Review of RMIT Pot Trial Performed to Determine Concentrations at which Dicamba Causes Stress in Lettuces

FINAL REPORT

Report Submitted To:

Melbourne Water
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1 Introduction

RMIT University has performed a lettuce growth study to determine concentrations at which dicamba may adversely effect the growth and development of lettuce crops exposed to irrigation water supplied by Melbourne Water (Porter et al, 2013).

Melbourne Water have requested Australian Environment Agency Pty Ltd undertake a review of the test report from this study.

2 Test method

There is no established test protocol for this type of study, and the test design including dose rates and replicates per treatment level were established through consultation with Melbourne Water.

Testing was undertaken in RMIT's controlled atmosphere glasshouse in the city of Melbourne. Ninety pots (250 mm in diameter) were filled with approximately 5 kg of all-purpose potting mix to which seven seeds were sown. The seedlings were progressively thinned out during the growing season, removing two at a time and recording their dry mass and growth.

The nominal application rates in were 1.6, 6.5, 15, 45, 90,180, 405 and 1215 µg/L. Application rates were analytically verified The lowest application rate is representative of concentrations which on occasions, have been found in the recycled water. There were 5 replicates of each application rate and 10 controls. Each of the pots was randomly assigned one of the treatments. Shoot mass, shoot length, root mass and root were measured at the end of growing period and at thinning. The plants were monitored on a daily basis for any observable changes in structure or growth patterns. The plants were harvested 94 days from sowing.

The test was undertaken using two series of plants. Series I consisted of plants being exposed to dicamba spiked irrigation water after reaching the 4-leaf stage. This corresponded to the first thinning event at 21 days after sowing. Series II plants were exposed to dicamba spiked irrigation water from sowing. As a result, these plants received a higher total dose during the course of the experiment due to several irrigations occurring between planting and the first thinning, after which Series I plants were also exposed.

Each of the herbicide solutions was prepared in 10 L volume container and thoroughly mixed. Each pot was irrigated with 350 -900 mL of the assigned solution. At 1, 7, 12 and 20 days after application, Series II plants were irrigated with dicamba spiked water while Series I plants were irrigated with non-spiked irrigation water. The irrigation water used in the experiment consisted of tap water plus 600 mg/L NaCl and nutrients designed to approximate the characteristics of irrigation water expected in the field.

Following the first thinning (21 days after sowing), plants from both series received irrigation of dicamba spiked water on days 28, 35, 41, 47, 53, 60, 64, 69, 74, 77, 80, 84 and 91. Irrigation amounts ranged from 350 mL/pot to 900 mL/pot. Of the total 17 irrigation events, the average water rate was 17 mm per week, equating to an overall irrigation volume of around 2.2 ML/ha.

Thinning out of seedlings was undertaken at 21, 37 and 56 days after sowing. At each thinning interval, two seedlings per pot were removed for the measurement of root length, shoot length, root weight and shoot weight. The roots were washed free of potting mix and dried prior to measuring dry biomass.

At the time of harvest (94 days after sowing) the remaining single plants per pot had measurements taken as above, including measuring fresh (wet) biomass.

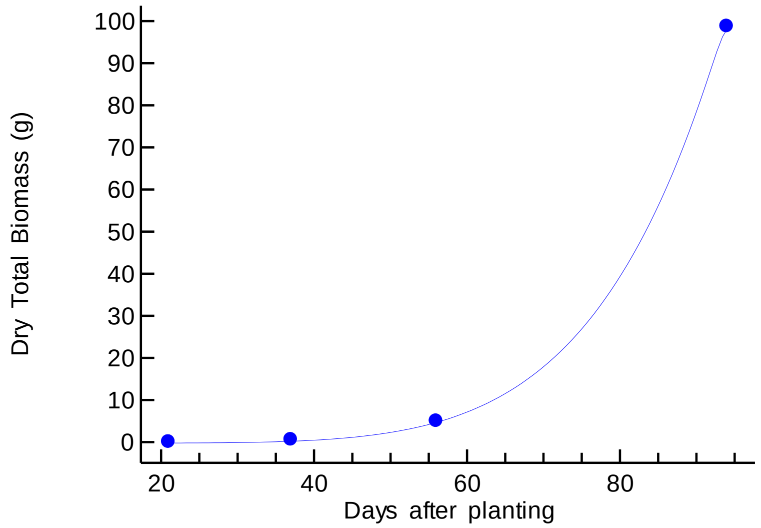
3 Results

Concentrations of dicamba were measured regularly at the range of nominal exposure concentrations. The mean measured concentrations for the different exposure groups were 1.9, 7.6, 16.8, 50.6, 102, 202, 447 and 1360 µg/L. The results will be assessed based on these mean measured concentrations. To calculate ECx results, results in terms of group mean inhibition percentages compared to the control group have been fitted using a sigmoidal dose-response model in the XLFit ver. 5.3.1.3 software package from ID Business Solutions Limited.

