Executive Summary

The herbicides MCPA and Dicamba have been reported to occur in low concentrations in the recycled water from the Western and Eastern treatment plants in Melbourne. To test for these compounds is expensive, and there is a time delay of several days before results of an analysis can be obtained. Consequently, Melbourne Water is looking for a fast screening method which can be used on a daily basis by the plant operators. This method would not replace the current testing regime but would provide greater confidence in the water quality between set testing times.

This report details the results from trials using a phytotoxicity test kit using three different seed different types as recommended by the manufacturer of the kits. The kits were purchased from a Canadian company called Environmental Bio-Detection Products Inc. (EBPI) and used as per the supplied instructions. The three seed types chosen were lettuce; garden cress and radish were chosen for their known fast germinating properties, lettuce was chosen to match pot trials which were being carried out simultaneously. Various cultivars of these seeds were tested to identify the cultivar with the highest response and fastest germination results.

The two herbicides used in this experiment were Dicamba (3,6-dichloro-2-methoxybenzoic acid) and MCPA (4-chloro-2-methylphenoxyacetic acid). The first set of trials used the compounds in their acidic form as that was the form reported in the analytical results. However, it is either the ester or salt forms that are used by farmers as they are readily soluble in water. Therefore the experiments were repeated using the more soluble forms.

Initial experiments were conducted using ETP recycled water spiked with MCPA and Dicamba to determine the most sensitive seed. The concentrations applied were 1.6, 6.5, 15, 45, 90 and 405 µg/L of Dicamba and 3, 10, 25, 224 and 675 µg/L of MCPA. ETP and WTP recycled waste waters and tap water were used as controls. The solutions were freshly prepared for each experimental trial and the concentration of random samples was checked by ALS Laboratories. Each kit holds 10 seeds and

each treatment was carried out in duplicate.

The data collected were germination rate and length of the root and shoot after 60 hrs in the dark at 25°C. The EC25 was calculated for each herbicide and the results were subjected to analysis of variance to assess statistical significance.

Of the seeds tested, only the Radish (*Champion*, Eden Seeds) and Garden Cress (Garden Greens extra curled, Eden Seeds) had a germination rate of greater than 90%. The overall findings were that Dicamba had no effect on the seed growth at concentrations up to 200 µg/L while MCPA showed an effect at 3 µg/L using reduced root growth as the indicator.

Introduction

Melbourne Water currently employs the services of ALS laboratories to test the recycled water from its Eastern Treatment Plant (ETP) and Western Treatment Plant (WTP) for the herbicides MCPA and Dicamba. Since the analytical method involves a number of procedures there is a time delay of several days between submitting the samples and obtaining the results. Melbourne Water would like to have a quick screening test which can be performed in-house by the plant operators. One option is to use a simple off-the-shelf phytoxicity kit. RMIT was engaged to trial such a kit with a range of seeds with the two herbicides, MCPA and Dicamba.

A commercial kit was obtained from Environmental Bio-Detection Products Inc.(EBPI). A total of 8 experiments was designed to capture the EC25 and No Observable Effect Concentration (NOEC). The first set of experiments was designed to identify the most sensitive plant species and test them with ETP recycled water spiked with increasing amounts of Dicamba and MCPA. The most sensitive species were then tested with WTP recycled water using Dicamba and MCPA. Initially, a follow up experiment was designed to investigate the suitability of other wetting media such as cotton wool and sterilized sand. However, this was not carried out as the results of the prior experiments showed that the foam set-up provided sufficient moisture holding capacity for the duration of the incubation time

and no specific advantage was to be gained by altering the media, especially when considering ease of use.

In the initial brief for the experiment a repeat of the above was to be carried out using WTP recycled water and Dicamba only at decreased concentrations. However, the results of all prior Dicamba experiments showed that the prescribed concentration range did not yield a response and so the experiment was not carried out. Instead, an experiment was added which to determine the minimum incubation time required for a recordable effect on radish seed growth using WTP spiked with MCPA against the control.

An extra set of experiments was carried out in Feb13. This time, the solutions were made up using WPT rather than MilliQ water to overcome any possible matrix interferences and MCPA was made up from the liquid ester form to see if there was any difference between the response to the solid form. All concentrations used were as in the previous trial.

Statistical analysis was carried out on all trials. Box and Whiskers graphs were constructed after the data has been filtered to a 99.7% confidence level, with only the exceeded minimum seed length being removed from the data set.

Experimental Design

The experiments were divided into 5 trials. Trials 1-3 were designed to determine the most sensitive species from a number of cultivars of lettuce, radish and garden cress. Solutions of dicamba and MCPA were made from their solid acid forms and diluted with MQ water. Trial 3 was a repeat of the Trial 2 using higher concentrations of dicamba and another cultivar of lettuce with MCPA only. Trial 4 tested MCPA with recycled water from WTP using radish seeds only. In Trial 5, the herbicides were diluted with WTP water rather than MQ water to test for any matrix effect and MCPA was made from the soluble ester formulation.

Germination Testing

Germination testing was undertaken to establish the fastest emerging seeds with a germination rate of greater than 90%.

Method – Phytotoxicity test kit components and assembly

Phytotoxicity kits were purchased from EBPI which consisted of a set of clear sleeves into which a custom made piece of foam (for maintaining moisture) and a piece of black absorbent paper (black to make the measurements easier) were inserted. Seeds were placed in individual compartments (10 per sleeve). Prior to the commencement of the main trials, the germination characteristics of a number of seed types were investigated. Incubation was carried out in 25ºC for 2 to 3 days depending upon the seeds.

In this experiment and in Trial 1, in house made foam and single filter paper was used. (At the time of the germination testing, not all the correct kit components were available.)

The seeds tested were:

- lettuce (*Lactuca sativa*, *All Year Round*, Mr. Fothergill's; Iceberg *Try These*, Mr. Fothergill's; *Great Lakes*, Mr, Fothergill's)
- radish (*Raphanus sativus, Scarlet Globe*, Mr. Fothergill's, *Champion*, Eden Seeds), and
- garden cress (Lepidium sativum, Garden Greens extra curled, Eden Seeds).

Initial tests were performed using tap water for an incubation time of 3 days. Subsequent tests included a test with recycled water from ETP.

Results:

1st Germination Test (lettuce and radish)

Design: 2 kits, total of 20 seeds, tap water only, 3 days incubation period.

- 1 x Lettuce "All Year Round", brand - Mr. Fothergill's
- 1 x Radish " Scarlet Globe", brand - Mr. Fothergill's

Table The number of germinated for radish and lettuce (lettuce, radish, 3 days incubation)

	Radish (*Scarlet Globe*)	Lettuce (*All Year Round*)
Tap water	10	7
Germination %	100	70

2nd Germination Test (lettuce, radish and garden cress)

Design: – 3 seed types, 2 kits per seed type - total 60 seeds, 1 x ETP water & 1 x Tap water, 2 days incubation period

- 2 x Garden Cress "Garden Greens" extra curled, Eden Seeds
- 2 x Lettuce Iceberg "Try These", Mr. Fothergill's
- 2 x Radish "Champion", Eden Seeds

Table of germination results for 2nd Germination test

Table Results for 2nd germination test (radish, lettuce, garden cress, 2 days incubation)

	Radish (Champion)	Lettuce (Iceberg, Try These)	Garden Cress (Garden Greens)
Tap water	10	6	10
ETP water	10	7	10
Germination %	100	65	100

3rd Germination Test (three lettuce cultivars)

Design: 3 lettuce cultivar types, 2 kits per cultivar type = total 60 seeds, 1 x ETP water & 1 x Tap water, 2 days incubation period

- 2 x Lettuce " Great Lakes, Mr, Fothergill's
- 2 x Lettuce "All Year Round", Mr. Fothergill's
- 2 x Lettuce "Iceberg", Country Value

Table Results for 3rd germination test (3 lettuce cultivars, 3 days incubation)

	Great Lakes	All Year Round	Iceberg
Tapwater	6	3	2
ETP water	4	6	0
Germination %	50	45	10

Conclusions from the germination experiments

The radish and garden cress seeds all germinated successfully within 2 days incubation. Lettuce seeds were not so successful. Lettuce *All Year Round* was selected for further experimentation as it had a 70% germination success rate over 3 days. The following seeds were selected for the bioassay experiment:

- Radish “Champion”, brand – Eden Seeds
- Garden Cress “Garden Greens” extra curled, brand – Eden Seeds
- Lettuce “All Year Round”, brand - Mr. Fothergill’s

Trials 1-3: Determination of the most sensitive seeds

In this series of experiments, the aim was to identify which of the above seeds were the most sensitive to MCPA and Dicamba in ETP recycled water as determined by having a statistically significant difference in the length of the root or the shoot. The medium used was sterilised foam with black filter paper. A first trial was run using some kit components made in-house while waiting for the arrival of fresh foam and filter inserts. However, this introduced a number of variables which could not be controlled effectively. Neverthelss, even in this preliminary trial, it was clear that lettuce seeds were not germinating consistently enough to be useful. Subsequent trials were conducted using radish and garden cress seeds only, with new materials purchased from EBPI.

Method

Six litres of ETP water was supplied by Melbourne Water and samples of the supplied recycled water were analysed by ALS Laboratories to determine if there were any detectable concentrations of Dicamba and MCPA already present. The concentration of Dicamba was 0.05 µg/L and MCPA 0.16 µg/L. The water was refrigerated at 4ºC.

Stock solutions of Dicamba (3,6-dichloro-2-methoxybenzoic acid) and MCPA (4-chloro-2-methylphenoxyacetic acid) were prepared by dissolving the acidic solid forms of both herbicides in milli-Q water. One gram of the solid was weighed, crushed and mixed with the water in a 1 L volumetric flask. Since the solids did not readily dissolve, the solutions were filtered to remove any undissolved solid and sent to ALS for analysis. Dicamba dissolved more readily than the MCPA with the final concentrations of the stock solutions being 824 mg/L of Dicamba and 280 mg/L of MCPA. The Dicamba stock solution was further diluted to give a secondary stock solution with a Dicamba concentration of 50 mg/L. These solutions were then refrigerated at 4ºC in 1 L amber bottles.

The stock solutions were further diluted to 500 ug/L for Dicamba and 750 ug/L for MCPA by taking 10 mL of the 50 mg/L Dicamba stock and 2.68 mL of the 280 mg/L MCPA stock and diluting it to 1 L in MilliQ water. These solutions were then used to make up the range of concentrations as per the tables below in 100 mL volumetric flasks.

