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Comparative Chronic Toxicity of Pyridine, α-Picoline, and β-Picoline to *Lemna minor* L. and *Chlorella vulgaris* B

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Two-third of known organic chemicals are heterocyclics (Kuhn and Suflita 1989; Kung et al. 1971). Pyridine and its derivatives are important representatives of heterocyclic compounds. Widely used as industrial solvents and raw material for pharmaceutical and agrochemical industries, they can make their way into the environment as wastewater and components of pesticide formulations (Higashio and Shoji 2004). Because pyridine, α-picoline and β-picoline have low octanol-water partition coefficients (K_{ow}) (Verschueren 1983) and high water solubility (Sax and Lewis 1987), they probably do not bioconcentrate in plants or aquatic organisms. However, no data on bioconcentration factors or biomagnifications in terrestrial or aquatic food chain were located. Most reports on comparative toxicity of such solvents towards test organisms deal with their effects on fish, aquatic invertebrates and algae (Majewsky et al. 1978; LeBlanc and Surprenant 1983; Stratton and Smith 1988). The US Environmental Protection Agency recommends maximum allowable limits of 0.05 % solvent for acute tests and 0.01 % for chronic tests (USEPA 1975), but the final concentration used vary among the different authors and are often higher than EPA limits due to problems associated with the use of small test volumes and toxicant solubility.

Algae and the common duckweed are essential components of aquatic ecosystems, hence any toxic effect on these can directly affect structure and function of an ecosystem (Kersting and Van der Brink 1997). Algae and duckweed are recommended organisms for estimating chronic toxicity (OECD 2000; Blinova 2004). The work reported here was done to compare the inhibitory effects of pyridine, α-picoline and β-picoline on *Chlorella vulgaris* Beijernik (Oocystaceae), a freshwater, unicellular green alga and the common duckweed *Lemna minor* L. (Lemnaceae), a free-floating, fast growing and wide spread vascular plant. Parameters, such as total chlorophyll, protein and biomass used in the toxicity assays were examined. This study will establish baseline data for adverse effect of pyridine and its derivatives, released from various industries, on phytoplankton in an aquatic ecosystem.

MATERIALS AND METHODS

Test chemicals used were of analytical grade. Pyridine, α-picoline and β-picoline were purchased from Sigma-Aldrich, Banglore, India.

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Table 1. Summary of the growth conditions for *L. minor* and *C. vulgaris*.

S.No.	Growth condition	*L. minor*	*C. vulgaris*
1.	Test type	Static	Static
2.	Temperature	25±2^{0}C	27±1^{0}C
3.	Light quality	Cool, white and fluorescent	Cool, white and fluorescent
4.	Light intensity	115 µmol photon PAR m^{-2} s^{-1}	90 µmol photon PAR m^{-2} s^{-1}
5.	Photoperiod	12 hr light/ 12 hr dark	12 hr light/ 12 hr dark
6.	Test vessels	120 x15 mm-petri-dish	50 mL Erlenmeyer flask
7.	Test solution volume	100 mL	15 mL
8.	Shaking rate	Twice daily by hand	Twice daily by hand
9.	Test specimen/vessel	50 colonies	8 x 10^5 cells mL^{-1}
10.	Dilutions/sample	8	8
11.	Dilution factor	0-0.05%	0-0.05%
12.	Dilution water pH & Hardness	Tap water 8.1±0.2; 245±14 mg/L	Tap water 8.1±0.2; 245±14 mg/L
13.	Test duration	14 days	14 days
14.	Test end point	Net frond number increase	Net cell number increase

The duckweed culture was collected from pollution free sites. Water sample were taken to confirm that the water bodies were not laden with pollutants. The duckweed culture had been acclimatized in the laboratory for 4 weeks. The duckweed plants were identified as *Lemna minor* (Hillman 1961). The duckweed test specimens were selected from the axenic stock culture 24 hr before the test began. The selection criteria was that the duckweed plants be healthy-looking and have two fronds of approximately equal size per colony. The stock solution of double strength (2X) algal growth medium was prepared following APHA (1998). Triplets of test plants were grown in double strength (2X) algal growth medium (without EDTA) under condition as summarized in Table 1. Pyridine, α-picoline and β-picoline concentrations (v/v) tested were 0.00125, 0.0025, 0.005, 0.0125, 0.025, 0.0375 and 0.05%). Cultures without pyridine, α-picoline and β-picoline were included as the control in all experiments. Harvesting of plants was done after 14 day. Total chlorophyll content was determined by acetone (80%) extraction (Arnon 1949). Protein content was determined in accordance with method described by Bradford (1976). Biomass was determined by the dry weight method (APHA 1998).

