Department of Environment and Geography

University of York

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Exam Number: Y3864317	**Module Code:** ENV00047M
Module Title: Ecotoxicology	**Assessment Deadline:** 26th March 2019

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ASSESSING THE TOXICITY OF CHLORTOLURON ON THE GROWTH RATE OF LEMNA MINOR

ABSTRACT

As herbicides becoming more widely used, their effect on aquatic environments needs to be studied in more detail. In this study *Lemna minor* was assessed on 0 days and 6 days to see if Chlortoluron had inhibited the *Lemna minor's* ability to grow.

For growth to be assessed, OEDC guidelines recommend assessing two variables: area of *Lemna minor* and the number of fronds.
The % of inhibition to growth was obtained by assessing area and frond number through set equations, whilst EC50 was obtained using R studio.

Analysis revealed that % of inhibition of growth increased as the concentration of herbicide increased. EC50 values suggested that the concentrations at which 50% of the growth of the *Lemna minor* is inhibited is 67.23μg l^{-1} (area) and 133.75μg l^{-1} (fronds), suggesting that Chlortoluron above these concentrations would cause considerable harm to the environment

Although a relationship was found between growth and concentrations there were several limitations to the study including algae contamination which has the potential to offset all data and results. Therefore, further studies similar to this are needed to ensure that the results collected are significant and reliable.

1. INTRODUCTION

Aquatic environments are becoming increasingly polluted due to anthropogenic activities (Moiseenko, 2008; Vorosmarty *et al.,* 2010), such as modern agriculture (Schwarenbach *et al.,* 2006; Valiente-Moro *et al.,* 2012).

A growing human population has increased agrochemical application, as there's an increased need for improved crop yields and protection of crops from pests to ensure food security (Jurado *et al.,* 2011). This increased use means that globally 140 billion kilograms of agrochemicals are now annually applied to crops (Schwarenbach *et al.,* 2006).

Agrochemicals are harmful to the environment and enter water bodies directly through run off, leaching and spray drift during the application process (Kloeppel *et al.,* 1997; Mensah *et al.,* 2013). Its been well documented that agrochemicals can have adverse effects on aquatic environments (He *et al.,* 2005; Boran *et al.,* 2007). The dispersion of agrochemicals into the environment has lead to the deaths of birds, fish and small mammals (Paoli *et al.,* 2015), as well as aquatic plants (Cedergreen & Streibig, 2005).

Aquatic plants play a vital role in aquatic ecosystems, as their presence effects flow velocity, nutrient uptake (Sand-Jensen & Krause-Jensen, 1997), acts as refuge for fish and providing a range of food webs (Wetzel, 1983), making aquatic plants essential for the correct functioning of aquatic ecosystems (Wetzel, 1983).

To minimize the effects that agrochemicals can have on aquatic environments, studies need to take place which assess the concentrations of agrochemicals and their inhibition of growth rate and also by assessing the EC50 value which finds the concentration which causes 50% reduction in plant growth (ChemSafetyPro, 2019).

Based on the knowledge that agrochemicals effect aquatic plant growth, the aim of this study is to determine the percentage inhibition of growth rate of *Lemna Minor* and EC50 values based on different concentrations of herbicide.

Although several aquatic plants could be used to assess inhibition of growth rate, *Lemna minor* is been assessed as it has a high tolerance to polluted water, is globally distributed (Landolt, 1986) and is often used in chemical toxicity tests (Lahive *et al.,* 2011; Ziegler *et al.,* 2016). Its also important that an aquatic plant is used for the purpose of this study as the herbicide, Chlortoluron, works by inhibiting photosynthesis (Valiente-Moro *et al*., 2012).

2. METHODS

2.1 LABORATORY ANALYSIS

To assess the growth rate at different concentrations on Chlortoluron different concentration solutions needed to be made. From the stock solution of 100mg l^{-1} chlortoluron in methanol, standard solutions of chlortoluron in growth media were created to give the concentrations 10, 30, 60, 150, 300μg l^{-1}. 15ml of each concentration was put in individual petri dishes, with duplicate dishes being created for each concentration. Within all individual dish, 2-3 colonies of *Lemna Minor,* was added. Duplicate control dishes were also created, which contained Gambourgs growth media and colonies of *Lemna Minor*. When the dishes had been prepared, photographs were taken of each sample. pH of each sample was also taken and recorded using an Acumat pH meter.

