (a) Roots; and (b) Shoots (data presented as mean ± SD, n = 3).

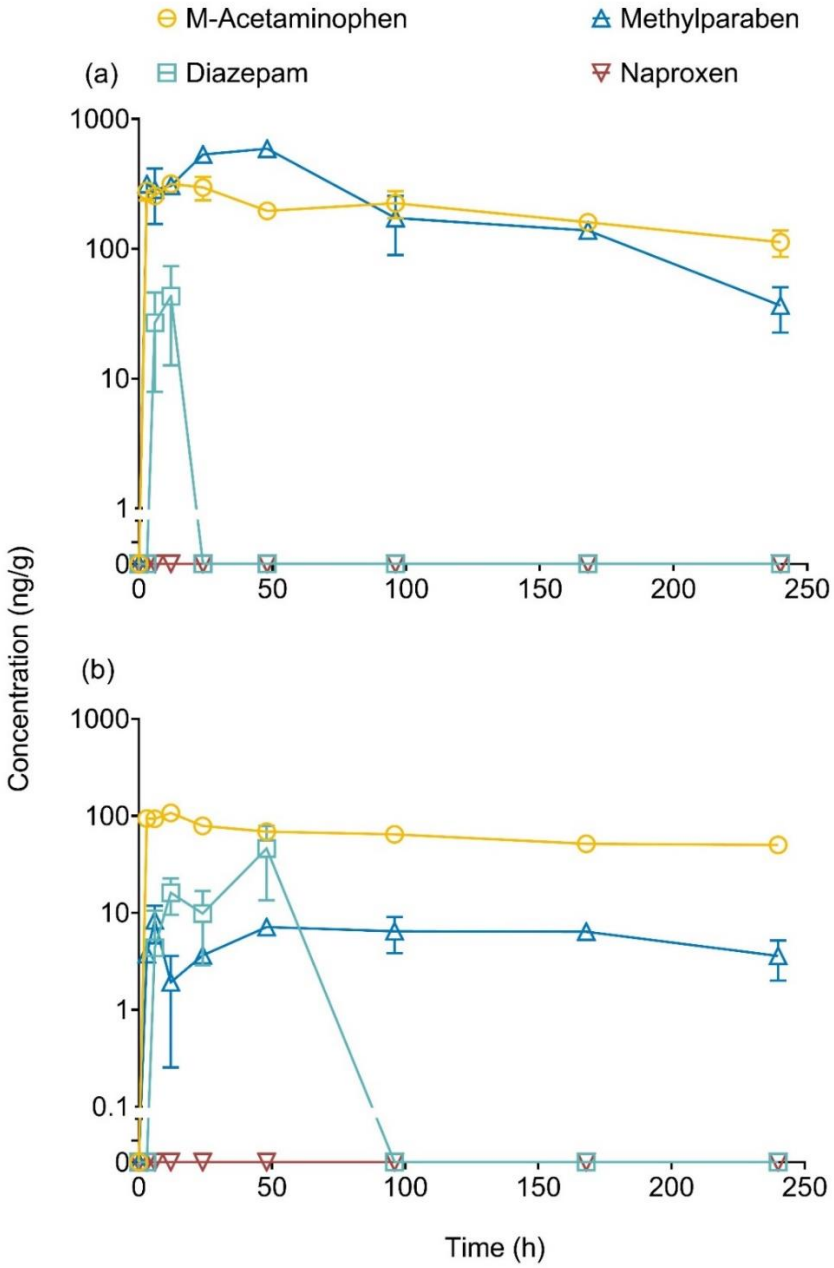

Figure 3-4 Formation of methylated TPs in wheat seedlings exposed to demethylated compounds: (a) Roots; and (b) Shoots (data presented as mean ± SD, n = 3).

Supplementary Information

Table S3-1. MRM transitions of target compounds on UPLC-MS/MS

Compound	MRM (m/z)			
	Quantification	CV/CE*	Qualification	CV/CE
ESI+				
Acetaminophen	151.97 > 109.99	38/22		
M-Acetaminophen	166.03 > 124.07	38/22	166.03 > 92.74	38/24
d4-Acetaminophen	156.03 > 113.99	40/12	156.03 > 96.75	40/22
DM-diazepam	271.03 > 139.99	56/28	271.03 > 165.03	56/28
Diazepam	285.03 > 154.02	56/26	285.03 > 193.09	56/32
d5-Diazepam	290.10 > 198.07	54/34	290. 10 > 154.11	54/26
ESI-				
DM-Methylparaben	137.09 > 93.08	34/15		
Methylparaben	151.05 > 92.03	38/20	151.05 > 136.00	38/14
d4-Methylparaben	155.05 > 96.05	36/20	155.05 > 140.01	36/14
DM-Naproxen	215.15 > 171.15	21/6	215.15 > 169.15	21/28
Naproxen	229.15 > 185.15	17/8	229.15 > 170.15	17/16
d3-Naproxen	232.18 > 188.10	14/5	232.18 > 173.14	14/18

*CV-cone voltage (kV), CE-collision energy (eV).

Table S3-2. Detection limits and recoveries of target compounds

Compound	LOQ*	Recovery (%)				
	ng/mL	*A. thaliana* cells	Wheat roots	Wheat shoots	Cell culture media	Wheat hydroponic solution
Acetaminophen	0.5	93.3 ± 7.7	83.2 ± 1.0	78.1 ± 0.7	112.0 ± 6.1	113.4 ± 5.7
M-Acetaminophen	0.2	63.4 ± 7.1	63.8 ± 13.3	63.2 ± 1.1	96.0 ± 2.7	98.8 ± 4.0
DM-Diazepam	0.2	82.5 ± 1.7	70.7 ± 3.8	60.0 ± 3.7	75.1 ± 2.3	77.3 ± 1.9
Diazepam	0.25	95.9 ± 8.9	83.3 ± 10.2	69.5 ± 11.5	114.9 ± 6.7	85.0 ± 13.0
DM-Methylparaben	3.0	80.8 ± 6.7	42.4 ± 10.1	54.8 ± 4.7	101.0 ± 5.8	100.8 ± 1.5
Methylparaben	1.5	64.3 ± 6.9	100.7 ± 2.8	97.4 ± 1.7	126.2 ± 3.2	97.3 ± 0.7
DM-Naproxen	3.0	65.0 ± 5.2	39.9 ± 7.3	42.6 ± 6.3	90.3 ± 2.5	90.3 ± 9.6
Naproxen	2.0	115.8 ± 3.0	89.1 ± 2.8	84.5 ± 3.6	80.3 ± 6.6	100.5 ± 1.3

*LOQ, limit of quantification.

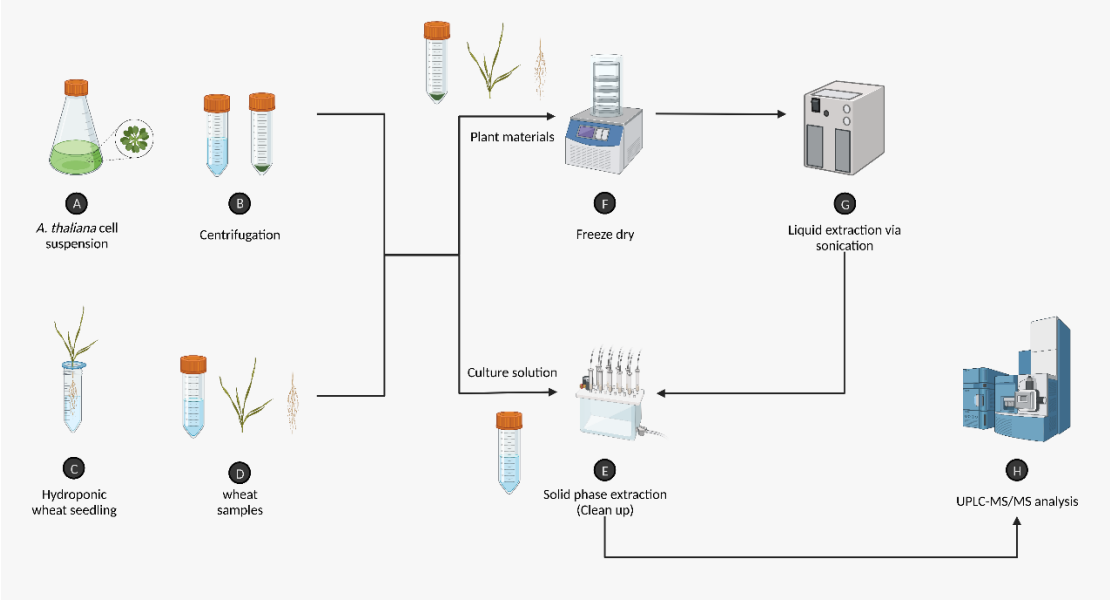

Figure S3-1. Scheme of the experimental design.

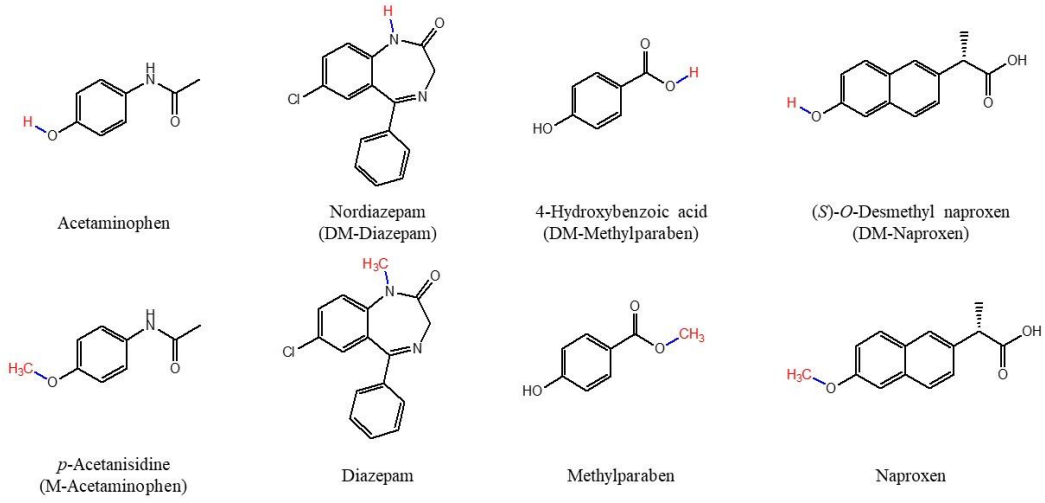

Figure S3-2. Chemical structures of the target compounds considered in this study.