In considering the inhibition or otherwise between treatment groups and the control at different intervals, it is worth considering the growth curve for the plants. The following graph shows the exponential growth of the lettuce plants (control group) with the main biomass increase occurring between the third thinning and final harvest. The final harvest values do not include control plants that succumbed to leaf rot.

While the values from the 1st, 2nd and 3rd thinning can be used to understand potential impacts on the plants in their earlier life stages, the much lower growth values at this time make differences in measurements more amplified. For the purpose of this study, it becomes therefore more difficult to interpret the results in terms of their adverse impact on lettuce growth over the course of the full study.

Figure 1: Growth Curve, Dry Biomass, Control Group Lettuce Plants



3.1 Validity criteria

No criteria were established to be able to determine the validity of the test. However, some guidance can be obtained from other test protocols. OECD Test Guideline 208 (OECD, 2006) describes the protocol for terrestrial plant testing for seedling emergence and seedling growth. This test guideline provides several validity criteria in the control group as follows:

- The seedling emergence is at least 70%;
- The seedlings do not exhibit visible phytotoxic effects (chlorosis, necrosis, wilting, leaf and stem deformations) and the plants exhibit only normal variation in growth and morphology for that particular species;

- The mean survival of emerged control seedlings is at least 90% for the duration of the study; and

- Environmental conditions for a particular species are identical and growing media contain the same amount of soil matrix, support media or substrate from the same source.

If these criteria were applied to the current study, the first and final criteria are met. The second criteria is met in that the control plants did not exhibit any visible phytotoxic effects. There was a high variation among the control plants. The potting mix used in the study was not characterised in terms of physical/chemical properties and microbial populations. These factors can vary and increase variability in the growth of plants (OECD, 2006). Given the purpose of this study, the variability among control plants should not invalidate the findings.

The mean survival of emerged control seedlings, while appearing to remain >90%, was confounded by the occurrence of leaf rot. While not addressed in the study report, it is possible the leaf rot was the result of the irrigation regime in the glass house leading to conditions being too wet. OECD (2006) recommends bottom watering for plant studies, but again, given the purpose of this study the method of watering was appropriate. The incidences of leaf rot in the control plants should not therefore invalidate the study.

3.2 Germination

In the tier II standard tests used to establish the initial guideline, the most sensitive result for lettuce was an EC25 of 13 g ac/ha based on a germination end-point. The test system used here did not demonstrate such adverse effects on germination. At the highest treatment rate of 1215 µg/L, germination averaged 86% across replicates with germination mostly being 100% in replicates at treatment rates less than this. Based on the first irrigation the day after sowing, plants in the highest treatment group received a dose as a rate per hectare of around 97 g ac/ha. However, in the standard tier II studies, the active substance would have been mixed in with the test soil as opposed to irrigation over the surface in this test, and this may have resulted in less actual exposure to the seeds.

3.3 1st Thinning

The first thinning took place after 21 days following planting. As expected, biomass measurements at this time were low with mean wet and dry biomass measurements from the control plants being 0.39 and 0.023 g respectively. Using group mean values, the following tables show the percent inhibition from Series I and Series II plants compared to the control plants:

Table 1: 1st thinning (21 days after planting) %inhibition results, Series I

Mean measured concentration (µg/L)	Wet weights (g)			Dry weights (g)			Shoot Length	Root Length
	Shoot	Root	Biomass	Shoot	Root	Biomass		
1.9	-1.3	10.5	-1.1	-7.4	11.5	-4.7	-1.5	7.3
7.6	-6.3	7.6	-6.2	-12.1	11.5	-8.7	-5.6	12.4
16.8	-22.0	14.4	-14.4	-31.1	34.7	-21.7	-12.9	17.4
50.6	-30.4	22.0	-25.9	-30.8	38.3	-20.9	-15.5	19.3
102	-6.6	28.9	-4.2	-12.0	44.3	-4.0	-7.4	14.8
202	-18.5	68.6	-16.2	-47.5	45.5	-34.3	-8.3	23.1
447	-20.5	17.5	-19.0	-20.6	31.4	-13.2	-11.0	27.1
1360	-8.2	24.0	-9.0	-24.4	36.8	-15.7	-6.2	23.1