The culture of *C. vulgaris* was obtained from the Department of Botany, Banaras Hindu University, Varanasi, India. Cells of *C. vulgaris* were propagated aseptically and photoautotrophically in a 50 mL Erlenmeyer flask containing 15 mL liquid BG-11 medium (Lintongan et al. 2004) under conditions as summarized in Table 1. For all experiments, 5 mL aliquots of the BG-11 medium containing green algae cells (initial cell concentration 8 x 10^5 mL^{-1}) were distributed to sterile 50 mL Erlenmeyer flasks containing different concentrations of pyridine, α-picoline and β-picoline. Pyridine, α-picoline and β-picoline concentrations (v/v) tested were 0.00125, 0.0025, 0.005, 0.0125, 0.025, 0.0375

and 0.05%. Cultures without pyridine, α- and β-picoline were included as the control in all experiments. All exposed cells of *C. vulgaris* were harvested after 14-day. Total chlorophyll content of *C. vulgaris* was determined by spectrophotometric measurement (GBC Cintra-40, Australia) of methanol extracts using the equations described by Lichtenthaler (1987). Protein content was determined from the data of nitrogen content, using the conversion factor 5.8, as described by Gnaiger and Bitterlich (1984), instead of the classical conversion factor, 6.25 (FAO/WHO 1970), since it was demonstrated that this new conversion factor is better for different kinds of samples, including, bacteria, algae, protozoa and invertebrates (Gnaiger and Bitterlich 1984). Algal cells were harvested by centrifugation at 3000 X *g* and frozen at –70 ^{0}C prior to lyophilisation for 24 hr. Biomass was determined by the dry weight method (Utting 1985). Data were statistically analysed by an overall one-way analysis of variance (ANOVA) and when differences observed were significant, the means were compared by the Tukey-Kramer Multiple Comparison Test. IC_{50} (concentration of compound to cause 50% inhibition of biological processes) values were graphically estimated.

RESULTS AND DISCUSSION

The initial chlorophyll content was 0.55 mg/g/Petri-dish in *L. minor* toxicity tests and it was 0.32 mg/g/Erlenmeyer flask in *C. vulgaris* toxicity tests. The data (Table 2) show the toxic effect of pyridine, α-picoline and β-picoline on the chlorophyll content of *L. minor* and *C. vulgaris*. The maximum chlorophyll content was recorded at 0.005 and 0.0025% concentration of pyridine in *L. minor* and *C. vulgaris*, respectively. In α-picoline exposure, the maximum chlorophyll content was recorded at 0.00125 and 0.005% concentration in *L. minor* and *C. vulgaris*, respectively, whereas maximum chlorophyll content was recorded at 0.00125 and 0.005% concentration of β-picoline in *L. minor* and *C. vulgaris*, respectively. The data (Table 3) show the toxic effects of pyridine, α- and β-picoline on the protein content of *L. minor* and *C. vulgaris*. The maximum protein content was recorded at 0.0125 and 0.005% pyridine in *L. minor* and *C. vulgaris*, respectively. In α-picoline exposure, the maximum protein content was recorded at 0.0025 and 0.005 % concentration in *L. minor* and *C. vulgaris* respectively, whereas maximum protein content was recorded at 0.005 and 0.0025% β-picoline in *L. minor* and *C. vulgaris*, respectively. Table 4 shows the toxic effect of pyridine, α-picoline and β-picoline on the biomass content of *L. minor* and *C. vulgaris*. The maximum biomass content was recorded at 0.005 % concentration of pyridine in both *L. minor* and *C. vulgaris*. In α-picoline exposure, the maximum biomass content was recorded at 0.00125 and 0.0125% concentration in *L. minor* and *C. vulgaris*, respectively, whereas maximum biomass content was recorded at 0.005 and 0.0025% concentration of β-picoline in *L. minor* and *C. vulgaris*, respectively.