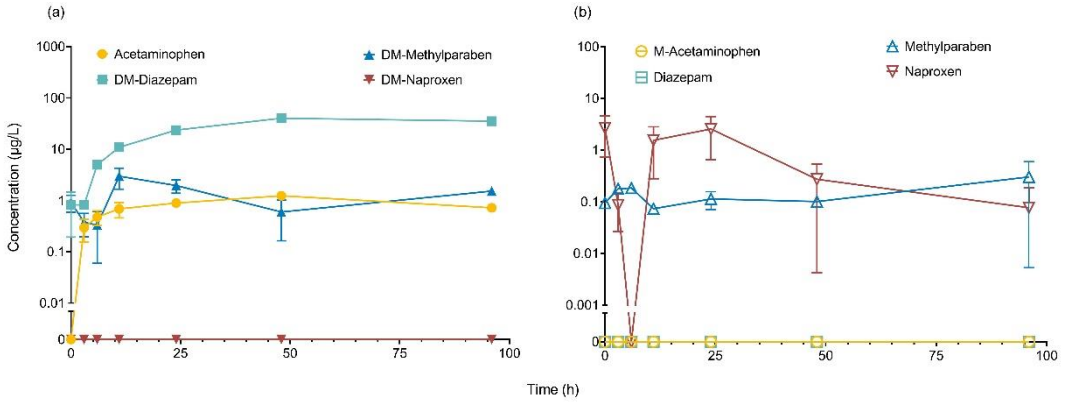

Figure S3-3. The formation of (a) demethylated TPs and (b) methylated TPs in *A. thaliana* cell media (data present as mean ± SD, n = 3).

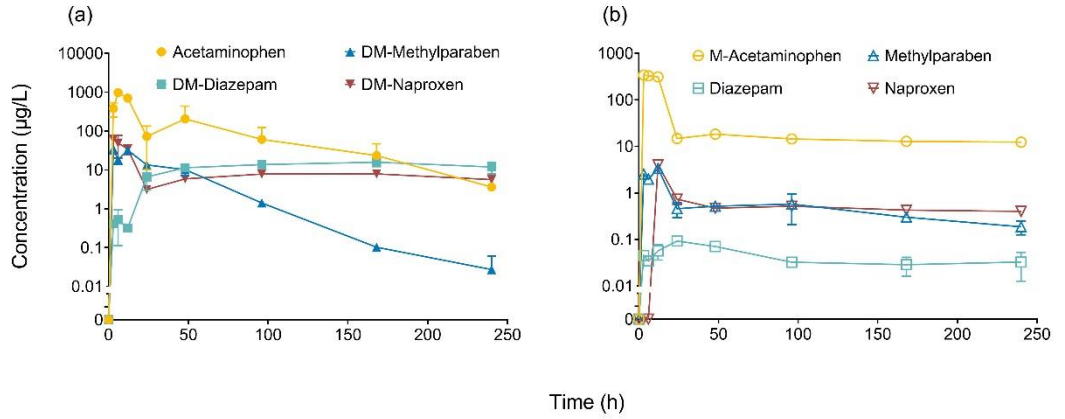

Figure S3-4. The formation of (a) demethylated TPs and (b) methylated TPs in wheat hydroponic solution (data presented as mean ± SD, n = 3).

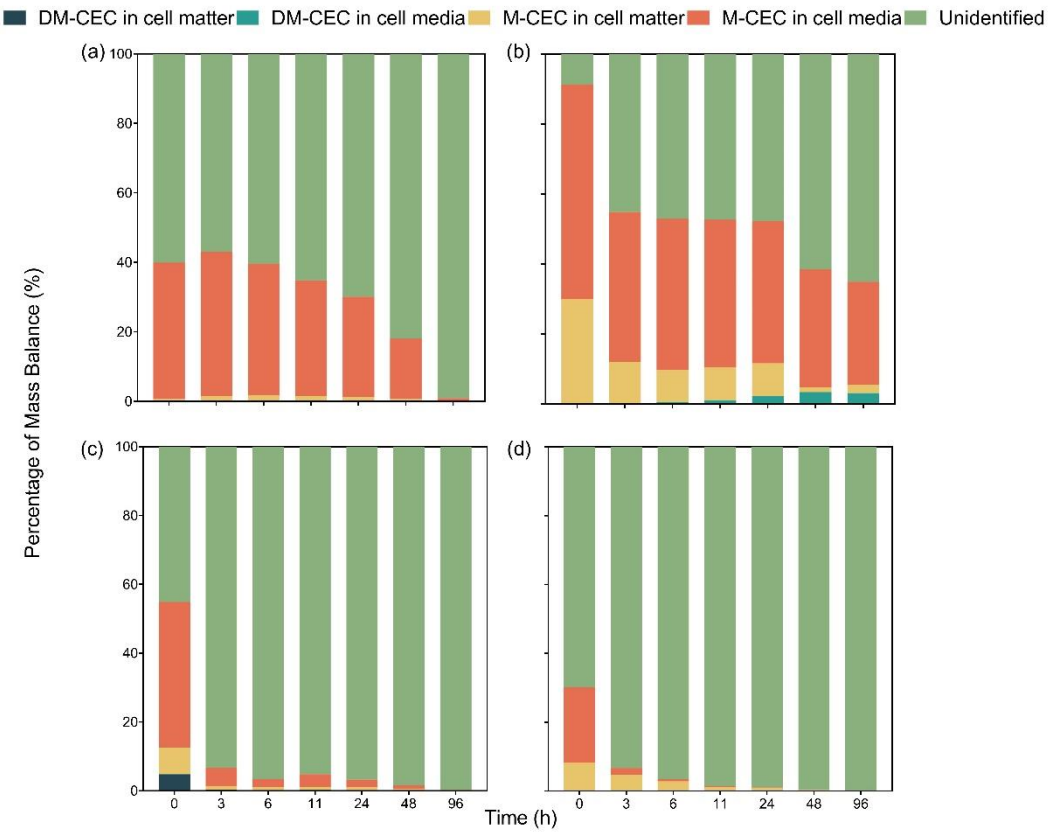

Figure S3-5. Chemical mass balance in *A. thaliana* cells exposed to methylated CECs (M-CEC). (a) M-acetaminophen, (b) diazepam, (c) methylparaben, and (d) naproxen. DM-CEC refers to the corresponding demethylated CEC in each pair.

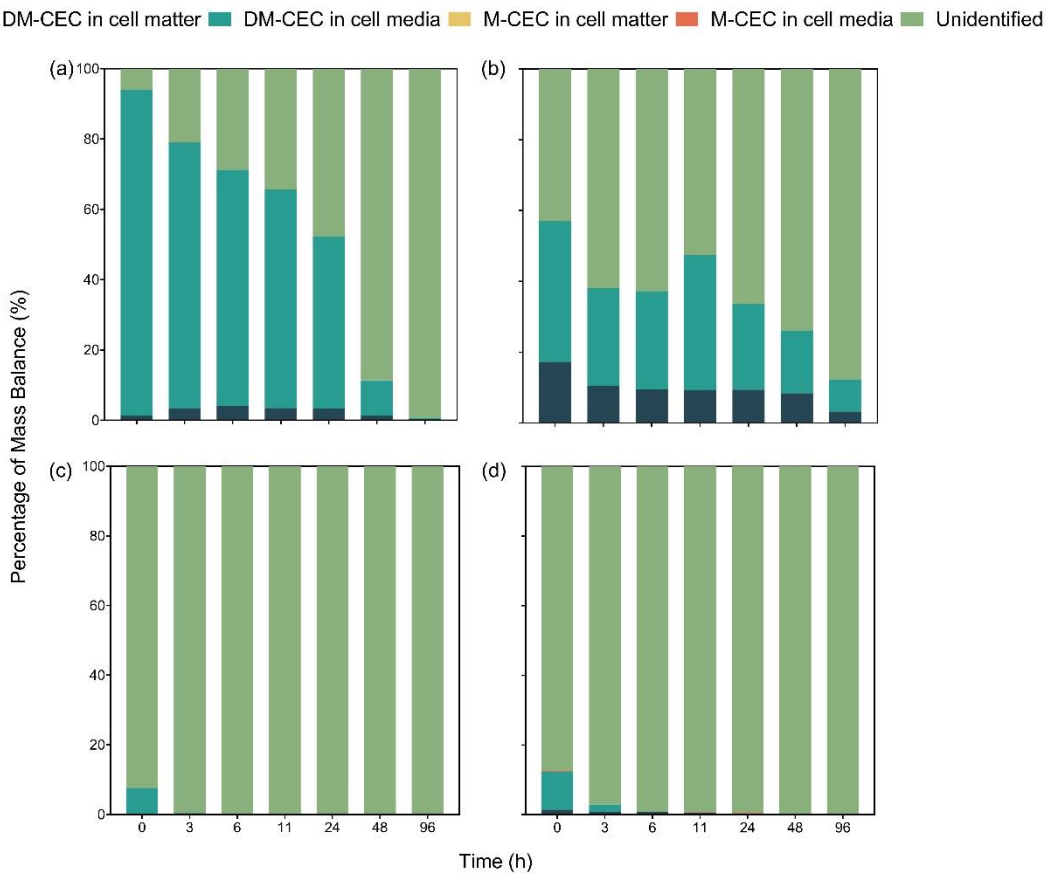

Figure S3-6. Mass balance of *A. thaliana* cells exposed to the demethylated CECs (DM-CEC) in the four pairs of target compounds, including (a) acetaminophen, (b) DM-diazepam, (c) DM-methylparaben, and (d) DM-naproxen. M-CEC refers to the correspondingly formed methylated CEC in each pair.

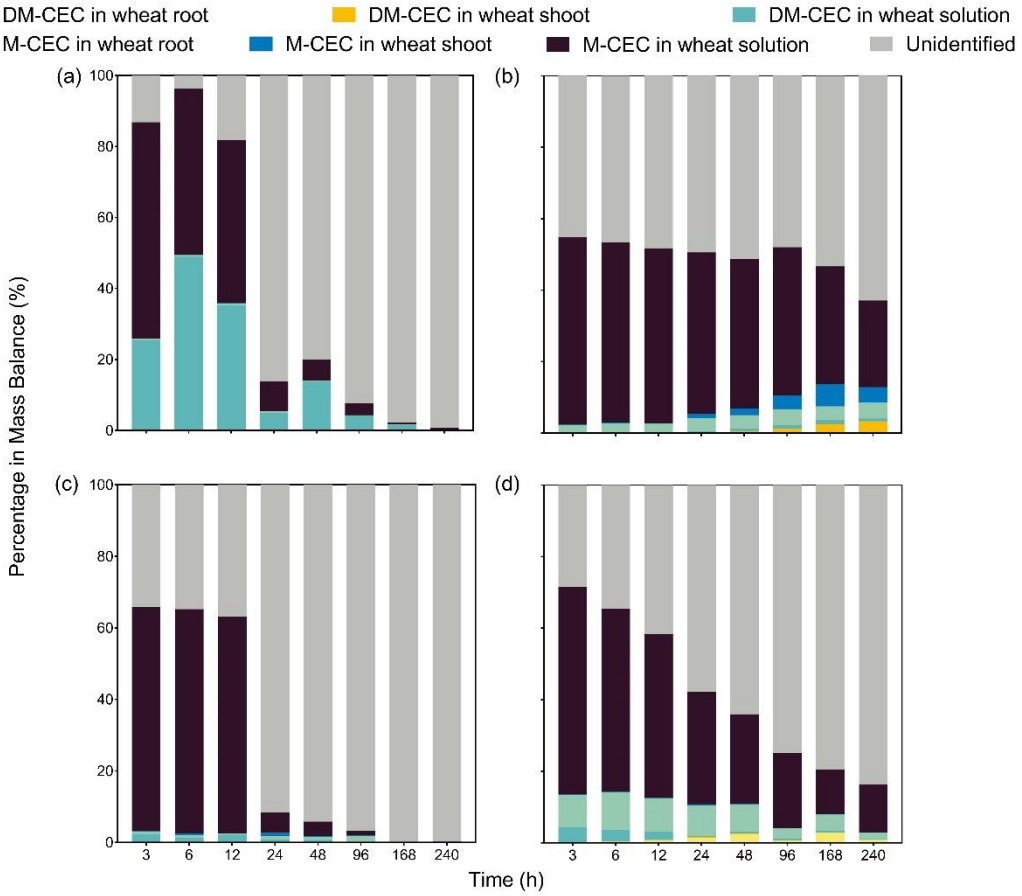

Figure S3-7. Mass balance of wheat seedlings exposed to the methylated CECs (M-CEC) in the four pairs of target compounds, including (a) M-acetaminophen, (b) diazepam, (c) methylparaben, and (d) naproxen. DM-CEC refers to the correspondingly formed demethylated CEC in each pair.

