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Legacy of agrochemicals in the circular food economy: Glyphosate-based herbicides introduced via manure fertilizer affect the yield and biochemistry of perennial crop plants during the following year

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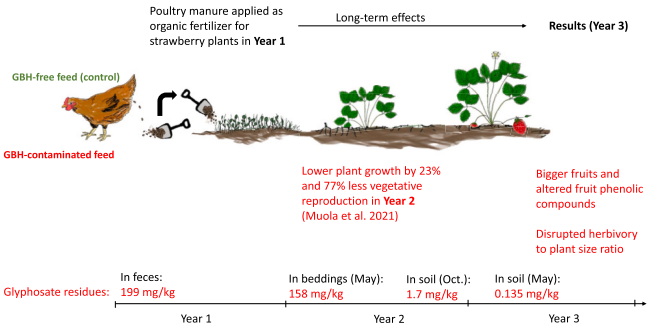
HIGHLIGHTS

- Glyphosate-based herbicides (GBH) can end up in poultry feces via their feed.
- Poultry feces used as manure fertilizers introduce GBH into cropping systems.
- GBH introduced via manure fertilizer caused long-term effects.
- Long-term effects were realized in pest interactions, yield and fruit biochemistry.
- We conclude persistent impact of GBH residues on plant physiology and ecology.

GRAPHICAL ABSTRACT



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ABSTRACT

Conventional agricultural practices favoring the use of glyphosate-based herbicides (GBHs) increase the risk of GBH residues ending up in animal feed, feces, and, eventually, manure. The use of poultry manure as organic fertilizer in the circular food economy increases the unintentional introduction of GBH residues into horticultural and agricultural systems, with reportedly negative effects on the growth and reproduction of crop plants. To understand the potential lasting effects of exposure to GBH residues via organic manure fertilizers, we studied strawberry (*Fragaria* x *vescana*) plant performance, yield quantity, biochemistry, folivory, phytochemistry, and soil elemental composition the year after exposure to GBH. Although plants exposed to GBH residues via manure fertilizer were, on average, 23% smaller in the year of exposure, they were able to compensate for their growth during the following growing season. Interestingly, GBH residue exposure in the previous growing season led to a trend in altered plant size preferences of folivores during the following growing season. Furthermore, the plants that had been exposed to GBH residues in the previous growing season produced 20% heavier fruits with an altered composition of phenolic compounds compared to non-exposed plants. Our results indicate that GBHs

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introduced via manure fertilizer following circular economy practices in one year can have effects on perennial crop plants in the following year, although GBH residues in soil have largely vanished.

1. Introduction

The poultry industry is the fastest-growing agricultural sub-sector (Kleyn and Ciacciariello, 2021; Mottet and Tempio, 2017), which has incentivized the use of poultry manure in agricultural production. Poultry manure is high in nutrients and organic compounds and is implemented as an eco-friendly fertilizer in sustainable crop production ("Circular Economy Action Plan," 2020; Deselnicu et al., 2018; Toop et al., 2017). Although poultry manure is promoted as an organic fertilizer in the circular food economy, the risks of chemical contamination have not been fully understood (World Health Organization. Regional Office for Europe, 2018). Agrochemicals, such as glyphosate-based herbicides (GBHs), are frequently used in animal feed production and have been shown to accumulate in poultry manure (Ruuskanen et al., 2019). Upon unintentional introduction of GBH residues via manure-based organic fertilizers into cultivation systems, we recently reported negative effects on perennial crop plants during the year of exposure (Muola et al., 2021). However, the effects of unintentionally introduced herbicides into crop production systems have rarely been studied in years after exposure, as most studies often focus on annual plants and immediate risks.

Glyphosate has been proclaimed safe for non-target organisms due to its rapid degradation and specific target site within the shikimate biosynthetic pathway, which efficiently kills plants. (Duke and Powles, 2008; Williams et al., 2000). However, many microbes also have the shikimate pathway, and an increasing number of studies has revealed that GBHs negatively affect plant and soil performance because of changes in their microbiome (Helander et al., 2012; Leino et al., 2021; Newman et al., 2016). Further, glyphosate residues persist in soil longer than assumed, especially in colder climates, which may affect soil processes, crop plants, and interacting species (Fuchs et al., 2021; Helander et al., 2012). For instance, recent studies have shown that conventional agricultural practices, including the use of GBHs, cause a lower functional diversity of the rhizosphere microbiome (Newman et al., 2016; van Bruggen et al., 2021). Furthermore, GBH residues in soil decrease plant biomass, alter crop plant hormonal homeostasis and modify plant defense, which in turn affects plant damage by herbivores (Fuchs et al., 2022; Muola et al., 2021; Ramula et al., 2022). Low glyphosate doses have been shown to affect the concentrations of phytochemicals derived from the shikimate pathway (Holländer and Amrhein, 1980) due to the inhibition of 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), a key enzyme in the shikimate pathway (Duke and Powles, 2008). Compounds derived from the shikimate pathway include phenylpropanoids, a compound group that is associated with plant defense responses against insect folivory (Vogt, 2010; Schott et al., 2022) and the antioxidant properties of fruits of various plant species (Neelam Khatkar and Sharma, 2020).

Beyond the direct effects of GBH exposure on plants, GBH residues may persistently change soil properties and thus have indirect effects on plants (Fuchs et al., 2021; Kanissery et al., 2019). In the past two decades, the importance of the soil legacy has become a prevalent concept, where past events shape the present conditions of the soil, including nutritional quality and the biotic (microbial and arthropod) diversity of soils (Bakker et al., 2018; Kostenko and Bezemer, 2020). Soil legacy can strongly shape plant performance through numerous mechanisms, either positively—such as beneficial microbes or enrichment of nutrients— or negatively, such as nutritional depletion or a high diversity of pathogenic microbes (Hannula et al., 2021; Yang et al., 2018). GBHs may change the elemental composition of soil and the availability of micronutrients for plants (Mertens et al., 2018). Furthermore, one degradation product of glyphosate is a functional phosphate unit, which can increase the phosphorus load in soil (Hébert et al., 2019). Thus, the unintended introduction of GBH residues via manure fertilizers may affect soil legacy, with long-term effects on plant holobiont and crop productivity (Saikkonen et al., 2020; Tang et al., 2021; Yang et al., 2018). In addition to the soil legacy, the performance of perennial (crop) plants can largely depend on their performance and fitness in the previous year (Ehrlén, 2000). Thus, for biannual and perennial plants, the consequences of exposure to GBHs in one year may have consequences for their performance in the following growing season. However, little is known about the effects of GBH history in soil on perennial plants in years after exposure, whether mediated by GBH-caused alterations in soil legacy, plant performance, or both. In this paper, the negative effects of GBH exposure on plant performance and the potential alteration of soil legacy are together referred to as 'GBH legacy.'