According to the data (Table 2) the 14 day IC_{50} of pyridine, α-picoline and β-picoline for chlorophyll content in *L. minor* and *C. vulgaris* were 0.068, 0.083 0.107 and 0.060, 0.088, 0.092 (%, v/v), respectively (Table 5). The 14-day IC_{50} of pyridine, α-picoline and β-picoline for total protein content in *L. minor* and *C. vulgaris* were 0.048, 0.059, 0.074 and 0.083, 0.125, 0.112 (%, v/v), respectively

Table 2. Effect of pyridine, α-picoline and β-picoline on total chlorophyll content (%) of *L. minor* and *C. vulgaris*.

Conc.	Pyridine		α-picoline		β-picoline	
(%)	*L. minor*	*C. vulgaris*	*L. minor*	*C. vulgaris*	*L. minor*	*C. vulgaris*
0.0	100.00	100.00	100.00	100.00	100.00	100.00
0.00125	108.60^{a}	100.84^{d}	101.73^{d}	100.76^{d}	140.91^{a}	102.54^{c}
	±0.98	±1.15	±1.05	±0.63	±0.95	±0.55
0.0025	113.13^{a}	108.60^{a}	101.02^{d}	105.47^{a}	131.09^{a}	104.58^{c}
	±1.25	±0.85	±0.88	±0.65	±0.62	±0.65
0.005	122.16^{a}	98.93^{a}	101.31^{d}	122.74^{a}	108.71^{a}	106.91^{c}
	±1.05	±0.80	±0.65	±0.55	±0.82	±0.74
0.0125	81.13^{a}	93.50^{a}	100.08^{a}	100.07^{a}	102.39^{a}	102.88^{a}
	±0.63	±0.65	±0.45	±0.88	±0.44	±0.78
0.025	68.51^{a}	91.06^{c}	85.14^{a}	92.04^{a}	88.00^{a}	100.94^{c}
	±0.45	±0.45	±0.78	±0.75	±0.55	±0.98
0.0375	65.23^{b}	75.86^{a}	78.80^{a}	79.88^{a}	80.69^{a}	89.38^{a}
	±0.35	±0.54	±0.66	±0.45	±0.65	±0.25
0.05	57.42^{a}	60.76^{a}	70.25^{a}	72.27^{a}	75.43^{a}	80.35^{a}
	±0.41	±0.35	±0.58	±0.65	±0.36	±0.52

All the values are mean (n=3)±S.D. [a] highly significant; ANOVA $p<0.001$, [b] significant; ANOVA $p<0.01$, [c] less significant; ANOVA $p<0.05$, [d] non significant; ANOVA $p>0.05$.

Table 3. Effect of pyridine, α-picoline and β-picoline on protein content (%) of *L. minor* and *C. vulgaris*.

Conc.	Pyridine		α-picoline		β-picoline	
(%)	*L. minor*	*C. vulgaris*	*L. minor*	*C. vulgaris*	*L. minor*	*C. vulgaris*
0.0	100.00	100.00	100.00	100.00	100.00	100.00
0.00125	120.75^{a}	101.89^{c}	110.47^{a}	105.65^{a}	105.65^{a}	102.37^{b}
	±0.36	±0.65	±0.88	±0.65	±0.66	±0.65
0.0025	125.07^{a}	103.79^{b}	112.79^{b}	109.02^{a}	108.97^{a}	108.91^{a}
	±0.45	±0.45	±0.65	±0.25	±0.74	±0.78
0.005	135.45^{a}	104.91^{d}	82.56^{a}	133.56^{a}	133.56^{a}	98.17^{a}
	±0.55	±0.36	±0.45	±0.68	±0.65	±0.25
0.0125	155.97^{a}	85.38^{a}	78.49^{a}	108.85^{a}	117.03^{a}	95.18^{a}
	±1.25	±0.45	±0.36	±0.88	±0.78	±0.65
0.025	74.25^{a}	81.01^{a}	70.35^{a}	106.75^{c}	104.89^{a}	91.36^{a}
	±0.88	±0.55	±0.25	±0.65	±0.36	±0.45
0.0375	62.88^{a}	74.50^{a}	66.28^{a}	104.89^{c}	102.28^{b}	90.56^{d}
	±0.65	±0.45	±0.35	±0.45	±0.64	±0.65
0.05	47.76^{a}	67.75^{a}	56.40^{a}	75.30^{a}	75.30^{a}	78.82^{a}
	±0.25	±0.75	±0.56	±0.55	±0.25	±0.45

