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Research Article
Growth Inhibitory Effect of 3,5-dichlorophenol on *Lemna gibba* (L.)

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Abstract

In the present study, growth inhibition effect of 3,5-dichlorophenol was studied against *Lemna gibba* for 7 days. The different concentration of 3,5-dichlorophenol was tested against *Lemna gibba*. After treatments the following parameters of average specific growth rate, growth inhibition and yield reduction were calculated on 0-4, 4-7 and 0-7 days. The test met all the validation criteria as per OECD guideline 221 especially average specific growth rate were 0.273 and 0.289 day^{-1} for frond number and dry weight of fronds, respectively in control. Section by section average growth rate were 0.273, 0.326 and 0.295 day^{-1} for 0- 4, 4-7 and 0-7 day, respectively in control. The maximum average specific growth rate was noticed in 4-7 day. There was no growth rate was observed in entire exposure period at 8 mg L^{-1} concentration. In case of dry weight the average specific growth rate (-0.005 day^{-1}) was reduced at 8 mg L^{-1} concentration of 3,5-dichlorophenol. The maximum growth inhibition rate (%) of 66.64 and 87.34% were observed for 0-4 and 0-7 days, respectively at 8 mg L^{-1} concentration. The effective concentration of 4.38 and 2.96 mg L^{-1} for growth inhibition rate (%) frond on 0-4 and 0-7 days, respectively. The 100% yield reduction of frond was recorded inall the section at 8 mg L^{-1} concentration. More than 80% inhibition of growth rate (%) and yield (%) were recorded at 8 mg L^{-1} concentration in dry weight of frond.

Key words: Average specific growth rate, growth rate inhibition (%), yield reduction (%), effective concentration

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

The propagation of chemicals in the aquatic environment occurs due to the increased industrialization, agricultural chemicals, insecticides and herbicides. Due to this, freshwater ecosystems are often susceptible to surface water contamination by drainage and accidental spills (Kumar and Han, 2011). Many aquatic organisms are affected; among them *Lemna* is the aquatic, small floating, common duckweeds which is found worldwide in the freshwater ecosystem. It forms uniform clones, reproduce rapidly by vegetative method and its small size made them use for environmental monitoring and ecology studies (Kumar and Han, 2010). *Lemna* is sensitive and it incorporates many pollutants from the growth medium through bottom of the leaf (Greenberg *et al*., 1992).

Lemna sp. were used as multipurpose environmental indicator and also used for phytoremediation of chemicals. *Lemna* sp., act as phytoremediator of heavy metal in waste water (Zaltauskaite *et al*., 2014). The *L. minor* also served as removal agent of ablated silver nanoparticles (AgNPs) from the test medium (Ucuncu *et al*., 2014).

In increasing population, there is need to provide food for living organism. Many chemicals were used to manage the different pest. The 3,5-dichlorophenol is mainly used as herbicide. Long term effect and biodegradability of 3,5-dichlorophenol was classified as toxic substance to aquatic organism (Zagorc-Koncan *et al*., 2002). *Lemna* are sensitive to heavy metals and pollutants (Hillman, 1959). It was also used for phytoremediation of cadmium and lead (Verma and Suthar, 2015). The growth parameters are the most commonly assessed ecotoxicological studies (Radic *et al*., 2010). Among the duckweeds, *L. gibba* is the largest with swollen fronds and easily maintained in controlled nutritional condition, temperature and light (Toro *et al*., 1988). Hence, *L. gibba* is used to evaluate the toxicity of 3,5-dichlorophenol by growth inhibition test.

MATERIALS AND METHODS

Culture of *Lemna gibba*: *Lemna gibba*, a small aquatic angiosperm maintained at BRF test facility and was used for the growth inhibition test. The test was performed according to OECD test guideline 221.

Fronds are the modified discoid stem of the *Lemna* plants which are inoculated into the 100 mL of sterilized 20X AAP medium in 500 mL beaker and maintained under the light intensity of 8500 lux at 24±2°C. The surface sterilized young and healthy fronds are sub cultured periodically in the fresh medium. The young, rapidly growing, without chlorosis comprising of two or more fronds is used for the growth inhibition test (OECD., 2006).