Table Volumes of Dicamba needed for the test solutions

Required concentration (µg/L)	Volume (mL) of stock (500 µg/L)	Volume of ETP recycled water mL (0.05 µg/L)
1.6	0.3	99.7
6.5	1.3	98.7
15	3	97
45	9	91
90	18	82
405	81	19
Total volume 100 mL; concentration of Dicamba measured in the ETP recycled µg/L water <0.05		

Table Volumes of MCPA needed for the test solutions

Required	Volume (mL) of	Volume of ETP

concentration (µg/L)	stock (750 µg/L)	recycled water mL (0.16 µg/L)
3	0.4	99.6
10	1.3	98.7
25	3.3	96.7
75	10	90
224	30	70
675	90	10
Total volume 100 mL; concentration of MCPA measured in the ETP recycled water 0.16 µg/L		

The experiments were run in triplicate with some variations as per the following table:

Table Summary of experiments to determine the most sensitive seed

Bioassay Trials	Seeds used	Herbicides used	Recycled Water Used	Incubation Period
1 – 04/09/2012*	Radish, Garden Cress, Lettuce	Dicamba, MCPA	ETP	60 hours
2 – 09/10/2012	Radish, Garden Cress	Dicamba, MCPA	ETP	60 hours
3 – 26/11/2012	Radish, Lettuce	Dicamba (excessive concentrations), MCPA	ETP	60 hours

* In bioassay trial 1, home-made foam and black paper inserts were used

The first experiment was the only trial in which all 3 seed types were used. For this experiment, Bioassay trial 1, 80 kits in total were used:

- Samples: 12 treatments (6 concentrations x 2 herbicides) x 2 replicates x 3 seed types. As there were only 80 kits available, one of the 405µg/L of Dicamba with radish seed was removed to enable 9 controls

- Controls: 3 seed types x (2 ETP water + 1 tap water)

All kits were randomly assembled into cardboard boxes (which held 6 kits each) and arranged over the top two shelves of the incubator. Incubation time was 60 hours at 25ºC in darkness.

For the following two trials, Bioassay trial 2 & 3, 56 kits in total were used for each

- Samples: 12 treatments x 2 duplicates x 2 seed types = 48 kits.

- Controls 2 seed types x 3, of which 2 duplicates were using 2 x ETP water and 1 x tap water only..

All kits were randomly assembled into cardboard boxes, which were able to hold 9-11 kits each and arranged over the top two shelves of the incubator. Incubation time was 60 hours at 25ºC in darkness.

The kit pieces consisted of 1 plastic tray and lid, foam to fit into one half of the kit, thick white filter paper to fit over the foam piece and 1 piece of thin black filter paper to sit on top of the white filter paper. A 15 mL aliquot of solution is syringed onto the thick white filter paper and the black filter paper is placed on top. Ten seeds are placed 1 cm from the middle half of the tray on the black filter paper. The seeds should match the 10 slots of the dividing middle of the tray, which provides space for the shoot to move through. Once all seeds are placed, the lid can be snapped into place. With the correct foam, board and filter paper assembly, the lid fits so that the seeds are held in place and pushed onto the moist filter paper. Sufficient pressure is created to maintain moisture from the water trapped in the foam and board pieces.

In the first trial, the foam and filter paper assembly was prepared in-house. Standard craft foam of 6 mm thickness was purchased and cut to size. The filter paper used was craft newspaper like paper, off-white in colour. However, the total thickness of the foam and filter paper was not sufficient to ensure constant wetting of the filter paper, which meant that some kits to dried out, compromising the results from the first experiment. All further experiments were conducted with foam, filter paper and black paper obtained from the manufacturer.

Results from Trials 1-3 – sensitivity testing

Trial 1 using using the in-house inserts (Dicamba and MCPA with radish, garden cress and lettuce seeds)

The results from this experimental trial were inconsistent and therefore did not offer a conclusive result on effect of herbicide on shoot growth. Photographs in Figure 1 show duplicate kits with radish seeds germinated with ETP recycled water spiked with 90µg/L of Dicamba. Both kits were saturated with the same volume of solution and the seeds were from the same batch. The incubation conditions were also identical, yet upon removal from the incubator, the kit with label “1D90-R” was dried out, whereas the duplicate kit “2D90-R” was still moist to the touch. The drying out of the first kit was most likely the

cause for the poor results rather than the Dicamba.

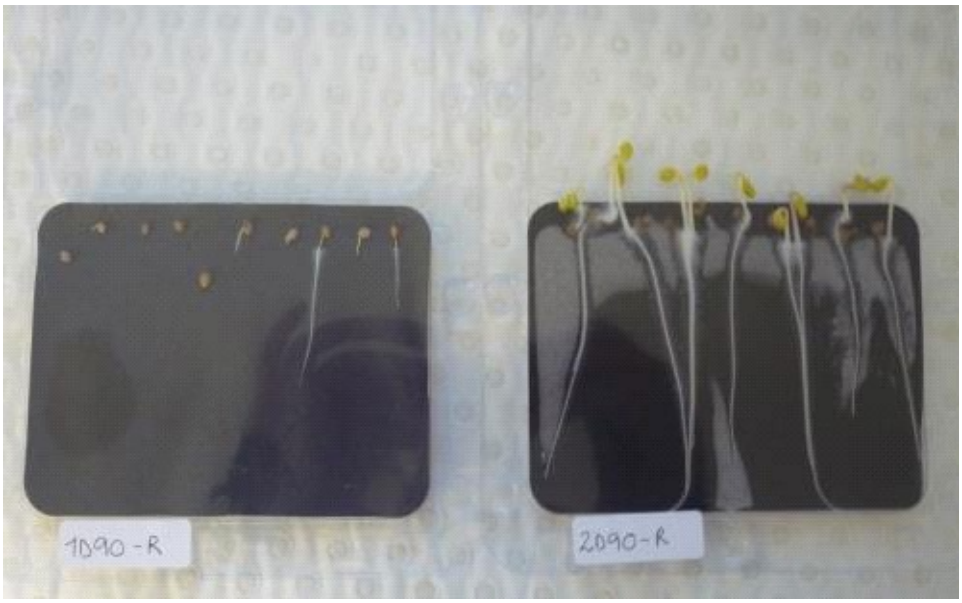


Figure Radish Seed 90 µg/L Dicamba applied

The identical problem was encountered with these kits with MCPA (Figure 2).

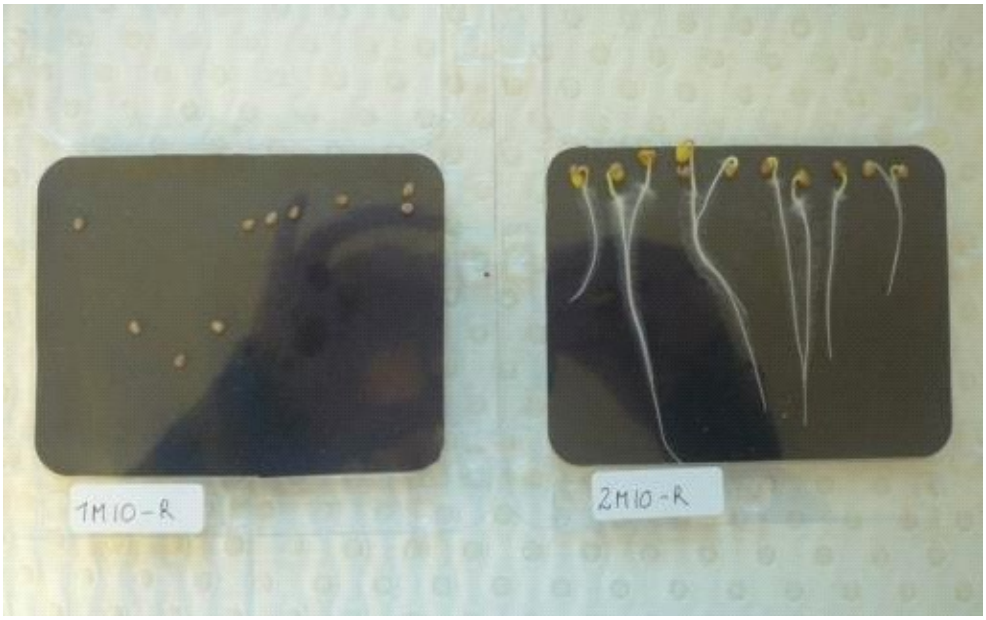


Figure Radish Seeds with 10 µg/L MCPA in ETP recycled water

The experiment was repeated with radish and garden cress seeds only as the germination of lettuce seeds was very poor.

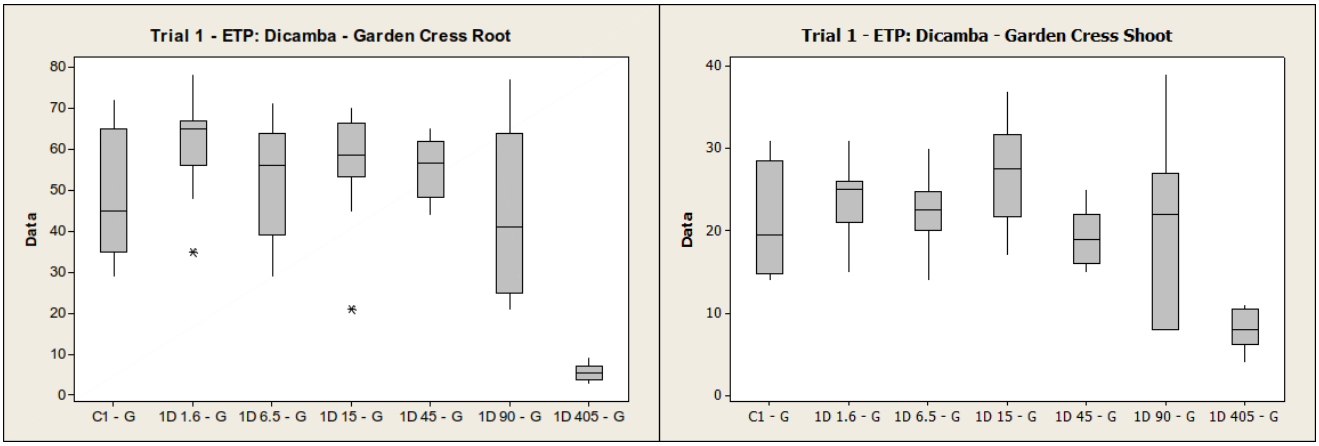
Trial 1 – Garden Cress, Radish and Lettuce with ETP water -Dicamba

Table7: Dicamba and Garden Cress with ETP

Dicamba ug/L	0	1.6	6.5	15	45	90	405
Log Conc	0	0.2	0.81	1.18	1.65	1.95	2.61
Average Root	52	51	44	42	53	38	5
Std Dev Root	16	23	19	25	10	22	2
Upper Limit	69	74	62	67	63	60	7
Lower Limit	36	28	25	16	42	16	3
Average Shoot	19	21	19	23	18	17	7
Std Dev Shoot	9	8	8	9	4	11	3
Upper Limit	28	28	27	32	22	28	10
Lower Limit	10	13	12	14	14	6	4
Average Seed Total	66	72	63	65	71	55	12
St Dev Total	27	30	25	33	13	32	5
Upper Limit	93	102	88	98	83	87	17
Lower Limit	39	42	37	32	58	23	6
Germination %	100	95	100	90	95	90	80
75th Percentile Root	39	39	39	39	39	39	39
75th Percentile Shoot	14	14	14	14	14	14	14
75th Percentile Total	49	49	49	49	49	49	49

This data set is based on the raw data; no seeds have been omitted from the average totals.

