Sensitivity of *Hydrangea paniculata* Plants to Residual Herbicides in Recycled Irrigation Varies with Plant Growth Stage

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Abstract: Recycling irrigation return flow is a viable option to achieve sustainability in horticultural production systems, but residual herbicides present in recycled water may be phytotoxic. The sensitivity of plants to residual herbicides may vary depending on the growth stage of the plant. If sensitive growth stages are avoided, the risk associated with using recycled water can be reduced. Here, we quantified the effect of residual oryzalin and oxyfluorfen exposure at various growth stages of *Hydrangea paniculata*. Exposure to both herbicides reduced plant growth, leaf visual rating, soil plant analysis development (SPAD) chlorophyll index, net photosynthesis, and light-adapted fluorescence of *H. paniculata*. Herbicide injury was greater for plants exposed to herbicides at early growth stages, however, the recovery rate of those plants was also rapid. For oxyfluorfen, plants produced healthy new growth immediately after the end of exposure, but for oryzalin, even newly formed leaves developed herbicide injury after the end of exposure, therefore leaf damage continued to progress before recovering. However, damage caused by residual herbicide exposure at all growth stages recovered over time. Physiological measurements such as the SPAD index, net photosynthesis, and light-adapted fluorescence responded quickly to herbicides exposure hence provided an early indicator of herbicide damage and recovery.

Keywords: Oryzalin; oxyfluorfen; nursery; ornamental crop; phytotoxicity

1. Introduction

Production of container-grown ornamental nursery plants is an intensive horticultural system that requires frequent inputs of water and agrochemicals to produce visually appealing plants. Irrigation in nurseries often generates substantial amounts of return flow, as 70–80% of applied water may be lost from nursery production areas [1–3]. Irrigation return flow generated from nurseries often contains various agrochemicals, which, if released without remediation, may degrade neighboring ecosystems. Public awareness of non-point source pollution is growing, and so are the regulations to reduce irrigation return flow. Several states, including California, Florida, Texas, Oregon, and Maryland restrict return flow from nurseries and other states will likely follow [4,5]. As water security, water accountability, and costs associated with water withdrawals are rising [6,7], recycling return flow is becoming environmentally sustainable and economically viable [5,8,9]. Therefore, nursery growers in states with and without mandatory return flow capture are starting to recycle water for irrigating ornamental crops.
Recycling nursery return flow for irrigation conserves water and can improve water security, but it also holds some degree of risk to growers. Residual pesticides in recycled water may be phytotoxic to sensitive crops [3,10], and some growers report evidence of phytotoxicity associated with pesticides (personal communication). Chronic, low-dose exposure to pesticides in irrigation water can result in reduced plant growth, chlorosis, leaf distortion, and other visible plant injuries. For example, pendimethalin (2.24 kg a.i./ha) reduced plant width in heather (Calluna vulgaris L.), and isoxaben (0.05 kg a.i./ha) reduced plant height in wintercreeper euonymus (Euonymus fortunei Turcz.), when applied as overhead spray [11]. Glyphosate residue in the rhizosphere reduced growth and biomass production in sunflowers (Helianthus annuus L.) [12], and the application of imazapyr and triclopyr for weed management in power transmission lines reduced germination rate and vegetative growth of non-target plants including yarrow (Achillea millefolium L.) and fireweed (Chamerion angustifolium L.) [13].

The concentration of pesticides in recycled water is orders of magnitude lower compared to standard application rates but still may cause sub-lethal effects on plants. Sensitivity of plants and their capacity to overcome injury may depend on the growth stages of plants [14,15]. Most leaves in young plants or actively growing shoots are new and have thinner cuticles compared to mature plants and shoots [16], hence young plants and new shoots are more prone to phytotoxicity compared to matured plants and shoots; however, this may not always be the case [17]. Phytotoxic symptoms produced by short term exposure to pesticides are either reversible or irreversible [14], and the latter is of most concern to growers. Peach seedlings sprayed with simazine at 3 mg/L and terbacil at 3 mg/L showed excellent recovery from damage, but the seedlings sprayed with oryzalin at 6 mg/L did not recover [18]. Trimec Classic (2,4-D + MCPA + dicamba) and glyphosate at 1.6 kg a.i./ha were applied as an overhead spray in rose plants, after which the plants were evaluated for pesticide-related injury. Injury by Trimec recovered after 11 weeks of exposure but the injury caused by glyphosate did not recover [19].

Herbicides used in container nursery production, including oryzalin and oxyfluorfen, are often found in nursery return flow [20–22]. Oryzalin is a pre-emergent herbicide belonging to the dinitroaniline family; it binds to free tubulin and restricts the formation of microtubules, arresting cells in the dividing stage, but when the herbicide is washed off the new microtubules reappear. After exposure to oryzalin, younger cells show quick recovery and reassembly of microtubules, while older cells take longer to recover [23]. Oxyfluorfen is a protoporphyrinogen oxidase (PPO) inhibitor and is applied as both pre and post-emergent herbicide. Photo-oxidative damage caused by oxyfluorfen can reduce net photosynthesis ($A$) and chlorophyll fluorescence [24]. Oxyfluorfen also causes disturbances in mitotic cell division, producing clastogenic and C-mitotic effects [25]. Plants may recover from phytotoxic damage caused by oxyfluorfen depending upon the length of exposure and time available for recovery. Complete recovery from phytotoxicity in rice was seen just a month after oxyfluorfen exposure [26]. Plant injury associated with oxyfluorfen exposure is often more acute when plants are exposed to oxyfluorfen at early growth stages compared to late growth stages [27,28].