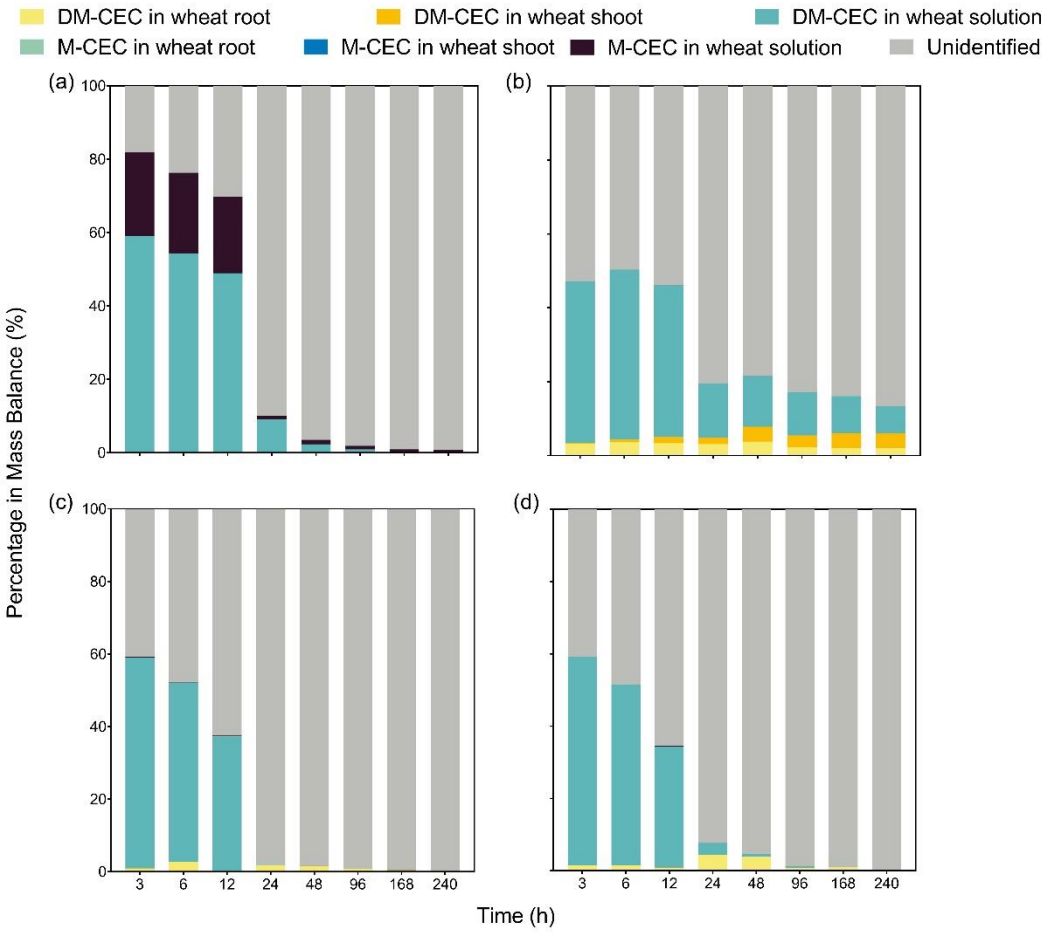

Figure S3-8. Mass balance of wheat seedlings exposed to the demethylated CECs (DM-CEC) in the four pairs of target compounds, including (a) acetaminophen, (b) DM-diazepam, (c) DM-methylparaben, and (d) DM-naproxen. M-CEC refers to the correspondingly formed methylated CEC in each pair.

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Chapter 4 Influence of Methylation and Demethylation on the Bioaccumulation and Acute Toxicity of Emerging Contaminants in *Daphnia magna*

Abstract

Contaminants of emerging concern (CECs) in the environment undergo various transformations, leading to the formation of transformation products (TPs) with modified ecological risk potential. Although the environmental significance of TPs is increasingly recognized, there has been relatively little research to understand influences of such transformations on subsequent ecotoxicological safety. In this study, we used four pairs of CECs and their methylated or demethylated derivatives as examples to characterize changes in bioaccumulation and acute toxicity in *Daphnia magna*, as a result of methylation or demethylation. The experimental results were further compared to quantitative structure-activity relationship (QSAR) predictions. The methylated counterpart in each pair generally showed greater acute toxicity in *D. magna*, which was attributed to their increased hydrophobicity. For example, the LC_{50} values of methylparaben (34.4 ± 4.3 mg L^{-1}) and its demethylated product (225.6 ± 17.3 mg L^{-1}) differed about 8-fold in *D. magna*. The methylated derivative generally exhibited greater bioaccumulation than the demethylated counterpart. For instance, bioaccumulation of methylated acetaminophen was about 33-fold higher than acetaminophen. *In silico* predictions via QSARs aligned well with the experimental results, and suggested increased persistence of methylated forms. The study findings underline the

consequences of simple transformations such as methylation and demethylation, and highlight the need to consider TPs to achieve a more holistic understanding of the environmental fate and risks of CECs.

4.1 Introduction

The occurrence of numerous contaminants of emerging concern (CECs) in the effluent from wastewater treatment plants (WWTPs) and impacted aquatic environments has been extensively reported.[1–4] However, most research has focused on the parent form of CECs while generally neglecting their transformation products (TPs) that are often in co-existence. Many CECs contain reactive functional groups, such as hydroxyl, carboxyl and amide groups, making them susceptible to various biotic and abiotic transformation reactions.[5–8] Simple transformations, such as methylation and demethylation, have been observed in various environmental matrices for many CECs.[6,9–13] For example, previous studies showed the presence of methylated TPs of triclosan and bisphenol A (BPA) in wastewater effluents and receiving streams.[13–15] The methyl ethers of tetrabromobisphenol A (TBBPA) were formed in aquatic environments in the presence of background methyl iodide.[7] Methylation of acetaminophen was observed in soil.[16] On the other hand, demethylation is a major metabolism pathway for CECs in organisms. For example, after oral administration in humans, naproxen and diazepam are demethylated to 6-*O*-desmethyl naproxen (DM-naproxen) and nordiazepam (DM-diazepam), respectively.[17,18]

Despite the fact that TPs seem to occur readily and co-exist with their parent forms in the environment, the ecotoxicological consequences of such transformations have not been adequately considered. Transformations such as the addition or loss of a methyl group can significantly change a compound's physicochemical properties, such as K_{ow} that is known to influence its fate and bioaccumulation.[19] Methylated products of diclofenac, BPA, and triclosan all displayed enhanced toxicity or bioaccumulation potential in aquatic organisms. [6,14,15,20]

In this study, we comparatively explored the behaviors of four typical CECs, i.e., acetaminophen, diazepam, methylparaben, and naproxen, and their methylated or demethylated TPs (M-acetaminophen, DM-diazepam, DM-methylparaben, and DM-naproxen) in *Daphnia magna*, by considering their bioaccumulation, acute toxicity, and interconversions. Quantitative structure-activity relationship (QSAR) models were further developed and used to describe the experimental results. The study findings highlight the importance of simple transformation reactions such as methylation and demethylation in understanding the overall ecological risks posed by CECs in aquatic environments.

4.2 Materials and Methods

4.2.1 Chemicals and Materials

The analytical standards (purity >98%) of the four pairs of compounds considered in this study were purchased from Sigma-Aldrich (St. Louis, MO), Santa Cruz (Dallas, TX), or Toronto Research Chemicals (Toronto, Ontario, Canada). Their physicochemical

properties are summarized in Table 1. Deuterated compounds d_4-acetaminophen, d_5-diazepam, d_4-methylparaben and d3-naproxen were purchased from Sigma-Aldrich, Toronto Research Center, or C/D/N isotopes (Pointe-Claire, Quebec, Canada), and used as internal standards. HPLC-grade methanol was purchased from Fisher Scientific (Fair Lawn, NJ). Ultrapure water was generated in-house using a Milli-Q water purification system (Millipore, Carrigtwohill, Cork, Ireland).

The pH of the test medium in this study was measured to be 8.50 ± 0.10. The neutral fraction (f_n) of the target compounds, their pH-adjusted octanol-water coefficients (log D_{ow}), and pH-adjusted lipid-water coefficients (log D_{lipw}) were calculated for this pH condition (Table 1). The calculation and related details are given in the Supporting Information (SI) in Text S1.

D. magna was purchased from Aquatic Research Organisms (Hampton, NH) and maintained following the OECD Guidelines.[21] Briefly, *D. magna* was raised in artificial freshwater (AFW) made by adding 58.5 mg of $CaCl_2 \cdot 2H_2O$, 24.7 mg of $MgSO_4 \cdot 7H_2O$, 13.0 mg of $NaHCO_3$ and 1.2 mg of KCl into 1 L of deionized water. *D. magna* in AFW was maintained in a growth chamber at 21 ° C with a 16:8 hour (light:dark) photoperiod, and fed daily with freshwater green algae (*Raphidocells subcapitata*). The medium was renewed twice a week to maintain a clear environment for the daphnids.

4.2.2 *D. magna* Acute Toxicity Tests

Acute toxicity testing of the target CECs and their derivatives to *D. magna* was carried out following the OECD Guidelines 202.[21] Preliminary tests were conducted for each target compound at widely spaced concentrations to identify the concentration range for deriving an accurate dose-response curve. The stock solutions of individual test compounds were prepared in methanol and diluted with 10 mL aerated AFW to different concentrations in 20 mL glass vials. The methanol volume was set to 100 μL in each vial as the solvent carrier, and a control group containing 100 μL methanol without the target compound was used as the carrier solvent control. Each test included at least five concentration levels, and the concentrations of test compounds in the medium were experimentally measured (Figure 1). Each treatment included four replicates. Five *D. magna*, < 24 h old at the beginning of the test and not the first brood progeny, were placed in each vial and maintained in the growth chamber under the same conditions as given above. Death of *D. magna* was determined by observing the lack of movement for 15 s after gentle agitation of the test vial, and the lethal rate of *D. magna* in each test vessel was recorded at 0, 24 and 48 h. The obtained dose-response data were fitted to a Boltzmann equation:[22]

$$Lethal\ rate\ (\%) = \frac{100}{1+10^{(logLC_{50}-logC)*Hillslope}} \quad (1)$$

where *C* is the measured concentration of the test compound. LC_{50} values at 48 h of the tested compounds were obtained from this equation.

