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Accumulation and phytotoxicity of perfluorooctanoic acid and 2,3,3,3-tetrafluoro-2-(heptafluoropropoxy)propanoate in *Arabidopsis thaliana* and *Nicotiana benthamiana*☆

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ABSTRACT

2,3,3,3-Tetrafluoro-2-(heptafluoropropoxy)propanoate (known as GenX) has been used as an alternative to perfluorooctanoic acid (PFOA) which was phased out of formulations for industrial and consumer product applications in 2015. While the effects of GenX on lab animals have been studied, little is known about its effects on plants. This study examined and compared the accumulation and toxicity of GenX and PFOA in the model plants *Arabidopsis thaliana* and *Nicotiana benthamiana*. Both plants showed reduction in biomass and root growth following exposure to PFOA or GenX in a dosage-dependent manner. The bioaccumulation factors (BFs) of GenX and PFOA were plant species-dependent, with higher BFs in *A. thaliana* compared to *N. bethanminana*. Additionally, GenX and PFOA were more readily accumulated into shoot tissues of *A. thaliana* than in *N. bethanminana*. Exposure to GenX also caused a reduction in chlorophyll content (18%) and total phenolic compounds (26%). However, GenX exposure increased superoxide dismutase activity and H_2O_2 content (1.6 and 2.6 folds increase, respectively) in *N. benthamiana*. Overall, our result suggest that GenX is bioaccumulative, and that its accumulation likely inhibits plant growth and photosynthesis as well as inducing oxidative stress.

1. Introduction

Perfluorooctanoic acid (PFOA) consists of a fully fluorinated alkyl C8 carbon chain and a carboxylic group. Due to its unique water- and oil-repellent properties and its thermal/chemical stability, PFOA has been widely used in industrial and consumer applications, such as nonstick cookware, food packaging, and water-repellent fiber coatings (EPA, 2017). PFOA is moderately soluble in water with low volatility. Unsurprisingly, it is detected in the environment, wildlife, biota, and humans (Giesy and Kannan, 2001). Soon after PFOA was shown to strongly accumulate in animals and plants with significant health concerns, the EPA launched the PFOA Stewardship Program, aiming to reduce and eliminate PFOA and its precursors by 2015 (EPA, 2006). Currently, PFOA is classified as a possible human carcinogen by The International Agency for Research on Cancer (IARC) (IARC, 2016).

Ammonium 2,3,3,3-tetrafluoro-2-(heptafluoropropoxy)propanoate, also known as GenX, has a density of 1.7 g/cm^3 and a very low vapor pressure (0.01 Pa at 20 °C) under standard conditions (Hoke et al., 2016). Since 2009, GenX has been used as an alternative to PFOA in fluoropolymer or resin manufacture (DuPont, 2010). GenX has been recently detected with a mean concentration of 663 ng/L in drinking water sources in North Carolina (Sun et al., 2016). The presence of GenX in river and coastal water sources have been also reported in China (up to 3.1 μg/L), Germany (up to 85 ng/L), and the Netherlands (up to 75 ng/L) (Heydebreck et al., 2015).

Based on an EPA draft risk assessment on GenX, the reference dose of GenX is 0.00008 mg/kg-day, which is higher than that of PFOA (0.00002 mg/kg-day) (EPA, 2018). However, the precise

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health and environmental risks of GenX relative to PFOA are still debated. Studies have reported acute toxicity of GenX in rats and mice, with median lethal dose (LC_{50}) of 5,200 mg/m^3 via inhalation, 5,000 mg/kg via dermal contact, and greater than 1,750−3,129 mg/kg through the oral route (ECHA, 2014). In addition, GenX may have adverse effects on fetal development as well as kidney, liver, and immune system function (EPA, 2018; López-Soldado et al., 2018; Caverly Rae et al., 2015).

Studying toxicity of GenX on freshwater fish *Oncorhynchus mykiss*, Hoke et al. reported a half maximal effective concentration (EC_{50}) < 96.9 mg/L, a no observed effect concentration < 8.89 mg/L, and a bioaccumulation factor < 30. This report further suggested that GenX posed a low risk to aquatic environments based on Globally Harmonized System (GHS) or European Classification, Labelling and Packaging of Substances (CLP) legislation (Hoke et al., 2016). Based on external exposure doses of PFASs, a wide range of PFAS-dependent absorption, distribution, metabolism, and elimination half-life values (ranging from few hours to more than hundred hours) in different animal species have been reported (Gannon et al., 2016; Lupton et al., 2012; Fasano et al., 2006; Gomis et al., 2018). In contrast to previous studies that used external dosing of these PFASs for toxicity assessment, Gomis et al. reported that GenX has a higher toxic potency than PFOA based on internal concentrations in serum and liver of male Wistar rat (Gomis et al., 2018).

Current studies have focused on the toxic effects of GenX on animals. To our knowledge, little is known about the bioaccumulation and effects of GenX on plants. This study examined bioaccumulation potential and phytotoxicity of GenX on two model plants, *Arabidopsis thaliana* and *Nicotiana benthamiana*. As two of the most extensively studied plant models, we deemed *Arabidopsis thaliana* and *Nicotiana benthamiana* as logical starting points for elucidating the accumulation rate and toxic effects of GenX *in planta*. These species are among the most well sequenced and genetically tractable organisms in modern biology. This fact improves the likelihood that our findings may lead to a genetic or molecular description of how plants tolerate, accumulate, or metabolize polyfluorinated compounds.

We monitored plant growth inhibition by recording plant biomass and shoot and root growth with or without exposure to each compound. Additionally, the accumulation rate and translocation efficiency of each compound in both plants was also measured. Several sub-lethal effects such as chlorophyll content, total phenolic compounds, superoxide dismutase (SOD) activity, and H_2O_2 content were also measured to provide a possible description of the mechanism through which GenX exerts its phytotoxicity.