The samples spent 6 days in a Sanyo Versatile Environmental Test Chamber at 20^0c in 24-hour light before been analysed by measuring pH, counting the number of fronds present in each sample and then being photographed again.

2.2 IMAGE ANALYSIS

The area of *Lemna minor* was assessed to establish if growth had been inhibited. The area of colonies was determined for the first set of photographs and the second set taken after the dishes had spent 6 days in a plant growth cabinet. The area of the colonies was determined using image analysis software Image J. Full details of the steps taken to determine the area of the colonies can be seen in **Appendix A**.

2.3 DATA ANALYSIS

A variety of different data analysis was used to determine the effect of Chlortoluron. This included an equation to assess the inhibition of growth (%) and growth rate. When growth rate had been determined the values were then inputted into the Inhibition of growth equation. Full detail on the equations used can be seen in **Appendix B**.

EC50 values were also determined using concentration response relationship curves using R studio. For the R code on how the concentration response relationships and the EC50 values were determined see **Appendix C.**

3. RESULTS

From the raw data in **Appendix D** a variety of significant results have been obtained.

3.1 INHIBITION OF GROWTH

Inhibition of growth increased as the concentration of herbicide increased, with this increase being observed for both fronds and area. Although an increase was seen for both factors, the percentage of inhibition based on fronds was relatively smaller than that of area (**Table 1**). The highest inhibition of growth was observed at the highest concentrations and the lowest inhibition of growth observed at 10 μg l^{-1}. There was little variation between the samples with the same concentration, however, variation was seen for the concentration of 60μg l^{-1}, as one sample of 60μg l^{-1} was 28.17% (fronds) and 57% (area), whilst the other 60μg l^{-1} sample was 12.21% (fronds) and 21.5% (area).

Table 1. The % inhibition of growth based on the number of fronds and the area of *Lemna minor* for different concentrations

Concentration	% inhibition based on fronds	% inhibition based on area
10	2.24	14
10	5.73	13.2
30	8.72	16.5
30	19.7	22.3
60	28.17	57
60	12.21	21.5
150	51.12	83.4
150	52.12	91
300	69.57	94.2
300	93.02	98.6

3.2 EC50 VALUES

The inhibition of growth (area) versus concentrations in **Figure 1** show that inhibition is either a low percentage or high percentage with only one value being between 30 and 80%. In **Figure 2** for the inhibition of growth (frond) its observed that there are fewer percentages of inhibitions in the upper quartile compared to **Figure 1.** The inhibition of fronds revealed a EC50 value of 133.75, whilst the percentage inhibition of area revealed a EC50 value of 67.23, suggesting that these are the concentrations where 50% of the *Lemna minor* growth is restricted.