Here, we studied whether GBHs unintentionally introduced via poultry manure as plant fertilizer in one year (Muola et al., 2021) have effects on strawberry (*Fragaria* × *vescana*) in the following growing season. In other words, we assessed whether 'GBH legacy' affects the growth, yield quantity, and chemistry of perennial strawberries. Furthermore, we explored the potential of 'GBH legacy' to alter the phytochemistry of strawberry and its interactions with herbivores. In the year of exposure, the strawberry plants fertilized with GBH-contaminated manure fertilizer were smaller and produced less vegetative propagation compared to plants growing in GBH-free substrates (Muola et al., 2021). Here, we tested whether this exposure to GBH residues via manure fertilizers affects (1) the soil elemental composition; (2) plant performance, yield, and folivory; and (3) leaf and fruit phenolic compound composition during the following year.

2. Materials and methods

2.1. Field experiment

We conducted a field experiment at Ruissalo Botanical Garden (Turku, Finland, 60°26′ N, 22°10′ E) using the strawberry cultivar 'Sara' (*Fragaria* × *vescana*) as the study species. 'Sara' is a hybrid cultivar between wild woodland strawberry (*Fragaria vesca*) and garden strawberry (*Fragaria* × *ananassa*). The field experiment was designed to examine how unintentional exposure to GBH residues via manure fertilizer affects a perennial crop plant both in the short term (Muola et al., 2021) and in the longer term via potential legacy effects (this study).

As manure fertilizer, we used beddings, including wood shavings, feces, and minor amounts of spilled feed collected from Japanese quails (*Coturnix japonica*) fed on GBH-contaminated or GBH-free control feed in a 12-month aviary experiment (Ruuskanen et al., 2019, 2020). The GBH feed consisted of organic feed for egg-laying chickens ("Luonnon Punaheltta," Danish Agro, Denmark) combined with Roundup Flex® equivalent to ca. 160 mg glyphosate/kg. This dose corresponds to the daily intake of 12–20 mg glyphosate per kilogram of body mass in full-grown Japanese quails. At 12 months of exposure, the average glyphosate concentration of the excreta samples was 199 mg kg^{-1} (S.E. = ±10.5 mg kg^{-1}) (Ruuskanen et al., 2019). The control group was fed the same organic feed with non-measurable glyphosate concentrations. During the aviary experiment, the beddings were changed bi-weekly, and the beddings used were collected regularly from 8 to 12 months of exposure, pooled per treatment, and stored in closed containers in a dry, dark storage room at 6 °C for a maximum of 8 months. For the present experiment, the beddings were spread on the experimental field as fertilizers on two occasions: August 2018 and May 2019. Altogether, 25 l (approx. 3.8 kg) beddings were spread on each 1 m^2 plot. To consider possible environmental gradients within the experimental

field, the control plots (N = 18) and GBH plots (N = 18) were arranged in a 6 × 6 chessboard grid consisting of 36 plots, each plot planted with two strawberry plants. The experimental field had no previous history of herbicide use, and it had not been used agriculturally during the last decade. The soil has been classified as stagnosol and has a sandy clay texture, with a relatively high proportion of organic material. Approximately one month after spreading the first beddings, at the end of September 2018, two *F.* × *vescana* were planted per plot. In 2020, no beddings or any other fertilizers were added to the experimental field.

A more detailed description of the experimental design, GBH treatments, and rearing conditions of the aviary experiment with Japanese quails appears in Ruuskanen et al. (2020, 2019). A more detailed description of the experimental design and the short-term effects of GBH exposure via manure fertilizer on *F.* × *vescana* cultivar 'Sara' appears in Muola et al. (2021).

2.2. GBH residues and basic elements

To determine the residues of glyphosate and its degradation products, gluphosinate and aminomethylphosphonic acid (AMPA), in the field soil, we collected soil samples in October 2019 and May 2020. Eight soil cores (2.5 cm diameter, 10 cm in depth) were taken per plot, and samples from six plots were randomly pooled to create three soil samples with a GBH history for residue analyses. All samples from the control plots were pooled into one sample for residue analysis. One bulk sample from control plots and three samples from glyphosate plots per time point were sent to Groen Agro Control (Delft, The Netherlands; certified laboratory). Before shipment, soil samples were stored in the dark at 4 °C. For soil element analysis, samples from three plots per treatment were pooled together, resulting in six samples from the control and six samples from glyphosate plots. Soil elemental composition was analyzed at the Eurofins laboratory for the following elements: Ca, P, K, Mg, S, Cu, Mn, and Zn. Nitrate (NO3) and ammonium (NH4) concentrations were also analyzed.

2.3. Plant growth and reproduction

To study whether exposure to GBH residues via organic fertilizer has legacy effects on the growth and reproduction of *Fragaria* × *vescana*, we measured plant size, runner production, and the fruit parameters—number, size, and biomass (yield)—during a period from May 2020 until August 2020. Plant size was measured as the diameter and height of the rosette of each individual plant on three occasions during the growing season: in early May before plants had started to grow, in July during the peak fruiting, and in late August at the end of the growing season. Diameter and height were then used to calculate the plant size index (diameter × height). To estimate *F.* × *vescana* investment in reproduction, we measured fruit and runner production. Fruit production was estimated by calculating the number of ripe fruits right before harvest. The harvested fruits were kept in a cool (approx. +5 °C) storage bag in the field, transported to the laboratory, and weighed on the day of harvest before freezing at −20 °C. Since all plots had a plant survival of 100%, we pooled the fruits from the two plants growing on the same plot and treated them as one replicate for the fruit phenolics analyses. For the statistical analysis, we calculated the average fruit weight and total yield. Both are traits of economic importance and contribute to the marketable value of yield (Klatt et al., 2014; Rindom and Hansen, 2009). We calculated the average fruit weight per plot by dividing the total weight of the fruits per plot by the number of fruits produced on that plot. The total yield per plant was then calculated by multiplying the average fruit weight of each plot with the number of fruits that the plant had produced. We recorded fruit damage by birds, which was negligible due to the exclusion of major frugivores with nets around all plants. On average, only 1.6 ± 0.2 fruits per plant were damaged by frugivores. To estimate runner production, runner biomass was collected at the end of August. Samples were dried at room temperature (approx. +22 °C) for two weeks, after which they were weighed.