2. Materials and methods

2.1. Chemicals

Perfluorooctanoic acid (95% pure, CAS no. 335-67-1) was obtained from Alfa Aesar, Ward Hill, MA, USA. 2,3,3,3-Tetrafluoro-2-(heptafluoropropoxy)propanoic acid (97% pure, CAS no. 13252-13-6) was purchased from SynQuest Labs, Alachua, FL, USA. Two isotope labeled standards, perfluoro-[1,2−^{13}C2]octanoic acid and 2,3,3,3-Tetrafluoro-2-(1,1,2,2,3,3,3- heptafluoropropoxy)-^{13}C3-propanoic acid, were obtained from Wellington Laboratories, Guelph, Canada. ACS reagent grade dichloromethane (DCM) was obtained from Acros Organics, Pittsburgh, PA, USA. HPLC grade methanol (MeOH), trace metal grade ammonium hydroxide (20%), H_2O_2 (30%), Na_2CO_3, acetone, and trichloroacetic acid (TCA) were obtained from Fisher Scientific, Pittsburgh, PA, USA. Na_2-EDTA was purchased from J.T. Baker, Phillipsburg, NJ, USA. Triton X-100 was obtained from Fluka analytical, Buchs, Switzerland. The graphitized non-porous carbon powder Supelclean™ ENVI-Carb™, HEPES buffer (99.5% pure), nitro blue tetrazolium (NBT, 98% pure), methionine (98% pure), riboflavin, 2-(N-morpholino) ethanesulfonic acid, and Folin-Ciocalteu's phenol reagent were obtained from Sigma-Aldrich, St. Louis, MO, USA. Murashige and Skoog (MS) powder and agar powder micropropagation Type I was purchased from Caisson Labs, East Smithfield, UT, USA. Sucrose was purchased from Macron Fine Chemicals, Center Valley, PA, USA.

2.2. Plants and growth conditions

Seeds of *Arabidopsis thaliana* wild-type Col-0 and *Nicotiana benthamiana* were surface-sterilized by soaking in 70% ethanol for 5 min and 50% bleach for 10 min, followed by rinsing with sterile deionized-water for five times. Seven sterilized seeds were placed on a petri dish containing half-strength Murashige and Skoog (½ MS) medium [1% (wt/vol)sucrose], pH 5.8, agar and different concentrations of GenX or PFOA (5 and 20 mg/L). The PFOA concentrations used in this study were guided by Yang et al. who had reported bioaccumulation of PFOA in *Arabidopsis thaliana* (Yang et al., 2015). The half-strength MS medium agar contains 2.23 g/L of MS premixed powder, 0.5 g/L of 2-(N-morpholino) ethanesulfonic acid, 5 g/L of sucrose, and 8 g/L of agar powder micropropagation Type I. Petri plates without GenX or PFOA were used as controls. Each treatment was conducted in triplicate. A total of 18 petri dishes were used. The seed-containing plates were sealed with 3M™ Transpore™ surgery tape and placed at 4 °C in dark for 24 h to synchronize seedling emergence rates. The plates were placed vertically on racks and incubated with a 16/8 h light/dark period with a light intensity of 100 μE m^{-2} s^{-1}at 21 ± 1 °C for 21 days.

Among the two plant species, one showed less tolerance to GenX observed in the agar-grown tests was selected for further investigation on the phytotoxicity of GenX in plant. Hydroponic-grown plants were used to assess sub-lethal effects of GenX on plant. Modified Hoagland solution (see Table S1 in supporting information) was used as growth medium. Given there were no prior studies on the sub-lethal effects of GenX in plant, a wide range of GenX concentrations (20, 50, 100, 200 mg/L) were applied in this study. Sterilized seeds were prepared as described above, treated at 4 °C for 24 h and placed on a piece of polyethylene fiber in each well of a 96-well plate of which the bottom of each well was removed. The seeded 96-well plate was then placed in a polyethylene container containing 45 mL of Hoagland solution with different concentrations of GenX (20, 50, 100, 200 mg/L). The plates were incubated under the same conditions as described above for 14 days. Plates containing Hoagland solution without GenX were used as controls.

2.3. Extraction of PFOA and GenX

After 21 days of incubation, the plant biomass (roots and shoots) and root length on the petri dishes were measured. Samples of roots and shoots were freeze-dried for 48 h before stored at −20 °C for later chemical extraction and measurements. The GenX and PFOA in the plant biomass was extracted as described by Blaine et al. (Blaine et al., 2013) with some modifications. Briefly, a mixture of 50:50 (v/v) of DCM and MeOH with 1% ammonia hydroxide (v/v) was prepared as extraction solvent. Ten mg of plant sample was transferred into a 15-mL polypropylene tube containing 3 mL of the

extraction solvent. After vortexed for 30 s, the tube was shaken on an orbital shaker (KS 260 basic, IKA®, USA) at 200 rpm at 37 °Cfor 1 h. After centrifugation at 1,500 rpm for 5 min (Sorvall™ Legend™ XTR, Thermo scientific, USA), the supernatant was then collected. The extraction process was repeated twice. The extracts were pooled, followed by drying under a gentle nitrogen stream. The dried extract was reconstituted with 1 mL of MeOH, and then mixed with 50 mg of ENVI-Carb for 20 s for cleaning up. The cleaned extract was then obtained by centrifugation at 13,000 rpm for 5 min (accuSpin^{TM}MicroR, Fisher scientific, USA). Based on spike-recovery tests, the average recovery of PFOA and GenX of this extraction process was 121.9% and 132.3%, respectively.

2.4. Chemical analysis for PFOA and GenX and potential metabolites

The concentrations of PFOA and GenX in the extract were analyzed using ultra-high performance liquid chromatography coupled to triplequadrupole mass spectrometry (LC-QqQ) as described previously (Huang et al., 2018). Briefly, the extract (10 μL) was injected into a LC-QqQ (Agilent 1290 Infinity II UHPLC coupled to an Agilent 6470 QqQ) equipped with a Jet Stream electrospray ionization source. The analytes were separated by an Agilent ZORBAX Eclipse Plus C-18 narrow bore (2.1 mm × 100 mm, 1.8 μm) HPLC column maintained at 40 °C and eluted with solvent A (5 mM ammonium acetate in water) and solvent B (95% MeOH and 5% water with 5 mM ammonium acetate) at a flow rate of 0.4 mL min^{-1}. The separation gradient method was as follows: 0–0.5 min (holding at 10% solvent B), 0.6–2 min (10% to 30% solvent B), 2.1–14 min (30% to 95% solvent B), 14.-14.5 min (95% to 100% solvent B), 14.6–16.5 min (holding at 100% solvent B), and stabilize column at 10% solvent B for 6 min. The MS parameters were optimized for PFOA and GenX under direct infusion at 0.4 mL min^{-1} to identify the MRM (multiple reaction monitoring) transitions (precursor/product fragment ion pair). ^{13}C isotopically labeled PFOA and GenX were used as internal standards. Sample acquisition and analysis were performed using Mass Hunter B.08.02 (Agilent). To identify possible metabolites from GenX, non-target analysis was performed using liquid chromatography-electrospray ionization-time of flight-mass spectrometry (LC-ESI-TOFMS) analysis as described previously by Stynar et al. (Strynar et al., 2015).