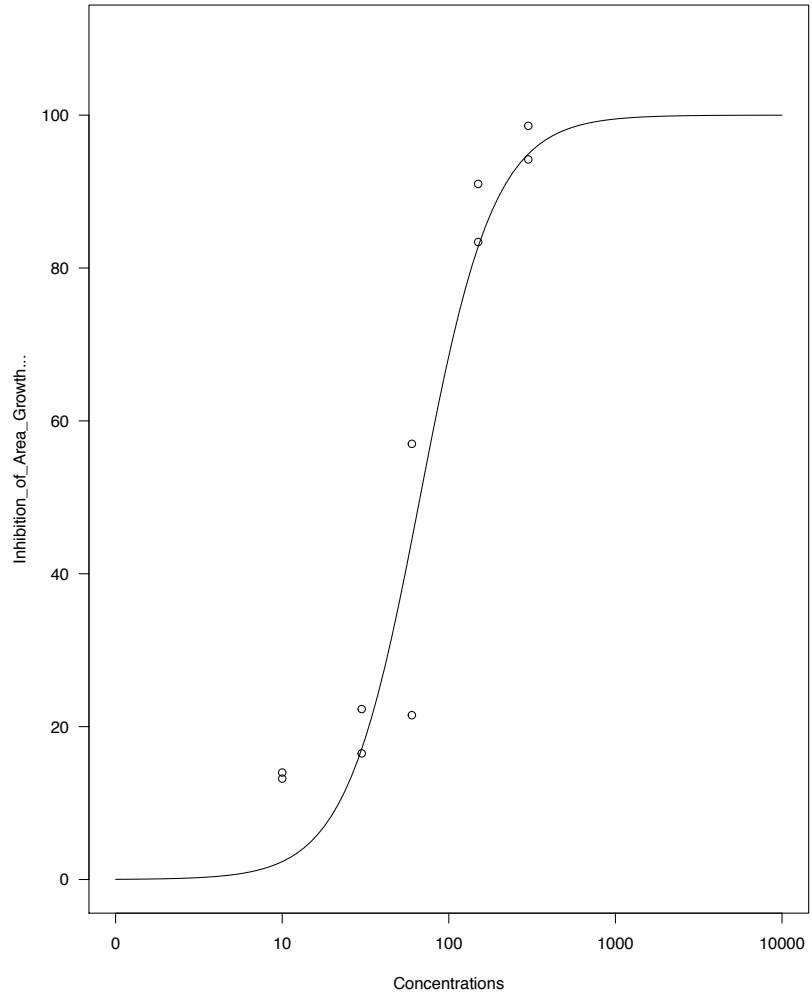

Figure 1. Concentration response relationship curve of % inhibition of growth based on area and concentrations.

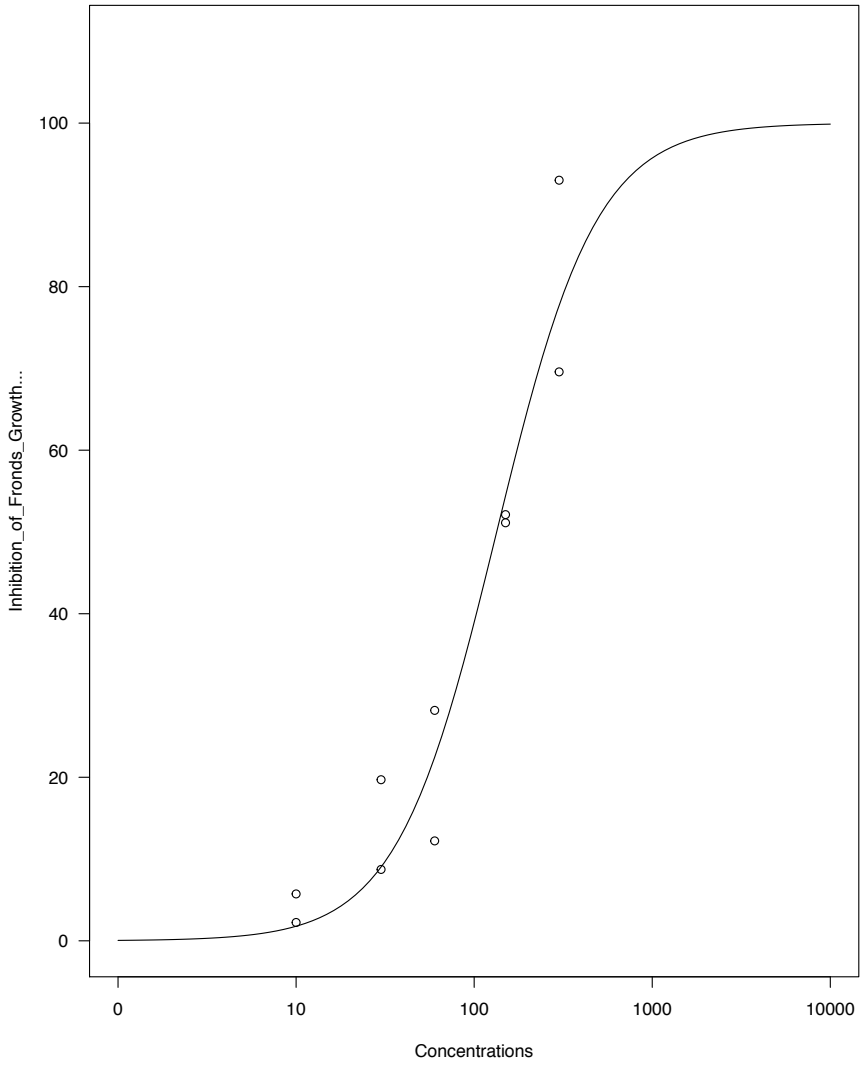

Figure 2. Concentration response relationship curve of % inhibition of growth based on number of fronds and concentration.