4.2.3 *D. magna* Bioaccumulation Experiments

The bioaccumulation experiments were conducted in 500-mL glass beakers and consisted of a 24 h uptake phase and a 24 h depuration phase. The 250 mL AFW was spiked with 0.25 mL of individual stock solution (1000 mg L^{-1} in methanol) at a nominal concentration of 1 mg L^{-1}, and triplicates were used for each target compound. For each beaker, 120 adult *D. magna* (21 d old) were added, and at 0, 2, 4, 8, 12 and 24 h, an aliquot of 10 *D. magna* and 1 mL of the test medium were withdrawn. After 24 h, the remaining *D. magna* were transferred to clean AFW to start the depuration phase. Similar to the uptake phase, 10 *D. magna* were withdrawn from each beaker at 2, 4, 8, 12 and 24 h. The wet weight of each *D. magna* sample was recorded and the samples were stored at −80 ° C prior to chemical analysis.

The depuration data were fitted to a first-order decay model to obtain the depuration rate constant (k_d, h^{-1})[23]:

$$C_{D.magna}(t) = C_i e^{-k_d t} \quad (2)$$

where $C_{D.magna}$ (μg kg^{-1}, wet weight, w.w.) is the internal concentration of the target compound in *D. magna*, and C_i (μg kg^{-1}, w.w.) is the initial concentration of the target compound in *D. magna* when the depuration phase started, which is also the concentration when uptake phase ended at 24 h. During the uptake phase, the concentration of the target compound in *D. magna* could be expressed as:[23]:

$$\frac{dC_{D.magna}}{dt} = k_u C_w(t) - k_d C_{D.magna}(t) \quad (3)$$

where k_u is the uptake rate constant (L kg^{-1} h^{-1}), and $C_w(t)$ is the concentration of the target compound (mg L^{-1}) in water. Since $C_{D.magna}$ at 0 h is zero, and C_w is constant during the experiment, equation (3) can be simplified to equation (4) to estimate k_u:[23]

$$C_{D.magna}(t) = \frac{k_u}{k_d} C_w(1 - e^{-k_d t}) \quad (4)$$

The dynamic bioconcentration factor (BCF, L kg^{-1}, w.w.) may be further calculated by the following relationship:[23]

$$BCF = \frac{k_u}{k_d}$$

(5)

Along with the dynamic BCF, steady-state BCF (L kg^{-1}, w.w.) was also calculated using the following equation:

$$BCF = \frac{C_{D.magna}}{C_w}$$

(6)

where $C_{D.magna}$ is the concentration of a target compound (μg kg^{-1}, w.w.) in *D. magna* at equilibrium, which was 24 h in this study (Figure 2).

4.2.4 Sample Preparation and Instrumental Analysis

Deuterated compounds were used as recovery surrogates. Before sample extraction, 10 μL of the stock solution containing the deuterated standard at 10 mg L^{-1} (in methanol) was added to the daphnid sample, and the samples were extracted by sonication in 1 mL methanol, followed by centrifugation at 14,000 rpm for 15 min. The same extraction was repeated for a total of three consecutive times, and the extracts were combined. The solvent extract was dried under nitrogen, recovered with 200 μL water:methanol (1:1, v/v), and centrifuged at 14,000 rpm for 15 min. An aliquot of 100 μL of the cleaned

extract was transferred to a 250-μL glass insert in a 2-mL LC vial for analysis on UPLC-MS/MS.

To obtain the actual concentration of target compounds in aqueous medium, 50 μL of the deuterated mixture was added to 1 mL solution sample in a 2-mL centrifuge tube, followed with 15 mg Cleanert PEP powder (70-90 μm, Agela Technologies, Torrance, CA). Samples were shaken by hand for 30 s and then subjected to vortex for 1 min. The centrifuge tubes were centrifuged at 14,000 rpm for 15 min, and the liquid phase was discarded. The remaining solid powder was subjected to the same extraction step with 1 mL methanol. The extract after centrifugation was transferred to another 2-mL centrifuge tube, dried under nitrogen, reconstituted in 1 mL water:methanol (1:1, v/v), and filtered through a 0.2-μm PTFE filter into a 2-mL glass LC vial for instrument analysis.

Instrument analysis of all compounds was carried out on a Waters ACQUITY TQD ultra-performance liquid chromatography-tandem quadrupole mass spectrometry (UPLC-MS/MS) (Waters, Milford, MA). Chromatographic separation was performed at 40 ° C using an ACQUITY BEH C18 column (100 × 2.1 mm i.d., 1.7 μm; Waters, Milford, MA). Mobile phase A was 0.01% formic acid in water (v/v) and mobile phase B was methanol. The mobile phases were programmed to the following gradient (with respect to mobile phase B): 0-1 min, 5-40%; 1-2 min, 40-90%; 2-4 min, 90-95%; and 4-6 min, 95-5%. The flow rate was maintained at 0.3 mL/min. The MRM transitions of all test compounds were optimized and are listed in Table S1. Data were processed using the TargetLynx XS software (Waters, Milford, MA).

4.2.5 *In silico* Predictions

To better understand the effects of methylation and demethylation on the environmental behaviors of CECs, *in silico* predictions were made using QSAR models for acute toxicity, bioaccumulation and persistence of the test compounds. The QSAR models computed the environmental behaviors of chemicals based on their chemical structures and available experimental datasets of compounds of similar structures. The consensus QSAR method in the U.S. EPA's Toxicity Estimation Software Tool (T.E.S.T., version 5.1.2) was used to predict the acute toxicity of the target compounds in *D. magna*.[24] The BCF and the biotransformation half-life values of test compounds were obtained using the BCFBAF™ model in the U.S. EPA's EPI Suite™ software (version 4.11). As a similar model is not available for aquatic invertebrates, *in silico* BCF values for lower trophic fish were predicted using the Arnot-Gobas method as an approximation for *D. magna*.[25] Similarly, *in silico* half-life values were derived from the estimated whole body primary biotransformation rate in fish and normalized to 10 g fish at 15 ° C as the inherent setting of the model.[26]

4.2.6 Quality Assurance and Quality Control

Recoveries and detection limits of all target compounds are shown in Table S2. Method blanks and matrix blanks were included during sample extraction to check for possible contamination. One solvent blank and one check standard (100 μg L^{-1}) were injected after every 10 samples to check cross-contamination and reproducibility (RSD <

20 %). No target compounds were detected in the method blanks, matrix blanks, and solvent blanks, indicating no background or cross-contamination during extraction and instrument analysis. Data in this study are presented as mean ± standard deviation (SD). The data were analyzed using SPSS Statistics 28 (IBM Corp, Chicago, IL) and graphed using GraphPad Prism 9 (La Jolla, CA). Statistical significance was generally derived by one-way analysis of variance (ANOVA) test, except that the calculated LC_{50} values were compared by the ratio test.[27] Significance level was set at $p < 0.05$.

4.3 Results and Discussion

4.3.1 Acute Toxicity of *D. magna*

To evaluate the influence of methylation or demethylation on the acute toxicity of CECs, *D. magna* was exposed to a range of aqueous concentrations of individual CECs and their methylated or demethylated TPs for 48 h. The derived dose-response curves are plotted in Figure 1, along with the calculated LC_{50} values. Methylation or demethylation changed the acute toxicity of most CECs, though the influence was compound-specific. For example, methylation of acetaminophen caused a significant decrease in acute toxicity ($p < 0.05$), with LC_{50} increasing from 21.2 ± 2.4 mg L^{-1} for acetaminophen to 32.1 ± 5.7 mg L^{-1} for M-acetaminophen. In contrast, methylation had the opposite effect on the acute toxicity of DM-methylparaben and DM-naproxen. For example, DM-methylparaben was found to be significantly less toxic to *D. magna* than methylparaben

($p < 0.05$), with approximately an 8-fold difference between their LC_{50} values. The LC_{50} of DM-naproxen was found to be 67.9 ± 6.0 mg L^{-1}, which was significantly greater compared to naproxen (32.1 ± 4.9 mg L^{-1}, $p < 0.05$). However, methylation and demethylation did not affect the acute toxicity of DM-diazepam and diazepam, and there was no significant difference between their respective LC_{50} values.

To better understand how methylation and demethylation affect the acute toxicity of CECs, the derived log LC_{50} values of the target compounds are plotted against their corresponding log D_{lipw} values (Figure S2a). A significantly negative linear relationship was observed, indicating that as log D_{lipw} increased, LC_{50} for *D. magna* generally decreased or the acute toxicity increased. Therefore, the changes in acute toxicity induced by methylation or demethylation of CECs may be partially attributed to changes in physicochemical properties, such as hydrophobicity. TPs with stronger hydrophobicity tend to exhibit greater acute toxicity as compared to their parent form. After methylation, log D_{lipw} of DM-methylparaben and DM-naproxen increased from -1.62 to 1.93, and -0.67 to -0.51, respectively, consistent with increases (7- and 2-fold changes in LC50, respectively) in their toxicity. In a previous study, methyl-diclofenac was found to be more toxic than diclofenac in *G. pulex* and *H. azteca*.[6] Methylated derivatives of bisphenol A were more toxic in zebrafish (*Danio rerio*) embryos.[20] It must be noted that the influence of methylation or demethylation on properties such as log D_{lipw} depends on the overall molecular structure of the compound and the position of the methyl group. In this study, the presence of a methyl group did not appreciably change the predicted log

D_{lipw} for DM-diazepam (3.16) and diazepam (3.06) (Table 1), which may explain their similar LC_{50} values for *D. magna* found in this study. Factors other than hydrophobicity may also regulate toxicity, such as metabolism and elimination. In this study, even though the methylated product of acetaminophen, *M*-acetaminophen, has a higher log D_{lipw} value (1.45) than acetaminophen (0.89), the derived LC50 was significantly larger for *M*-acetaminophen (32.1 ± 5.7 mg/L) than acetaminophen (21.2 ± 2.4 mg/L) (Table 2). Likewise, in previous studies, the methylated ethers of TBBPA were found to be less toxic than TBBPA in earthworms after 72 h exposure on filter paper (*Eisenia fetida*), or after 14 d exposure in soil (*Metaphire guillelmi*), or in zebrafish embryos following aqueous exposure for 28 d.[28,29]

Observations from this and other studies indicate that the effect of simple transformation reactions such as methylation and demethylation on toxicity is complex and depends closely on the specific molecular structure of the compound undergoing the transformation. Different modes of action may contribute to the acute toxicity of CECs to *D. magna* after methylation or demethylation.[30] CECs contain different functional groups that may have specific interactions with specific cellular components like enzymes or receptors in *D. magna*.[31] However, the observed general correlation between hydrophobicity and acute toxicity in *D. magna* in this study implies that bioaccumulation driven by hydrophobicity was likely an important cause for the methylation or demethylation-induced changes in non-target toxicity.