In order to manage risks associated with recycled water for irrigation, we need to develop an improved understanding of the basis of plant injury from chronic low-dose pesticide exposure. Recycling irrigation return flow water for irrigation is a viable option and, if the sensitive growth stages are avoided, the risk associated with irrigation from recycled water can be minimized. Quantifying chlorophyll fluorescence and $A$ of plants exposed to the herbicide can reveal physiological herbicide injury [24,29–32] and can be used to monitor herbicidal stress in plants. In addition to physiological performance, growth, visual appearance, and flower quality are also essential attributes of ornamental plants as customers are more likely to buy visually appealing plants. Therefore, morphological assessments, in addition to physiological performance, can provide a complete picture of herbicide injury in plants. We hypothesized that plants exposed to residual herbicides in irrigation water early in the growth cycle (i.e., shortly after leaf-out) would show greater responses than plants exposed later in the growth cycle. Further, we hypothesized that physiological responses would provide a
rapid indicator of plant damage and recovery. This study focused on: (1) quantifying the physiological and morphological effects of residual oryzalin and oxyfluorfen in simulated recycled water at various growth stages of *Hydrangea paniculata* Siebold ‘Limelight’ (panicle hydrangea); (2) identifying variation in sensitivity among growth stages of plants to residual herbicide exposure; and (3) determining the time required to recover from herbicide damage. We used *Hydrangea paniculata* as model plant because it is one of the most popular shrubs in the U.S. [33,34] and is sensitive to residual herbicide in irrigation water [10].

2. Materials and Methods

2.1. Plant Material and Treatments

This study was conducted in a greenhouse at the Michigan State University Horticulture Teaching and Research Center (HTRC) located in Holt, Michigan, USA (42.67° N, 84.48° W). *Hydrangea paniculata* Siebold. ‘Limelight’ (panicle hydrangea) plants were obtained from Spring Meadow nursery (Spring Meadow, Inc., Grand Haven, MI, USA) and grown in composted pine bark (Renewed Earth, Kalamazoo, MI, USA) and peat moss substrate (80:20; volume:volume) in 11.3 L black plastic containers. All plants were dormant and were pruned consistently, leaving only three shoots of 10 cm length per plant before the start of the study.

Plants were brought into the greenhouse on 15 January 2019, and were top-dressed with 60 g of controlled-release fertilizer (18–5–8; N-P2O5-K2O with micronutrients, 5–6 months, ICL Specialty Fertilizers, Summerville, SC, USA) and irrigated for 10 min with well water that supplied the greenhouse via a drip irrigation system. Each pot received a maximum of 2 L of water every day. The temperature in the greenhouse ranged from 22 °C to 27 °C, relative humidity was between 30–70% and plants received natural sunlight. Buds began to sprout on plants on 27 January 2019, and by 6 February 2019, all the plants had visible leaves on at least six different nodes. As all the plants had initiated growth by 6 February 2019, this day was referenced as ‘initiation of growth’ for the study.

Plants were assigned at random to two treatment groups, with one set receiving simulated recycled irrigation containing 0.02 mg/L of oxyfluorfen (Goal 2XL; Dow AgroSciences LLC, Indianapolis, IN), and the other set receiving simulated recycled irrigation containing 8 mg/L of oryzalin (Surflan AS; United Phosphorus Inc., King of Prussia, PA). We prepared the desired concentration of oxyfluorfen and oryzalin solution (simulated irrigation water) by dissolving the appropriate amount of each herbicide in 50 L of water. Two 100 L black plastic tanks were used to prepare and store herbicide solution. A submersible sump pump was used to agitate the herbicide solution and to manually apply herbicide solution as overhead irrigation on all the leaves of the plant and on the substrate. Herbicide solution was applied daily with an irrigation wand (Yardworks®Front Trigger Red 7-Pattern Nozzle, Model Number: 56715) and lasted for a minute. Herbicide solutions were freshly prepared two times a week. We selected 0.02 mg/L of oxyfluorfen and 8 mg/L of oryzalin as herbicide treatments, as these are the maximum concentrations reported in nursery irrigation return flow or retention reservoirs [20,22,35].

Each treatment group was further divided into five sub-groups, with five individual plants (replication) per sub-group. One sub-group of plants served as an untreated control and received local well water that supplied the greenhouse via drip irrigation system; the remaining four groups received herbicide exposure at four different growth stages (GS), i.e., five days after initiation of growth (GS +5; maximum of two nodes per branch), 15 days after initiation of growth (GS +15; maximum of five nodes per branch), 25 days after initiation of growth (GS +25; maximum of seven nodes per branch), and 35 days after initiation of growth (GS +35; maximum of nine nodes per branch). A flow chart for herbicide exposure is described in Table 1. Plants were temporarily isolated with foam panels during the spraying process to avoid cross-contamination and then put back in place. After 10 days of continuous exposure, plants were returned to the drip irrigation system and observed for damage and recovery over the course of the next 20 days and also at the end of the study. Each time the plants were
evaluated for treatment responses, simultaneous observations of plants from the control group were also carried out for comparison.