2.4. Folivory

Folivory, that is, damage to the leaves, was scored in mid-August to assess the accumulated herbivore damage throughout the season (i.e. total damage) by counting the number of insect-damaged leaves. Sawfly larvae caused most of the leaf damage, but we recorded minor damage caused by other Hymenoptera, Lepidoptera, and/or Coleoptera. Leaves were considered damaged if at least 2% of the leaf area (by visual inspection) indicated damage due to being chewed by folivore. Leaves for phenolic compound analysis were sampled at the same time to allow the testing of potential association between leaf phenolics and folivory.

2.5. Phenolic compound extraction from fruit and qualitative and quantitative analyses

Fruits of each experimental plant were first counted and then harvested from both plants per plot. After harvest, fruits were weighed, and then frozen until biochemical analyses. Fruits from 10 plots per treatment with the highest cumulative fruit weight (added weight of fruits of both plants) were chosen for the analysis of phenolic compounds. Fresh fruit material was homogenized before phenolic extraction. Fruit samples of 20 g were weighed into 50 mL falcon tubes and extracted with 3 × 20 mL of extraction solvent (acetone: water: formic acid, 70/28.5/1.5, *v/v/v*). The extraction was assisted by 15 min of ultrasonic bath (at room temperature) and 10 min of centrifugation (at 1500×*g*). After centrifugation, the supernatants from the three-time extraction were combined and evaporated to completely dry with a vacuum rotary evaporator at +30 °C. The residues were redissolved in 5 mL of extraction solvent and filtered through an RC 0.45 μm filter. All extracts were stored at −80 °C until further analysis.

The identification of phenolic compounds was conducted on a Waters Acquity ultra-performance liquid chromatography-mass spectrometry (UPLC-MS) system, consisting of a 2996 diode array detector (DAD), an electrospray ionization interface (ESI), and a Waters Quattro Premier mass spectrometer (MS, Waters Corp., Milford, MA, U.S.A.). A Phenomenex Aeris peptide XB-C18 column (150 × 4.6 mm, 3.6 μm, Torrance, CA, U.S.A.) was applied in the liquid chromatographic separation of phenolics. The mobile phases were Milli Q water (as solvent A) and acetonitrile (solvent B), both containing 1.0% formic acid (*v/v*). The binary gradient program was set as: 0–50 min with 4–22% solvent B, 50–55 min with 22–60% B, 55–57 min with 60−4% B, and 57–60 min with 4% B. The strawberry extracts (5 μL) were injected into the LC system at 25 °C with a total flow rate of 0.5 mL min^{-1}. LC chromatography was recorded at wavelengths of 280 nm (for flavan-3-ols and ellagitannins), 320 nm (phenolic acids), 360 nm (flavonols), and 520 nm (anthocyanins).

For ESI-MS analysis, a positive ion mode was conducted with a mass range scanned from 100 to 1000 *m/z*. The source temperature was set to 120 °C. Capillary voltage, cone voltage, and extractor voltage were 3.2 kV, 60 V, and 6 V, respectively. The MS data analysis was performed using Masslynx 4.1 software (Waters Corp., Milford, MA, U.S.A.).

A Shimadzu LC-30AD liquid chromatograph equipped with an SPD-M20A photodiode array detector was applied to quantify the phenolic compounds. The chromatographic condition was the same as that described in the UPLC-MS identification. The HPLC analysis was performed in triplicate. All identified compounds were quantified using the content–LC peak area calibration curves of external reference standards. The selection of external reference standards and all information on calibration curves are provided in Supplementary Table 1.

2.6. Phenolic compound extraction from leaves and qualitative and quantitative analyses

A subset of 15 plants per treatment was randomly selected for

analysis of the leaf chemical composition of phenolic compounds. On 18th of August, one leaf per plant of similar size and age was removed and frozen at −20 °C until lyophilization. All leaf samples were freeze dried for 72 h and subsequently finely ground with a metal matrix in a ball mill (Retsch GmbH) for 5 min at 21 beats per second. The leaf powder was weighed to 20 mg into a 2 ml reaction tube (Eppendorf GmbH) before the extraction procedure. Subsequently, leaf powder was extracted with 2 × 1.4 mL acetone/water (80:20, *v/v* on a rotary shaker for 2 × 3 h (280 rpm), followed by centrifugation for 10 min. The supernatant was transferred to a new microcentrifuge tube and evaporated to the water phase in an Eppendorf concentrator (5301, Eppendorf AG). Aqueous samples were frozen at −20 °C and lyophilized. Prior to analysis, the freeze-dried phenolic extract was dissolved in 1 mL of Milli-Q purified water, vortexed for 5 min, and filtered with a 0.20 μm PTFE filter into UPLC vials. The sample was 5 × diluted with water and analyzed according to (Engström et al., 2014, 2015) on an Acquity UPLC system (Waters Corp., Milford, MA, USA), interfaced to a Xevo TQ triple-quadrupole mass spectrometer with electrospray ionization (ESI) (Waters Corp., Milford, MA, USA). The UPLC system consisted of an auto sampler, a binary solvent manager, a 100 mm × 2.1 mm i.d., 1.7 μm Acquity UPLC BEH Phenyl column (Waters Corp., Wexford, Ireland), and a diode array detector. The mobile phase consisted of two solvents: acetonitrile (A) and 0.1% aqueous formic acid (B) with the following gradients: 0.1% A in B (0–0.5 min, isocratic), 0.1–30% A in B (0.5–5.0 min, linear gradient), 30–35% A in B (5.0–6.0 min, linear gradient), and column wash and stabilization (6.0–9.5 min). The flow rate was 0.5 mL min^{-1}. Data collection for both UV and MS occurred continuously from 0 to 7 min. Negative ESI mode was used with the following conditions: capillary voltage, 2.4 kV; desolvation temperature, 650 °C; source temperature, 150 °C; desolvation and cone gas (N_2), 1000 and 100 L h^{-1}; and argon as collision gas. The stabilities of the UHPLC retention times and the *m/z* values of the MS detector were monitored with a flavonoid mix stock solution containing 4 μg mL^{-1} each of kaempferol-7-*O*-glucoside, kaempferol-7-*O*-neohesperoside, kaempferol-3-*O*-glucoside, quercetin-3-*O*-galactoside, and quercetin-3-*O*-glucoside in acetonitrile/0.1% aqueous formic acid (1:4, *v/v*). The stability of the MS/MS response was monitored by injecting 1 μg mL^{-1} catechin solution (in 1/4 acetonitrile/0.1% formic acid *v/v*) five times before and after every batch of 10 samples. Quantitative results were corrected for possible fluctuations in the system's quantitative performance within each analysis set as well as among different sets.