All the values are mean (n=3)±S.D. [a] highly significant; ANOVA $p<0.001$, [b] significant; ANOVA $p<0.01$, [c] less significant; ANOVA $p<0.05$, [d] non significant; ANOVA $p>0.05$.

(Table 5). The 14 day IC_{50} of pyridine, α-picoline and β-picoline for biomass content in *L. minor* and *C. vulgaris* test were 0.062, 0.089, > 0.05 and 0.102, 0.112, 0.094 (%, v/v), respectively. *L. minor* cultures exposed to pyridine showed an IC_{50} value 0.068 for chlorophyll content, which was 1.22 times of α-picoline

Table 4. Effect of pyridine, α- and β-picoline on biomass content (%) of *L. minor* and *C. vulgaris*.

Conc.	Pyridine		α-picoline		β-picoline	
(%)	*L. minor*	*C. vulgaris*	*L. minor*	*C. vulgaris*	*L. minor*	*C. vulgaris*
0.0	100.00	100.00	100.00	100.00	100.00	100.00
0.00125	100.21[d]	100.48[d]	124.46[a]	103.59[a]	101.11[d]	101.56[d]
	±0.12	±0.38	±0.54	±0.25	±0.10	±0.54
0.0025	105.10[a]	101.43[d]	114.67[a]	110.34[a]	103.60[c]	104.84[a]
	±0.10	±0.55	±0.78	±0.35	±0.98	±0.55
0.005	140.28[a]	103.34[c]	105.43[a]	129.96[a]	107.20[a]	101.58[a]
	±0.68	±0.65	±0.55	±0.85	±0.98	±0.65
0.0125	84.17[a]	99.52[a]	103.26[b]	166.88[a]	104.43[b]	99.77[c]
	±0.55	±0.45	±0.45	±0.35	±0.65	±0.66
0.025	81.19[a]	92.05[a]	91.30[a]	116.46[a]	100.28[a]	90.98[a]
	±0.45	±0.65	±0.25	±0.11	±0.68	±0.45
0.0375	76.62[a]	88.57[a]	83.70[a]	108.44[a]	101.66[d]	88.60[b]
	±0.35	±0.85	±0.30	±0.95	±0.78	±0.36
0.05	58.34[a]	80.07[a]	73.37[a]	98.90[a]	101.39[a]	93.67[a]
	±0.65	±0.55	±0.35	±0.85	±0.67	±0.45

All the values are mean (n=3)±S.D. [a] highly significant; ANOVA $p<0.001$, [b] significant; ANOVA $p<0.01$, [c] less significant; ANOVA $p<0.05$, [d] non significant; ANOVA $p>0.05$.

Table 5. IC_{50} values (%, v/v) 14 day of pyridine, α- and β-picoline against *L. minor* and *C. vulgaris*.

Compounds	IC_{50} value					
	L. minor			*C. vulgaris*		
	Chlorophyll	Protein	Biomass	Chlorophyll	Protein	Biomass
Pyridine	0.068	0.048	0.062	0.060	0.083	0.102
	±0.004	±0.003	±0.002	±0.003	±0.005	±0.003
α-picoline	0.083	0.059	0.089	0.088	0.125	0.112
	±0.004	±0.003	±0.002	±0.004	±0.003	±0.002
β-picoline	0.107	0.074	>0.05*	0.092	0.112	0.094
	±0.003	±0.004		±0.002	±0.003	±0.005

* not calculated, ± S.D.