2.5. Tolerance index (TI), bioaccumulation factor (BF) and translocation factor (TF)

The effects of PFOA or GenX on plants were assessed using three indices: tolerance index, bioaccumulation factor and translocation factor. Tolerance index (TI) is based on changes of phenotypes such as biomass or root length of plants after exposure to a known contaminant over a period of time. The TI was estimated using Eq. A (Mishra and Choudhuri, 1999; Rabie and Almadini, 2005; Sari-Gorla et al., 1999) as described below.

$$Tolerance\ Index\ (TI) = \frac{\textbf{\textit{mean phenotype measurement of treatment}}}{\textbf{\textit{mean phenotype measurement of control}}} \quad \text{(Eq. A)}$$

The tendency of PFOA or GenX to accumulate in plants can be assessed as bioaccumulation factor (BF) which is defined as the ratio of the concentration of PFOA or GenX in plants over the concentration of PFOA or GenX in medium. The unit of BF is mg/kg dry weight (DW) of plant/mg/L agar medium, and can be estimated using Eq. B (Arnot and Gobas, 2006; Soda et al., 2012).

$$Bioaccumulation\ factor\ (BF) = \frac{\textbf{\textit{chemical concentration in plant biomass}}\ (\text{mg/kg (DW)})}{\textbf{\textit{Initial chemical concentration in medium}}\ (\text{mg/L agar medium})} \quad \text{(Eq. B)}$$

Translocation factor (TF) explains the ability of a plant to translocate a compound of interest from the root system to the shoot system of the plant. The TF of PFOA or GenX in different plants was determined by using equation Eq. C (Soda et al., 2012) as described below. The higher the TF values, the more effective of the plant to translocate the compound from roots to shoots.

$$Translocation\ factor\ (TF) = \frac{\textbf{\textit{chemical concentration in shoot}}}{\textbf{\textit{chemical concentration in root}}} \quad \text{(Eq. C)}$$

2.6. Sub-lethal effect assays

2.6.1. Superoxide dismutase (SOD) assays

The crude enzymes of SOD in the plant biomass were extracted as follows. Fresh weight (FW) of 100 mg plant tissues was first homogenized using 0.1 g of silica beads (0.1 mm), followed by addition of 1 mL of extracting solution containing 50 mM HEPES and 0.1 M Na_2EDTA. The mixture was then vortexed for 2 min and then centrifuged at 4,500 rpm at 4 °C for 15 min. The supernatant containing the crude SOD enzymes was collected for measurement of SOD activity based on the inhibition of photochemical reduction of nitro blue tetrazolium (NBT) (Yu et al., 1998). Briefly, the SOD assay was performed by adding 100 μL of crude enzymes into 5 mL of reaction mixture containing 63 μM NBT, 50 mM HEPES, 0.1 mM Na_2EDTA, 50 mM Na_2CO_3, 13 mM methionine, 0.025% (w/v) Triton X-100, and 1.3 μM of riboflavin. The assays were then exposed to visible light for 15 min and absorbance was measured using a spectrophotometer (Spectronic 20D+, Thermo, USA) at 560 nm wavelength. One unit of SOD was defined as the amount of enzyme that can inhibit 50% of NBT reduction.

2.6.2. Measurement of H_2O_2 content

Hydrogen peroxide (H_2O_2) content in plant tissues was determined using a colorimetric method described previously (Junglee et al., 2014). Briefly, 100 mg of homogenized fresh plant tissues was mixed with 2 mL of reaction solution containing 5% (w/v) TCA, 10 mM KH_2PO_4, and 1 M KI. After vortexing for 30 s and centrifugation at 4,500 rpm for 15 min, the supernatant was collected and incubated in the dark for 20 min. The intensity of yellow color was measured at 390 nm using a spectrophotometer (Spectronic 20D+, Thermo, USA). A standard curve was established by using H_2O_2 solution with concentration ranging from 50 μM to 1 mM.

2.6.3. Quantification of total chlorophyll content in plant

Total chlorophyll content in plant tissues was extracted from 100 mg of fresh tissues (in small pieces) in 10 mL acetone (80%, v/v) for 1 min, followed by centrifugation at 4,500 rpm for 10 min. The supernatant was collected for absorbance measurement at wavelength 645 (D_{645}) and 663 (D_{663}) nm using a spectrophotometer (Spectronic 20D+, Thermo, USA). The total chlorophyll content was then determined using - Arnon equation (Arnon, 1949) as shown in Eq. D.

$$Total\ Chlorophyll\ (mg/g\ FW) = 20.6\ D_{645} + 8.02\ D_{663} \quad \text{(Eq. D)}$$

2.6.4. Measurement of total phenolic compounds

Total phenolic compounds in the plant tissues were measured using the gallic acid equivalence (GAE) method (Marinova et al., 2005). Briefly, 100 mg of fresh tissues (in small pieces) was mixed with 2.5 mL of Folin-Ciocalteu's phenol reagent. After incubation at 37 °C for 5 min, the mixture was spiked with 2.5 mL of 7.5% Na_2CO_3 (w/v) and 5 mL of dd-H_2O to bring the mixture to a total volume of 10 mL. After incubating at room temperature for 2 h, the total amount of phenolic compounds in the mixture was determined as absorbance at 750 nm using a spectrophotometer (Spectronic 20D+, Thermo, USA). The reagent solution was used as blank. Gallic acid was used as the standard compound to establish calibration curves. The total phenolic compounds in sample was expressed as GAE per gram of fresh plant tissue weight.