Table 1. Flow chart for herbicide exposure for each herbicide applied. On each assessment of herbicide-exposed plants, control plants were also assessed, simultaneously.

| Treatment Groups | GS +5 | GS +15 | GS +25 | GS +35 |
|------------------|-------|--------|--------|--------|
| Before Herbicide exposure | Before Herbicide exposure | Before Herbicide exposure | Before Herbicide exposure | Before Herbicide exposure |
| Recovery phase | Recovery phase | Recovery phase | Recovery phase | Recovery phase |
| Day 0 | Day 0-5 | Day 5-15 | Day 15-25 | Day 25-35 |
| Day 35-45 | Day 45-55 | Day 55-65 |

In addition to the plants mentioned above, three sets of H. paniculata with three plants per set were grown separately until bloom under drip irrigation following a similar management strategy as the aforementioned plants. When flower panicles were approaching complete bloom, flowers on the first set were sprayed with oxyfluorfen and flowers on the second set were sprayed with oryzalin using the same application rates, durations, and methods as for the whole-plant exposure experiments above. Flowers on the third set were not treated hence acted as a control.

2.2. Assessment of Physiological and Morphological Effect of Herbicide

Herbicide injury was assessed for each growth stage at the end of each 10-day herbicide exposure period. Injury was also assessed at 10 and 20 days after cessation of herbicide exposure to determine plant recovery. One final assessment was conducted at the end of the study i.e., 65 days after initiation of growth. On each assessment of phytotoxicity, control plants were also assessed simultaneously to compare with the herbicide exposure group.

At each assessment, leaves were examined for the visible damage (e.g., discoloration, stunting, and curling) and scored on the scale of zero (all dead leaves) to 10 (no leaf damage). Growth index (GI; an average of plant height and two perpendicular widths) was measured on each plant.

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Growth \ Index \ (GI) = \frac{\text{height} + \text{width}_1 + \text{width}_2}{3}
\]  

A portable photosynthesis system (LI-6400 XT, Li-Cor, Inc., Lincoln, NE, USA) mounted with a leaf chamber fluorometer (LI-6400-40, Li-Cor, Inc., Lincoln, NE, USA) was used to measure \(A\) and light-adapted fluorescence (\(F'_{v}/F'_{m'}\)). A section of a fully mature leaf on either the 3rd or 4th node from the shoot of each plant was used for physiological measurements. Photosynthetically active radiation (PAR) in the chamber was set to 1500 \(\mu\)mol m\(^{-2}\) s\(^{-1}\), block temperature was set to 25 °C, and 400 ppm of \(CO_2\) was supplied, and relative humidity in the chamber varied between 40%-60%. Each leaf was then acclimatized for five minutes. We first measured \(A\) and then \(F'_{v}/F'_{m'}\). We also measured the soil plant analysis development (SPAD) chlorophyll index (leaf chlorophyll index) on three leaves per plant on either the 3rd or 4th node from the top using a portable SPAD meter (SPAD-502; Minolta corporation, Ltd., Osaka, Japan). At the end of the study, plants were harvested and dried in an oven (45 °C), to determine total above-ground biomass (TDB; the weight of leaves and stem after drying in an oven at 45 °C for three days).
Flowers were assessed for herbicide phytotoxicity on nine additional plants. After 10 days of herbicide exposure, panicles were left to completely bloom (for 12 days) and then were assessed for their GI (average of height and two perpendicular widths), total flower mass (TFM; the weight flower after drying in an oven at 45 °C for 3 days), and visual injury (on the scale of zero to 10).

2.3. Statistical Analysis

The experiment was conducted as a completely randomized design with five replications per treatment. All the statistical analyses were carried out using SAS version 9.4 (SAS Institute, Inc., Cary, NC). On each assessment of herbicide-exposed plants, control plants were also simultaneously assessed. Before data analysis, all the data except visual injury were converted to percentage based on means of the control. Converting actual values to percentage provided better insights in response to treatments by allowing direct comparisons across the various growth stages. The mean value of control was assumed to be 100 percent, and the variation in control was also calculated based on mean value of 100 percent. However, the actual values are also provided in Supplementary Tables S1 and S2. Preliminary analyses indicated significant differences in some evaluation parameters for herbicide × herbicide and growth stages × herbicide interactions; therefore, data were analyzed separately using one-way ANOVA for each herbicide. For each herbicide, we analyzed visual leaf injury, and percentage change in GI, \( A, \frac{F'v}{F'm'} \), and SPAD compared to control at 1, 10, and 20 days after herbicide exposure for all four growth stages. For final data, 65 days after the initiation of growth, TDB, visual leaf injury, GI, \( A, \frac{F'v}{F'm'} \), and the SPAD index were analyzed as recorded, using one-way ANOVA. A Tukey honest significant difference (HSD) post-hoc mean separation test was carried out for ANOVA with \( p \)-value of 0.05 or less to determine a difference within treatments for each herbicide.