2.7. Statistical analysis

We used general linear models (GLM) to test whether exposure to GBH residues via organic fertilizer has legacy effects on the growth and reproduction of *Fragaria* × *vescana*. We analyzed the potential legacy effect of GBH exposure on plant growth using a repeated-measures model. The plant size index measured in May, July, and August was used as a repeated measurement of plant growth. Fruit weight and total yield were used as measures of sexual reproduction. Runner biomass was used as a measure of vegetative reproduction.

Treatment (control vs. GBH) was used as a between-subject factor in the repeated model and as a fixed factor in the models testing the effects of the GBH legacy on plant reproduction. During the previous year, *F.* × *vescana* plants that were exposed to GBH residues via organic fertilizer were, on average, 23% smaller compared to the control plants. Since previous year growth is likely to affect resource allocation, and thus growth, and reproduction of perennial (crop) plants—such as *F. vescana*—during the following year, we used the plant size index from August 2019 as a covariate.

We used a general linear model (GLM) to test whether exposure to GBH residues has legacy effects on *F. vescana*'s ability to attract or repel folivores. The number of folivore-damaged leaves in mid-August was used as a response variable in the model that tested for the plant's ability to attract/repel folivores. Treatment (GBH vs. control) was used as a fixed factor. Since larger strawberry plants are known to attract more folivores, we used the plant size index measured in mid-August as a covariate in the model to control for the potential effect of plant size on folivory.

In all models, the interaction between the covariate and the fixed factor, and in the case of the repeated-measures model, all interactions between the covariate and explanatory factors were tested, and the non-significant interactions were left out of the final models. In cases where the covariate (i.e., plant size) had a significant effect on the trait of interest, we used regression analysis to confirm this association. The normality and homoscedasticity of the residuals were checked by visual examination and Levene's test, respectively. All models were constructed using SAS Enterprise Guide 7.1 software.

Metabolite concentration data showed a normal distribution, and variances were homogeneous. Consequently, we applied Student's t-test with Bonferroni correction for treatment comparison of metabolite concentrations per metabolite in leaves and fruit. We used GLM to test whether average fruit size can explain the difference between individual phenolic compounds per treatment. To test for potential association between leaf phenolic concentrations and folivory, we conducted Pearson correlation with folivore damage and the concentrations of the phenolic compounds grouped as hydrolysable tannins, flavonols, proanthocyanidins and total polyphenols in leaves.

3. Results

3.1. GBH residues and basic elements in soil

There was no difference in soil elemental composition between the treatments. The soil pH was 7.4 on average. The average concentration of soil elements was as follows: calcium 6700 mg l^{-1}, phosphorus 85.8 mg l^{-1}, potassium 334.2 mg l^{-1}, magnesium 267.5 mg l^{-1}, sulfur 21.1 mg l^{-1}, copper 11.9 mg l^{-1}, manganese 22.6 mg l^{-1}, zinc 12.3 mg l^{-1}, nitrate 9.1 mg l^{-1}, ammonium 2.0 mg l^{-1}, and ammonium nitrate 11.0 mg l^{-1}. Glyphosate, AMPA, and gluphosinate residues were constantly very low in the control soil during both years (Table 1). In the GBH soil,

Table 1
Concentration of glyphosate and its degradation products aminomethylphosphonic acid (AMPA) and gluphosinate in bedding material (May 2019) and soil samples (Oct 2019 & May 2020). Values in May 2019 represent concentrations in bedding material on the day of spreading in the field; data retrieved from Muola et al., 2021. Limit of detection of glyphosate ≤0.01 mg kg^{-1}, AMPA ≤0.05 mg kg^{-1} and gluphosinate <0.01.

	May 2019		Oct. 2019		May 2020	
	Control	GBH	Control	GBH	Control	GBH
Glyphosate	0.17	158	0.014	1.7	0.019	0.135
AMPA	0.05	1.3	0.07	0.47	0.091	0.29
Gluphosinate	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01

Mean concentrations in mg kg^{-1}.

Table 2
Effect of GBH legacy on plant (*Fragaria* × *vescana*) size, reproduction (total yield, runner production, and fruit size) and folivory. The previous year's plant size (2019) was used as a covariate to control its effect on all measured parameters except folivory, which commonly correlates with prevalent plant size (2020).