2.7. Statistical analyses

Student's t-test was used to evaluate if there was a significant difference in plant growth between the treatment groups (i.e., exposing to PFOA or GenX) and the controls. The Shapiro-Wilk test was selected for normality statics test since the sample size is less than 5,000. The statistical analysis of data was performed using the software Sigmaplot 14.0 (Systat Software, USA).

3. Results and discussion

3.1. Effects of PFOA and GenX on the growth of A. thaliana and N. benthamiana

A. thaliana and *N. benthamiana* showed no growth defects when exposed to 5 mg/L of GenX, but the growth of their shoots were inhibited when exposed to 5 mg/L of PFOA. Moreover, when exposed to 20 mg/L of PFOA over the course of 21 days, the growth and development of both shoots and roots of *A. thaliana* and *N. benthamiana* were significantly inhibited based on the photos shown in Fig. 1A. While significant growth inhibition was observed in *N. benthamiana* after exposure to 20 mg/L GenX for 21 days, a similar severity of inhibition was not observed in *A. thaliana* based on the photos shown in Fig. 1B, suggesting that *N. benthamiana* may be more sensitive to GenX than *A. thaliana*.

Tolerance index (TI) is a parameter that has been widely used to evaluate the capacity of plants and fungi to withstand stressors such as heavy metals, drought, and salinity (Mishra and Choudhuri, 1999; Rabie and Almadini, 2005; Sari-Gorla et al., 1999). Higher TI values (up to 100%) correspond with better stressor tolerance by the species. In this study, TI values were determined using shoot weight, root weight, and root length to evaluate the inhibition

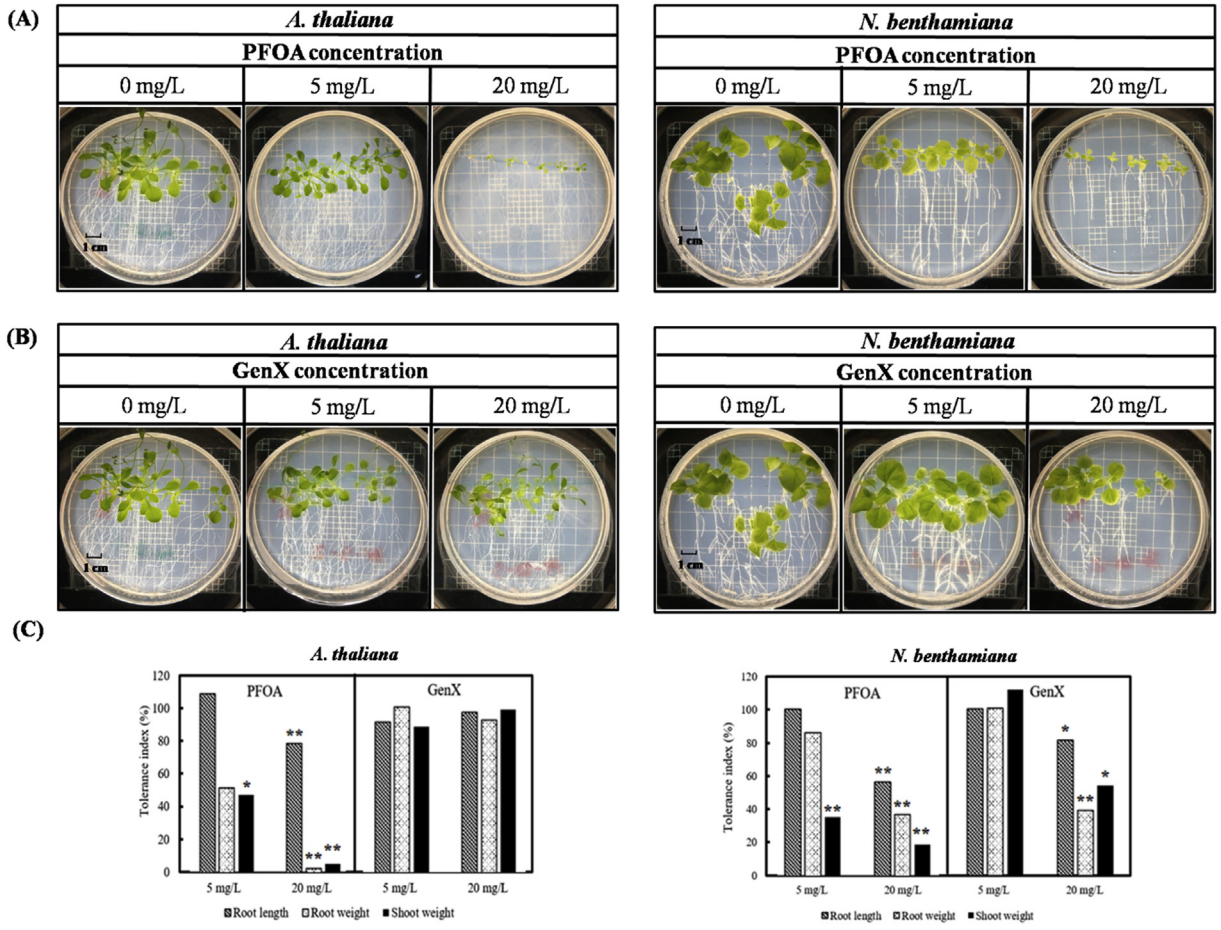

Fig. 1. *A. thaliana* and *N. benthamiana* grown on half-strength Murashige and Skoog medium agar plate containing 0, 5, or 20 mg/L concentrations of PFOA(A) or GenX (B). Tolerance index (TI) of *A. thaliana* and *N. benthamiana* to PFOA or GenX was estimated using changes in shoot weight, root weight and root length (C). Asterisks indicate significant differences between the treatments and the control, where $p < 0.05$ (*) and $p < 0.001$ (**).

effects of PFOA and GenX on plant growth and development (Fig. 1C). When exposed to 20 mg/L of PFOA, all three TI values suggested significant growth inhibition of both plant species, where the weight-based TI values decreased to 5–10% in *A. thaliana* and 20–40% in *N. benthamiana*, respectively.