3. Results

3.1. Morphological Responses to Herbicide Exposure

Exposure to simulated recycled irrigation containing oryzalin reduced GI of \( H.\paniculata \) compared to plants that were not exposed, for all but GS +25 (Figure 1). The largest reduction in GI (20%) was observed immediately after the end of oryzalin exposure, when plants were exposed at the earliest growth stage i.e., GS +5. However, plants exposed to oryzalin at GS +5 recovered quickly compared to other growth stages. Oryzalin exposure at GS +15, GS +25, and GS +35 resulted between in a 9–12% reduction in GI immediately after the end of the exposure. Reduction in GI caused by oxyfluorfen exposure was similar to that of oryzalin. Reduction in GI, immediately after the end of oxyfluorfen exposure, was highest (32%) when plants were exposed to oxyfluorfen at GS +5 and least (7.5%) when plants were exposed to oxyfluorfen at near maturity i.e., GS +35. GI of plants receiving oxyfluorfen exposure at GS +25 and GS +35 recovered completely in just 10 days after the end of oxyfluorfen exposure. Plants receiving oxyfluorfen exposure at GS +5 and GS +15 did not recover, completely even after 20 days from the end of oxyfluorfen exposure (Figure 1).
Relative growth index of *H. paniculata* ‘Limelight’ in response to oryzalin (8 mg/L; top) or oxyfluorfen (0.02 mg/L; bottom) following 10 days of herbicide exposure at various stages of plant growth. Growth stage (GS) GS +5 received herbicide exposure five days after initiation of growth, GS +15 received herbicide exposure 15 days after initiation of growth, GS +25 received herbicide exposure 25 days after initiation of growth, and GS +35 received herbicide exposure 35 days after initiation of growth. Standard errors of the means are denoted by vertical ‘T’ lines. Mean separations for each herbicide were carried out using Tukey’s honest significant difference post-hoc mean separation test. Means within each herbicide across all growth stages that are followed by the same letters are not significantly different at $p = 0.05$.

The location of leaf injury was similar for both herbicides and occurred on younger leaves towards the tip of the stem. In contrast, the type of damage caused by each herbicide was different. Oryzalin exposure distorted leaf shape and produced random yellow patches in leaves, while oxyfluorfen exposure reduced leaf size and caused complete or interveinal necrosis (Figure 2). Immediately after the end of oryzalin exposure, plants exposed at GS+5 had the lowest leaf visual rating (7.4), while plants exposed at all other growth stages had lower (8.2 to 8.8) leaf damage (Figure 3). Plants did not recover from leaf injury immediately after end of oryzalin exposure, and even 10 days after the end of oryzalin exposure newly formed leaves continued to produce herbicide damage. Therefore, leaf visual rating declined from one day after the end of exposure to 10 days after the end of exposure. Leaf injury across all growth stages started recovering (by the growth of healthy new leaves) rapidly thereafter, and was only 10% lower compared to control on the 20th day after the end of oryzalin exposure (Figure 3). Leaf injury for oxyfluorfen exposure followed a similar pattern as GI. Immediately after the end of oxyfluorfen exposure, the lowest leaf visual rating (4.8) was observed on plants exposed...
to oxyfluorfen at GS+5, whereas leaf visual rating was highest (8) on plants exposed to oxyfluorfen at GS+25. Unlike oryzalin, plants exposed to oxyfluorfen at all growth stages started recovering (by growth of healthy new leaves) immediately after the end of oxyfluorfen exposure. After 20 days from the end of oxyfluorfen exposure, plants exposed at GS +5 had lowest leaf injury, while plants exposed at GS +35 had maximum leaf injury. However, leaf visual rating for plants receiving oxyfluorfen exposure at all growth stages was still 6–13% lower compared to the control even on the 20th day after the end of oxyfluorfen exposure (Figure 3).

Figure 2. Representative herbicide damage immediately after the end of oxyfluorfen exposure (A) and 10 days after the end of oryzalin exposure (B). Plants were exposed to oxyfluorfen or oryzalin at growth stage (GS), GS +15 for 10 days. Both plants received a score of seven out of ten for leaf visual rating.
Figure 3. Leaf visual rating of *H. paniculata* ‘Limelight’ in response to oryzalin (8 mg/L; top) or oxyfluorfen (0.02 mg/L; bottom) following 10 days of herbicide exposure at various stages of plant growth. Growth stage (GS) GS +5 received herbicide exposure five days after initiation of growth, GS +15 received herbicide exposure 15 days after initiation of growth, GS +25 received herbicide exposure 25 days after initiation of growth, and GS +35 received herbicide exposure 35 days after initiation of growth. Standard errors of the means are denoted by vertical ‘T’ lines. Mean separations for each herbicide were carried out using Tukey’s HSD post-hoc mean separation test. Means within each herbicide across all growth stages that are followed by the same letters are not significantly different at \( p = 0.05 \).