Plants in this series had not yet received a dose of dicamba. This is reflected in the above table where results, while variable, did not show any treatment related effect on any of the measured end-points. It demonstrates the issue of variability in plant toxicity testing that, even with no exposure, there is significant "inhibition" in some cases such as root length and mass compared to control plants, while for shoot measurements, plants were always larger in terms of mass than control plants.

Table 2: 1st thinning (21 days after planting) %inhibition results, Series II

Mean measured concentration (µg/L)	Wet weights (g)			Dry weights (g)			Shoot Length	Root Length
	Shoot	Root	Biomass	Shoot	Root	Biomass		
1.9	53.3	35.0	10.6	22.1	24.0	22.4	2.6	25.5
7.6	52.6	76.7	25.4	29.9	44.3	32.0	-3.7	26.8
16.8	55.5	74.2	28.4	26.7	33.2	27.7	2.9	23.3
50.6	37.7	56.0	3.9	3.3	20.7	5.8	0.3	24.1
102	45.7	59.7	19.2	10.2	33.8	13.6	-2.1	32.4
202	40.4	46.9	26.4	30.5	33.5	30.9	-1.6	31.6
447	74.3	62.9	65.1	66.0	52.9	64.1	19.2	32.4
1360	97.7	88.1	85.0	90.2	54.7	85.2	51.7	46.4

In contrast to the Series I plants, at this first thinning there were negative effects on both shoot and root measurements at all concentrations. In terms of dry weight biomass, an EC25 of 103 µg/L has been calculated for the first thinning data. The first thinning took place 21 days after sowing. Based on the irrigation regime provided in the report, and mean measured concentrations, the EC25 equates to a cumulative rate of around 46 g ac/ha.

3.4 2nd Thinning

The second thinning was undertaken at 37 days post sowing. Plant biomass at this stage was still relatively small as the plants had not yet entered their exponential growth phase. The mean dry weight biomass from the control plants was 0.56 g.

Table 3: 2nd thinning (37 days after planting) %inhibition results, Series I

Mean measured concentration (µg/L)	Wet weights (g)			Dry weights (g)			Shoot Length	Root Length
	Shoot	Root	Biomass	Shoot	Root	Biomass		
1.9	-38.1	-74.2	-73.1	-26.9	-47.8	-46.7	-1.5	7.3
7.6	-46.0	-76.8	-75.8	-44.7	-54.2	-53.7	-5.6	12.4
16.8	32.5	-8.2	-6.9	27.3	-12.7	-10.7	-12.9	17.4
50.6	-3.0	-16.8	-16.4	-1.3	-33.9	-32.2	-15.5	19.3
102	-36.6	-45.5	-45.3	-55.6	-64.1	-63.6	-7.4	14.8
202	-21.1	-12.3	-12.5	-40.5	-32.4	-32.8	-8.3	23.1
447	-53.6	-23.6	-24.5	-83.9	-44.6	-46.5	-11.0	27.1
1360	-67.9	-0.2	-2.3	-105.2	-2.9	-8.1	-6.2	23.1

Plants in this series had received two irrigations with spiked water. While inhibition relative to control values was observed on root length measurements, results overwhelmingly indicated no treatment related impact at this stage on plant development with negative inhibition generally observed at all treatment rates.