4.3.2 Bioaccumulation in *D. magna*

To further understand the effect of methylation and demethylation on the acute toxicity to *D. magna*, bioaccumulation of the CECs and their methylated or demethylated counterparts was measured in adult organisms. The concentrations of target compounds remained relatively constant in the aqueous media during the 24 h uptake phase, with RSDs ranging from 2.8% to 18.4% (Figure S1). Therefore, the mean measured concentrations of target compounds in the water phase were used as C_w to fit Equations (5) and (6) to derive BCF values. The bioaccumulation kinetics of target compounds are shown in Figure 2. The concentrations of CECs and their methylated or demethylated TPs generally showed an increasing trend at the beginning of the uptake phase and reached an apparent equilibrium in 24 h. Upon transferring the exposed *D. magna* to clean AFW to initiate the depuration phase, the concentration of test compounds gradually declined over time. With the exception of diazepam, methylated derivatives consistently showed much higher concentrations in *D. magna* than their demethylated counterparts. For example, after 2 h of exposure, the concentrations of acetaminophen and M-acetaminophen in *D. magna* were found at 308.7 ± 42.6 ng g^{-1} (w.w.) and 8730.7 ± 2900.9 ng g^{-1}(w.w.), respectively, a 28-fold difference (Figure 2a). This was consistent with the fact that methylated acetaminophen has a higher log D_{lipw} than acetaminophen (Table 1). In addition, at pH 8.5, acetaminophen was expected to be partially ionized in the aqueous media, while M-acetaminophen should be completely in its neutral state (Table 1). Methylparaben also displayed a much higher accumulation (2216.3 ± 85.7 ng

g^{-1}, w.w.) than DM-methylparaben (682.7 ± 91.5 ng g^{-1}, w.w.) in *D. magna* at the end of the uptake phase (24 h, Figure 2c). The 3-fold change also coincided with the difference in log D_{lipw} between DM-methylparaben (-1.62) and methylparaben (1.93) (Table 1). The level of DM-naproxen in *D. magna* was below LOD, and therefore its bioaccumulation may be deemed negligible (Figure 2d). In contrast, significant accumulation of naproxen in *D. magna* was observed, again suggesting a pronounced effect by hydrophobicity induced by methylation. It is also likely that DM-naproxen was rapidly metabolized due to the presence of an exposed hydroxyl group (Table 1). The presence of the hydroxyl group in DM-naproxen may facilitate its conjugation with an amino acid or glucose in *D. magna*,[32,33] contributing to its rapid metabolism and reduced bioaccumulation. Unlike the other three pairs, there was no significance in the bioaccumulation between DM-diazepam and diazepam in *D. magna* (Figure 2b), with 6792.5 ± 1215.8 ng g^{-1}(w.w.) and 7599.7 ± 1470.3 ng g^{-1}(w.w.) detected in *D. magna* after 24 h, respectively. This may be attributed to the fact that methylation or demethylation does not result in a great change in their physicochemical properties and that both compounds have similar log K_{ow} or log D_{lipw} values (Table 1).

The derived kinetic parameters of target compounds are given in Table S3. In general, the methylated derivative in each pair had a larger k_u than the corresponding demethylated counterpart. The dynamic BCF values, calculated as the ratio of k_u and k_d, showed a strong correlation with the BCF values derived from the steady state (Figure S3, R^2=0.98, $p < 0.01$), suggesting enhanced bioaccumulation for most methylated CECs.

For example, the dynamic BCF of M-acetaminophen was 10.0 ± 0.0 in *D. magna*, which was significantly higher than the dynamic BCF of acetaminophen (0.3 ± 0.0). For DM-diazepam and diazepam, however, the BCF values in *D. magna* were not significantly different from each other, which again coincided with their generally similar physicochemical properties.

For aquatic organisms, increased bioaccumulation of contaminants is often attributed to a compound's hydrophobicity, as bioaccumulation is driven by lipids in an organism and is positively related to hydrophobicity or log K_{ow} for neutral compounds.[15,19,24,34,35] Increased bioaccumulation after methylation was previously observed for diclofenac in aquatic invertebrates. Bioaccumulation of methylated diclofenac was found to be 25-110-fold that of diclofenac in *H. azteca* and *G. pulex*.[6] In this study, methylation generally increased log K_{ow} of CECs, and further log D_{ow} and log D_{lipw}, although the relative increases are specific to the individual compounds. The generally enhanced bioaccumulation in *D. magna* was also in agreement with the effect of methylation on CEC bioaccumulation in plants.[19] Methylation of CECs could occur in natural water bodies due to the presence of methyl iodide,[7] during wastewater treatment,[36] and during biological transformations in soil,[16] plants,[37] and earthworms.[28] Therefore, methylated derivatives of CECs may be prevalent in the environment and should be considered in a holistic risk assessment because of their different behaviors and biological activities, such as increased bioaccumulation potentials.

4.3.3 Interconversion Between CECs and Their Derivatives

Biologically mediated transformations such as methylation and demethylation may also occur in organisms such as *D. magna* after their uptake of CECs, which may further influence their toxicity. Methylation and demethylation in *D. magna* were investigated after exposing *D. magna* to the individual compounds. Methylation of the selected demethylated CECs was negligible, as no methylated product was detected in *D. magna* after its exposure to the corresponding demethylated counterpart. However, demethylation of diazepam, methylparaben and naproxen in *D. magna* was evident (Figure 3a), while acetaminophen was not detected in *D. magna* exposed to M-acetaminophen. The demethylation of methylparaben was limited, with a peak concentration of DM-methylparaben at 0.5 ± 0.0 nmol g^{-1} (w.w.) in *D. magna* after 12 h of exposure to 1 mg L^{-1} methylparaben. This represented only about 2.0% of the molar equivalent of methylparaben in *D. magna*. The demethylation of diazepam was found at similar levels, with DM-diazepam at 4.4% molar equivalent of diazepam. Interestingly, the molar equivalents of the demethylated derivatives increased over time during the depuration phase, even though the overall concentrations generally decreased over time. For example, the molar equivalents of DM-diazepam and DM-methylparaben reached 33.5% and 54.8% at the end of depuration, respectively. This may be attributed to the fact that demethylation continued during the depuration phase, which may have influenced the apparent depuration of these compounds (Table S3).

The demethylation of naproxen in *D. magna* was the most pronounced among the four methylated compounds, with DM-naproxen generally detected at levels higher than naproxen itself during both the uptake and depuration phases (Figure 3a). DM-naproxen was formed quickly in *D. magna* after exposure to naproxen, with 21.5 ± 2.7 nmol g^{-1} (w.w.) after 12 h into the uptake phase, which was significantly higher than that of the parent naproxen (6.5 ± 0.4 nmol g^{-1}, w.w.). Similar to DM-diazepam and DM-methylparaben, the molar equivalent of DM-naproxen also continued to increase during the depuration phase. At the end of depuration, DM-naproxen accounted for approximately 88.9% of the total naproxen and DM-naproxen residues in *D. magna*. The high proportion of DM-naproxen in *D. magna* also suggested that demethylation was the primary metabolism pathway of naproxen in *D. magna*.

To better understand the demethylation of CECs in *D. magna*, the formation rates of DM-diazepam, DM-methylparaben and DM-naproxen were estimated (Figure 3b) by simulating their formation over the initial 12-h period, during which good linear relationships between their formation and time were present (Figure S4). Formation rates showed no significant differences between DM-diazepam and DM-methylparaben. However, the formation rate of DM-naproxen (1.5 ± 0.3 nmol g^{-1} h^{-1}) was significantly greater than DM-diazepam or DM-methylparaben. Based on their respective chemical structure (Table 1), the demethylation of diazepam and naproxen appears to differ slightly from that of methylparaben. While the demethylation of methylparaben involves the removal of a methyl group from a carboxyl group, which may be catalyzed by

carboxylesterases,[38,39] CYP450s,[40] or through non-enzymatic hydrolysis,[39,41] the demethylation of M-acetaminophen, diazepam and naproxen reflects the removal of a methyl group from an amide or hydroxyl group, which likely is catalyzed mainly by CYP450s.[18,42] Previous studies showed that carboxylesterases play a more important role in drug metabolism in invertebrates due to the lower activity of CYP450s.[43] The more significant demethylation observed for naproxen in comparison to methylparaben suggests that CYP450s may also play an important role in the metabolism of such substrates in aquatic invertebrates. The observed significant differences in the demethylation rates of diazepam and naproxen imply that CYP450s in aquatic invertebrates like *D. magna* may exhibit different levels of activity towards different CECs.

4.3.4 *In silico* Predictions

QSAR models are often employed for predicting the environmental fate of man-made chemicals for which experimental data are not available, enabling a preliminary assessment of their environmental risks. In this study, several environmental parameters of CECs and their methylated or demethylated derivatives were predicted using QSAR models and the predicted values were further compared against the experimentally derived data (Table 2). The LC_{50} values computed by the T.E.S.T. software aligned well with experimental data for the neutral compounds, including acetaminophen, M-acetaminophen, DM-diazepam and diazepam (R^2=0.95, $p < 0.05$). For example, *in vivo* LC_{50} of DM-diazepam and diazepam in *D. magna* were 4.4 ± 1.1 mg L^{-1} and 3.0 ± 0.3

mg L^{-1}, respectively, while the *in silico* values were 5.4 mg L^{-1} and 4.2 mg L^{-1} for DM-diazepam and diazepam, respectively. However, for the partially ionized compound methylparaben and the fully ionized compounds DM-methylparaben, DM-naproxen and naproxen, *in silico* predicted acute toxicity was greater as compared to the *in vivo* results. For example, the predicted LC_{50} of DM-naproxen in *D. magna* was 9.5 mg L^{-1}, which was much smaller than the experimental value of 67.9 ± 6.0 mg L^{-1}. However, the relative potency, as determined by dividing the LC_{50} of the demethylated derivative in each pair by that of its methylated counterpart, [24] suggested that the influence of methylation or demethylation on the acute toxicity of CECs in *D. magna* may be predicted using *in silico* methods (R^2=0.94, $p < 0.05$).

In silico BCF values were obtained for lower trophic fish, in lieu of *D. magna*, using the BCFBAF™ model in the U.S. EPA's EPI suite™ software (v 4.11). Since the derived BCF values could not be directly compared with the *in vivo* BCF values obtained for *D. magna* in this study, a relative bioaccumulation ratio was calculated by dividing the BCF of the demethylated derivative in each pair by that of its methylated counterpart. The tendency of bioaccumulation after methylation or demethylation of CECs predicted by the QSAR models generally agreed with the *in vivo* results, although the correlation was not statistically significant, likely due to the small sample size. The *in silico* predictions in this study showed that QSARs may underestimate the increases in bioaccumulation potential of CECs from methylation. For instance, the BCF of acetaminophen rose by

approximately 33-fold in *D. magna* after methylation, while the *in silico* approach projected only a 50% increase in small fish.

In vivo half-lives of the test compounds were derived from the depuration rate (k_d, h^{-1}) during the 24-h depuration phase in *D. magna*. The *in silico* half-life was estimated from the primary biotransformation rate in fish and normalized to a 10 g fish at 15 ° C based on the inherent characteristics of the QSAR model.[25,26] Similar to BCF values, *in vivo* and *in silico* half-lives could not be compared directly between the different organisms. Hence, the relative persistence of test compounds was calculated for evaluation. As shown in Table 2, *in silico* predictions suggest that methylation may prolong the persistence of CECs in fish. This was in contrast to the *in vivo* results in *D. magna*, which showed that methylation generally shortened the persistence of CECs. As mentioned above, methylated CECs generally accumulated faster with a larger k_u value during the uptake phase, but dissipated rapidly during the depuration process. Considering that biota residing in wastewater effluent-dominated streams often experience pseudo-persistent exposure to CECs due to the constant discharge of effluents from WWTPs, uptake rates may be more important in regulating the accumulation of CECs in aquatic organisms dwelling in the impacted system. The prolonged biotransformation half-lives of methylated CECs should be validated under field conditions.