### 3.2. Physiological Responses to Herbicide Exposure

Exposure to both herbicides reduced the SPAD chlorophyll index at various growth stages. The SPAD index was reduced on plants across all growth stages, except GS +5, on 10th day after the end of oryzalin exposure. For oxyfluorfen exposure, the SPAD index was reduced on all growth stages, except GS +25 on first day after the end of the exposure. However, on the 20th day after the end of both herbicide exposure, the SPAD index of exposed plants was similar to control plants regardless of when plants were exposed to herbicide (Figure 4).
Figure 4. Relative SPAD index of *H. paniculata* ‘Limelight’ in response to oryzalin (8 mg/L; top) or oxyfluorfen (0.02 mg/L; bottom) following 10 days of herbicide exposure at various stages of plant growth. Growth stage (GS) GS +5 received herbicide exposure five days after initiation of growth, GS +15 received herbicide exposure 15 days after initiation of growth, GS +25 received herbicide exposure 25 days after initiation of growth, and GS +35 received herbicide exposure 35 days after initiation of growth. Standard errors of the means are denoted by vertical ‘T’ lines. Mean separations for each herbicide were carried out using Tukey’s HSD post-hoc mean separation test. Means within each herbicide across all growth stages that are followed by the same letters are not significantly different at \( p = 0.05 \).

Exposure to each herbicide at all growth states reduced \( A \) at one or ten days after exposure, or both (Figure 5). However, \( A \) recovered to the same level as non-exposed plants for all plants regardless of exposure dates or herbicide by day 10 or 20 (Figure 5). Net photosynthesis did not decrease immediately after the end of oryzalin exposure on GS +5 and GS +25 plants. However, \( A \) was consistently lower across all growth stages on the 10th day from the end of oryzalin exposure. The largest reduction in \( A \) (36%) occurred when plants were exposed at GS +15, immediately after the end of oryzalin exposure. However, plants receiving oryzalin exposure across all growth stages had similar \( A \) compared to control, 20 days after the end of oryzalin exposure. For oxyfluorfen exposure, a reduction in \( A \) was observed for all growth stages, immediately after the end of oxyfluorfen exposure. However, unlike oryzalin, plants across all the growth stages slowly and progressively recovered in next 10 days, at which time the \( A \) of plants exposed to oxyfluorfen and the control plants were similar.
Figure 5. Relative net photosynthesis of *H. paniculata* 'Limelight' in response to oryzalin (8 mg/L; top) or oxyfluorfen (0.02 mg/L; bottom) following 10 days of herbicide exposure at various stages of plant growth. Growth stage (GS) GS +5 received herbicide exposure five days after initiation of growth, GS +15 received herbicide exposure 15 days after initiation of growth, GS +25 received herbicide exposure 25 days after initiation of growth, and GS +35 received herbicide exposure 35 days after initiation of growth. Standard errors of the means are denoted by vertical ‘T’ lines. Mean separations for each herbicide were carried out using Tukey’s HSD post-hoc mean separation test. Means within each herbicide across all growth stages that are followed by the same letters are not significantly different at \( p = 0.05 \).

Oryzalin exposure at did not reduce \( F_{v'}/F_{m'} \) except for GS +15. For GS +15, a 15% reduction in \( F_{v'}/F_{m'} \) was observed on the first day after exposure (Figure 6). Oxyfluorfen exposure at all growth stages, except GS +25, immediately reduced \( F_{v'}/F_{m'} \) (Figure 6). However, 10 days after the end of oxyfluorfen exposure, \( F_{v'}/F_{m'} \) completely recovered for three out of four growth stages, except GS +25, which completely recovered by 20 days of the end of oxyfluorfen exposure (Figure 6).
Figure 6. Percent reduction in light-adapted fluorescence of *H. paniculata* ‘Limelight’ in response to oryzalin (8 mg/L; top) or oxyfluorfen (0.02 mg/L; bottom) following 10 days of herbicide exposure at various stages of plant growth. Growth stage (GS) GS +5 received herbicide exposure five days after initiation of growth, GS +15 received herbicide exposure 15 days after initiation of growth, GS +25 received herbicide exposure 25 days after initiation of growth, and GS +35 received herbicide exposure 35 days after initiation of growth. Standard errors of the means are denoted by vertical ‘T’ lines. Mean separations for each herbicide were carried out using Tukey’s HSD post-hoc mean separation test. Means within each herbicide across all growth stages that are followed by the same letters are not significantly different at \( p = 0.05 \).

3.3. Final Evaluation

Plants receiving oryzalin exposure at different growth stages had similar TDB, GI, SPAD index, \( A \), and \( Fv'/Fm' \) at 65 days after the initiation of growth. They only differed in the leaf visual rating. Leaf injury did not recover completely on plants receiving oryzalin exposure at GS +15, GS +25, and GS +35. Sixty-five days after initiation of growth, leaf visual rating was lowest for plants in treatment group GS +35 and GS +25 (Table 2).

Sixty-five days after initiation of growth, plants receiving oxyfluorfen exposure at GS+5 and GS +15 had 31% and 15% lower TDB compared to control, respectively. Plants receiving oxyfluorfen at GS +15, GS +25 and GS +35 had TDB similar to that of control. In contrast, the leaf visual rating was lower for plants receiving oxyfluorfen exposure at later growth stages. Leaf visual rating for GS +35, GS +25 and GS +15 was reduced by 17%, 8%, and 4%, respectively. Sixty-five days after initiation of growth, GI, SPAD index, \( A \), and \( Fv'/Fm' \) completely recovered in all the plants exposed to oxyfluorfen (Table 2).
**Table 2.** Total dry above-ground biomass (TDB), leaf visual rating (VR), soil plant analysis development chlorophyll index (SPAD), growth index (GI), and light-adapted chlorophyll fluorescence (Fv’/Fm’) of *H. paniculata* ‘Limelight’ at 65 days after initiation of leaf growth. Plants were exposed to either oryzalin (8 mg/L) or oxyfluorfen (0.02 mg/L) at various growth stages (GS) for 10 days. GS +5 received herbicide exposure five days after initiation of growth, GS +15 received herbicide exposure 15 days after initiation of growth, GS +25 received herbicide exposure 25 days after initiation of growth, and GS +35 received herbicide exposure 35 days after initiation of growth. Mean separations for each herbicide were carried out using Tukey’s honest significant difference post-hoc mean separation test. Means within each herbicide that are followed by the same letters are not significantly different at given p-value.