Dependent variable	Effect	F	Df	P
Plant size	GBH legacy	0.37	1, 68	0.5437
	time	84.21	2, 67	<.0001
	GBH legacy × time	0.29	2, 67	0.7525
	Plant size (2019)	26.77	1, 68	<.0001
	Plant size × time	9.95	2, 67	0.0002
Total yield	GBH legacy	1.15	1, 67	0.2882
	Plant size (2019)	17.10	1, 67	0.0001
	GBH × Plant size	2.30	1, 67	0.1342
Runner production	GBH legacy	0.01	1, 67	0.9156
	Plant size (2019)	0.11	1, 67	0.7448
Fruit weight	GBH legacy	8.76	1, 68	0.0042
	Plant size (2019)	13.95	1, 68	0.0004
Fruit number	GBH	2.29	1, 67	0.1350
	Plant size (2019)	10.87	1, 67	0.0016
	GBH × Plant size	2.65	1, 67	0.1083
Folivory	GBH legacy	2.56	1, 68	0.1144
	Plant size (2020)	14.4	1, 68	0.0003
	GBH × Plant size	3.64	1, 68	0.0608

the concentrations were approximately 13 times higher in October 2019 compared to May 2020, but both values were very low compared to glyphosate residues in the bedding material from May 2019 (Table 1). Control soil had trace amounts of glyphosate and AMPA, which is attributed to leaching from GBH stripes caused by heavy rains.

3.2. Plant performance and folivore damage

Plant size was not affected by the GBH legacy in soil (Table 2). The effect of the previous year's plant size differed among the three time points plant size was measured (May $r^2 = 0.198$, $p \leq 0.0001$, July $r^2 = 0.275$, $p \leq 0.0001$ and August $r^2 = 0.211$, $p \leq 0.0001$), the effect being strongest during the peak flowering in July. Total yield, number of fruits, and runner production were not affected by the legacy of GBH exposure (Table 2). On average, the total yield was 82 ± 6.4 g, fruit number 35.7 ± 2.3, and runner biomass 25.3 ± 1.2 g. Interestingly, plants that were exposed to GBH residues during the previous year produced slightly heavier fruits compared to the controls (control fruit 2.04 g ± 0.10 g, GBH-exposed fruit 2.45 g ± 0.10 g; Table 2). The previous year's plant size (2019) affected the total yield and number of fruits but not runner production (Table 2). Plants that were larger at the end of the previous growing season produced higher yields (association between plant size and total yield $r^2 = 0.161$, $p = 0.0005$, data not shown), more, and larger fruits (association between plant size and fruit number $r^2 = 0.119$, $p = 0.0032$, and plant size and fruit size $r^2 = 0.103$, $p = 0.0064$, data not shown; Table 2).

Plants in the control treatment had an average of 54.0 ± 2.6 folivore-damaged leaves, whereas plants that had been exposed to GBH residues had 48.3 ± 2.7 folivore-damaged leaves. However, the difference between the treatments was not statistically significant ($F = 2.56$, $df = 1, 68$, $p = 0.1144$, Table 2, Fig. 1a). Interestingly, the GBH legacy had a marginally significant, indirect effect on folivory via plant size (Table 2).

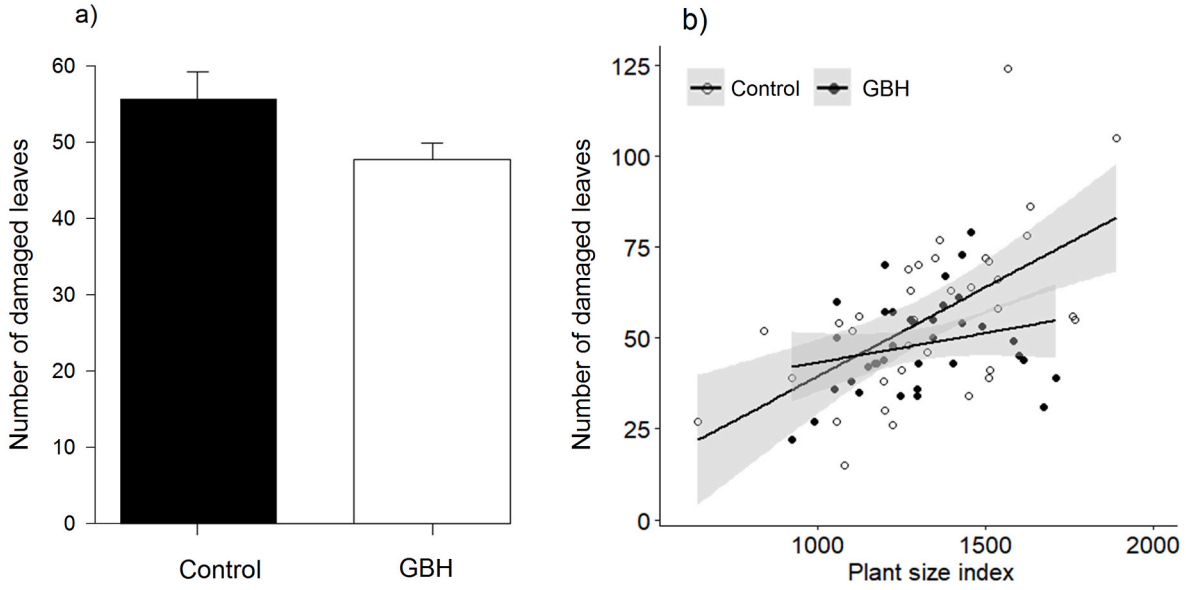

Fig. 1. Legacy effect of manure fertilizer containing glyphosate-based herbicide (GBH) residues on damage caused by chewing folivores on *Fragaria* × *vescana*. Damage, a) shown as the number of folivore-damaged leaves on plants growing in control treatment or in treatment with GBH legacy ($F = 2.56$, $df = 1, 68$, $p = 0.1144$; lsmean ± SE). b) Marginally significant ($p = 0.0608$) interaction effect between plant size and the number of chewing folivore-damaged leaves on *Fragaria* × *vescana* in control treatment and in treatment with the legacy of GBH residues via manure fertilizer (regression line ± confidence interval).