Figure : Graphs of Root and Shoot lengths response of Garden Cress to Dicamba



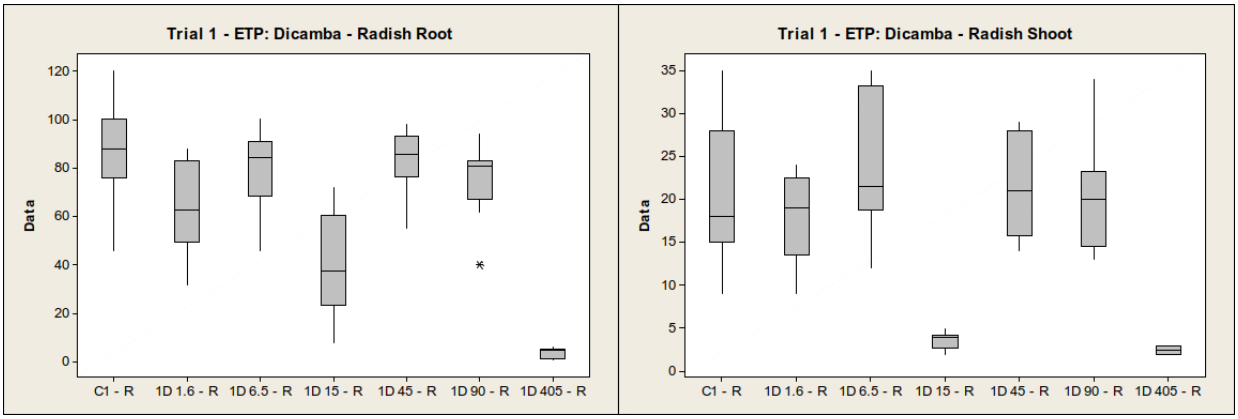
Box and Whiskers Graphs: Trial 1 – Dicamba on Garden Cress

The root length dropped below the 75th percentile measurement at 90 µg/L and the shoot length between 90 and the maximum concentration of 405 µg/L.

Table 8: Dicamba and Radish with ETP

Dicamba ug/L	0	1.6	6.5	15	45	90	405
Log Conc	0	0.2	0.81	1.18	1.65	1.95	2.61
Average Root	84	44	67	28	51	57	4
Std Dev Root	26	30	27	24	41	34	2
Upper Limit	110	73	94	53	92	91	6
Lower Limit	58	14	41	4	10	22	2
Average Shoot	26	16	20	4	19	19	3
Std Dev Shoot	21	7	10	1	9	8	1
Upper Limit	47	23	30	5	27	27	3
Lower Limit	4	9	10	3	10	11	2
Average Seed Total	109	52	87	29	64	71	5
St Dev Total	41	38	34	26	52	44	3
Upper Limit	150	90	121	55	116	115	8
Lower Limit	69	14	53	3	12	27	2
Germination %	100	95	95	80	85	75	50
75th Percentile Root	63	63	63	63	63	63	63
75th Percentile Shoot	19	19	19	19	19	19	19
75th Percentile Total	82	82	82	82	82	82	82

This data set is based on the raw data; no seeds have been omitted from the average totals.



The Dicamba and Radish seed series of kits of the first Trial were compromised by the in-house manufactured components. The controls for the Radish seeds were one of the few duplicates that did not dry out and therefore does not make it a useful comparative set against the Radish and Dicamba results which generally had one if not both of the duplicate kits affectedFigure 4:Radish Controls and 1.6µg/L - Trial 1 (Figure 4). Due to the mix of variables other than the Dicamba concentrations, this data set is not a true representation of the effect of Dicamba on Radish seeds.

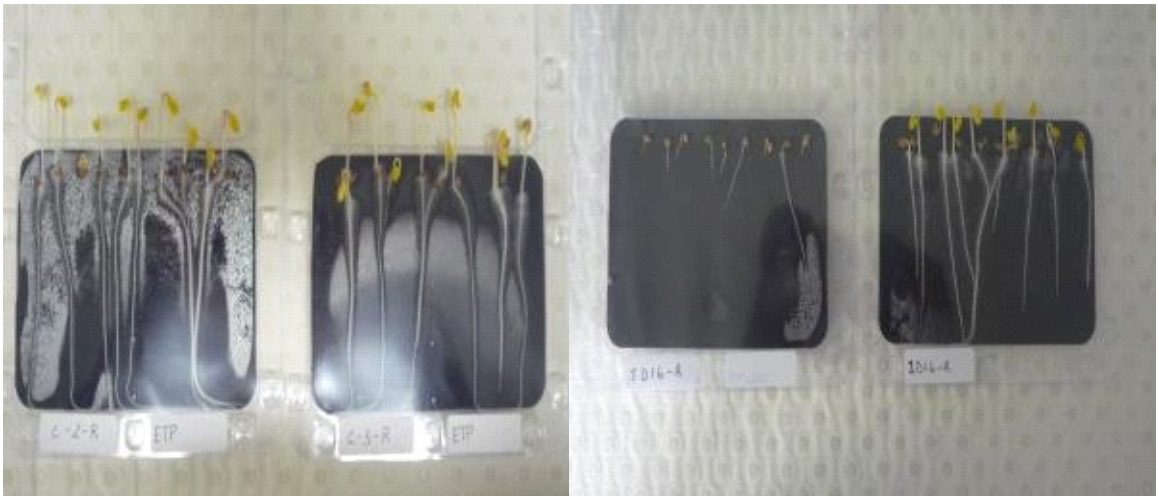


Figure :Radish Controls and 1.6µg/L - Trial 1

These photographs show the variance in kit results which contributed to the inconsistency in this set. The control kits both had sufficient moisture to ensure that the seeds could grow and not dry out over the 3 day incubation period. The second photograph shows the kit labelled “1D1.6 –R” having dried out and as a result, most seeds did not grow. The duplicate kit marked “2d1.6-R” did grow

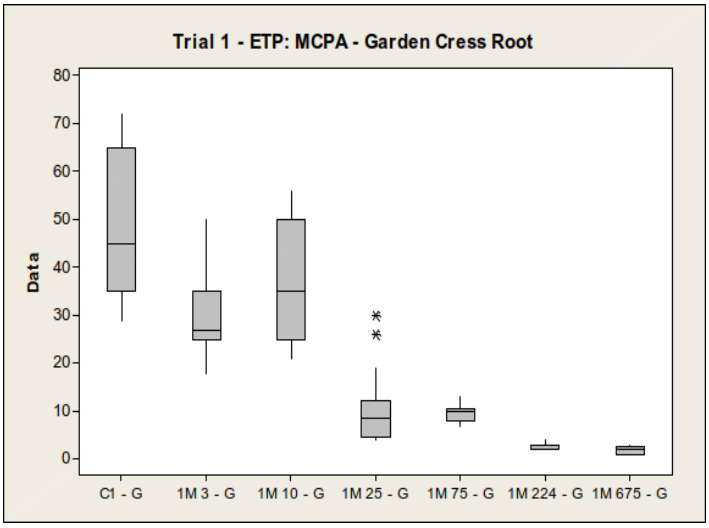
Trial 1 – Garden Cress, Radish and Lettuce with ETP water -MCPA

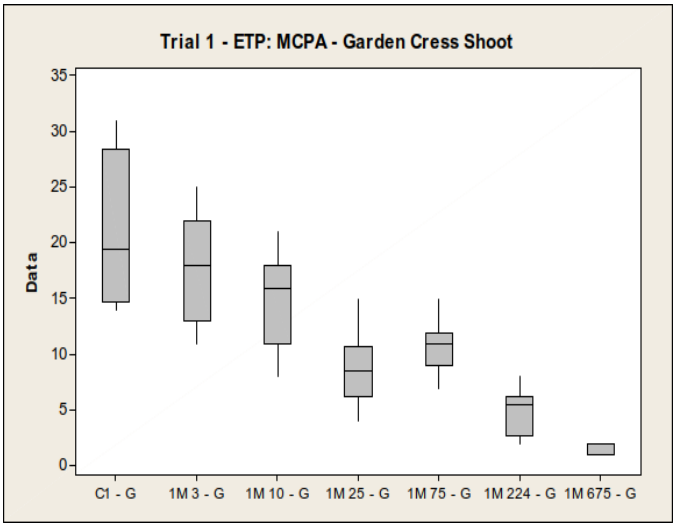
Table 9: MCPA and Garden Cress with ETP

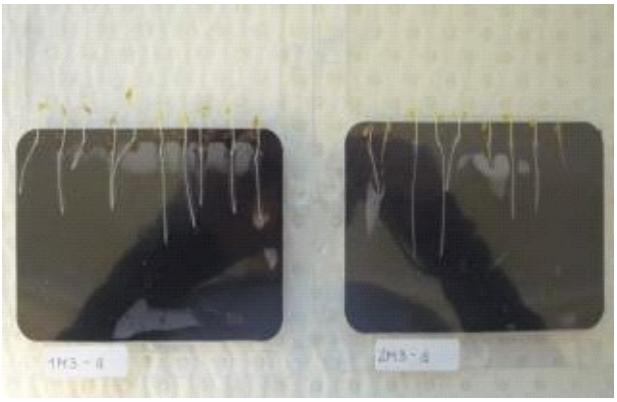
MCPA ug/L	0	3	10	25	75	224	675
Log Conc	0	0.48	1	1.4	1.88	2.35	2.83
Average Root	43	27	32	10	9	2	2
Std Dev Root	20	12	16	8	2	1	1
Upper Limit	62	38	48	18	11	3	3
Lower Limit	23	15	16	3	7	1	1

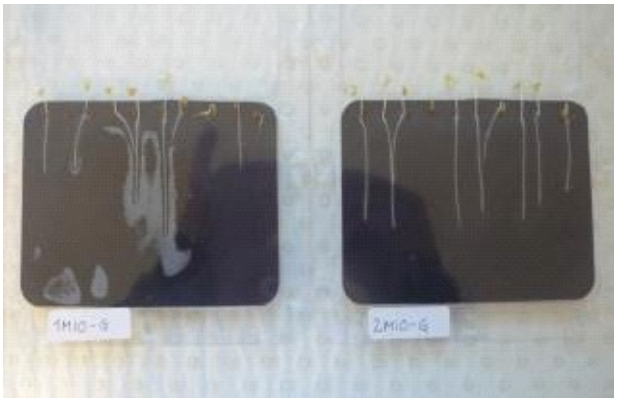
Average Shoot	18	16	13	7	9	4	2
Std Dev Shoot	8	6	6	4	4	2	1
Upper Limit	26	22	19	11	13	7	2
Lower Limit	10	10	7	3	5	2	1
Average Seed Total	54	41	42	16	17	6	1
St Dev Total	31	17	23	8	7	3	2
Upper Limit	86	57	65	23	24	9	3
Lower Limit	23	24	19	8	11	2	-1
Germination %	95	95	95	100	100	95	100
75th Percentile Root	32	32	32	32	32	32	32
75th Percentile Shoot	14	14	14	14	14	14	14
75th Percentile Total	41	41	41	41	41	41	41

This data set is based on the raw data; no seeds have been omitted from the average totals.