No GenX-mediated growth inhibition was observed in *A. thaliana* regardless of concentration (Fig. 1C). By contrast, *N. benthamiana* was more sensitive to GenX as indicated by the root- and shoot-weight -based TI values which decreased from 100% to 40% and 55%, respectively. The TI values suggested that the growth inhibition occurred to *N. benthamiana* when exposed to 20 mg/L of GenX. These results also suggest that tolerance of PFOA and GenX is a plant species dependent phenotype.

Previous studies have investigated the toxicity of PFOA in animals and plants. For example, the EC_{50} of PFOA was found to be 44–51 mg/L in aquatic animals (Le and Peijnenburg, 2013) and 8–11 mg/L in plants (Yang et al., 2015). The reported EC_{50} of PFOA values in plants are supported by the observations in this study that both plant species experienced significant growth inhibition in the presence of 5 mg/L of PFOA (Fig. 1a and b). Similarly, our observations were consistent with previous findings that PFOA had little effects on the root length, but still significantly impacted the shoot and root weights of exposed *A. Thaliana* plants (Yang et al., 2015).

3.2. Bioaccumulation and translocation of PFOA and GenX in *A. thaliana* and *N. benthamiana*

GenX and PFOA were accumulated at different levels in the roots and the shoots of *A. thaliana* and *N. benthamiana* (Fig. 2). While higher PFOA concentrations were observed in the shoots of *A. thaliana* (Fig. 2A), higher PFOA concentrations were observed in the roots of *N. benthamiana* (Fig. 2B). Similar to the PFOA accumulation trends observed in these two plant species, higher GenX concentrations in the shoots of *A. thaliana* (Fig. 2C) and in the roots of *N. benthamiana* were observed (Fig. 2D). Several labs have shown that not only PFOA but also other short and long chain PFASs are accumulated differently in different plant species such as tomato, cabbage, zucchini, spring wheat, oat, maize, potato, and ryegrass grown in soil and hydroponic system (Wen et al., 2016; Felizeter et al., 2014; Stahl et al., 2009). However, GenX accumulation in plants has not been studied previously. Our result was consistent to previous studies suggesting that different plant species accumulate PFOA differently. This phenomenon was also observed for GenX-exposed plants in our study.

For both plant species, the BFs of PFOA (67.0 in *A. thaliana* and 16.4 in *N. benthamiana*) are about two-fold higher than the BFs of GenX (29.0 in *A. thaliana* and 7.7 in *N. benthamiana*) as shown in Table 1 and Tables S2–S3. These results not only suggested that PFOA was more bioaccumulative than GenX in both plant species but also implied that either the uptake rate of GenX was lower than that of PFOA or the eliminating rate of GenX was higher than that of PFOA in plant. This implication was indirectly supported by the fact that no suspected metabolites were detected in plant samples during non-targeted analyses.

Previous studies have shown that PFOA can be excreted out of from animal cells via organic anion transporters such as OAT2 and

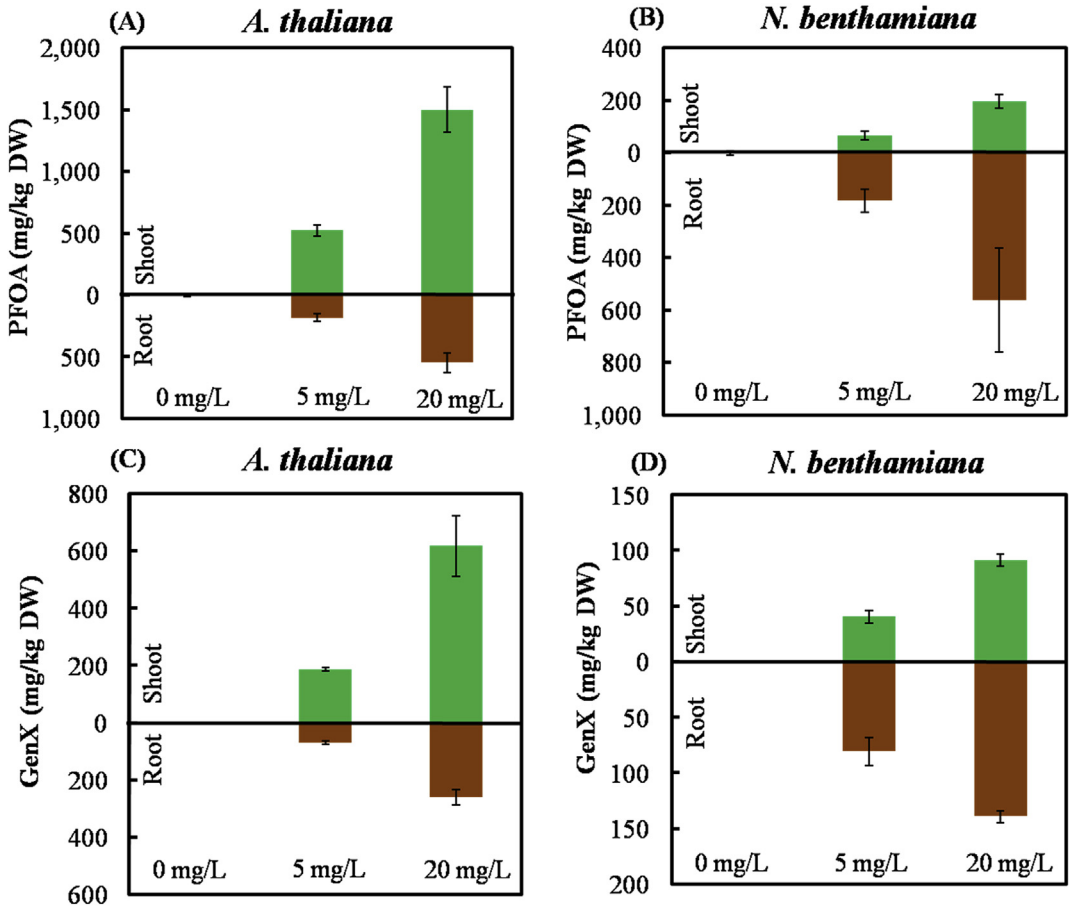

Fig. 2. PFOA and GenX bioaccumulation in plant roots and shoots after 21 d of growth on half-strength Murashige and Skoog medium agar plate containing 0, 5 or 20 mg/L PFOA or GenX. Higher PFOA concentrations were observed in the shoots of *A. thaliana* (A) while higher PFOA concentrations were detected in the roots of *N. benthamiana* (B). A similar trend was observed for GenX concentrations measured in *A. thaliana* (C) and in *N. benthamiana* (D).