Table 4: 2nd thinning (37 days after planting) %inhibition results, Series II

Mean measured concentration (µg/L)	Wet weights (g)			Dry weights (g)			Shoot Length	Root Length
	Shoot	Root	Biomass	Shoot	Root	Biomass		
1.9	-25.3	-20.9	-21.0	27.0	1.3	2.6	2.6	25.5
7.6	85.7	23.0	24.9	46.6	21.6	22.8	-3.7	26.8
16.8	55.4	40.2	40.6	43.8	35.5	35.9	2.9	23.3
50.6	45.7	30.3	30.8	11.4	-7.1	-6.2	0.3	24.1
102	63.6	49.9	50.3	25.7	27.1	27.0	-2.1	32.4
202	60.0	61.0	60.9	51.6	54.6	54.5	-1.6	31.6
447	92.5	95.7	95.6	75.0	91.7	90.9	19.2	32.4
1360	99.6	100.0	100.0	96.6	99.9	99.7	51.7	46.4

Again, significant impacts on plant growth were observed in these treatments where plants had been exposed since planting. At the top rate there was essentially complete inhibition of plant growth compared to control plants and with the exception of the lowest treatment group, inhibition exceeding 25% of control values was frequent. In terms of dry weight biomass, an EC25 of 109 µg/L has been calculated for the second thinning data, which is consistent with the findings from the first thinning. The second thinning took place 37 days after sowing. Based on cumulative doses received in terms of g/ha by this time, the EC25 equates to a cumulative rate of around 84 g ac/ha.

3.5 3rd Thinning

The final thinning was undertaken 56 days after sowing. At this stage, plants were entering their exponential growth period, however, calculations of inhibition are still based on relatively low control biomass values with a group mean of 4.97 g.

Table 5: 3rd thinning (56 days after planting) %inhibition results, Series I

Mean measured concentration (µg/L)	Wet weights (g)			Dry weights (g)			Shoot Length	Root Length
	Shoot	Root	Biomass	Shoot	Root	Biomass		
1.9	4.0	-7.1	3.6	-10.3	-65.1	-13.8	5.8	5.2
7.6	-17.4	-16.3	-17.3	-1.2	-8.9	-1.7	2.2	9.7
16.8	-0.9	10.4	-0.5	22.0	15.1	21.5	4.4	25.8
50.6	1.8	9.3	2.0	24.6	23.6	26.2	3.7	23.6
102	-16.6	-24.3	-16.8	6.0	-27.0	3.9	3.3	17.0
202	-47.0	-39.8	-46.7	-4.4	2.0	-4.0	5.0	16.5
447	-23.2	-72.9	-25.0	19.7	-9.5	17.8	12.7	-2.3
1360	74.1	-3.6	71.2	77.7	37.9	75.1	26.0	38.0

It is interesting that, at this stage, there is still no discernible treatment related effect with the exception of the highest treatment rate. Apart from the highest treatment rate, inhibition tended to remain <25% for all other doses for all measurements.

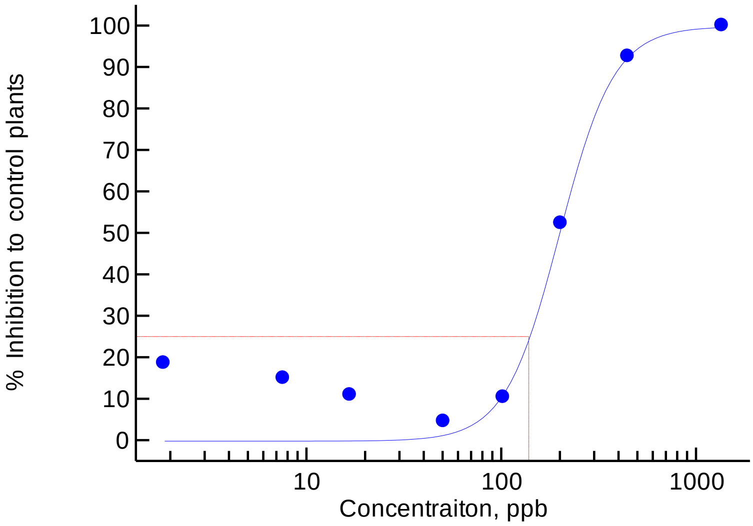
Table 6: 3rd thinning (56 days after planting) %inhibition results, Series II

Mean measured concentration (µg/L)	Wet weights (g)			Dry weights (g)			Shoot Length	Root Length
	Shoot	Root	Biomass	Shoot	Root	Biomass		
1.9	-1.6	-15.5	-2.1	21.0	-15.8	18.6	5.8	20.6
7.6	-19.2	1.4	-18.4	14.5	21.4	15.0	-4.2	23.7
16.8	-21.8	-16.2	-21.6	9.9	25.1	10.9	-1.9	12.5
50.6	-20.6	-8.8	-20.1	4.3	7.3	4.5	-2.7	14.0
102	-7.7	-8.2	-7.7	10.1	13.9	10.4	-6.7	12.0
202	23.1	19.8	23.0	51.9	59.1	52.3	7.0	17.1
447	90.7	82.0	90.4	92.5	92.8	92.6	48.4	44.0
1360	100	100	100	100	100	100	100	100