Trial 1 - ETP: MCPA - Garden Cress Shoot
Data
35
30
25
20
15
10
5
0
C1 - G
1M 3 - G
1M 10 - G
1M 25 - G
1M 75 - G
1M 224 - G
1M 675 - G





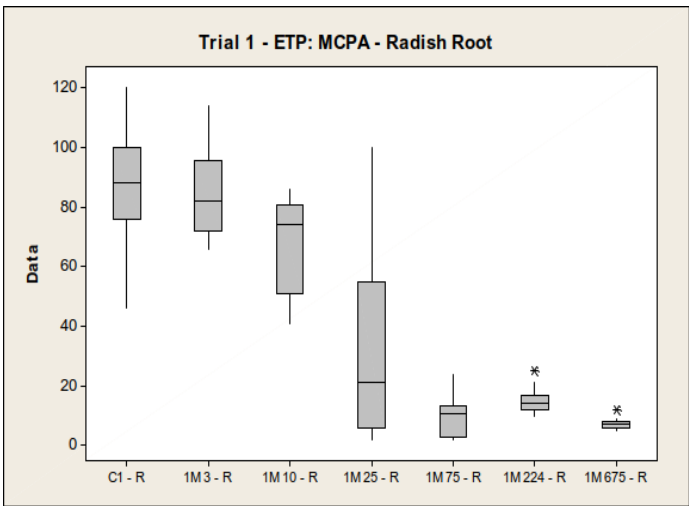
In this series, the MCPA shows an effect at 3 µg/L on root growth. Shoot is still within the 75th percentile range at 3µg/L; however, it dips below the line before 10 µg/L. This photo shows the decrease in root growth and the variation within the kits themselves. Not all seeds were affected evenly which suggests possible natural variation within the seed batch to the effect of MCPA at that level. At the 10 µg/L the average root length increases slightly and particularly in the kit labelled "2M10-G", the germinated seeds looked to have more uniform lengths. The kit conditions were noted to be of dry filter paper and seedling and moist foam for the first kit. The duplicate had dry filter paper and moist foam.

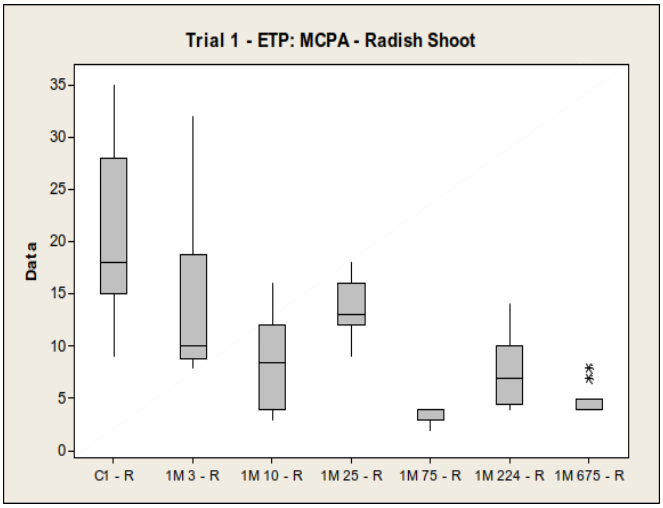
Table 10: MCPA and Radish with ETP

MCPA ug/L	0	3	10	25	75	224	675
Log Conc	0	0.48	1	1.4	1.88	2.35	2.83
Average Root	70	74	62	30	8	13	6
Std Dev Root	35	27	23	31	7	5	3
Upper Limit	104	101	85	61	15	18	9

Lower Limit	35	47	39	-1	1	8	4
Average Shoot	17	13	8	11	3	7	5
Std Dev Shoot	10	7	4	5	1	4	1
Upper Limit	26	20	13	16	4	10	6
Lower Limit	7	6	4	6	2	3	3
Average Seed Total	86	87	35	30	8	18	11
St Dev Total	43	31	40	35	8	8	3
Upper Limit	129	118	76	66	17	26	14
Lower Limit	43	56	-5	-5	0	10	7
Germination %	100	100	50	80	95	95	100
75th Percentile Root	52	52	52	52	52	52	52
75th Percentile Shoot	12	12	12	12	12	12	12
75th Percentile Total	65	65	65	65	65	65	65

This data set is based on the raw data; no seeds have been omitted from the average totals.





Trial 2 – Radish and Garden Cress seeds with MCPA and Dicamba, with ETP water

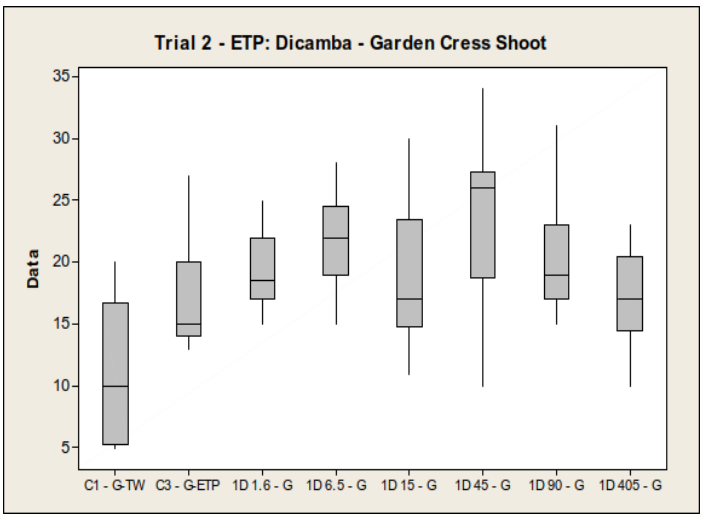
Table 10: Garden Cress with Dicamba

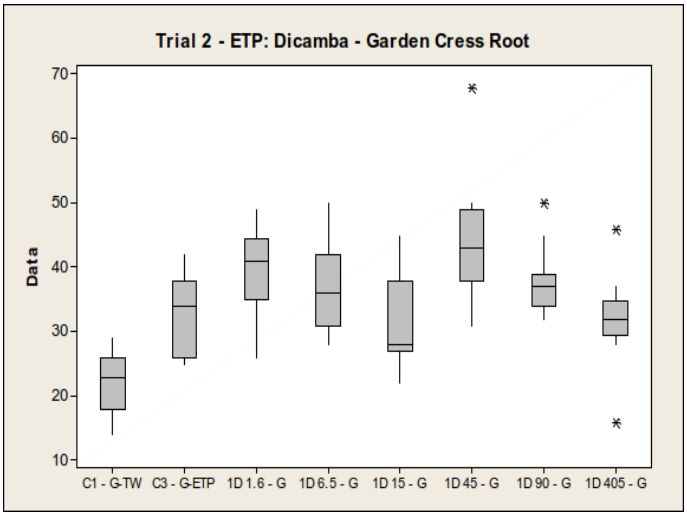
Dicamba ug/L	0	1.6	6.5	15	45	90	405
Corrected conc (µg/L)	0	0.2	0.8	1.9	5.2*	10.4	50
Log Conc	0	-0.7	-0.1	0.28	0.72	1.02	1.7
Average Root	30	38	33	29	41	38	30
St dev Root	10	10	11	10	10	10	10
Upper Limit	40	48	44	38	51	48	40
Lower Limit	21	28	23	19	31	27	20
Average Shoot	17	17	19	16	20	17	15
St Dev Shoot	4	5	6	7	8	6	6
Upper Limit	21	22	25	23	28	23	21
Lower Limit	12	12	13	9	11	11	9
Average Total seed	46	55	52	45	61	52	45
St Dev seed	14	14	15	15	17	15	15
Upper Limit	60	69	67	60	77	67	60
Lower Limit	32	41	38	30	44	37	30
Germination %	85	90	90	90	95	90	55
75 percentile Root	23	23	23	23	23	23	23
75 percentile Shoot	12	12	12	12	12	12	12
75 percentile Total	35	35	35	35	35	35	35

*The 45 and 405 µg/L were checked by ALS and it would appear that there had been an error in making up these solutions. Based on the results for the two checked solutions, all others have been corrected by a factor of 50/405. They have been included in the report for extra information but the experiment was repeated.

None of these concentrations caused either the root length or shoot length to fall below 75% of the maximum growth.

Graphs: Garden Cress and Dicamba – 2nd Trial (commercial kit components)


Trial 2 - ETP: Dicamba - Garden Cress Shoot
Data
35
30
25
20
15
10
5
C1 - G-TW
C3 - G-ETP
1D 1.6 - G
1D 6.5 - G
1D 15 - G
1D 45 - G
1D 90 - G
1D 405 - G


Trial 2 - ETP: Dicamba - Garden Cress Root
Data
70
60
50
40
30
20
10
C1 - G-TW
C3 - G-ETP
1D 1.6 - G
1D 6.5 - G
1D 15 - G
1D 45 - G
1D 90 - G
1D 405 - G

These results showed that even at maximum concentration of 50 µg/L of Dicamba added, the growth was not affected sufficiently to go below the 75th Percentile value for root, shoot and total seed length.

Trial 2 – Radish Seed and Dicamba

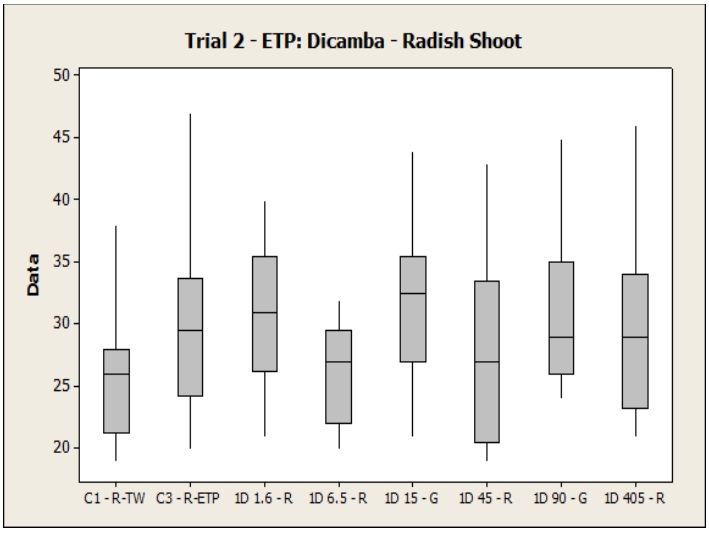
Table 11: Results for radish seed and Dicamba with ETP

Dicamba ug/L	0	1.6	6.5	15	45	90	405
Corrected conc (µg/L)	0	0.2	0.8	1.9	5.2*	10.4	50
Log Conc	0	-0.7	-0.1	0.28	0.72	1.02	1.7
Average Root	69	68	64	67	70	73	63
Std Dev Root	14	16	8	15	17	10	11
Upper Limit	83	84	73	82	88	84	74
Lower Limit	55	52	56	52	53	63	53
Average Shoot	27	27	24	28	25	27	26
Std Dev Shoot	10	6	9	11	9	8	9
Upper Limit	36	33	33	38	34	35	36
Lower Limit	17	21	16	17	16	19	17
Average Seed Total	95	95	88	94	95	100	90
St Dev Total	23	15	19	24	23	16	17
Upper Limit	118	110	108	118	118	117	107
Lower Limit	72	81	69	71	73	84	72
Germination %	100	100	100	100	100	100	100
75th Percentile Root	51	51	51	51	51	51	51
75th Percentile Shoot	20	20	20	20	20	20	20
75th Percentile Total	71	71	71	71	71	71	71

*The 45 and 405 µg/L were checked by ALS and it would appear that there had been an error in making up these solutions. Based on the results for the two checked solutions, all others have been corrected by a factor of

50/405. They have been included in the report for extra information but the experiment was repeated.