Table 1
Bioaccumulation factor, translocation factor, total plant mass, and PFOA or GenX uptake by 21-day-old *A. thaliana* and *N. benthamiana*. Plants were grown on half-strength Murashige and Skoog medium agar containing 0, 5, or 20 mg/L of PFOA or GenX.

Species	*A. thaliana*				*N. benthamiana*			
	PFOA		GenX		PFOA		GenX	
Bioaccumulation factor[a]	67.0		29.0		16.4		7.7	
Translocation factor[b]	2.77		2.52		0.35		0.58	
	5 mg/L	20 mg/L	5 mg/L	20 mg/L	5 mg/L	20 mg/L	5 mg/L	20 mg/L
Total plant mass[c] (mg)	12.3 ± 3.7	4.0 ± 0.3	13.9 ± 0.8	13.8 ± 0.1	10.7 ± 3.2	9.8 ± 3.3	15.1 ± 0.8	8.2 ± 6.6
Uptake of PFOA or GenX by plant[d] (μg)	5.15 ± 0.51	3.98 ± 0.51	2.18 ± 0.07	7.32 ± 1.19	1.03 ± 0.27	2.6 ± 0.58	0.78 ± 0.11	0.83 ± 0.04

[a] Bioaccumulation factor (BF) = concentration of PFOA (or GenX) in plant/concentration of PFOA (or GenX) in medium agar. Average concentration of PFOA (or GenX) in plant was used in the calculation.
[b] Translocation factor (TF) = concentration of PFOA (or GenX) in shoot/concentration of PFOA (or GenX) in root.
[c] Total biomass = shoot DW + root DW.
[d] 0.075 mg and 0.3 mg of GenX (or PFOA) was added initially in 5 mg/L and 20 mg/L treatment, respectively.

OAT3 (Katakura et al., 2007). Although organic anion transporters responsible for eliminating PFOA or GenX in the plant are still unknown, these transporter proteins are believed to be ubiquitous not only in animal and but also in plant cells. In rhizosphere, transporter proteins play an important role in excluding organic anion in order to (i) enhance the availability of nutrients, (ii) reduce the concentration of toxic cation in rhizosphere, and (iii) reduce the accumulation of toxic substances in cytoplasm (Ryan et al., 2001). More studies need to be conducted to understand the elimination rate and mechanisms of PFOA and GenX accumulation in plant cells.

As shown in Table 1, interesting patterns of PFOA and GenX translocation were observed in both plant species. Comparable translocation factors (TFs) of PFOA and GenX (2.77 and 2.52, respectively) were observed in *A. thaliana*. Similarly, in *N. benthamiana*, the TFs of PFOA and GenX were 0.35 and 0.58, respectively. Different translocation factor of PFASs between plant species was also reported in several studies. Felizeter et al. proposed a possible mechanism of PFAAs translocation (Felizeter et al., 2014). When plants uptake of PFAAs from soil or nutrient solution by root, the majority of the PFAAs, except for long chain PFAAs ($C > 10$), are carried with the transpiration stream from stem to the leaves. After that, PFAAs are transported from leaves to fruit and storage organs via phloem sap. This suggested that transpiration rate of different plants might play an important role in the mobility of PFOA and GenX. In addition, Wen et al. found that the TF of PFOA and PFOS correlate positively with the ratio of protein content in shoots and those in the roots showing that the importance of protein on PFOA and PFOS translocation (Wen et al., 2016).

After 21 days of incubation, *A. thaliana* was observed to have accumulated more PFOA relative to its biomass than *N. benthamiana* independent of the concentration used (Table 1). This trend was also observed in the patterns of GenX uptake by these two plant species with an approximately 10-fold higher uptake of GenX observed in *A. thaliana* relative to *N. benthamiana*.

Knowledge about PFAS uptake, translocation, and bioaccumulation in plants, particularly in agricultural crops, is important to assess potential human exposure to PFAS through consumption of PFAS-contaminated crops. Stahl et al. examined the carryover of PFOA from soil to plants including spring wheat, oats, potatoes, maize, and perennial ryegrass exposed to PFOA ranging from 0.25 to 50 mg/kg (Stahl et al., 2009). PFOA was accumulated up to 341,000 mg/kg in straws but was only accumulated up to 1,440 mg/kg in grains. Interestingly, a higher level of PFOA was present in the peels of potatoes (2–234 mg/kg) but a small amount of PFOA as detected in the potato tubers (7–52 mg/kg). However, Lechner and Knapp reported that the majority of PFOA was accumulated in the vegetative tissues (103.6–796.6 mg/kg) while only a small amount of PFOA was present in the peels (7.7–29.3 μg/kg) and peeled edible parts (2.9–31.3 μg/kg), when examining the uptake of PFOA in carrot (*Daucus carota* ssp. *Sativus*), potato (*Solanum tuberosum*), and cucumber (*Cucumis Sativus*) in soil mixed with PFAS-contaminated sewage sludge (Lechner and Knapp, 2011). Felizeter et al. (2012) reported that hydroponically-grown lettuce (*Lactuca sativa*) with 10 μg/L PFOA accumulate less PFOA in the edible parts (i.e. foliage). The foliage to root concentration factors of PFOA was observed around 0.1 suggested that PFOA trended to accumulate in roots of lettuce. Our results showed that the translocation of PFOA and GenX is dependent on plant species and these observations were consistent with reports from previous studies. Our studies also provide foundations to screen plant species with a high uptake and accumulation rate, which could be potentially used as an environmentally friendly and effective phytoremediation means to alleviate the PFOA and GenX contamination. Notably, *A. thaliana* is a weed mustard commonly seen and easily grown in the wild.