Interestingly, by the time of this third thinning there appeared to considerable recovery in plants exposed to treatments up to 102 µg/L, and even at the next highest rate of 202 µg/L in terms of inhibition at 56 days compared to that observed at 37 days (2nd thinning). This recovery was more noticeable in the wet weight measurements.

The most sensitive end-point from the 3rd thinning data from these Series II plants was for dry weight biomass.

Figure 2: Inhibition of Series II Plants, Biomass dry weight, 3rd Thinning



The growth inhibition EC25 for dry weight biomass is calculated at this thinning stage to be 138 µg/L.

The third thinning occurred at 56 days after planting. Based on cumulative doses received in terms of g/ha by this time, the EC25 equates to a cumulative rate of around 167 g ac/ha.

3.6 Final Harvest

The final harvest occurred at 94 days post planting. Plants had undergone their exponential growth period. In undertaken the following analysis, those plants that had succumbed to leaf rot were excluded from the analysis. From the control group, replicates 1c, 1h and 1j were removed. From the Series I plants, replicates 2d (1.9 µg/L), 3c (7.6 µg/L), 4b and 4d (16.8 µg/L), 6 a-c (90 µg/L) and 7 a-c (180 µg/L) were not used in the analysis. This reduced several exposure concentrations to only two to three replicates, but for consistency with treatment of the control group plants was considered necessary. In some cases, plants with minor leaf rot infections were retained in the data set to allow at least 2 replicates per treatment. There did not seem to be as much concern with leaf rot in the Series II plants, and replicates 11a and 11 d (7.6 µg/L), 12 d (16.8 µg/L), 13 d (50.6 µg/L) and 14 b (102 µg/L) were removed from the analysis.

At concentrations higher than 102 µg/L, plants noted as being infected with leaf rot were not omitted as there was also significant treatment related effects at these concentrations.

The EC25 and EC10 have been calculated for the final harvest results. Terrestrial plant studies can be highly variable both between replicates within a treatment, and between treatments themselves. It is not uncommon to find, due to this variability, that study NOECS exceed the EC25 values and in some jurisdictions, for example the USA and Canada, the EC25 value is used without an assessment factor as the view is taken that plant communities can tolerate up to 25% adverse effects (including both lethal and sub-lethal) and still recover.

This is apparent in the current study where Series II plants showed significant inhibition during early thinning, and appeared to recover at lower treatment rates as time progressed. In standard regulatory non-target plant studies, the exposure period is only 21 days following treatment, which tends to occur at the 4-leaf stage.

The purpose of this particular study is to consider dicamba in irrigation water and how it may impact lettuce growth over the growth cycle of the crop. The measurements are taken well beyond the 21 d exposure period used for non-target terrestrial plant toxicity study results used in establishing the interim guideline. Given the purpose of the study and the longer growth cycle considered, both EC25 and EC10 values have been calculated.

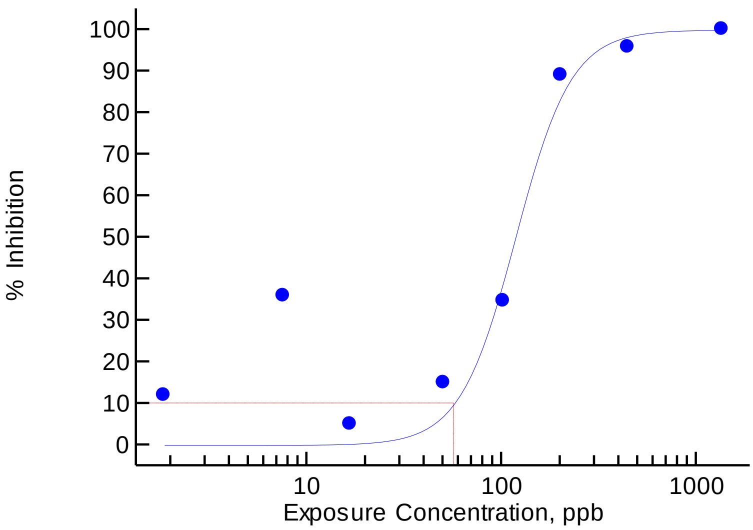
Table 7: Final Harvest (94 days after planting) %inhibition results, Series I