Graphs Effect of Dicamba on Radish Seed



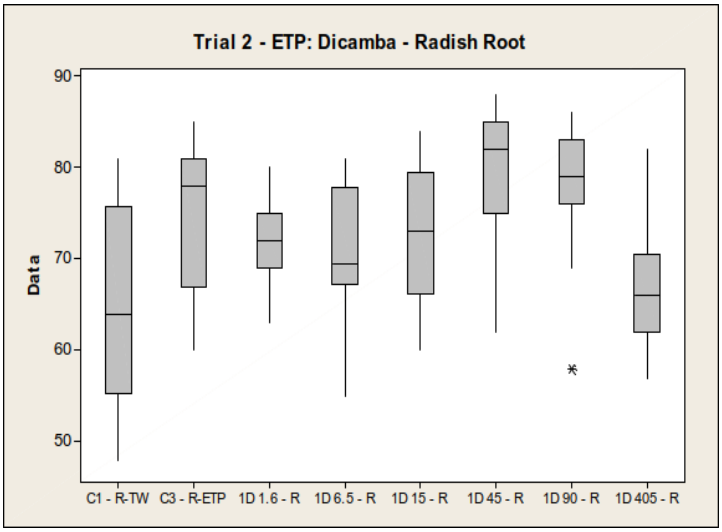


Table 12: Garden Cress and MCPA using ETP recycled water

MCPA ug/L	0	3	10	25	75	224	675
Corrected Conc	0	3.7	12.4	31	93	277	1200
Log Conc	0	0.57	1.09	1.49	1.97	2.44	3.08
Average Root	30	22	14	6	6	4	4
Std Dev Root	10	11	6	2	5	1	1
Upper Limit	40	34	20	9	11	5	5
Lower Limit	21	11	8	4	1	2	3
Average Shoot	17	19	19	13	12	4	0
Std Dev Shoot	4	6	8	3	4	2	0
Upper Limit	21	26	27	17	16	6	0
Lower Limit	12	13	11	10	9	1	0
Average Seed Total	46	42	32	20	18	4	4
St Dev Total	14	16	13	4	8	5	1
Upper Limit	60	58	45	24	26	9	5
Lower Limit	32	25	20	15	9	-1	3
Germination %	85	95	95	90	90	50	10
75th Percentile Root	23	23	23	23	23	23	23

75th percentile Shoot	12	12	12	12	12	12	12
75th percentile Total	35	35	35	35	35	35	35

*The 75 and 675 µg/L were checked by ALS and it would appear that there had been an error in making up these solutions. Based on the results for the two checked solutions, all others have been corrected by a factor of 93/75. The problem is in trying to prepare the standards from the acid form which has a very low solubility. They have been included in the report for extra information but the experiment was repeated later in trial 5 using the commercial formula.

Graphs Effect of MCPA on Garden Cress

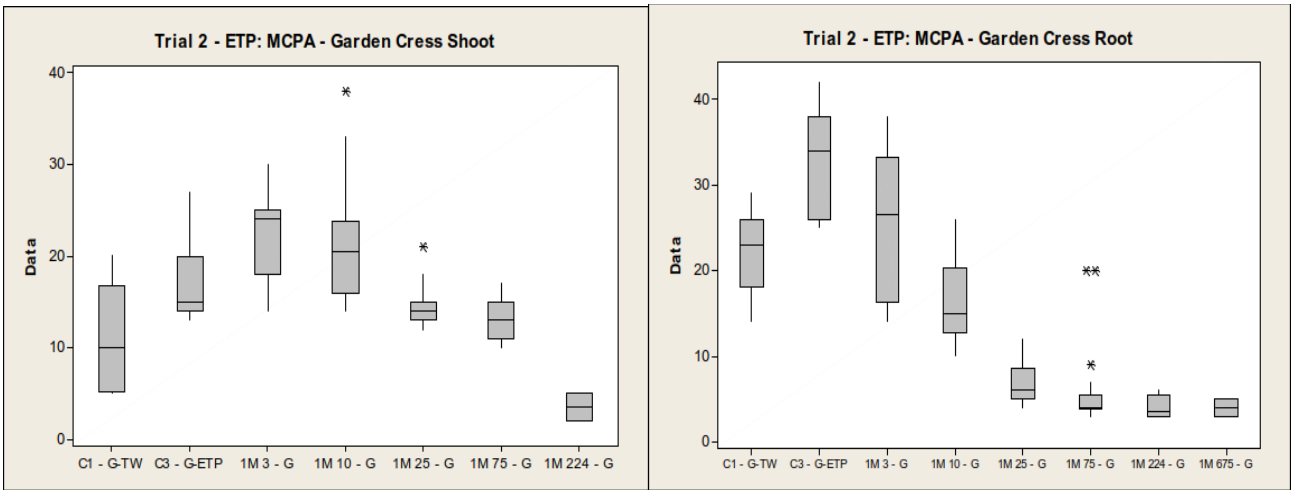
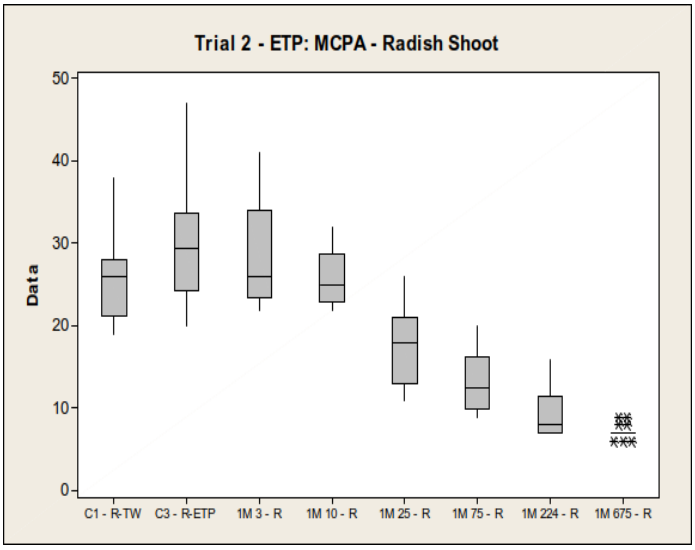


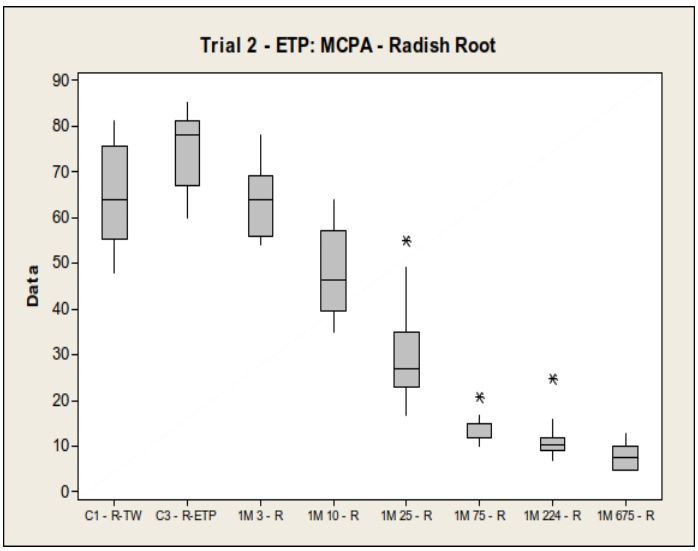
Table 13: Radish and MCPA using ETP

MCPA ug/L	0	3	10	25	75	224	675
Corrected Conc	0	3.7	12.4	31	93	277	1200
Log Conc	0	0.57	1.09	1.49	1.97	2.44	3.08
Average Root	69	57	44	26	13	10	7
Std Dev Root	14	16	12	12	4	5	3
Upper Limit	83	73	56	38	17	15	10

Lower Limit	55	41	32	14	9	6	5
Average Shoot	27	27	24	15	12	9	7
Std Dev Shoot	10	8	11	6	4	3	1
Upper Limit	36	35	35	21	16	12	8
Lower Limit	17	19	14	9	8	6	6
Average Seed Total	95	84	68	41	23	19	14
St Dev Total	23	20	19	15	8	7	3
Upper Limit	118	103	87	56	32	26	17
Lower Limit	72	64	49	26	15	12	12
Germination %	100	100	100	100	100	100	100
75th Percentile Root	51	51	51	51	51	51	51
75th Percentile Shoot	20	20	20	20	20	20	20
75th Percentile Total	71	71	71	71	71	71	71

Graphs Effect of MCPA on Radish using ETP





Trial 3: Dicamba at high concentrations with Radish and MCPA with "Grand Slam" lettuce seeds

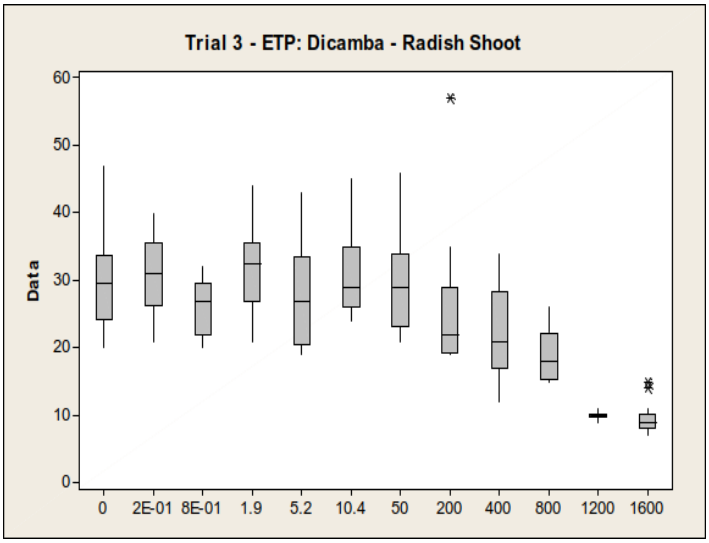
Table 14: Radish with added excessive Dicamba concentrations of 200µg/L, 400µg/L, 800µg/L, 1200µg/L and 1600µg/L