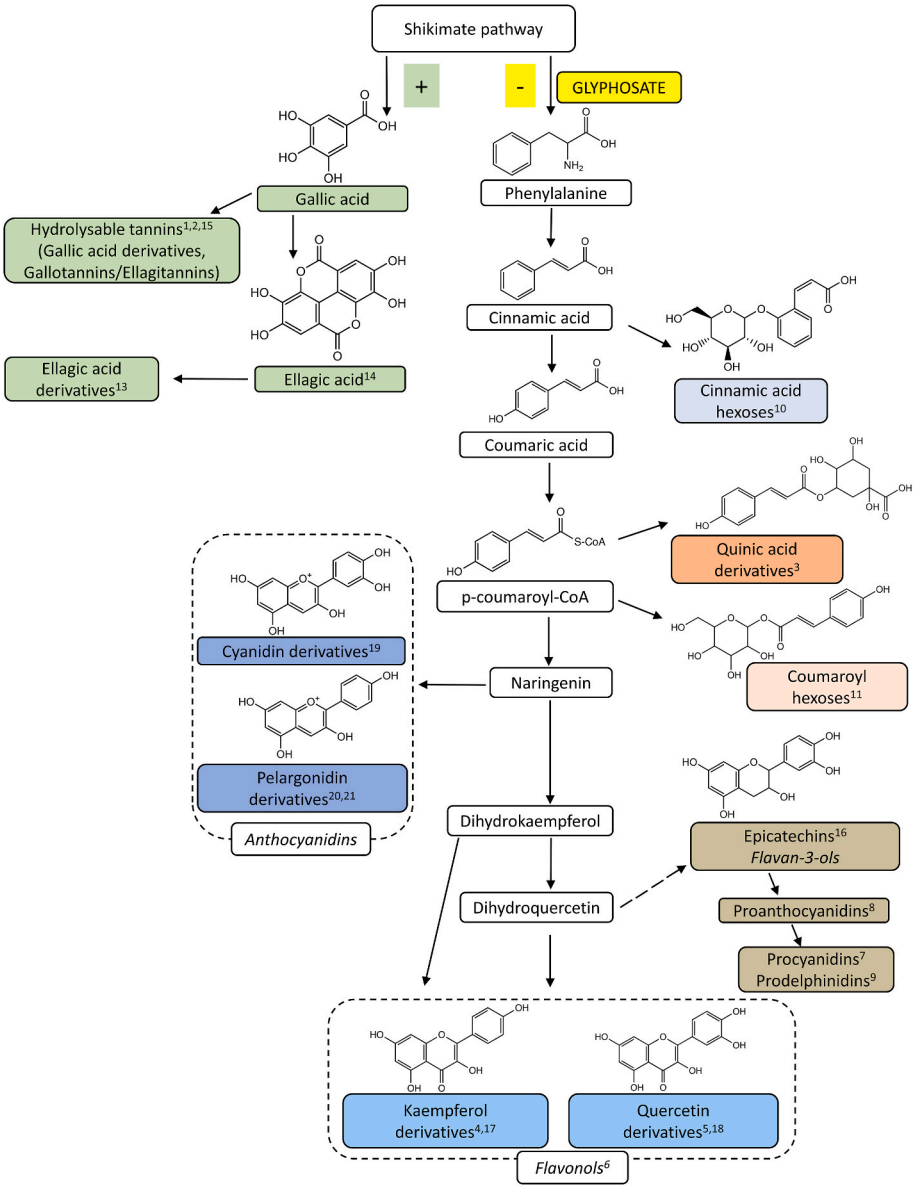

Fig. 2. Schematic visualization of biosynthetic pathways of the analyzed compounds derived from the shikimate pathway in the leaves and fruits of strawberries. Colors and indexed numbers represent the metabolic affiliation and correspond to metabolites presented in Table 3 (leaf metabolites 1–9) and Table 4 (fruit metabolites 10–22). Glyphosate blocks 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), an enzyme needed for the biosynthesis of aromatic amino acids such as phenylalanine (Maeda and Dudareva, 2012) (reduced biosynthesis indicated with -), and consequently precursors are more available for alternative pathways independent of the EPSPS enzyme (Ossipov et al., 2003; Zabalza et al., 2017) (induced biosynthesis indicated with +). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

The number of folivore-damaged leaves tended to increase as the plant size increased for the plants growing in the control plots but not for the plants in GBH plots ($F = 3.64$, $df = 1, 68$, $p = 0.0608$, Fig. 1 b).

3.3. Leaf chemistry

Exposure to GBH did not influence the phenolic compounds in strawberry leaves. The strawberry leaf extracts were analyzed for their polyphenol profiles using group-specific multiple reaction monitoring methods (Engström et al., 2014, 2015) (Table 3, Fig. 2). None of the measured polyphenol subgroups showed significantly different concentrations between the treatments. The extracted polyphenols were mainly composed of hydrolysable tannins (56%) and proanthocyanidins (36%) (Table 3). Folivore damage and concentrations of grouped leaf phenolic compounds were not associated (hydrolysable tannins: $t = 0.97174$, $df = 28$, $p = 0.3395$; flavonols: $t = -1.5971$, $df = 28$, $p = 0.1215$; proanthocyanidins: $t = -0.75705$, $df = 28$, $p = 0.4553$; total polyphenols: $t = 0.28194$, $df = 28$, $p = 0.7801$.

3.4. Fruit chemistry

We selected 13 major peaks (Supplementary Fig. 1) present in all strawberry fruit extracts, out of which 10 compound peaks were tentatively identified via a comparison of the mass spectra from the literature, and three compounds were tentatively assigned to a compound group based on their specific fragmentation (Supplementary Table 2, Fig. 2). Fruits from plants subjected to the GBH legacy showed higher concentrations of an ellagic acid pentoside and marginally significant higher concentrations of ellagic acid (Table 4). We recorded lower concentrations of pelargonidin 3-*O*-rutinoside, the dominating anthocyanin in the strawberry fruits produced by the plants subjected to the GBH legacy. Furthermore, a marginally lower concentration of pelargonidin 3-*O*-glucoside was also observed (Table 4). The remaining nine compounds did not show different compound concentrations of the compounds between the treatments (Table 4). Average fruit weight did not explain the recorded differences between the treatments.

Table 3
Legacy effect of manure fertilizer containing glyphosate-based herbicide (GBH) residues on polyphenol concentrations in leaf extract of *Fragaria* × *vescana* growing in control treatment (C) or in treatment with GBH legacy (GBH). Shown are concentrations in mg g^{-1} dry weight (mean ± S.E.). Colors and numbers indicate the pathway affiliation in Fig. 2. T-test showed no significant differences between the treatments.