3.3. Phytotoxicity of GenX on N. benthamiana

3.3.1. GenX dose-response curve and EC_{10} and EC_{50} in N. benthamiana

The phytotoxicity of GenX in *N. benthamiana* was further investigated, as *N. benthamiana* had shown growth inhibition when exposed to 20 mg/L GenX (Fig. 1C). Hydroponically grown *N. benthamiana* was used to establish a dose-response curve of GenX in planta. As shown in Fig. 3, compared to controls (i.e., no GenX exposure), *N. benthamiana* started to show growth inhibition when exposed to 20 mg/L of GenX. Sever growth inhibition was observed when exposed to 200 mg/L of GenX. Spotted white lesions on the plant leaf surface were also observed, which are likely due to the toxicity-triggered cell death and disruption of chloroplast function *in planta*. According to the curve of GenX dose-response to *N. benthamiana*, the EC_{10} of GenX was estimated to be 34.7 mg/L and EC_{50} was 107.5 mg/L (Fig. 3). No GenX metabolites were detected through LC/MS non-target analysis. The EC_{50} of GenX was 102 mg/L in *Daphnia* and 96.9 mg/L in trout (Hoke et al., 2016). In this study, the value of the observed EC_{50} of GenX in *N. benthamiana* is comparable to that in *Daphnia*.

3.3.2. Sub-lethal effects of GenX on N. benthamiana

In addition to dose-response relationships that assess the mortality of each species due to exposure, a few studies examined sub-lethal effects of toxic chemicals to plants and animals using phenotypic markers (Yu et al., 1998; van Doorn and Ketsa, 2014; He et al., 2010). No studies have evaluated the sub-lethal effects of GenX to plants. In this study, four markers - chlorophyll content,

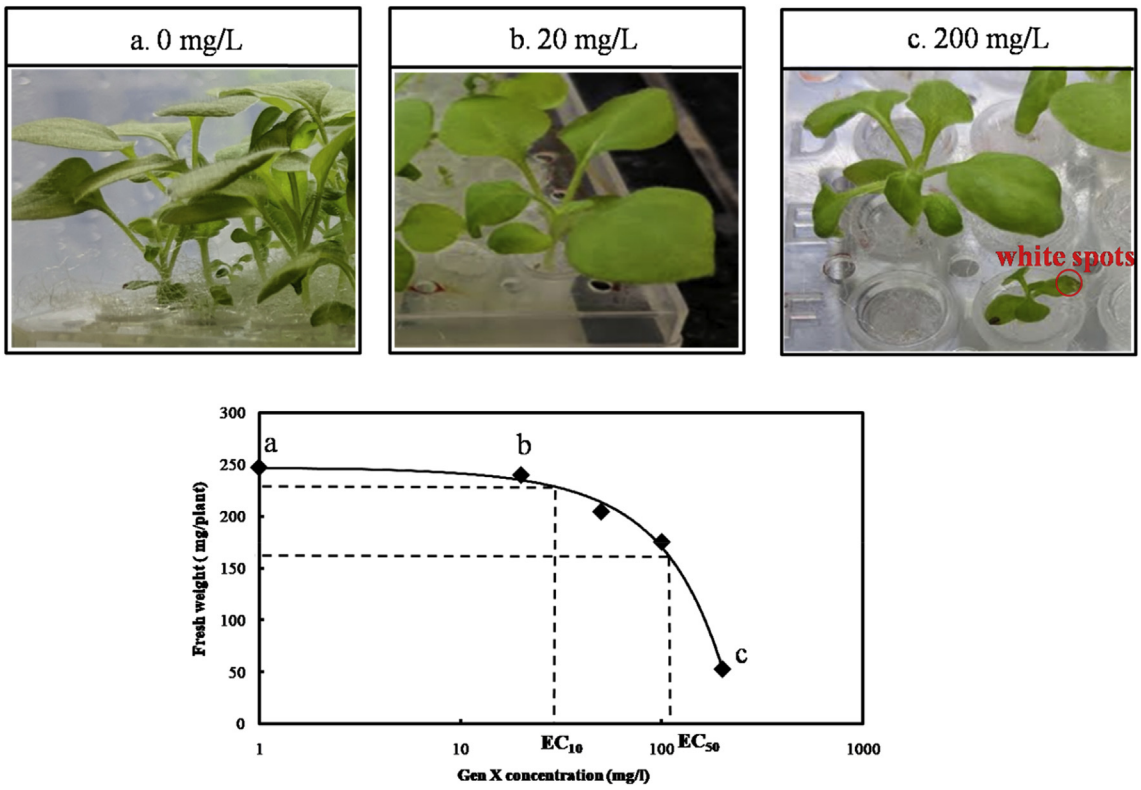

Fig. 3. The dose-response curve was produced using fresh weight of *N. benthamiana* plants grown hydroponically with 0, 20, 50, 100, or 200 mg/L of GenX. Plant mass decreased significantly when GenX concentration was greater than 20 mg/L. White spots were observed on the leaves of *N. benthamiana* exposed to 200 mg/L of GenX. Average values were used in constructing the curve. Error bars were included in the figure but too small to be seem. The data (including error bars) are available in Table S4.

total phenolic compounds, SOD activity, and H_2O_2 concentration-were chosen to assess the sub-lethal effects of GenX on plants (Fig. 4). Chlorophyll content and total phenolic compounds decreased 40% and 18% when the plants were exposed to 100 mg/L of GenX (a concentration close to the estimated EC_{50}) (Fig. 4A and B). Meanwhile, an increasing trend of SOD activity and H_2O_2 content was observed as GenX concentrations increased. The SOD activity and H_2O_2 content increased 1.6 and 2.7-fold in the plant tissues, respectively, when exposure to 100 mg/L of GenX comparing to controls. (Fig. 4C and D).