| GS     | TDB (g) | VR       | SPAD     | GI (cm) | A (µmol m⁻² s⁻¹) | Fv’/Fm’ |
|--------|---------|----------|----------|---------|------------------|---------|
| Control| 143 ± 10| 10 ± 0a  | 36 ± 0.48| 96 ± 1.1| 15.9 ± 0.7       | 0.6 ± 0.03 |
| GS+5   | 124 ± 6 | 9.7 ± 0.13ab | 37 ± 0.59 | 92 ± 2.1 | 16.1 ± 0.6       | 0.59 ± 0.01 |
| GS+15  | 126 ± 5 | 9.5 ± 0.23abc | 35 ± 0.57 | 91 ± 0.8 | 14.7 ± 1.4       | 0.6 ± 0.02 |
| GS+25  | 119 ± 8 | 9.3 ± 0.2bc | 36 ± 1.33 | 89 ± 2.1 | 15.2 ± 1.6       | 0.6 ± 0.03 |
| GS+35  | 127 ± 6 | 9 ± 0.16c | 35 ± 1.23 | 90 ± 3.2 | 15.5 ± 1.4       | 0.58 ± 0.02 |
| p-value| NS      | <0.0005  | NS       | NS      | NS               | NS      |

**Oxyfluorfen exposure**

| GS     | TDB (g) | VR       | SPAD     | GI (cm) | A (µmol m⁻² s⁻¹) | Fv’/Fm’ |
|--------|---------|----------|----------|---------|------------------|---------|
| Control| 128 ± 5a| 10 ± 0a  | 37 ± 0.4 | 91 ± 1.1| 16.5 ± 1.9       | 0.56 ± 0.02 |
| GS+5   | 86 ± 13b| 10 ± 0a  | 36 ± 0.61| 88 ± 2.3| 15.9 ± 1.9       | 0.53 ± 0.02 |
| GS+15  | 104 ± 7ab| 9.6 ± 0.1b | 35 ± 0.61| 91 ± 2.8| 15.2 ± 1.7       | 0.55 ± 0.02 |
| GS+25  | 108 ± 8ab| 9.2 ± 0.13c | 36 ± 0.56| 89 ± 1.2| 15.7 ± 1.8       | 0.52 ± 0.02 |
| GS+35  | 128 ± 9ab| 8.7 ± 0.13d | 37 ± 0.42| 92 ± 1.6| 15.4 ± 1       | 0.54 ± 0.02 |
| p-value| <0.05   | <0.0005  | NS       | NS      | NS               | NS      |

NS = Not Significant at p-value of 0.05.

### 3.4. Evaluation of Flowers

GI, TFM, and visual rating of flowers of *H. paniculata* were not affected (p > 0.05) by exposure to either oxyfluorfen or oryzalin. GI for control, oryzalin, and oxyfluorfen exposure were 17.49 ± 0.2 g, 7.19 ± 0.38 g, and 7.41 ± 0.18 g, respectively, and visual rating for flowers with and without herbicide exposure was similar (10 out of 10).

### 4. Discussion

#### 4.1. Morphological Response Depends on the Growth Stage of Plant

Studies evaluating the effect of herbicides at specific leaf stages of weed and crops are common [36,37]. However, researchers acknowledge that studies concerning herbicide sensitivity at various stages of plant growth are comparatively rare [38]. Some herbicides may injure younger leaves, while others may produce damage on older leaves [39,40]. In our study, we increased the duration of herbicide exposure but reduced the concentration of herbicide compared to general herbicide application practice to simulate irrigation with recycled water. Both oxyfluorfen at 0.02 mg/L and oryzalin at 8 mg/L produced phytotoxicity in *H. paniculata*, and injury was primarily observed in younger and growing leaves for all four growth stages. However, the maximum morphological damage occurred for GS+5 plants; hence it was the most sensitive growth stage for both herbicides. Younger leaves adsorb, retain, and translocate higher concentration of herbicides because of thinner cuticle and wax layers compared to mature leaves [28,41], and a higher number of exposed leaves due to lesser
canopy density in younger leaves increases pesticide interception [42]. Antioxidant capacity of leaves is known to increase herbicide resistance and is relatively low in younger leaves [43,44]. All leaves in GS +5 plants were young, rapidly growing, and had open canopy at the time of herbicide exposure; therefore, they sustained maximum herbicide damage. Plants that received herbicide at GS +15, GS +25, and GS +35, had some mature leaves that were increasingly tolerant to residual concentration of herbicide, resulting in lower herbicide injury. Dithiopyr, a similar herbicide as oryzalin, produced a greater reduction in growth when applied at early growth stages [45], and oxyfluorfen injury was also found to be higher on plants exposed to oxyfluorfen at early growth stages compared to late growth stages [27,28]. In our study, after the end of herbicide exposure, leaf visual rating on plants exposed to oxyfluorfen started to recover immediately. This is consistent with oxyfluorfen mode of action, as it is minimally translocated from the application site and mostly works as a contact herbicide [46]. In contrast, for plants exposed to oryzalin, herbicide damage increased from one to ten days after the end of exposure, as oryzalin is readily absorbed and sometimes translocated from newly growing leaves [47,48]. Therefore, even after the end of oryzalin exposure, oryzalin absorbed and retained in leaves was affecting newly forming and enlarging leaves. Another reason for the difference in recovery may be due to the mode of action of these herbicides. Oxyfluorfen produces reactive molecules that disrupt cell membranes and cause cell death; this reaction is immediate in the presence of light [49,50], while oryzalin restricts the formation of microtubules that do not produce immediate visual injury or other effects [51]. Oxyfluorfen has minimal impact on cell division and growth, while the mode of action for oryzalin is predominantly related to cell division and growth; therefore, the effect of oryzalin is delayed and persists longer.