	Metabolite group	Treatment		t-test	
		C (mean ± S.E.)	GBH (mean ± S.E.)	p value	% of total Polyphenols
1	Galloyl derivatives	2.26 ± 0.33	2.06 ± 0.13	0.58	2
2	HHDP derivatives	51.41 ± 3.93	49.13 ± 2.48	0.62	53
3	Quinic acid derivatives	3.37 ± 0.15	3.64 ± 0.13	0.17	4
4	Kaempferol derivatives	1.45 ± 0.17	1.21 ± 0.12	0.25	1
5	Quercetin derivatives	2.45 ± 0.22	2.83 ± 0.26	0.28	3
6	Myricetin derivatives	-	-	-	-
7	Procyanidins	33.01 ± 3.81	35.23 ± 2.69	0.64	36
8	Prodelphinidins	0.13 ± 0.02	0.16 ± 0.02	0.35	<0.1
9	Proanthocyanidins	33.11 ± 3.82	35.41 ± 2.70	0.64	36
	Polyphenols sum	94.10 ± 2.95	94.10 ± 1.28	0.98	100

Table 4
Legacy effect of manure fertilizer containing glyphosate-based herbicide (GBH) residues on concentrations of polyphenols in fruit extract of *Fragaria* × *vescana* growing in control treatment (C) or in treatment with GBH legacy (GBH). Shown are concentrations in μg g^{-1} fresh weight (mean ± S.E.). Colors and numbers indicate the pathway affiliation in Fig. 2. Significant ($p < 0.05$) and marginally significant ($p < 0.1$) values are marked in bold.

	Metabolite	Treatment		t-test
		C	GBH	p value
10	Cinnamic acid-hexose	72.4 ± 3.92	76.8 ± 3.79	0.43
11	Coumaric acid-hexose	59.8 ± 4.46	56.0 ± 3.45	0.37
12	Unknown phenolic acid	19.4 ± 0.90	19.3 ± 0.96	0.94
13	Ellagic acid-pentose	13.3 ± 0.62	15.4 ± 0.60	**0.02**
14	Ellagic acid	8.21 ± 0.62	10.3 ± 0.77	**0.05**
15	Unknown ellagitannin	32.3 ± 1.24	32.5 ± 1.87	0.95
16	(+)-Catechin	245 ± 13.5	255 ± 17.5	0.65
17	Quercetin 3-*O*-glucuronide	2.79 ± 0.002	2.82 ± 0.002	0.87
18	Kaempferol 3-*O*-glucuronide	5.82 ± 0.02	5.67 ± 0.02	0.63
19	Cyanidin 3-*O*-glucoside	15.5 ± 0.84	15.3 ± 0.50	0.86
20	Pelargonidin 3-*O*-glucoside	257 ± 8.17	235 ± 8.14	**0.07**
21	Pelargonidin 3-*O*-rutinoside	12.5 ± 0.74	10.6 ± 0.41	**0.04**
22	Unknown compound	115 ± 8.36	124 ± 8.35	0.46

4. Discussion

Our results reveal how the previous year's GBH-contaminated poultry manure fertilizer can cause unforeseen consequences for perennial crop plants during the next growing season. Our previous study showed that plants exposed to GBH residues via manure fertilizer were, on average, 23% smaller in the year of exposure (Muola et al., 2021). Here, we discovered that the same plants were largely able to compensate for this difference, as there was no difference in size, vegetative propagation, or total yield during the following growing season. Interestingly, the plants exposed to GBH residues in the previous growing season suffered slightly less from folivory, but even more interesting is the observation that GBH residue exposure in the previous growing season tended to disrupt folivore preference for larger strawberry plants. Further, GBH-exposed plants produced, on average, 20% heavier fruits with an altered composition of phenolic compounds compared to fruits of non-GBH-exposed plants. Such effects recorded in the year after GBH exposure on perennial (crop) plants have been inadequately encompassed in the majority of risk assessment analyses due to the lack of multi-year studies on perennial crops (Brühl and Zaller, 2021).

We propose that the repercussions of exposure to GBH residues via manure fertilizer, referred to as 'GBH legacy', detected in this study were caused by indirect effects rather than direct toxic effects of long-persisting GBH residues in soil (Brühl and Zaller, 2019; Fuchs et al., 2021; van Bruggen et al., 2021). Previous studies have revealed that GBH residues in feed pass through the digestive tract of birds accumulate in manure (Ruuskanen et al., 2020) and may remain in soil even for years, depending on edaphic and climatic conditions (Carlisle and Trevors, 1988; Laitinen et al., 2006, 2009; Roy et al., 1989; Stenrød et al., 2005). We were able to trace relatively high amounts of glyphosate residues in soil five months after the application of contaminated manure. Similar concentrations of GBH residues are found in the topsoil layer of agricultural fields in the European Union (Silva et al., 2018).

However, the content of GBH residues in the experimental field decreased substantially by the beginning of the second growing season, indicating a fast degradation (Singh et al., 2020). Although we still detected relatively low concentrations of glyphosate and its main degradation metabolites in exposed soils in the second year, they did not affect the elemental composition of soil or plant growth or the foliar composition of phenolic compounds during the second year. Our results on altered folivore preference, fruit size, and phenolic compound concentrations support the possible indirect effects of GBH history through changes in the plant, which include modulated resource allocation to growth, reproduction, and secondary metabolite production, or changes in the indigenous plant microbiomes (Fuchs et al., 2021; Rainio et al., 2020; Ramula et al., 2021; van Bruggen et al., 2021). Topsoil of agricultural fields in the EU show similar concentrations of glyphosate and AMPA (Silva et al., 2018), which demonstrates that the results from our study may be a present issue on many farms.

Since the GBH legacy did not reduce the number of flowers (data not shown), bigger fruits in plants exposed to GBH indicate that pollination may have been more successful on these plants (Wietzke et al., 2018). Insect pollination is known for its positive impact on strawberry fruit quality (Andersson et al., 2012; Klatt et al., 2014). Together with other traits contributing to fruit quality, successful insect pollination has been shown to increase fruit weight, which contributes to the higher commercial value of strawberries (Andersson et al., 2012; Castle et al., 2019; Klatt et al., 2014). However, given that we did not study pollinators or pollination, we cannot conclude whether or not the heavier fruits on plants exposed to the GBH legacy are linked to more successful insect pollination. Alternatively, the GBH legacy may have caused an increased investment of plant resources into reproduction. GBH residues in soil have been shown to induce plant stress-related hormones (Fuchs et al., 2022), which may alter plant resource allocation toward larger fruits (Kumar et al., 2014; Medeiros et al., 2015).