Dicamba ug/L	0	1.6*	6.5*	15*	45*	90*	200	400	405*	800	1200	1600
Corrected Conc	0	0.2	0.8	1.9	5.2	10.4			50			
Log Conc	0	-0.7	-0.1	0.28	0.72	1.02	2.3	2.6	1.7	2.9	3.08	3.2
Average Root	69	68	64	67	70	73	46	52	63	31	30	22
St dev Root	14	16	8	15	17	10	13	17	11	15	12	7
Upper Limit	83	84	73	82	88	84	59	69	74	46	42	30
Lower Limit	55	52	56	52	53	63	33	35	53	15	17	15
Average Shoot	27	27	24	28	25	27	21	21	26	13	9	9
St Dev Shoot	10	6	9	11	9	8	11	8	9	6	2	2
Upper Limit	36	33	33	38	34	35	32	29	36	19	11	11
Lower Limit	17	21	16	17	16	19	10	13	17	8	6	7
Average Total seed	95	95	88	94	95	100	67	73	90	44	38	32
St Dev seed	23	15	19	24	23	16	18	22	17	18	13	8
Upper Limit	118	110	108	118	118	117	85	95	107	62	51	40
Lower Limit	72	81	69	71	73	84	50	51	72	26	26	24
Germination %	100	100	100	100	100	100	95	100	100	100	100	95
75th	51	51	51	51	51	51	51	51	51	51	51	51

Percentile Root												
75th Percentile Shoot	20	20	20	20	20	20	20	20	20	20	20	20
75th Percentile Total	71	71	71	71	71	71	71	71	71	71	71	71

*The 45 and 405 µg/L were checked by ALS and it would appear that there had been an error in making up these solutions. Based on the results for the two checked solutions, all others have been corrected by a factor of 50/405. They have been included in the report for extra information but the experiment was repeated. All unadjusted solutions were part of a consequent trial which had no solutions analysed by ALS.

Graphs Effect of Excessive Dicamba on Radish Seeds – two trials combined as per the data table on prior page



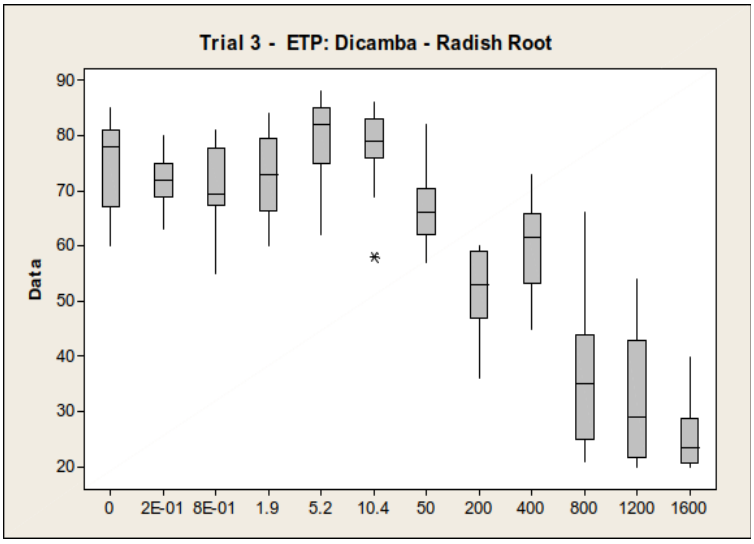
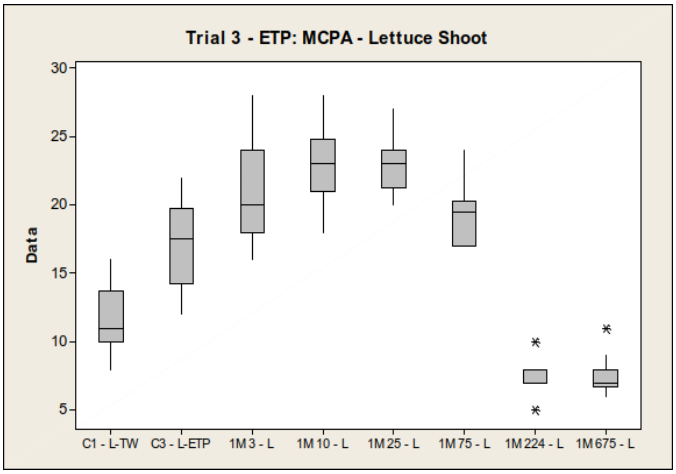
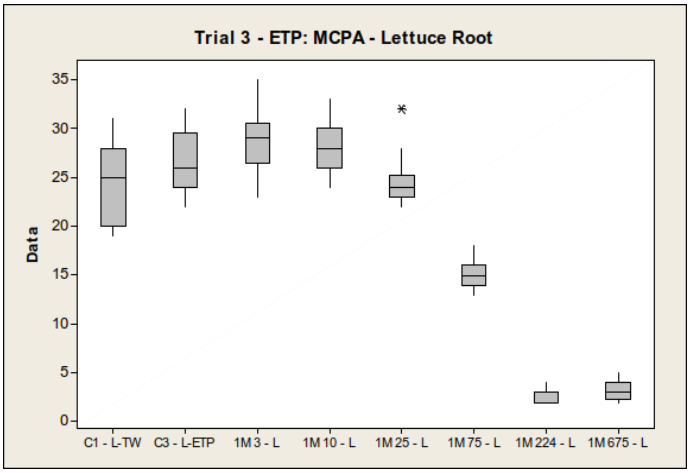


Table 15: "Grand Slam" lettuce seeds, coated, and MCPA with ETP recycled water

MCPA ug/L	0	3	10	25	75	224	675
Log Conc	0	0.48	1	1.4	1.88	2.35	2.83
Average Root	26	27	27	24	15	3	3
Std Dev Root	4	5	3	3	2	1	1
Upper Limit	30	32	30	27	16	3	4
Lower Limit	22	22	23	21	13	2	2
Average Shoot	15	19	21	21	19	7	7
Std Dev Shoot	5	5	5	4	3	2	2
Upper Limit	20	24	26	25	21	8	9
Lower Limit	10	14	16	17	16	5	5
Average Seed Total	39	46	47	45	33	9	10
St Dev Total	11	8	7	6	3	3	2
Upper Limit	50	54	55	51	37	12	12
Lower Limit	29	38	40	40	30	6	8
Germination %	95	95	95	100	100	95	100
75th Percentile Root	19	19	19	19	19	19	19
75th Percentile Shoot	11	11	11	11	11	11	11
75th Percentile Total	30	30	30	30	30	30	30

Graphs Effect of MCPA on “Grand Slam” lettuce seeds

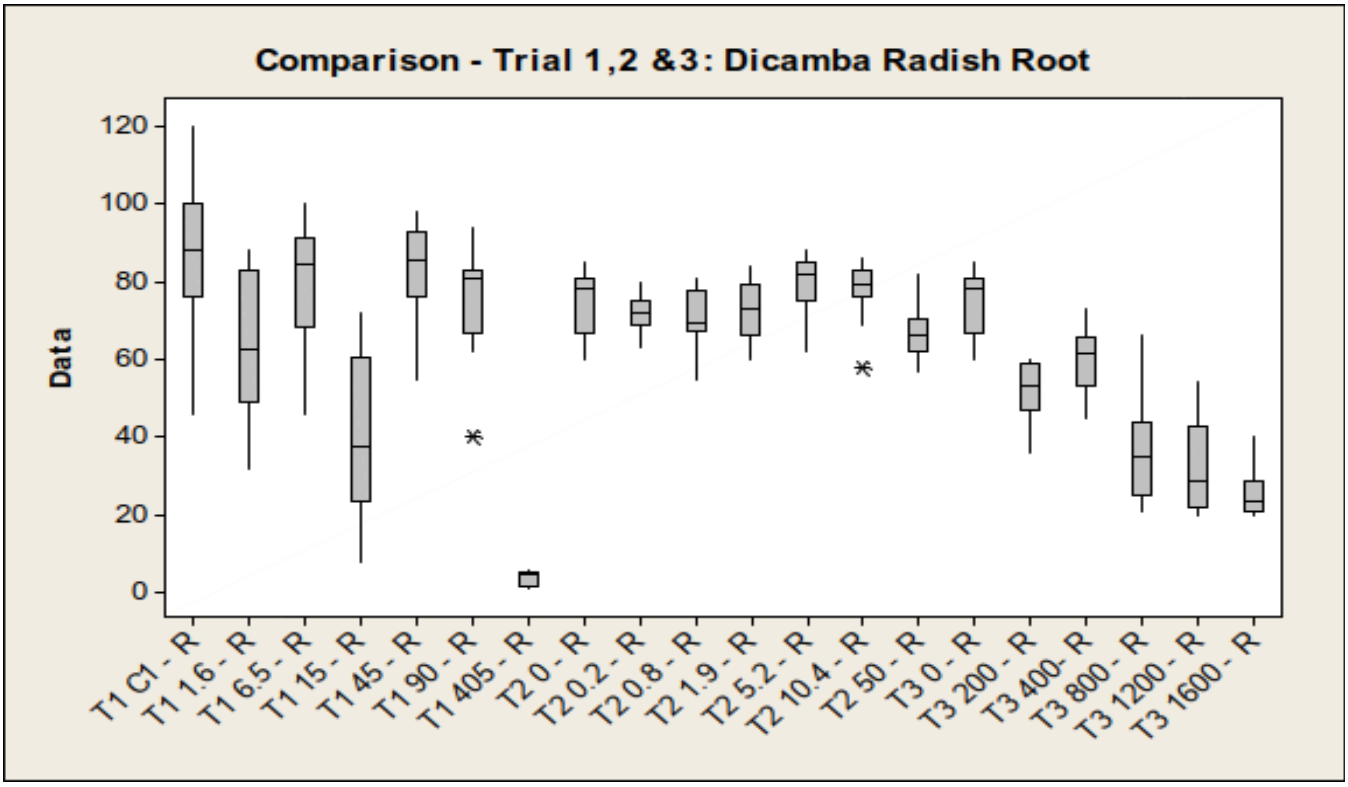


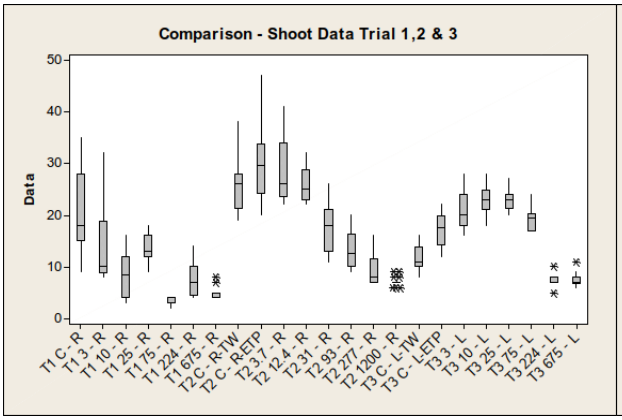


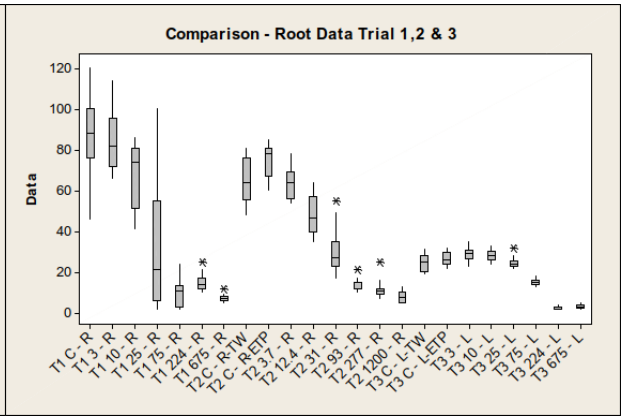
Comparison data between Trials 1, 2 and 3