Mean measured concentration (µg/L)	Wet weights (g)			Dry weights (g)		
	Shoot	Root	Biomass	Shoot	Root	Biomass
1.9	23.5	18.5	23.2	13.2	-31.4	11.9
7.6	21.8	19.6	21.6	37.5	-17.5	35.8
16.8	-3.8	6.1	-3.3	5.8	-24.2	4.9
50.6	5.2	8.5	5.4	14.6	25.7	14.9
102	34.0	21.4	33.3	35.7	-1.0	34.6
202	62.9	36.2	61.4	89.7	64.7	89.0
447	91.7	78.2	91.0	96.2	80.9	95.7
1360	100	100	100	100	100	100
EC25	89.8	129.7	90.8	81.3	143.8	82.0
EC10	53.4	70.0	53.3	56.9	113.5	57.1

The most sensitive end-point was for shoot dry weight with an EC10 of 56.9 µg/L. The increased inhibition at the two lowest treatment groups is unlikely to be treatment related due to lower inhibition at the next three treatment levels.

In terms of total dry weight biomass, the following dose/response was observed:

Figure 3: % Inhibition, dry weight biomass, Final harvest, Series I plants.



In terms of the EC10 of 57.1 µg/L, the 95% confidence intervals are wide due to the high inhibition at the two lowest treatment rates with a 95% CI of 0-115 µg/L. However, these confidence intervals are tightened considerably if only the results from 16.8 µg/L and above are included in the analysis, with an EC 10 of 57.7 µg/L and 95% CI of 33.4-82.0 µg/L.

In terms of the cumulative dose received by plants in Series I, the EC25 and EC10 correspond to cumulative rates of 174 g ac/ha and 121 g ac/ha respectively.

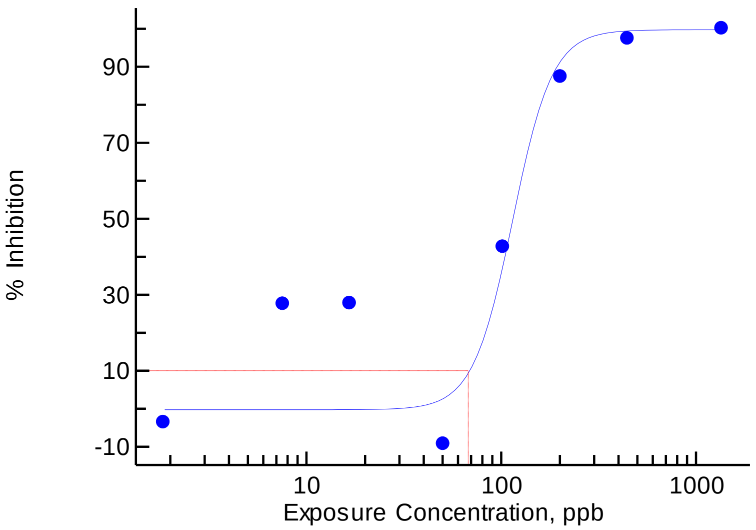
Table 8: Harvest (94 days after planting) %inhibition results, Series II

Mean measured concentration (µg/L)	Wet weights (g)			Dry weights (g)			Shoot Length	Root Length
	Shoot	Root	Biomass	Shoot	Root	Biomass		
1.9	13.5	7.6	13.2	-3.0	-24.0	-3.7	1.5	-3.0
7.6	4.3	-2.6	3.9	29.2	-28.8	27.5	-3.7	-4.8
16.8	13.8	27.8	14.6	27.3	38.6	27.7	-2.7	6.3
50.6	7.2	-7.4	6.4	-9.4	-7.4	-9.3	3.4	-2.1
102	15.0	7.2	14.1	43.1	23.8	42.5	-1.2	9.0
202	47.0	20.3	45.6	88.4	52.1	87.3	10.5	-2.7
447	93.9	79.6	93.1	97.6	88.8	97.3	48.9	47.1
1360	100	100	100	100	100	100	100	100
EC25	136	215	140	87.5	118	87.5	312	417
EC10	90.7	154.8	94.2	68.5	73.9	67.6	218	387

While the Series II plants had shown increased inhibition compared to control plants than the Series I plants during the 1st, 2nd and 3rd thinning stages, at the time of final harvest they appeared to have been impacted less than the Series I plants where exposure commenced at the 4-leaf stage. As with the Series I plants, the most sensitive end-points related to dry weight shoot and biomass measurements, and the results between the two series were not noticeably different with these end-points.