The effect of a sub-lethal dose of herbicide may vary depending on the growth stage of plants [52]. In our study maximum visual damage was observed when plants were exposed to herbicides at early growth stage and similar to our finding, soybean plants also had a maximum visual injury at early growth states when exposed to a sub-lethal dose of 2,4-D [53]. Recovery in GI and leaf visual rating was rapid in plants exposed to herbicides at GS +5, because cell multiplication and growth are rapid at early vegetative stages compared to other stages of plant growth [54,55].

4.2. Physiological Measurements Provide a Rapid Indicator of Herbicide Damage and Recovery

In our study, physiological measurements (SPAD index, A, and Fv’/Fm’) responded to herbicide exposure. The SPAD index mirrored leaf visual rating, in the sense that the largest reduction in SPAD index occurred 10 days after exposure for oryzalin and immediately after exposure for oxyfluorfen. Oxyfluorfen directly reduces chlorophyll formation but oryzalin does not have a direct impact on chlorophyll, and this was evident in our study through the SPAD index. The reduction in the SPAD index was higher for oxyfluorfen compared to oryzalin, during the early growth stage i.e., GS +5 (statistical comparison not shown).

Physiological measurements, such as A and Fv’/Fm’, may be used as early indicators of herbicide damage [56,57]. In our study, both A and fluorescence parameters had a faster and greater response to herbicide exposure compared to visual injury and growth, at later growth stages from GS +15 to GS +35, and GI and leaf visual rating had a greater response at early growth stage i.e., GS +5. However, Fv’/Fm’ for oryzalin exposure was not as responsive as A. For oryzalin, leaf visual rating was lowest 10 days after the end of the exposure, but A and Fv’/Fm’ had already started to recover. The increase in A and Fv’/Fm’ was followed by visual leaf recovery evident on the 20th day after the end of oryzalin exposure. Thus, leaf damage by herbicides, such as oryzalin, that do not produce immediate visible damage can be identified quickly by using physiological tools, such as A, and those tools can be used as early indicators for herbicide damage, preferably at later growth stages when visible damage may take some time to appear. In contrast to our results, exposure to oryzalin did not reduce A for dwarf gardenia (Gardenia jasminoides ‘Radicans’ Thunb.) and fountain grass (Pennisetum rupelli Steud.) probably because the concentration used was eight times lower compared to ours [58]. As discussed earlier, recovery from injury associated with oxyfluorfen exposure was faster than recovery from
oryzalin and was obvious during physiological evaluations. Photosynthesis and \( Fv'/Fm' \) accurately and clearly reflected oxyfluorfen damage across all growth stages (expect GS +25 for \( Fv'/Fm' \)), therefore they can provide quick identification of oxyfluorfen injury. Both \( A \) and \( Fv'/Fm' \) completely recovered from herbicide damage as early as 10 days after the end of exposure and was followed by morphological recovery. Thus, physiological tools can also be efficiently applied to detect herbicide recovery in addition to herbicide damage. Physiological recovery of plants from oryzalin exposure was slower compared to oxyfluorfen. Physiological parameters, such as \( A \) and \( Fv'/Fm' \), were either the same or lower on 10th day after the end of oryzalin exposure compared to a day after the end of oxyfluorfen exposure, expect on GS +15 for \( Fv'/Fm' \). Overall, plants exposed to oryzalin took somewhere between 10–20 days for \( A \) and \( Fv'/Fm' \) to completely recover. However, this was still quicker than recovery of GI and visible symptoms.

4.3. Flowers Were Not Damaged by Residual Oryzalin and Oxyfluorfen

Both oxyfluorfen and oryzalin exposure did not produce any effect on flowers in \( H. \) paniculata. Oxyfluorfen mode of action requires the presence of chlorophyll within the chloroplast; in flowers (petals) there are chromoplasts instead of chloroplast, which is the main reason behind the resistance of flowers to oxyfluorfen [59,60]. Stomatal density in flowers is lower compared to leaves [61] and lower stomatal density also reduces herbicide penetration and damage. Thus exposing flowers to these herbicides did not produce injury. Oryzalin application impacts flower morphology at a cellular level by swelling the tip of conical cells, changing the epidermal cell angle and producing shorter cells [62], but this was not observed at the morphological scale in our study.