3.3 PH

When pH was tested after 6 days it was observed that pH had become more acidic for all concentrations including the control samples (**Figure 3**). There's little deviation of pH for different concentrations, with all concentrations having similar pH values to the control samples.

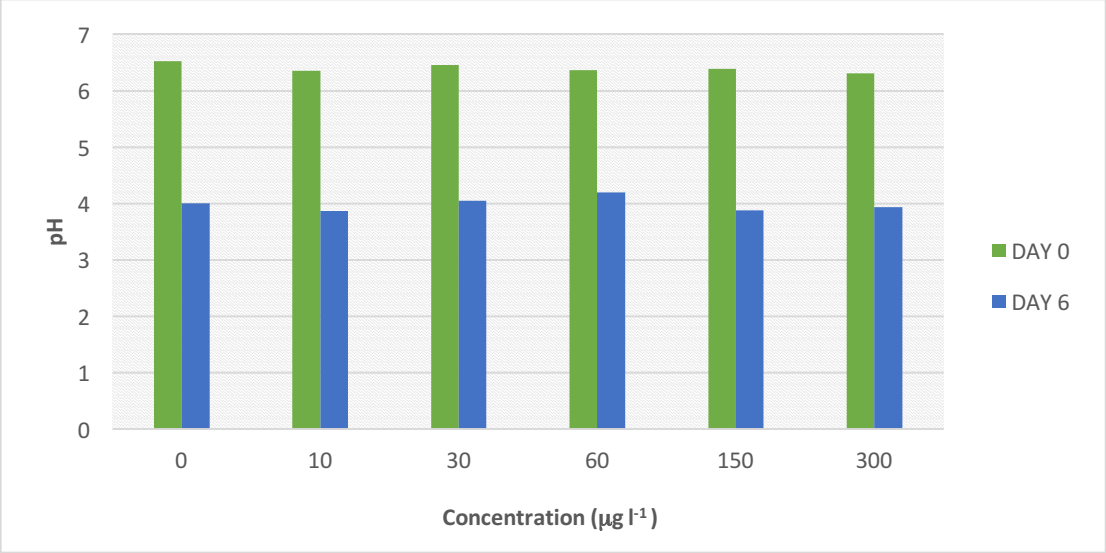

Figure 3. A bar graph to show pH levels at all concentrations at 0 days and after 6 days.

3.4 OBSERVATIONS

After 6 days it was observed that several samples including one of the 60µg l^{-1} had been contaminated by algae. This has the potential to effect the growth of the *Lemna minor*, as the other 60µg l^{-1} sample did not have the same growth limitations. There was also seen to be some discoloration in the samples which had been subject to high concentrations. This may be a result of the herbicide or the presence of algae.

4. DISCUSSION

Herbicides are widely used and can pose as a threat to aquatic environments, therefore its essential for tests to be carried out so environmentalists can be aware of the potential harm and offer guidance to those using these chemicals.

4.1 EC50 VALUES OF DIFFERENT HERBICIDES

EC50 aims to determine the concentration at which 50% of growth of a plant is hindered. Based on the OECD guidelines (2006) two different variables should be tested as some variables are often more effected by substances than others. The variables used in this study differed in EC50 values, however they're both suitable variables to be considered, therefore both values of 133.75µg l^{-1} (fronds) and 67.23µg l^{-1} (area) are valid. However, according to the OECD guidelines (2006), variables such

as area can be more sensitive to substances, suggesting that the lower value should be used for management and application purposes of Chlortoluron to prevent harm.