For the Series II plants, the most sensitive end-point was dry weight biomass with an EC25 and EC10 of 87.5 µg/L and 67.6 µg/L respectively. In terms of a total cumulative rate, based on the irrigation regime and measured concentrations, the ER25 and ER10 equate to 226 g ac/ha and 176 g ac/ha respectively.

Figure 4: % Inhibition, Total dry weight biomass, Final harvest, Series II plants.



It cannot be concluded that the inhibition found at 7.6 µg/L and 16.8 µg/L are treatment related due to the low inhibition found at the next highest treatment level. However, the higher inhibition at these two lower treatment rates results in relatively high confidence intervals and the 95% CI for the EC10 value is calculated to be 3.9 µg/L to 131 µg/L.

3.7 Visual Observations

Visual observations on plant health were undertaken with rankings on the severity of effects made for different observations including leaf cupping, stem twisting, leaf strapping, leaf curling and wilting. The ratings scale had the range of no symptoms; some replicates showing observable symptoms; observable symptoms; and clearly noticeable severe symptoms.

At the four leaf stage of both Series I and Series II plants, symptoms were noted from the 45 µg/L (nominal) concentration and higher, with the severity of symptoms increasing with increasing concentration.

The study NOEC based on visual observations is therefore 15 µg/L nominal (16.8 µg/L mean measured) concentration. This is lower than the lowest EC10 value calculated from the quantitative data.

4 Discussion and Conclusion

A considerable amount of information can be obtained from this study. Due to the number of different time periods at which quantitative measurements were obtained, and the range of concentrations tested it is possible to draw firmer conclusions as to the likely impacts of dicamba in irrigation water to the growth and development of lettuce plants.

In the absence of other information, the interim guideline of 1.6 µg/L was based on terrestrial plant test data derived in terms of an application rate (grams per hectare), as a cumulative dose over the course of the crop growth cycle and based on a particular irrigation regime (Lee-Steere et al, 2012). The current test provides a good basis for concluding that the limiting factor in setting the guideline will be the exposure concentration in water. To demonstrate this, the results from the Series II plants can be used. These plants received a dose of dicamba in the irrigation water from seed. At the final harvest, the most sensitive end-point was shown to be dry biomass, so the dry biomass results from all three thinnings and the final harvest are shown below, along with the cumulative dose received by plants in terms of an application rate in g/ha.

Table 9: EC25 and ER25 (cumulative dose) results, Series II plants

Days after Sowing	Measurement time	EC25 (µg/L)	ER25 (g/ha, cumulative dose)
1	1st thinning	103	46
21	2nd thinning	109	84
37	3rd thinning	138	167
94	Final harvest	87.5	226

The relative consistency in findings through the course of the study in terms of water concentration indicate this is the main influence on toxicity, not the cumulative dose which increases over time due to additional irrigation periods. It is also observed that at the time of the final harvest, the results from both series of plants were similar. This is despite plants in Series II receiving dicamba doses from seed while those in Series I received them from the 4-leaf growth stage. Consequently, Series I plants received a lower overall cumulative dose but the outcomes in terms of water concentration were very similar. This further supports a conclusion that the water concentration is the main factor influencing toxicity rather than the cumulative dose.

In terms of the EC10 (water concentration that can be expected to result in inhibition to 10% of plants), the lowest value at the final harvest was 56.9 µg/L for shoot dry weight in the Series I plants. However, visual impacts showed more sensitivity than the quantitative measurements with 16.8 µg/L being the highest treatment level that did not result in any visual symptoms of toxicity.

This level is approximately an order of magnitude higher than the interim guideline level of 1.6 µg/L and indicates that the initial guideline level could be relaxed. The findings in this report relate only to lettuce and do not account for potential effects of mixtures of herbicides that may be found in irrigation water.

5 References

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