Injury of plants due to environmental stresses such as high light intensity, pathogens, salts and heavy metals have been demonstrated to induce the production of reactive oxidative species (ROS) including superoxide anion radicals and H_2O_2 (Mishra et al., 1995; Hernández et al., 1995; Ádám et al., 1995). These free radicals can cause lipid peroxidation, protein denaturation, and DNA mutation (Yu et al., 1998). Anti-oxidative enzymes such as SOD can remove free radical. They are thus play a critical role in preventing oxidative stress. Accordingly, an increased SOD or catalase activity can be used as an oxidative stress marker in stressed organisms. Exposure to PFOA can also cause oxidative stress in *A. thaliana* as indicated by an increase of oxidative stress marker malondialdehyde (MDA) and H_2O_2 concentration (Yang et al., 2015). The exposure to EC_{50} or higher doses of GenX resulted in a nearly 2-fold increase in SOD activity and H_2O_2 content, suggesting that the accumulation of GenX may induce oxidative stress to damage cells that led to disruption of normal cell functions in plants.

4. Conclusions

This study suggests that GenX can bioaccumulate in plants, translocate into different plant tissues, inhibit plant growth, induce oxidative stress, and cause a reduction in plant chlorophyll content. In several cases, these phenomena were observed to be species-dependent, as different bioaccumulation levels and overall uptake of PFOA or GenX was observed in the two plant models in this study. While *A. thaliana* was observed to tolerate high concentrations of GenX (20 mg/L), it also accumulated GenX more readily than *N. benthamiana*. Based on study which correlated toxic effects based on measured internal dose (Gomis et al., 2018), there might be a higher risk of GenX exposure through consumption of GenX contaminated crops which are predisposed to its accumulation. While *A. thaliana* is not an important agricultural crop, it is closely related to several important crops including canola, cabbage, turnips, and broccoli. Similarly, *N. benthamiana* is a species of tobacco which is related to agronomically important crops such as peppers, tomatoes, and potatoes. Preferential accumulation of PFOA or GenX in roots or in shoots also implicates an urgent need to fill knowledge gaps on the bioaccumulation and translocation of GenX in different food-related crops. On the other hand, plants similar to *A. thaliana* that can tolerate high GenX concentrations and accumulate higher levels of GenX would be ideal candidates for phytoremediation agents in PFOA-contaminated soils.

The mechanisms responsible for the observed differential distribution of PFOA and GenX within plant tissues are not well understood in either model plant. Future studies are needed to elucidate the factors contributing to the translocation of these chemicals in each plant species. Future studies examining potential synergistic effects of accumulation and phytotoxicity of PFOA and GenX on plants, as well as mixtures of PFASs, are essential to develop better strategies to manage the environmental and human health risks of PFAS.

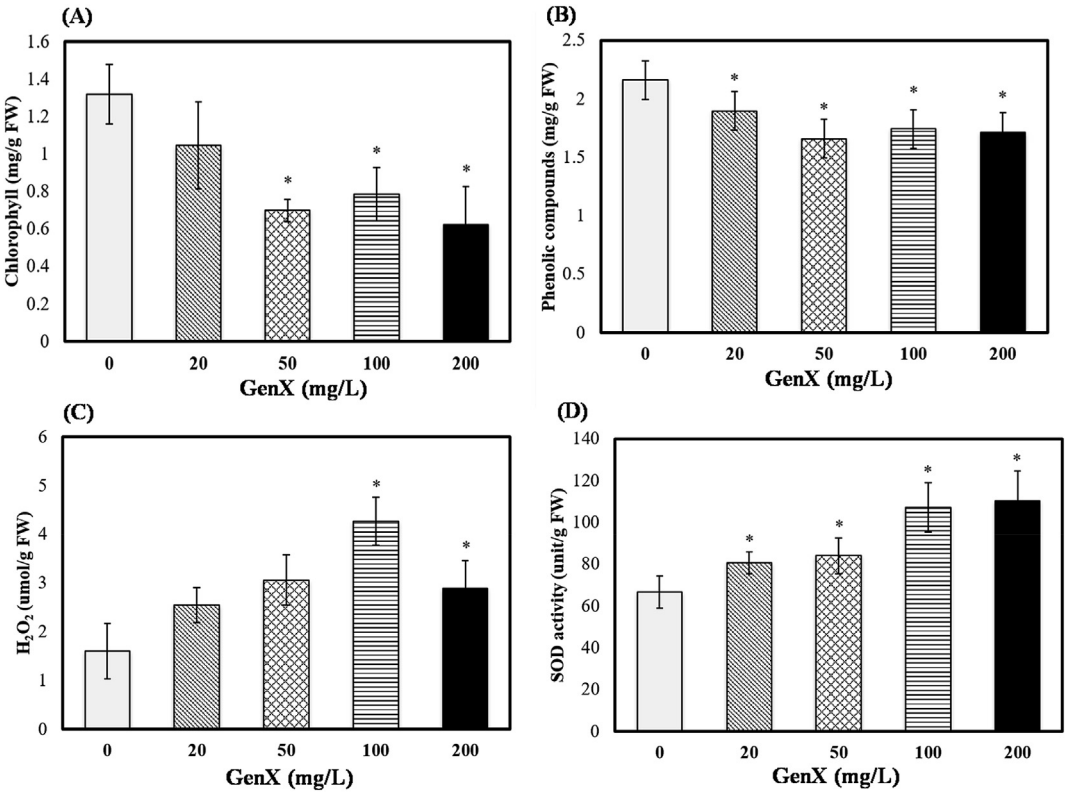

Fig. 4. Sub-lethal effects with respect to the changes in *chlorophyll* content (A), total phenolic compounds (B), H_2O_2 content(C), and SOD activity(D) of *N. benthamiana* grown hydroponically in medium containing 0, 20, 50,100 or 200 mg/L of GenX for 14 days. Asterisks (*) indicate significant differences ($p < 0.05$) between the treatments and the controls.

CRediT authorship contribution statement

Chih-Hung Chen: Investigation, Methodology, Validation, Formal analysis, Writing - original draft. **Shih-Hung Yang:** Investigation, Methodology, Validation, Formal analysis, Visualization, Writing - original draft. **Yina Liu:** Validation, Writing - review & editing. **Pierce Jamieson:** Writing - review & editing. **Libo Shan:** Writing - review & editing. **Kung-Hui Chu:** Conceptualization, Supervision, Writing - review & editing.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2019.113817.

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