4.4. Leaf Visual Injury Takes the Longest to Recover

Oryzalin did not reduce TDB at the end of vegetative growth stage (65 days after initiation of growth), which possibly is because oryzalin had lower leaf damage and less reduction in the SPAD index compared to oxyfluorfen. At the end of the vegetative growth stage, GI for oxyfluorfen exposure was the same across all growth stages but TDB was lower for GS +5. Therefore, oxyfluorfen exposure at early growth stages (GS +5) will increase radial growth but reduce plant canopy density. Reduction in TDB caused by oxyfluorfen application at early growth stages did not recover even after 50 days of oxyfluorfen exposure, but exposure at later growth did not reduce final TDB. Similarly, in other studies, oxyfluorfen application in strawberry (to control broadleaf weeds) produced transient foliar injury that usually did not translate to yield loss [63], and oryzalin application at 1 mg/L did not reduce root and shoot weight in dwarf gardenia [58].

Visual leaf injury for plants exposed to both herbicides at GS +15, GS +25, and GS +35 were still present at the end of the vegetative growth stage, and the leaf injury from oxyfluorfen exposure was higher (4–13%) compared to oryzalin (5–10%) exposure. Therefore, leaf injury needs the longest time to recover compared to other morphological and physiological parameters. In other studies, oryzalin applications at various rates on sweet potato produced sustained leaf distortion (<10%) and plant stunting (<12%) [64], and oxyfluorfen produced lasting leaf injury in cabbage (\( Brassica \) oleracea L.), tomato (\( Lycopersicon esculentum \) Mill.), cucumber (\( Cucumis sativus \) L.), and lettuce (\( Lactuca sativa \) L.) [65]. However, leaf injury produced by herbicide at the early growth stages may completely recover. Visual leaf injury caused by oryzalin application, immediately after transplanting, in sweet potato reversed and did not translate to a reduction in yield [64], and foliar injury in broccoli produced by post-emergence application of oxyfluorfen completely recovered in late-maturing varieties, while early maturing varieties had sustained foliar injury and yield loss [66]. Similarly, in our study visual leaf injury for GS +5 for oxyfluorfen and GS +5 and GS +15 for oryzalin completely recovered, while visual leaf injury cause by herbicide exposure at later growth stage did not recover.
5. Conclusions

In this study, we found that the sensitivity of plants to residual herbicide in recycled irrigation water was dependent on the growth stage of the plants at the time of exposure. Residual herbicides, such as oxyfluorfen or oryzalin, present in recycled water may produce sub-lethal effects on woodyamentals when used for irrigation. Young and growing leaves are more susceptible to herbicidal injuries compared to mature leaves. Early growth stages of plants have a higher ratio of young to mature leaves and therefore are more prone to herbicide damage. Leaf injury from some herbicides will immediately begin to recover, while leaf injury from others will continue to increase before starting to recover. Physiological measurements of herbicide damage can be assessed earlier compared to morphological measurements, particularly for herbicides that do not produce damage immediately after exposure, and can reflect immediate plant performance. Physiological measurements are more sensitive to herbicide injury at later growth stages, while morphological measurement may be sensitive at early growth stages. Hence, those tools can be used as an early indicator of damage and recovery. Damage caused by herbicides, such as oxyfluorfen, that directly destroy photosynthesis apparatus is more severe and may permanently reduce TDB if plants are exposed at early growth stages. Flowers were not affected by 0.02 mg/L of oxyfluorfen and 8 mg/L of oryzalin exposure because of the differences in cell structure compared to leaves. The limitation of our study is the use of only one plant taxon and two herbicides; results may be different if different taxa or herbicides with a different mode of action are used.

Supplementary Materials: The following are available online at http://www.mdpi.com/2073-4441/12/5/1402/s1, Table S1: Actual values of growth index (GI), soil plant analysis development chlorophyll index (SPAD), photosynthesis (A) and light-adapted chlorophyll fluorescence (Fv'/Fm') of H. paniculata ‘Limelight’. Plants were exposed to oxyfluorfen (0.02 mg/L) at various growth stages (GS) for 10 days and measurements of GI, SPAD, A, and Fv'/Fm' was recorded at 1, 10, and 20 days after oxyfluorfen exposure (DAE). GS +5 Exposed received herbicide exposure five days after initiation of growth, GS +15 Exposed received herbicide exposure 15 days after initiation of growth, GS +25 Exposed received herbicide exposure 25 days after initiation of growth, and GS +35 Exposed received herbicide exposure 35 days after initiation of growth, Table S2: Actual values of growth index (GI), SPAD index (SPAD), photosynthesis (A) and light-adapted chlorophyll fluorescence (Fv'/Fm') of H. paniculata ‘Limelight’. Plants were exposed to oryzalin (8 mg/L) at various growth stages (GS) for 10 days and measurements of GI, SPAD, A, and Fv'/Fm' was recorded at 1, 10, and 20 days after oryzalin exposure (DAE). GS +5 Exposed received herbicide exposure five days after initiation of growth, GS +15 Exposed received herbicide exposure 15 days after initiation of growth, GS +25 Exposed received herbicide exposure 25 days after initiation of growth, and GS +35 Exposed received herbicide exposure 35 days after initiation of growth.

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