Overall, *in silico* predictions and experimental measurements were in agreement for the influences introduced by methylation or demethylation. This highlights the feasibility

of incorporating QSAR models to evaluate the potential influence of common transformations such as methylation and demethylation on the environmental risks of CECs to non-target organisms in impacted ecosystems.

4.3.5 Conclusions and Environmental Implications

Simple transformations such as methylation and demethylation contribute to the proliferation of the numbers of CECs and diverse structures in environmental compartments impacted by e.g., wastewater effluent.[10,13–15] This study showed that these transformations can alter the physicochemical properties of CECs, resulting in changes in their environmental processes such as bioaccumulation and acute toxicity in aquatic organisms. These transformations of man-made chemicals may also take place within a non-target organism after their accumulation from the ambient environment. Certain transformations, like methylation, likely result in enhanced bioaccumulation and increased toxicity in non-target organisms. Although not considered in this study, halogenation of man-made chemicals, such as gemfibrozil, 4-nonylphenol and naproxen, during the disinfection process in WWTPs, has also been reported, and the halogenated products generally exhibited increased bioaccumulation and toxicity to aquatic invertebrates.[24,44,45] Due to the presence of numerous CECs in sources such as wastewater effluents and sediments, the co-existence of various TPs presents an additional challenge in addressing the overall environmental risks of man-made chemicals.

It is important to note that high concentrations of test CECs and their corresponding methylated or demethylated TPs were used in this study in order to derive the LC_{50}

values and examine conversions in *D. magna*; these concentrations were above environmentally relevant levels. However, previous studies suggested that BCFs may be greater at lower exposure concentrations.[23] Therefore, the effect of methylation or demethylation on bioaccumulation of CECs may be more pronounced than what was observed in this study. The environmental occurrence and concentrations of methylated or demethylated TPs are largely unknown for most CECs. Further research into the occurrence of TPs in different environmental compartments is needed to gain knowledge about the realistic exposure levels and to refine risk assessment.

A major challenge in comprehensively assessing environmental risks is the sheer number of CECs and their TPs. It is unrealistic to experimentally evaluate transformation-induced changes in their environmental behaviors and toxicological profiles for all CECs.[46] The incorporation of well-established QSAR models to predict essential chemical properties and environmental risk markers, such as hydrophobicity and lipophilicity, bioaccumulation potential, and acute toxicity, may help prioritize TPs with enhanced biological activities.[47–49] This approach can be used to more effectively direct future research efforts to better understand the environmental significance of common transformation reactions for CECs.

Table 4-1. Physicochemical properties of selected CECs and their methylation/demethylation counterparts

Compound	logK_{ow}[a]	pK_a[c]	pH 8.50		
			f_n	logD_{ow}[d]	logD_{lipw}[e]
Acetaminophen	0.46	9.38	0.9	0.41	0.89
M-Acetaminophen	1.03	1.5[b]	1	1.03	1.45
DM-Diazepam	2.93	2.85[b]	1	2.93	3.16
Diazepam	2.82	3.4	1	2.82	3.06
DM-Methylparaben	1.58	4.54	0	-2.38	-1.62
Methylparaben	1.96	8.34[c]	0.41	1.57	1.93
DM-Naproxen	2.84[b]	4.34[b]	0	-1.32	-0.67
Naproxen	3.18	4.18	0	-1.14	-0.51

[a]Measured values from PubChem: https://pubchem.ncbi.nlm.nih.gov/.
[b]Predicted by ChemAxon or retrieved from The Human Metabolome Database: https://hmdb.ca/.
[c]Measured value from CompTox Chemicals Dashboard: https://comptox.epa.gov/dashboard/chemical/properties/DTXSID4022529.
[d]Calculated logD_{ow} values crosschecked with the logD_{ow} values predicted by ChemAxon: https://disco.chemaxon.com/calculators/demo/plugins/logd/.

Table 4-2. The comparison between *in vivo* experimental results and *in silico* predictions for acute toxicity, dissipation and bioaccumulation of CECs and their methylated or demethylated counterparts.

Compound	LC_{50}-48 h (mg L^{-1})			
	in vivo	relative potency[a]	*in silico*	relative potency
Acetaminophen	21.2 ± 2.4	1	27.1	1
M-Acetaminophen	32.1 ± 5.7	0.7	61	0.4
DM-Diazepam	4.4 ± 1.1	1	5.4	1
Diazepam	3.0 ± 0.3	1.5	4.2	1.3
DM-Methylparaben	225.6 ± 17.3	1	55.7	1
Methylparaben	34.4 ± 4.3	6.6	10	5.6
DM-Naproxen	67.9 ± 6.0	1	9.5	1
Naproxen	32.1 ± 4.9	2.1	13.8	0.7
	BCF (L kg^{-1}, w.w.)			
	in vivo	ratio[b]	*in silico*	ratio
Acetaminophen	0.3 ± 0.0	1	1	1
M-Acetaminophen	10.0 ± 0.0	33.3	1.5	1.5
DM-Diazepam	9.8 ± 0.3	1	44.5	1
Diazepam	9.0 ± 0.4	0.9	37.2	0.8
DM-Methylparaben	0.9 ± 0.5	1	2.8	1
Methylparaben	2.8 ± 0.2	3.1	3.9	1.4
DM-Naproxen	0	N/A	19	1
Naproxen	1.5 ± 0.6	N/A	84.2	4.4
	Half-life (h)			
	in vivo[c]	ratio	*in silico*	ratio
Acetaminophen	1	1	0.3	1
M-Acetaminophen	0.4	0.4	1.6	5.3
DM-Diazepam	5.8	1	12.5	1
Diazepam	1.5	0.3	18.8	1.5
DM-Methylparaben	2.2	1	1.4	1
Methylparaben	2.7	1.2	0.5	0.4
DM-Naproxen	N/A	N/A	6.5	1
Naproxen	4.3	N/A	41.8	6.4

[a]Relative potency was calculated as the ratio of LC_{50} of demethylated derivative over methylated derivative.

[b]Ratio was calculated as the value of methylated derivatives over that of the demethylated counterparts;

[c]*In vivo* half-life values were derived from the depuration rate in Table S4-3 in SI.

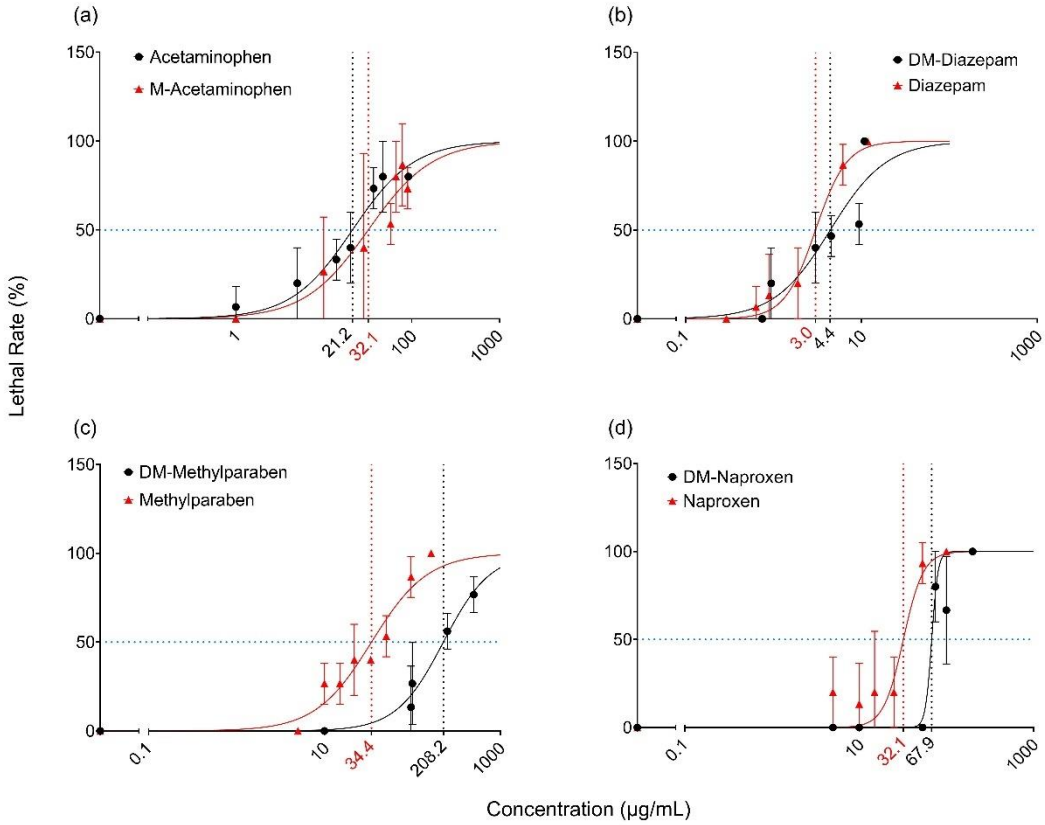

Figure 4-1. Concentration-response curves of (a) acetaminophen and M-acetaminophen, (b) DM-diazepam and diazepam, (c) DM-methylparaben and methylparaben, and (d) DM-naproxen and naproxen for *D. magna* over 48 h acute exposure.

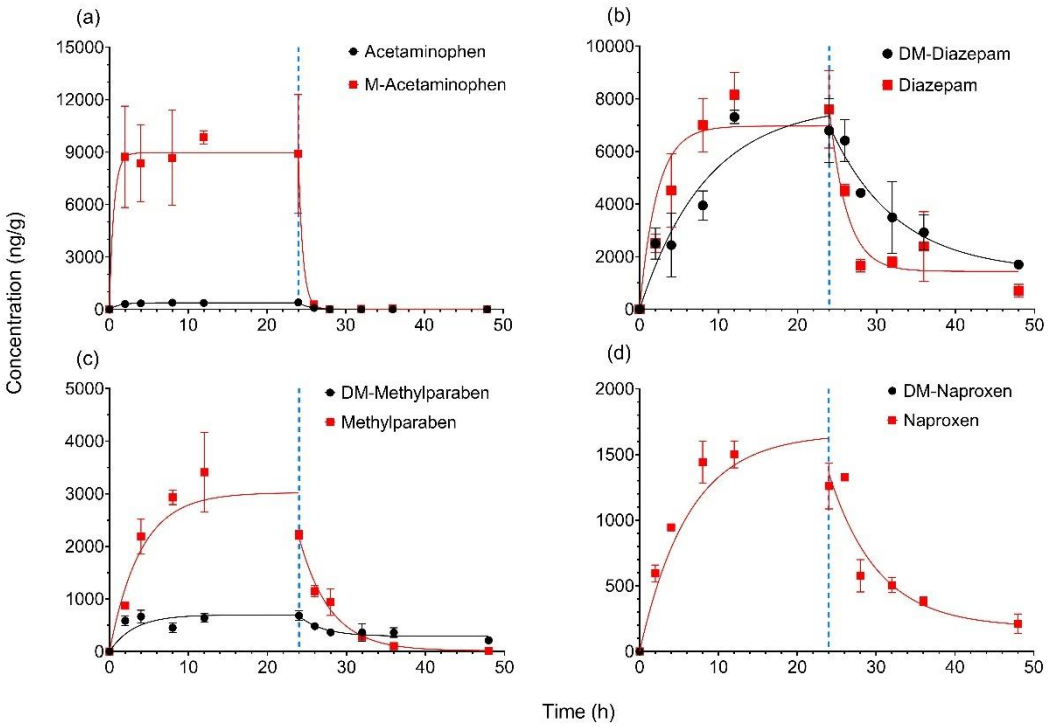

Figure 4-2. Bioaccumulation kinetics of the four pairs of CECs and their methylated/demethylated derivatives in *D. magna*: (a) acetaminophen and M-acetaminophen, (b) DM-diazepam and diazepam, (c) DM-methylparaben and methylparaben, and (d) DM-naproxen and naproxen.