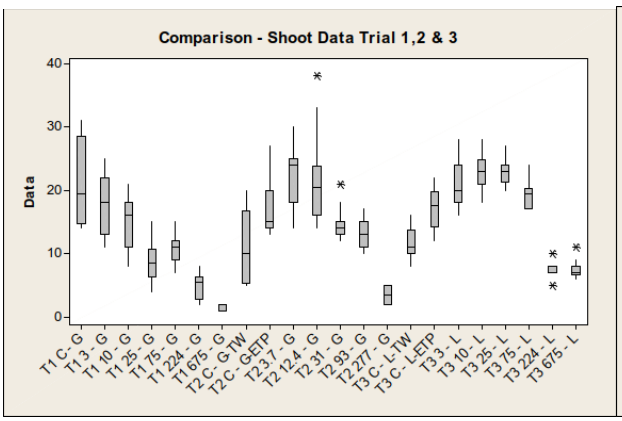
The comparison data is separated into the two herbicides, Dicamba and MCPA.

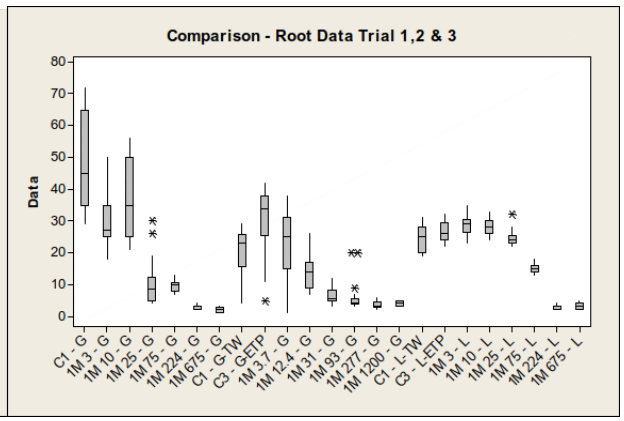
The purpose of the comparisons was to see if any trends can be identified. As only Trial 2's solutions were tested by ALS, there has to be an assumption that either the other trials solutions were at the correct level or should be corrected according to the analytical results. As each of the trial solutions were freshly prepared, a link would have to be shown between the analysed solutions and the other solutions by comparing the results and possible effect of dicamba on the seeds. Another desirable outcome of the comparisons was to see if there are any obvious trends between the three trials as they all had differences. Trial 1 has the in-house produced kit components which compromised the consistency of the kit conditions and introduced variables such as the kit drying out part way through the incubation. Trial 2 had significantly lower concentrations according to the external laboratory analyses and had all the concentrations corrected accordingly. The third trial was in response to the lack of a response to the dicamba and was designed to increase the concentrations to 4 times the highest level used throughout the Bioassay experiments. The concentrations ranged from 200µg/L to 1600µg/L.


Comparison - Trial 1,2 &3: Dicamba Radish Root
Data
120
100
80
60
40
20
0
T1 C1 - R
T1 1.6 - R
T1 6.5 - R
T1 15 - R
T1 45 - R
T1 90 - R
T1 405 - R
T2 0 - R
T2 0.2 - R
T2 0.8 - R
T2 1.9 - R
T2 5.2 - R
T2 10.4 - R
T2 50 - R
T3 0 - R
T3 200 - R
T3 400 - R
T3 800 - R
T3 1200 - R
T3 1600 - R


Comparison - Shoot Data Trial 1,2 & 3
Data
50
40
30
20
10
0
T1 C - R
T1 3 - R
T1 10 - R
T1 25 - R
T1 75 - R
T1 224 - R
T1 675 - R
T2 C - R-TW
T2 C - R-ETP
T2 3.7 - R
T2 12.4 - R
T2 31 - R
T2 93 - R
T2 277 - R
T2 1200 - R
T3 C - L-TW
T3 C - L-ETP
T3 3 - L
T3 10 - L
T3 25 - L
T3 75 - L
T3 224 - L
T3 675 - L


Comparison - Root Data Trial 1,2 & 3
Data
120
100
80
60
40
20
0
T1 C - R
T1 3 - R
T1 10 - R
T1 25 - R
T1 75 - R
T1 224 - R
T1 675 - R
T2 C - R-TW
T2 C - R-ETP
T2 3.7 - R
T2 12.4 - R
T2 31 - R
T2 93 - R
T2 277 - R
T2 1200 - R
T3 C - L-TW
T3 C - L-ETP
T3 3 - L
T3 10 - L
T3 25 - L
T3 75 - L
T3 224 - L
T3 675 - L





There is a striking similarity between the behaviour of garden cress and radish with the radish growing more strongly. There is a clear change in response between 12 and 25 µg/L of MCPA. In the case of dicamba there is a response between 200 and 400 µg/L.

Trial 4 and 5 Effect of MCPA with WTP water

The aim of these experiments was to assess the effect of MCPA with Western Treatment Plant recycled water (WTP) using average root, shoot and total seed length to determine the EC25 and to determine the minimum time needed in order to conduct an incubation experiment using radish only.

Method

Bioassay Trial	Seeds used	Herbicides used	Recycled Water Used	Incubation Period
4 – 16/11/2012	Radish	MCPA	WTP	48 Hours
5 – 02/02/2013	Radish	Dicamba, MCPA	WTP	60 Hours

For Bioassay Trial 4, the following variations to the method applied:

WTP recycled water used and the incubation period was 48 hours only. The experiment was started at 9.30 am and then observed for growth at 4pm on the first day, then again at 9am and 4pm the second day. The kits were then removed and the roots and shoots were measured at 9.30am on the following day after completing the total of 48-hour incubation period.

For the Bioasssay trial 5 the following variation was applied to the preparation of the solutions. All solutions with the various concentrations of Dicamba and MCPA using the soluble ester of MCPA and diluting with WPT water rather than MilliQ water. This was done to overcome any potential matrix issues that may arise due to the large variance between the stock solution and water ratios as the concentrations increase.

Observations and results:

To not disturb the kit assemblies, only visual assessments were made at each observation, backed by photographs.

14/11/12 – 4pm – no visible shoot growth
15/11/12 – 9am - no significant growth
15/11/12 – 4pm – no visible effect on growth at that stage

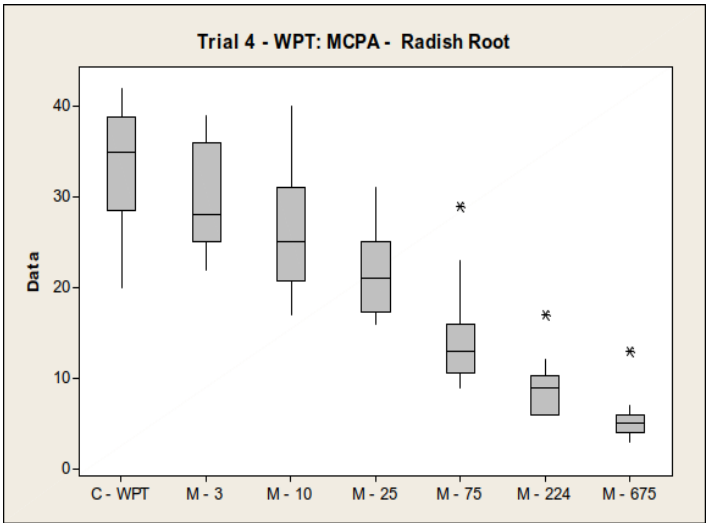
16/11/12 – 10.00am – All kits removed and measured. Effects of MCPA and Dicamba now visually observable.

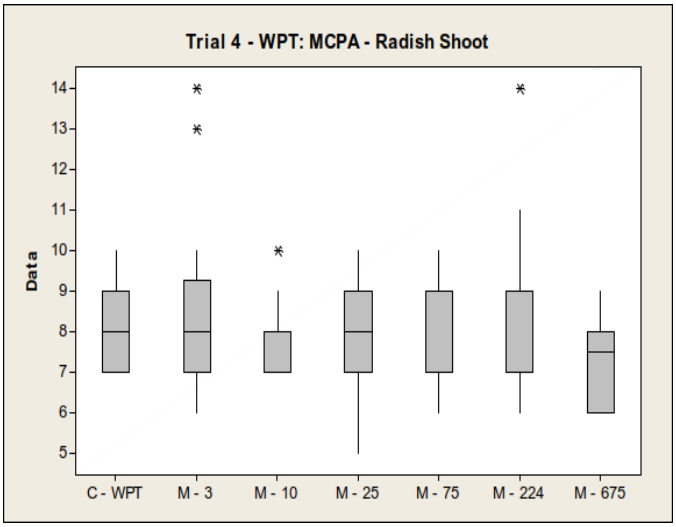
Trial 4 – Radish, with MCPA and WTP recycled water with timed observations over 36 hours to identify minimum incubation time required for visible effects

Table 16: Trial 4 - MCPA and Radish seed with WTP

MCPA ug/L	0	3	10	25	75	224	675
Log Conc	0	0.48	1	1.4	1.88	2.35	2.83
Average Root	29	27	23	19	13	8	5
Std Dev Root	10	7	10	6	6	3	2
Upper Limit	38	35	32	26	19	12	7
Lower Limit	19	20	13	13	8	5	3
Average Shoot	8	8	7	7	7	7	7
Std Dev Shoot	2	2	1	2	2	2	1
Upper Limit	11	10	8	9	9	10	8
Lower Limit	6	6	6	5	4	5	6
Average Seed	36	35	30	26	20	16	12

Total							
St Dev Total	11	8	10	8	6	4	2
Upper Limit	47	44	40	34	26	20	14
Lower Limit	26	27	20	18	14	12	10
Germination %	98	100	19	100	100	100	100
75th Percentile Root	21	21	21	21	21	21	21
75th Percentile Shoot	6	6	6	6	6	6	6
75th Percentile Total	27	27	27	27	27	27	27