Based on EC50 of other herbicides, Chlortoluron is middle of the toxicity scale, suggesting that its not highly toxic, but is also not mildly toxic, as its still a relatively low concentration that causes for 50% of the *Lemna Minor* growth to be limited. Park *et al*., (2017) examined different herbicides and their effect to inhibit growth, which found that Diuron had an EC50 value of 24.3μg l^{-1}, suggesting that its a toxic herbicide and lower concentrations are needed to see inhibition of growth. Atrazine was considerably less toxic with EC50 of 342.2μg l^{-1}. When comparing the EC50 values to the study by Park *et al*., (2017), the EC50 values of Chlortoluron are not severely toxic, but are not acute either as EC50 values of 67μg l^{-1} and 133μg l^{-1} were obtained.

Although EC50 values vary for different herbicides, this may be influenced by the herbicides mode of action to restrict growth and its chemical composition (Tu *et al.,* 2001), as herbicides treat different plants species so need a different mode of action (Marin-Morales *et al.,* 2013; Armstrong, n.d.).

4.2 PERCENTAGE OF INHIBITION OF *LEMNA MINOR*

Percentage inhibition of growth of *Lemna Minor* differed based on the concentration of Chlortoluron that was present. As the concentration increased the inhibition of growth based on frond number and area also increased, suggesting that the herbicide effected the *Lemna minor* ability to grow. Limitations to growth at higher concentrations will have been visible as Chlortoluron kills plants through inhibiting photosynthesis of the plant (Valiente-Moro *et al.,* 2012). Although clear inhibition to growth was seen at high concentrations, some herbicides at low concentrations don't limit growth straight away and develop over a long period of time. As some inhibition of growth was observed at lower concentrations, Chlortoluron may reduce the lifespan of the *Lemna Minor* in the long run (Nehls & Segner, 2001).

Inhibition to growth at 60 μg l^{-1} varied between the two samples, however this was a result of contamination of algae in one of the samples. Algae competes with *Lemna minor* and causes for the number of fronds and area to decrease (Roijackers *et al.,* 2004), thus causing a higher percentage of inhibition to growth at that concentration.

4.4 ENVIRONMENTAL VARIABLES

pH measured during this study decreased from neutral to acidic after 6 days, but there was no variation between the control samples and the samples containing herbicide, suggesting that the pH was not effected by the herbicide. Although pH values changed this won't have played part in inhibiting the growth of *Lemna Minor*, as its been observed that *Lemna minor* can withstand a wide range of pH (Haung *et al.,* 2007).

4.4 LIMITATIONS

Several limitations occurred, including the contamination of samples which caused discolouration and a reduction in the number of fronds in samples which did not have high concentrations. This has the potential to interfere with the results, meaning that they weren't reliable when producing EC50 values. It also has the potential to interfere with the EC50 values, particularly as the EC50 value for area was 67$\mu g\ l^{-1}$, which may have been a result of one sample at 60$\mu g\ l^{-1}$ having a high % of inhibition to growth. In future studies it would be worth excluding that data from the contaminated sample.

Concentration of the samples was also not tested again and should have been based on OECD guidelines (2006), as the end concentration should be within 80% of the starting concentration.

As there were a variety of irregularities observed during the study, it would be beneficial for the the same study to be completed again so that the results of this study can be tested against those of the new study (Quadir & Rahman, 2014).

5. CONCLUSION

Although there's a variety of limitations that had the potential to cause unreliable results, the overall results have proved to be quite significant. It can be concluded that Chlortoluron does have a relatively high toxicity in comparison to other herbicides, and as a result of this observed toxicity, the concentrations at which it starts to impact upon 50% of the *Lemna minor* should be taken into consideration, particularly if this herbicide is being applied to land near water bodies containing a large variety of aquatic plants.

Word Count: 1947

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APPENDIX A: IMAGE J ANALYSIS STEPS

1. Connect camera to laptop and transfer images to PC
2. Open Image J
3. Import image by selecting *File* then *Open*
4. Convert the image to 8 Bit by selecting *Image*, *Type*, *8-Bit*
5. Convert the image to binary format by selecting *Process*, *Binary*, *Make binary*

(if you photo's are clear, the lemna should now all be coloured black – if not, repeat the binary processing)

6. Set the scale by firstly drawing a line along the ruler image using the *straight line selections* button (e.g. 1 cm), then selecting *Analyse*, *Set scale*. You will then be asked to input the measurement of the line
7. Now draw a line around the Lemna colonies using the *freehand selections* button
8. Determine the area of the colonies by selecting *Analyse*, *Analyse Particles* – you will be asked for an output and select *summary* (you can also select show: outlines to check the analysis makes sense)
9. The software will the provide a total area for the image – note this in your lab books
10. This process should be repeated for each image.