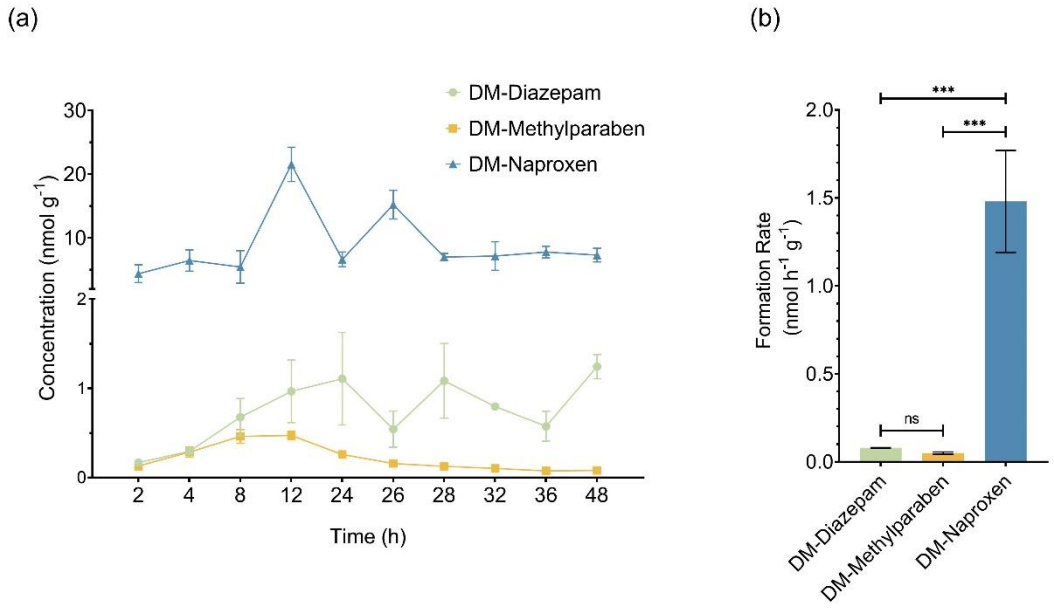

Figure 4-3. Formation of demethylated TP in *D. magna* exposed to diazepam, methylparaben or naproxen: (a) concentration kinetics over the exposure time period; (b) formation rates of demethylated TPs during the first 12-h period.

Supplementary Information

Text 4-1. Calculation of physicochemical property parameters

The pH of the artificial freshwater environment for *D. magna* was measured as 8.10 ± 0.10. The fraction of neutral species (f_n) for the test compounds was calculated as the following equation:[1–3]

$$f_n = \frac{1}{1+10^{i(pH-pK_a)}} \qquad (1)$$

where i is 1 for acids and -1 for bases. The pH-adjusted octanol-water coefficient log D_{ow} was estimated as:

$$logD_{ow} = logK_{ow} + logf_n \qquad (2)$$

The pH-adjusted liposome-water partition coefficient (log D_{lipw}) was calculated using the following equation:[4]

$$logD_{lipw} = 0.9 * logD_{ow} + 0.52 \qquad (3)$$

The physicochemical parameters of all target compounds are summarized in Table 1. The relationship between log D_{lipw} of the target compounds and their corresponding

acute toxicity (LC_{50}) and bioconcentration factor (BCF) was evaluated through linear regression analysis (Figure S2).

Table S4-1. MRM transitions for test compounds on UPLC-MS/MS

Compound	MRM (m/z)			
	Quantification	CV/CE*	Qualification	CV/CE
ESI+				
Acetaminophen	151.97 > 109.99	38/22		
M-Acetaminophen	166.03 > 124.07	38/22	166.03 > 92.74	38/24
d4-Acetaminophen	156.03 > 113.99	40/12	156.03 > 96.75	40/22
DM-diazepam	271.03 > 139.99	56/28	271.03 > 165.03	56/28
Diazepam	285.03 > 154.02	56/26	285.03 > 193.09	56/32
d5-Diazepam	290.10 > 198.07	54/34	290. 10 > 154.11	54/26
ESI-				
DM-Methylparaben	137.09 > 93.08	34/15		
Methylparaben	151.05 > 92.03	38/20	151.05 > 136.00	38/14
d4-Methylparaben	155.05 > 96.05	36/20	155.05 > 140.01	36/14
DM-Naproxen	215.15 > 171.15	21/6	215.15 > 169.15	21/28
Naproxen	229.15 > 185.15	17/8	229.15 > 170.15	17/16
d3-Naproxen	232.18 > 188.10	14/5	232.18 > 173.14	14/18

*CV-cone voltage (kV), CE-collision energy (eV).

Table S4-2. Recoveries and limits of quantification (LOQ) of test compounds.

Compound	LOQ (ng/mL)	Recovery (%)	
		D. magna	AFW
Acetaminophen	0.5	84.6 ± 5.3	87.4 ± 1.8
M-acetaminophen	0.2	62.0 ± 5.7	99.6 ± 2.3
DM-diazepam	0.2	103.5 ± 11.0	105.0 ± 2.7
Diazepam	0.25	128.4 ± 3.2	94.3 ± 2.3
DM-methylparaben	3.0	51.9 ± 7.3	72.6 ± 12.3
Methylparaben	1.5	95.8 ± 3.2	116.8 ± 1.8
DM-naproxen	3.0	60.9 ± 12.6	119.8 ± 2.2
Naproxen	2.0	127.6 ± 1.1	106.4 ± 1.3

Table S4-3. Bioaccumulation kinetic parameters of the target CECs in *D. magna*.

Compound	k_u (L kg^{-1} h^{-1})	R^2	k_d (h^{-1})	R^2
Acetaminophen	0.2 ± 0.0	0.991	0.8 ± 0.0	1.000
M-Acetaminophen	17.3 ± 0.4	0.982	1.7 ± 0.0	1.000
DM-Diazepam	1.2 ± 0.1	0.900	0.1 ± 0.0	0.968
Diazepam	4.1 ± 0.3	0.879	0.4 ± 0.2	0.926
DM-Methylparaben	0.3 ± 0.0	0.620	0.3 ± 0.1	0.895
Methylparaben	0.7 ± 0.1	0.855	0.2 ± 0.0	0.986
DM-Naproxen	_	_	_	_
Naproxen	0.2 ± 0.0	0.855	0.2 ± 0.1	0.868

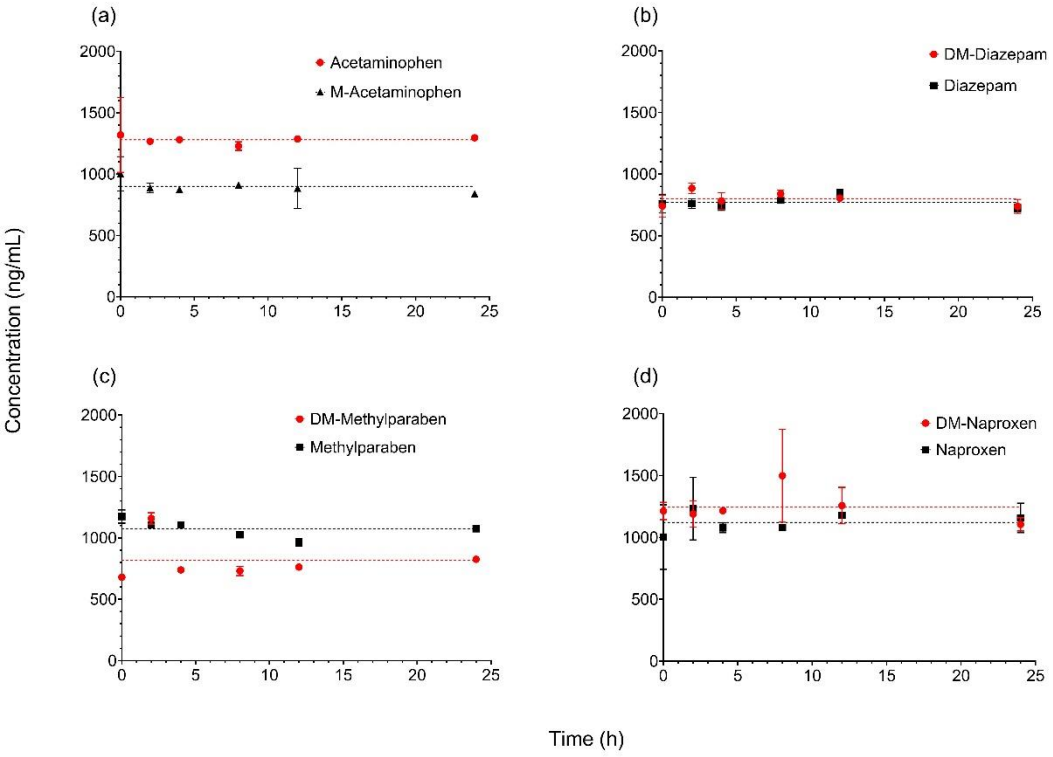

Figure S4-1. Concentrations of test compounds in the artificial freshwater during the uptake phase.

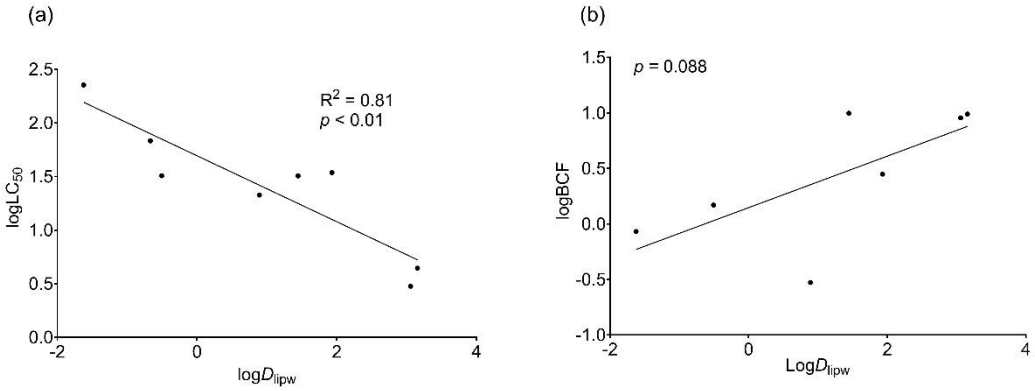

Figure S4-2. Relationships between logD_{lipw} and (a) logLC_{50} and (b) logBCF.