Preparation of 20X AAP medium: The 20X AAP medium was used as growth and test medium. It was prepared by different stock solution of A1: 20 mL (Sodium nitrate 26 g L^{-1}, magnesium chloride hexahydrate 12 g L^{-1}, Calcium chloride dihydrate 4.4 g L^{-1}), stock solution A2: 20 mL (Magnesium sulphate heptahydrate 15 g L^{-1}), stock solution A3: 20 mL (di-kalium hydrogen phosphate 3-hydrate 1.4 g L^{-1}) stock solution B: (Boric acid 0.19 g L^{-1}, manganese chloride tetrahydrate 0.42 g L^{-1}, ferrous chloride hexahydrate 0.16 g L^{-1}, disodium EDTA 0.30 g L^{-1}, zinc chloride 3.3 mg L^{-1}, cobaltous chloride hexahydrate 1.4 mg L^{-1}, disodium molybdate dihydrate 7.3 mg L^{-1}, copper chloride dihydrate 0.012 mg L^{-1}), stock solution C: 20 mL (Sodium bicarbonate 15 g L^{-1}) into 850 mL of sterile distilled water. The pH of the medium was 7.5±0.1 adjusted with 1 M HCl or NaOH. Then sterile distilled water was added to the prepared solution to attain final volume of 1 L. The prepared growth medium was sterilized by membrane filtration using 0.2 µm pore sized membrane (OECD., 2006).

Growth inhibition test: The different concentrations of 3,5-dichlorophenol were prepared in the growth medium. An amount of 8 mg of 3,5-dichlorophenol was dissolved in 100 mL of growth medium, considered as initial stock. Further the concentrated stock solution is diluted to reach concentrations of 8, 4, 2 and 1 mg L^{-1}. Growth medium without 3,5-dichlorophenol was considered as control. The 100 mL of control and the test medium was taken in each 250 mL glass beaker. The control and treatments were maintained in three replicates. The *Lemna* were inoculated into each test and control beaker with colony consist of 2-4 young fronds and totally 9-12 fronds. The cultures were incubated in test chamber with 8500 lux at 24±2°C as same as during culture. The culture beakers were repositioned frequently for even distribution of light intensity to the fronds (OECD., 2006).

Observations: The incubated cultures were subjected to frond count in section by section on 0th, 4th and 7th day of the test. The pH of the medium, fronds dry weight and root number were observed on initial and final day of the experiment. The pH of the test and control medium was should not deviate by ±1.5 units on end of the experiment.

Average growth rate and yield: The section by section average specific growth rate of frond number and frond dry weight was calculated for each replicates of control and treatment (OECD., 2006):

$$\mu_{i-j} = \frac{\text{In } (N_j) - \text{In}(N_j)}{t}$$

Where:

μ_{i-j} = Average specific growth rate from time i to j
N_i = Measurement variable in the test or control vessel at time i
N_j = Measurement variable in the test or control vessel at time j
t = Time period from i to j

The percent inhibition of growth rate of frond number and frond dry weight was calculated for each treatment replicates by:

$$\%I_r = \frac{(\mu_C - \mu_T)}{\mu_c \quad \mu_c} \times 100$$

Where:

$\%I_r$ = Percent inhibition in average specific growth rate
μ_C = Mean value for μ in the control
μ_T = Mean value for μ in the treatment

The percent inhibition in yield was calculated for each treatment replicates by:

$$\%I_y = \frac{(b_C - b_T)}{b_c \quad b_c} \times 100$$

Where:

$\%I_y$ = Percent reduction in yield
b_C = Final biomass minus starting biomass for the control group
b_T = Final biomass minus starting biomass in the treatment group

Statistical analysis: The obtained results of growth inhibition (%) and average specific growth rates were presented in Mean±SD and average specific growth rate was presented in graph with linear regression. The EC_{50} value was calculated from NCSS software (NCSS., 2001).

RESULTS

The pH of the medium was 7.5-7.6 on initial day and 7.5-7.9 on final day of the experiment. Hence, there was no deviation in the medium pH during the test period. Initially 10 fronds were inoculated in each beaker. The maximum average specific growth rate of (Frond) 0.273, 0.323 and 0.295 day^{-1} were recorded for 0-4, 4-7 and 0-7 days, respectively in control. I n all the exposure day at 8 mg L^{-1} concentration of 3,5-dichlorophenol did not showed any growth. The linear regression values on 0-4 day showed perfect concentration dependant growth rate reduction (Frond) than 0-7 and 4-7 day Fig. 1.

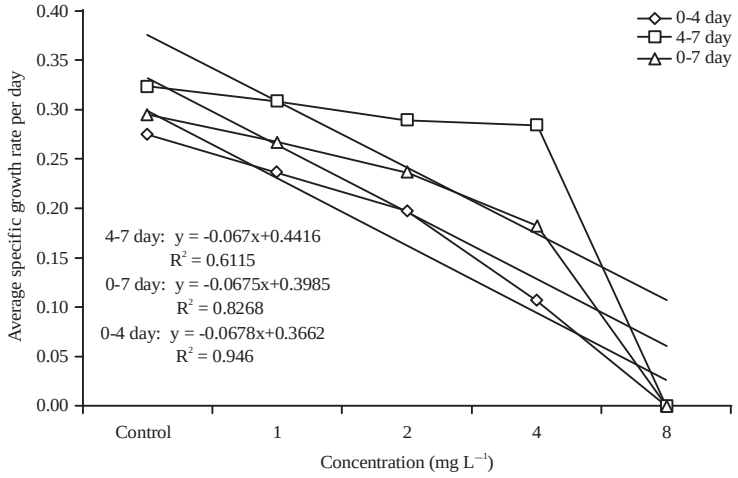

Fig. 1: Average specific growth rate of *Lemna gibba* (Frond) after treatment of 3,5-dichlorophenol