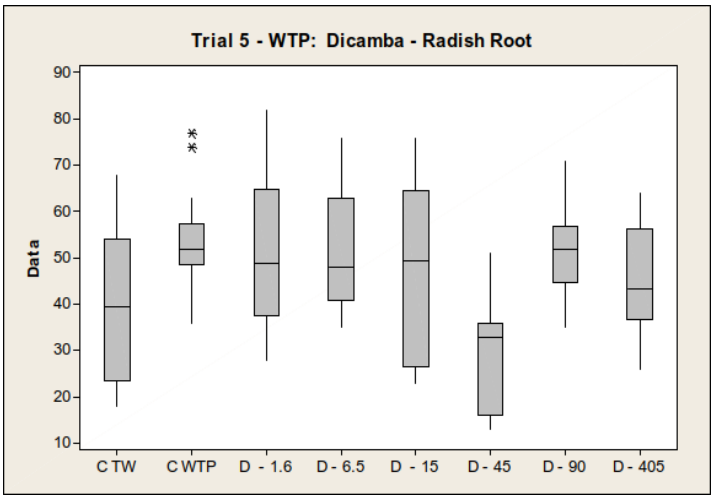
Trial 5: Dicamb and MCPA with Radish Seeds using WTP

Table 17: Trial 5 - Dicamba and Radish seed using WTP

Dicamba ug/L	0	1.6	6.5	15	45	90	405
Corrected conc	0	2.99	8.67	18.51	53.23	105.33	469.97
Log Conc	0	0.48	0.94	1.27	1.73	2.02	2.67
Average Root	50	49.8	45	43.1	26.1	42.4	41.1
St dev Root	15.3	19.1	18	22	15.9	16.9	14.5
Upper Limit	65.3	68.9	63	65.1	42	59.3	55.5
Lower Limit	34.8	30.7	26.9	21.1	10.2	25.4	26.6
Average Shoot	24.2	27.3	27.4	27.5	18.2	25	17.8
St Dev Shoot	10.6	9.7	7.9	8.3	8.9	9.4	6.7
Upper Limit	34.7	37	35.3	35.8	27	34.4	24.5
Lower Limit	13.6	17.6	19.5	19.2	9.3	15.6	11.1
Average Total	73	77.1	72.3	70.6	44.3	67.4	58
St Dev seed	23.6	26.4	22.8	28.3	22.2	23.9	19.3
Upper Limit	96.6	103.5	95.1	98.9	66.5	91.3	77.3
Lower Limit	49.3	50.7	49.5	42.3	22.1	43.4	38.6
Germination %	100	100	100	100	100	100	100
75th Percentile	37.5	37.5	37.5	37.5	37.5	37.5	37.5

75th Percentile	18.1	18.1	18.1	18.1	18.1	18.1	18.1
75th Percentile	54.7	54.7	54.7	54.7	54.7	54.7	54.7

Dicamba secondary stock was analysed by ALS and found to be 580µg/L. The WPT had a pre-existing concentration of Dicamba of 1.2µg/L. The correction to the concentration took those two results into consideration.



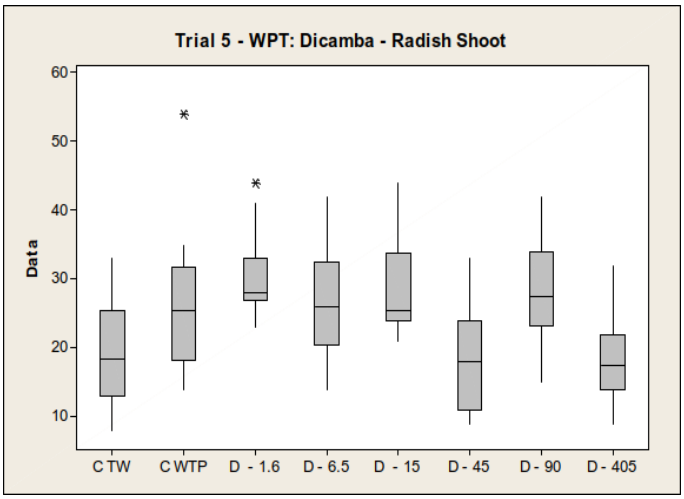
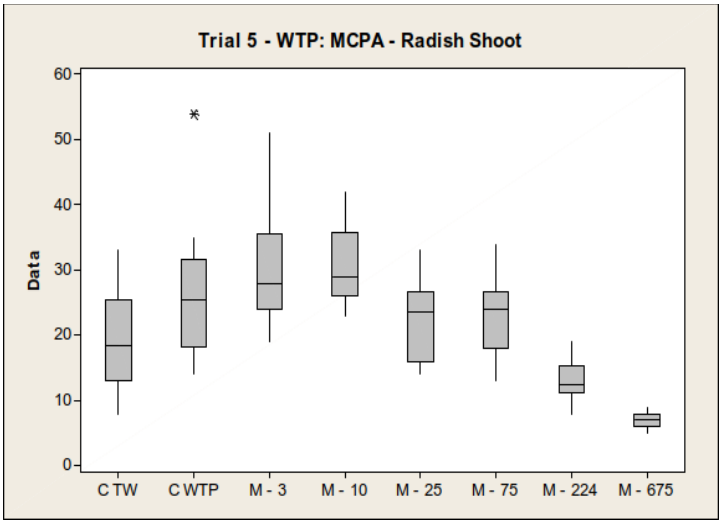
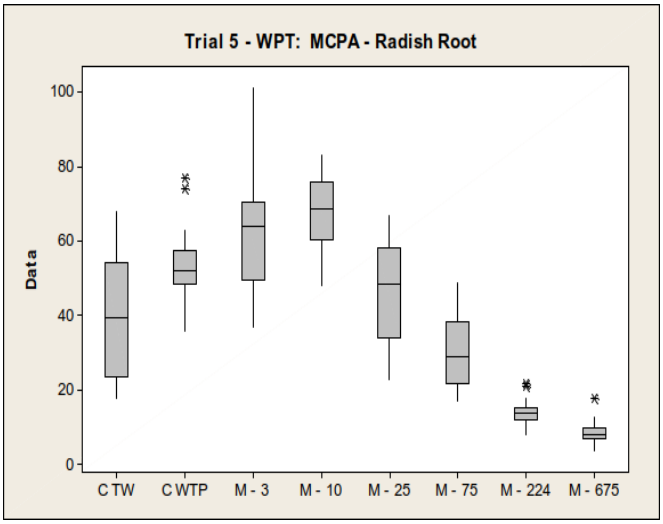


Table18: Trial 5 - MCPA and Radish Seed with WTP

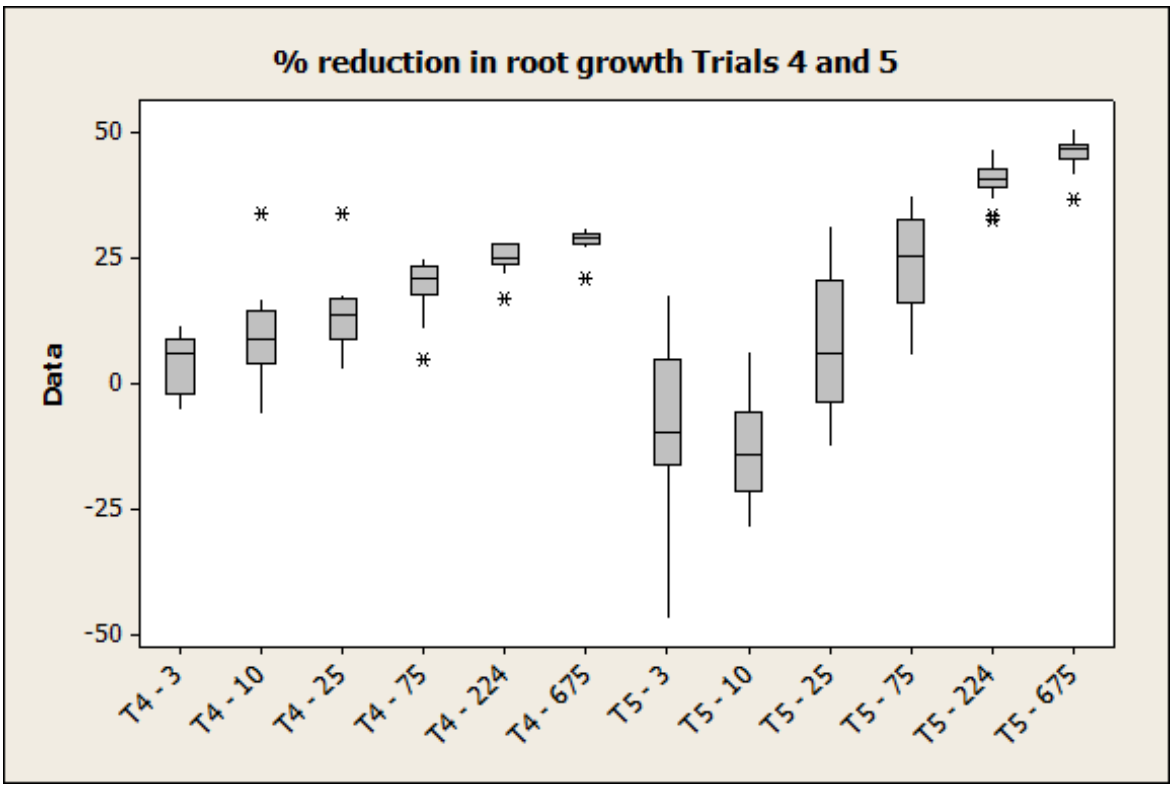
MCPA ug/L	0	3	10	25	75	224	675
Log Conc	0	0.48	1	1.4	1.88	2.35	2.83
Average Root	5	6	6	5	3	1	1
Std Dev Root	2	3	2	2	1	0	0
Upper Limit	7	9	8	6	4	2	1
Lower Limit	4	3	4	3	2	1	1
Average Shoot	2	3	3	2	2	1	1
Std Dev Shoot	1	1	1	1	1	0	0
Upper Limit	3	4	4	3	3	2	1
Lower Limit	1	2	2	1	1	1	1
Average Total	7	9	9	7	5	2	2
Std Dev Total	2	3	3	2	2	1	0
Upper Limit	10	11	12	9	6	3	2
Lower Limit	5	6	5	5	3	2	1
Germination %	100	100	100	100	100	95	100
75th Percentile	4	4	4	4	4	4	4
75th Percentile	2	2	2	2	2	2	2
75th Percentile	5.5	5.5	5.5	5.5	5.5	5.5	5.5


Trial 5 - WTP: MCPA - Radish Shoot
Data
60
50
40
30
20
10
0
C TW
C WTP
M - 3
M - 10
M - 25
M - 75
M - 224
M - 675


Trial 5 - WPT: MCPA - Radish Root
Data
100
80
60
40
20
0
C TW
C WTP
M - 3
M - 10
M - 25
M - 75
M - 224
M - 675

Comparison between trials 4 and 5 using dicamba

In the figure below the % reduction in root was determined for each of the two trials using MCPA. They show the same pattern with root growth being affected at about 25 µg/L.



Conclusions

- Radish is the most sensitive seed to use in these experiments as it is fast growing and has a consistently high percentage germination
- The root was a more sensitive indicator than the shoot
- These tests were not sensitive enough to show any effect of dicamba until the concentration reached over 200 µg/L
- The tests did show sensitivity to MCPA at concentrations down to 3 µg/L