The observed investment in larger fruits may reflect plant responses to adverse growth conditions in the previous year. It is well known that the previous year's plant performance can affect the performance and reproduction of perennial crops during consecutive growing seasons (Ehrlén, 2000). In our case, the previous year's plant size correlated positively with the current year's plant size, irrespective of whether the plants were grown in control or GBH-contaminated soils. Interestingly, plants exposed to the GBH legacy in soil produced on average 20% heavier fruits. However, heavier fruits did not result in a higher total yield, since control plants produced on average 11% more fruits, which is likely attributed to the inverse relationship between fruit number and fruit size (Guardiola and García-Luis, 2000). The introduction of GBH residues via manure fertilizer did not affect the soil legacy concerning elemental composition, which indicates that glyphosate did not act as a chelating agent (Mertens et al., 2018). Further, it indicates that GBH residues did not add any permanent additive load of phosphate to the soil (Hébert et al., 2019; Wang et al., 2005). In general, fertilization with manure causes relatively high nutrient conditions in the soil, which may mask the minor effects elicited by glyphosate residues on soil elements.

GBH targets the shikimate pathway, which is the basis for the biosynthesis of many specialized plant metabolites, essential drivers for plant interactions with their biotic environments, such as herbivorous and predatory insects (Fuchs et al., 2021). Folivore damage showed a marginally significant positive association with plant size in the control plants but not for the plants that had been exposed to GBH residues during the previous year. According to the plant vigor hypothesis, larger plants are more attractive to folivores (Price, 1991). This pattern has been found in previous studies that quantified folivore damage to strawberries (Koski et al., 2021; Muola and Stenberg, 2018), and the same trend was observed in this study for strawberries grown in GBH-free soil. Folivore damage was not negatively associated with leaf phenolic compound concentrations indicating that neither there was a strong deterrence effect by higher concentrations nor did folivory induce the biosynthesis of the studied metabolites. Interestingly, the observed trend for positive association between plant size and folivore damage was not found for plants growing in soil with a GBH legacy. Furthermore, although GBH residues can affect plant defense metabolites and, thus, affect plant–herbivore interaction (Fuchs et al., 2021, 2022; Ossipov et al., 2003), our findings indicate that GBH legacy did not affect the studied plant defense metabolites. None of the analyzed defense metabolites in leaves, such as gallotannins, differed between the treatments, which calls for other explanations for the mechanism behind the disrupted plant size–folivory association. One possibility is that the GBH legacy may affect bioactive phytochemicals other than those analyzed and change plant nutritional quality or plant volatile organic compounds, which affect the attraction of folivores and their enemies (D'Alessandro et al., 2006; Fuchs et al., 2021; Kanissery et al., 2019; Letourneau and van Bruggen, 2006). In contrast to leaves, the GBH legacy changed the composition of phenolic compounds of fruits. Our results demonstrate an increase in ellagic acid and an ellagic acid derivative in fruits from plants growing in soil with a GBH legacy, with a decrease in two pelargonidin derivatives in fruits of the same treatment. Ellagic acid either derives from the shikimate pathway prior to the EPSPS enzyme or is a product of ellagitannin hydrolysis (Fig. 2), which means that its biosynthetic origin is independent of EPSPS and, thus, the mode of action of glyphosate (Ossipov et al., 2003; Zabalza et al., 2017). On the contrary, the biosynthesis of pelargonidin derivatives is dependent on products derived from the shikimate pathway after the EPSPS enzyme (Fuchs et al., 2021; Vogt, 2010), indicating that the GBH legacy affected the shikimate pathway and phenolic compound biosynthesis, even though glyphosate residues in soil were negligibly low. Although phenolic compounds play a minor role in composing fruit quality for human consumption, they can function as antioxidants (Neelam Khatkar and Sharma, 2020). Further studies are needed to assess how GBH residues in soil affect fruit quality by analyzing sugar content and fruit flavor.

5. Conclusions

Our study provides a unique and holistic view of the consequences of the use of GBH-contaminated manure fertilizer on the plant performance, yield, and biochemistry of strawberry as a perennial crop during the year after GBH exposure. Although GBH residues virtually disappeared from one year to the next, GBH history caused legacy effects across the years, which impacted fruit size, fruit composition, and the relationship between plant size and folivore damage. The altered composition may, in turn, lead to the modification of fruit quality. GBH-exposed plants were able to compensate by increasing their size compared to that of the previous year, with no consequences for the total yield. Our study highlights the multifaceted and long-lasting effects of GBH exposure via manure fertilizer on cropping systems, especially with perennial crop plants. This, together with the increased availability of poultry manure from the fast-growing poultry industry and the endeavor toward the circular food economy promoting the use of poultry manure as organic fertilizer, calls for a better understanding of the extent of the effects of GBH residues in organic fertilizers. Making use of waste products—such as livestock manure—in the circular food economy is a step toward sustainability, but it needs to take the exaggerated use of agrochemicals in the livestock industry into account to fully estimate its circular and long-term impact on plant health and ultimately food security. Future studies are needed to investigate the longevity of other common (agro)chemicals that may compromise the safety and quality of food products, as well as the sustainability of the circular food economy, as well as legacy effects that can persist after respective bioactive compounds have decomposed.

CRediT authorship contribution statement

Benjamin Fuchs: Conceptualization, Data curation, Formal Analysis, Writing - original draft, Funding acquisition. **Kari Saikkonen**:

Conceptualization, Supervision, Writing review & editing, Funding acquisition. **Marjo Helander**: Conceptualization, Supervision, Writing - review & editing, Funding acquisition. **Ye Tian**: Investigation, Formal analysis. **Baoru Yang**: Investigation, Writing - review & editing. **Marica T. Engström**: Investigation, Data curation, Formal analysis. **Juha-Pekka Salminen**: Formal analysis, Writing - review & editing. **Anne Muola**: Conceptualization, Methodology, Writing - original draft, Funding acquisition.

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Declaration of competing interest

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

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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