APPENDIX B: EQUATIONS FOR DATA ANASYIS

The inhibition of growth (in %) should then be calculated, using:

$$I = \frac{(Ac - At)}{Ac}.100$$

Where:

Ac = ln (final area of the control) – ln (starting area of the control)

At = ln (final area of the treated Lemna) – ln (starting area of the treated Lemna)

I is the % inhibition in growth

You should also determine the % inhibition based on growth rate. Growth rate is calculated as:

$$GR = \frac{Ln(Nj) - \ln(Ni)}{tj - ti}$$

Where:

GR = average specific growth rate

Ni = number of fronds determined in test at time i

Nj = number of fronds determined in test at time j

ti = moment time for start of the period

tj = moment time for end of the period

The inhibition data (based on frond number and area) should then be used to derive EC50 values.

APPENDIX C: R CODE FOR EC50

```
EC50 <- read.table(file.choose(), header=TRUE)
EC50
attach(EC50)
library(drc)

#Determining concentration-response curve
fronds.drc<drm(Inhibition_of_Fronds_Growth...~Concentrations,data=E5
 0,fct=LL.3(fixed=c(NA,100,NA)))
summary(fronds.drc)
plot(fronds.drc,ylim=c(0,110),xlim=c(0,10000),type=c("all"))

area.drc<drm(Inhibition_of_Area_Growth...~Concentrations,data=EC50,
 fct=LL.3(fixed=c(NA,100,NA)))
summary(area.drc)
plot(area.drc,ylim=c(0,110),xlim=c(0,10000),type=c("all"))

#Lack-of-fit Test
modelFit(fronds.drc)
modelFit(area.drc)

#EC50 Analysis
ED(fronds.drc,50,interval="delta")
ED(area.drc,50,interval="delta")

detach(EC50)
```

APPENDIX D: RAW DATA

Appendix D.1: Area of *Lemna minor*

standard	Area (day 0)	Area (day 6)
control A	0.485	1.162
Control B	0.299	1.407
10a	0.296	0.836
10b	0.285	0.817
30a	0.281	0.772
30b	0.291	0.746
60a	0.245	0.486
60b*	0.326	0.847
150a	0.379	0.462
150b	0.424	0.47
300a	0.382	0.406
300b	0.361	0.367

Appendix D.2: Number of fronds and colonies of *Lemna minor* on day 0 and day 6

day 0			day 6		
standard	no. colonies	no. fronds	standard	no. colonies	no. fronds
control A	4	10	control A	7	34
control B	4	11	Control B	7	36
10a	3	8	10a	11	26
10b	3	9	10b	7	28
30a	3	9	30a	7	27
30b	3	11	30b	9	29
60a	3	8	60a	5	19
60b	2	8	60b*	7	23
150a	3	10	150a	4	18
150b	3	9	150b	2	16
300a	2	9	300a	2	13
300b	4	11	300b	2	12

Appendix D.3: Growth Rate and % inhibition of growth of fronds and area

growth rate			% inhibition	
standard	growth rate	% inhibition (fronds)	standard	% inhibition (area)
10a	0.196	2.244	10a	14
10b	0.189	5.735	10b	13.2
30a	0.183	8.728	30a	16.5
30b	0.161	19.7	30b	22.3
60a	0.144	28.1795	60a	57
60b*	0.176	12.219	60b*	21.5
150a	0.098	51.122	150a	83.4
150b	0.096	52.119	150b	91
300a	0.061	69.576	300a	94.2
300b	0.014	93.017	300b	98.6

Appendix D.4: Average PH on day 0 and Day 6 for all Concentrations

	PH	
Concentration	DAY 0	DAY 6
0	6.52	4.005
10	6.35	3.87
30	6.45	4.055
60	6.37	4.195
150	6.39	3.88
300	6.31	3.94