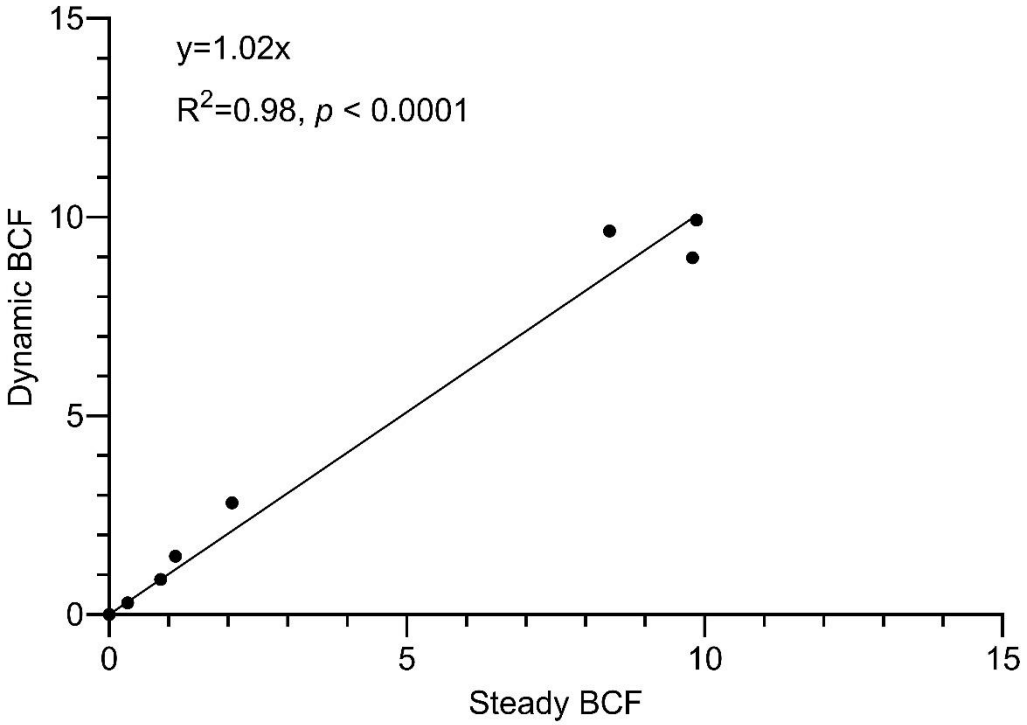

Figure S4-3. The correlation between steady state BCF and dynamic BCF in *D. magna*

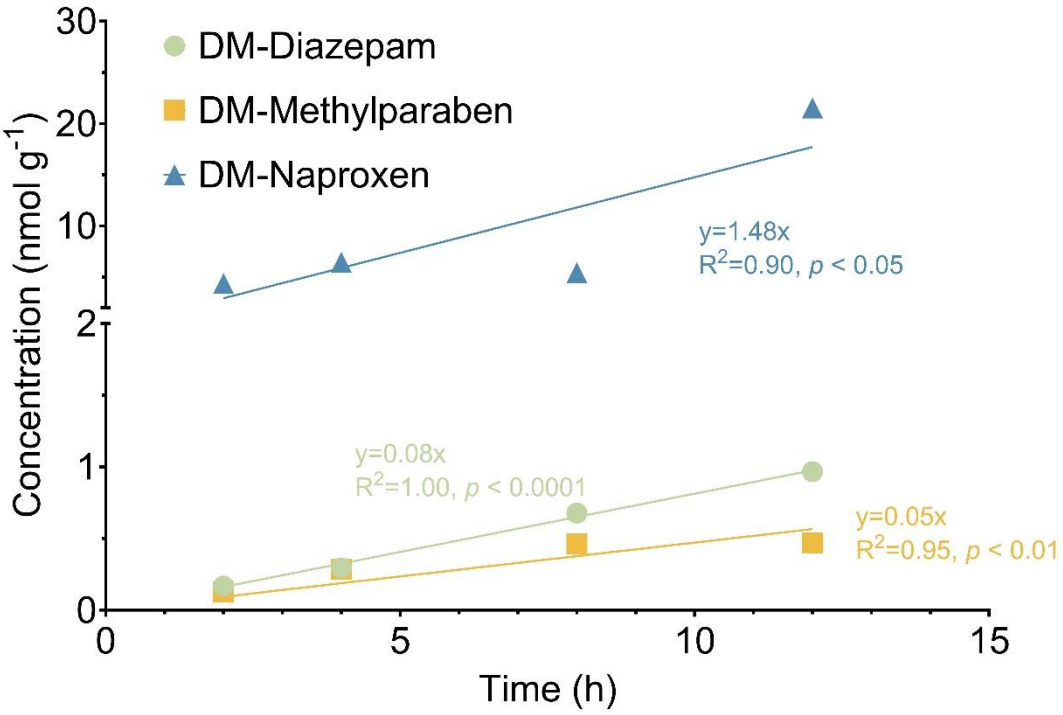

Figure S4-4. Linear correlations between the concentration of the formed demethylated derivatives in *D. magna* and the exposure time to the corresponding methylated parent compounds.

References

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Chapter 5 Summary of Findings and Future Work

5.1 Summary of Findings

This dissertation project evaluated the effects of methylation and demethylation on the environmental behaviors of CECs in higher plants and aquatic invertebrates from multiple angles, and explored the potential interconversions between CECs and their methylated or demethylated TPs after bio-uptake. The experimental data were further correlated with molecular properties and compared with *in silico* predictive results. The primary findings and conclusions are briefly summarized below.

5.1.1 Influence of Methylation and Demethylation on Plant Uptake of Emerging Contaminants

Four CECs and their methylated or demethylated TPs were comparatively evaluated for their uptake into *A. thaliana* cells or by wheat seedlings. The methylated compounds, generally more hydrophobic with a greater log K_{ow} and log D_{ow}, often displayed a greater accumulation potential in both plant models as compared to their demethylated counterparts, with the exception of acetaminophen/M-acetaminophen in *A. thaliana* cells. The influence of methylation and demethylation on the translocation of CECs in wheat plants was molecular-specific. Methylation caused a significant increase in the translocation of acetaminophen, but a significant decrease for DM-diazepam. Methylation also generally prolonged the persistence of CECs in both *A. thaliana* cell

culture media and wheat seedling hydroponic solution. A significant linear relationship was observed between log D_{ow} and log BCF, indicating that the generally increased accumulation of methylated compounds may be attributed to their higher hydrophobicity. Results from this study suggested that common transformations such as methylation and demethylation may affect the persistence and accumulation of CECs in plants, and their role should be considered to obtain a more comprehensive understanding of the risks of CECs in the terrestrial environment including agro-food systems.

5.1.2 Methylation and Demethylation of Emerging Contaminants in Plants

The interconversions between CECs and their methylated or demethylated TPs were evaluated in *A. thaliana* cells and wheat seedlings after their uptake. The methylation-demethylation cycle was observed in both plant models, with demethylation generally taking place at a greater degree than methylation. The rate of methylation or demethylation appeared to be molecule -specific. Computation results showed that the chemical bond strength between the methyl group and the major molecular fragment in the methylated CECs followed a general order of methylparaben < diazepam < naproxen < M-acetaminophen, a pattern reflective of experimental observations for demethylation in *A. thaliana* cells. Future studies considering more chemical structures would help strengthen such QSAR models so that the potential for simple transformations such as methylation and demethylation may be predicted in the absence of experimental data.

5.1.3 Influence of Methylation and Demethylation on the Bioaccumulation and Acute Toxicity of Emerging Contaminants in *Daphnia magna*

The acute toxicity of selected CECs and their methylated or demethylated TPs was further assessed by exposing *D. magna* to individually compounds. Methylation or demethylation resulted in changes in the acute toxicity for most CECs, and the influence was compound-specific. Methylation led to a significant increase in the acute toxicity of DM-methylparaben and DM-naproxen, but a decrease for acetaminophen. A significant negative linear relationship was observed between log LC_{50} values and log D_{lipw} values, indicating that as log D_{lipw} increased, the acute toxicity generally increased. Methylation increased the bioaccumulation in *D. magna* for acetaminophen, DM-methylparaben and DM-naproxen, and the increased bioaccumulation likely underlined the increases in acute toxicity for methylated compounds. In *D. magna*, active demethylation of diazepam, methylparaben and naproxen was observed, with the demethylation of naproxen especially pronounced, suggesting that enzymes in *D. magna* exhibited different levels of activity towards different substrates. QSAR models were used to predict changes in acute toxicity and bioaccumulation as a result of methylation, and the predicted values were in good agreement with experimental observations.

5.1.4 Overall Conclusions

The exploratory research presented in this dissertation clearly showed that simple transformations such as methylation and demethylation can significantly change the

physicochemical properties of CECs and subsequently cause changes in their environmental behaviors such as accumulation by plants and aquatic organisms, toxicity and persistence. Methylation generally leads to increased hydrophobicity and further greater bioaccumulation and acute toxicity. However, exceptions were also observed in this study, suggesting that specific molecular structures may respond differently to the impact of simple transformations. QSAR models using molecular descriptors have the capability to predict the easiness of transformation reactions such as methylation and demethylation, the subsequent changes in physicochemical properties from such transformations, and further, the ensuing changes in bioaccumulation, translocation, and toxicity. Such models should be calibrated with more experimental observations and by the inclusion of more diverse structures. Such predictive tools are extremely valuable, given the enormous number of CECs and their transformation products, which renders experimentation-based approaches largely infeasible. This dissertation research highlights the prevalence of simple transformations such as methylation and demethylation in the environment, and the need to consider such transformations in achieving a more comprehensive understanding of the environmental fate and risks of CECs.

5.2 Future Research

Results from this dissertation research and a few other studies showed that simple transformations can effectively influence the environmental behaviors of CECs, and the effect is specific to molecular structures. Changes in bioaccumulation and toxicity due to transformations should be further evaluated under environmentally relevant conditions.

The greatest challenge to understanding the environmental risks of CECs is the sheer number of CECs and their metabolites. In the absence of experimental data, predictive tools such as QSAR models and computational chemistry should be used to predict the possibility for the occurrence of transformations as well as the changes in physicochemical properties accompanying these transformations. Likewise, modeling may be also used to estimate changes in environmental behaviors and risks for CECs that are susceptible to transformations. It must be noted that only methylation and demethylation were considered in this research. Other common transformations may also be of great importance to improve our understanding of environmental risks of CECs. For example, halogenated CECs can be produced during the disinfection process that is commonly used in treating wastewater and drinking water, and such halogenated derivatives may have very different biological activity profiles as well as environmental behaviors from their precursors. Conjugation with endogenous biomolecules has been widely observed for biologically mediated CEC transformations. For example, conjugates of CECs and/or their metabolites are common in higher plants. Enzymes such as glucuronidases, aminoacylases, and dipeptidases in the human gut and intestine may hydrolyze these conjugates, releasing the parent or metabolites in their free form. Future research is needed for these unique TPs of CECs to obtain a more comprehensive understanding of the environmental fate and risks of CECs.