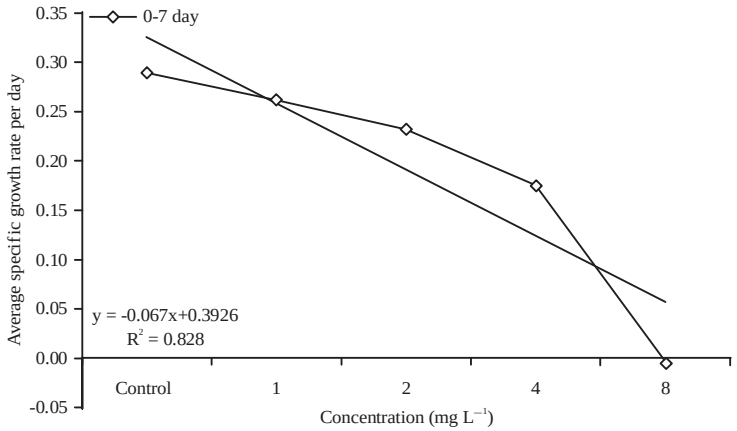

Fig. 2: Average specific growth rate of *Lemna gibba* (Dry weight) after 7th day treatment of 3,5-dichlorophenol

Table 1: Growth inhibition effect of 3,5-dichlorophenol on *Lemna gibba* (Frond)

	Growth inhibition rate (%)	
Concentration (mg L^{-1})	0-4	0-7
1	14.38±3.50	18.15±2.71
2	26.57±5.11	33.77±2.34
4	48.84±4.27	54.44±4.77
8	66.64±1.11	87.34±0.16
EC_{50} 95% confidential limit (Lower-upper)	4.38 (3.94-4.82)	2.96 (2.75-3.17)
EC_{90} 95% confidential limit (Lower-upper)	25.04 (31.89-18.19)	11.41 (9.63-13.19)
Regression equation	y = 3.91+1.69x	y = 3.96+2.18x

Mean±SD, 3 replicate

Table 2: Effect of 3,5-dichlorophenol on *Lemna gibba* (Frond) yield reduction (%)

	Yield reduction (%)		
Concentration (mg L^{-1})	0-4	4-7	0-7
1	21.53±5.01	20.18±5.94	20.78±3.11
2	39.81±7.21	38.02±1.41	38.67±2.75
4	73.28±6.01	57.85±7.73	62.34±5.50
8	100.0±0.00	100.0±0.00	100.0±0.00
EC_{50} 95% confidential limit (Lower-upper)	2.16 (2.04-2.28)	2.46 (2.31-2.61)	2.36 (2.22-2.50)
EC_{90} 95% confidential limit (Lower-upper)	5.89 (5.32-6.46)	7.43 (6.61-8.25)	6.96 (6.22-7.70)
Regression equation	y = 4.01+2.94x	y = 3.95+2.67x	y = 3.97+2.73x

Mean±SD, 3 replicate

In the present study 3,5-dichlorophenol exhibited growth inhibition (%) of *L. gibba* at concentration dependent manner. The maximum growth rate inhibition of 66.64 and 87.34% were recorded in 0-4 and 0-7 day, respectively, at 8 mg L^{-1} concentration. At 4 mg L^{-1} concentration exhibited 48.84 and 54.44% growth inhibition (%) on 0-4 and 0-7 day of the treatment period (Table 1). The EC_{50} values were 4.38 and 11.41 mg L^{-1} concentration for growth inhibition rate (%) of *L. gibba* for 0-4 and 0-7 day, respectively (Table 1).

The 3,5-dichlorophenol exhibited concentration dependent yield reduction (%) of *L. gibba*. The maximum yield reduction of 100% was recorded at 8 mg L^{-1} concentration of 3,5-dichlorophenol in all the observation period (Table 2). At 1 mg L^{-1} concentration also showed more than 20% yield reduction in 0-4, 4-7 and 0-7 day. During the test period more than 50% yield reduction was observed in 4 mg L^{-1} concentration. The EC_{50} values of 2.16, 2.46 and 2.36 mg L^{-1} concentration for 50% yield reduction of *L. gibba* for 0-4, 4-7 and 0-7 day, respectively.

Table 3: Effect of 3,5-dichlorophenol on *Lemna gibba* (dry weight)

Concentration (mg L^{-1})	Yield reduction (%)	Growth inhibition rate (%)
1	20.52±2.40	17.81±2.10
2	37.62±1.56	32.65±1.26
4	62.98±5.86	54.66±4.99
8	100.53±0.46	87.27±0.17
EC_{50} 95% confidential limit (Lower-upper)	2.76 (2.48-3.04)	2.99 (2.78-3.2)
EC_{90} 95% confidential limit (Lower-upper)	12.78 (8.8-16.76)	11.35 (9.61-13.09)
Regression equation	y = 4.14+2.76x	y = 3.94+2.21x

Mean±SD, 3 replicate

Dry weight of the frond: In the present study, 3,5-dichlorophenol reduced the growth rate of *L. gibba*. Figure 2 showed concentration dependent average specific growth rate. Maximum average specific growth rate of 0.289 day^{-1} was recorded in control and minimum of -0.005 day^{-1} in 8 mg L^{-1} concentration. The linear regression R 0.828 also supported that concentration dependent activity.

The growth inhibition (%) of *L. gibba* was reduced by effect of 3,5-dichlorophenol during the test period. The maximum growth rate inhibition was 87.27% at 8 mg L^{-1} concentration on 0-7 day. The minimum reduction of 17.81% was recorded in 1 mg L^{-1} concentration. The EC_{50} value of 2.99 mg L^{-1} concentration was obtained for 50% growth inhibition rate (Table 3).

Table 3 shows 3,5-dichlorophenol at 8 mg L^{-1} concentration inhibit the complete yield reduction of *L. gibba*

on 0-7 day period. The minimum yield reduction was noticed in 1 mg L^{-1} concentration. The EC_{50} value of 2.76 mg L^{-1} concentration was obtained for 50% yield reduction.

Root number: The root number of *L. gibba* was varied from control to treatments. In control many new roots were emerged while 8 mg L^{-1} concentration showed there was no new roots.

DISCUSSION

In the present study, 3,5-dichlorophenol exhibited reduced frond number and dry weight of frond and also chlorosis was observed in fronds of *L. gibba*. The present study coincide with earlier finding of Boudreau *et al*. (2003) who stated that perfluorooctane sulfonate inhibited frond number and showed chlorosis of *L. gibba*. In the present study growth inhibition rate was reduced for wet and dry weight of frond; EC_{50} value were 2.96 and 2.99 mg L^{-1} concentration of 3,5-dichlorophenol. Similar, results was obtained by Seeland *et al*. (2012) who tested pyrimethanil on *L. gibba* and the EC_{50} was 7.8 mg L^{-1} concentration. The toxicity effect of surfactant, alcohol ethoxylate on *L. minor* (frond count) was studied and revealed that the EC_{10} was 0.101 mg L^{-1} (Ivankovic and Hrenovic, 2010). The most recommended drug, acetaminophen was studied against *Lemna* sp. and found that the EC_{50} was 446.6 mg L^{-1} (Nunes *et al*., 2014).

In the present study, growth of the *L. gibba* was affected by 3,5-dichlorophenol. The result harmonize with finding of Coronado-Posada *et al*. (2013) who reported that methanolic coal dust extract inhibited yield and growth rate by reduced frond production of *L. minor* and sign of toxicity. Diclofenac sodium, nicotine and 3,5-dichlorophenol inhibit the frond number of *L. minor* (Fekete-Kertesz *et al*., 2015). Waste untreated and biologically treated water were reduced the growth rate of *L. minor* and reduced the frond number and finally death of *Lemna* (Zaltauskaite *et al*., 2014). Sodium chloride and methyl parathion reduced growth of *L. minor* by reduced the frond number when compared to control (Keppeler, 2009). The uranium affects the growth of *L. minor* such as frond number and dry weight (Horemans *et al*., 2016).

In the present study, 8 mg L^{-1} concentration of 3,5-dichlorophenol completely inhibits the *L. gibba* growth rate. Similar result was found by Khellaf and Zerdaoui (2010) who reported that different concentration of copper and nickel inhibit the growth of *L. gibba* and complete inhibition was noticed at 0.5 and 1 mg L^{-1} concentration, respectively. Keppeler (2009) has studied Methyl Parathion against *L. minor* and the IC_{50} was 49.48 mg L^{-1}.

CONCLUSION

In the present study, 3,5-dichlorophenol inhibit 100% average specific growth rate of *L. gibba*. While the concentration increases, frond number was reduced. The emergence of new root was 100% inhibited at 8 mg L^{-1} concentration of 3,5-dichlorophenol. Our study clearly stated that 3,5-dichlorophenol was highly toxic. In future, reduce the usage of 3,5-dichlorophenol in agriculture as well as other industrial purpose to maintain proper environment without alteration of food chain.

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