(IC_{50} 0.083) and 1.65 times of β- picoline (IC_{50} 0.107). *C. vulgaris* showed an IC_{50} value of 0.060 for pyridine which was 1.48 times of α- picoline (IC_{50} 0.088) and 1.52 times of β- picoline (IC_{50} 0.092) treated culture (Table 5). In terms of protein content, *L. minor* showed an IC_{50} value of 0.048, 0.059 and 0.074 for pyridine, α-picoline and β-picoline, respectively and *C. vulgaris* showed an IC_{50} value 0.083, which was 1.5 and 1.38 times more than α- picoline and β-picoline, respectively. β-picoline supported (ANOVA, $p<0.001$) the biomass of *L. minor* at each tested concentrations whereas, pyridine and α-picoline, both, significantly (ANOVA, $p<0.001$) inhibited growth at concentrations >0.005%. The toxicity pattern of the tested compounds was as follows: pyridine > α-picoline > β-picoline. Protein was the most sensitive and biomass was the least sensitive parameter for *L. minor*. However, in *C. vulgaris* the sensitivity pattern was chlorophyll > protein > biomass. *L. minor* was found to be more sensitive than *C. vulgaris* in term of protein and biomass content. Whereas, *C. vulgaris* was found to be more sensitive

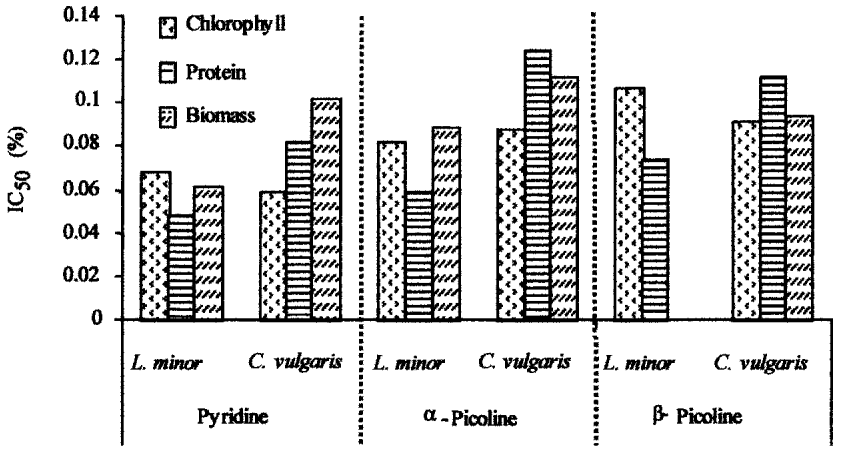

Figure 1. IC_{50} values of pyridine, α-picoline and β-picoline for different parameters of *L. minor* and *C. vulgaris*.

than *L. minor* in term of chlorophyll content (Fig. 1). Due to high water solubility of pyridine (K_{ow} 1.04), α-picoline (K_{ow} 1.06) and β-picoline (K_{ow} 1.2), the cells of *L. minor* and *C. vulgaris* have an affinity to these molecules (Reddy and Locke 1996). Our results obtained are in agreement with the hypothesis of Reddy and Locke (1996). Since, pyridine and picoline containing herbicides specifically exert phytotoxic action on photosystem II; changes in the chlorophyll content can be a reliable indicator of toxicity (Mayer et al. 1997). In this study, the results suggest that pyridine, α-picoline and β-picoline may affect biomass (Table 4) and protein content (Table 3) in the same manner as chlorophyll (Table 2). At each lower concentrations, pyridine, α- and β-picoline stimulated production of chlorophyll, protein and biomass content as well. Mayer and Jensen (1995) have also reported triazine, an n-heterocyclic ring containing herbicide, induced increases of chlorophyll and biomass in the alga *Selenastrum capricornatum*. This process may result from homeostasis triggered, as a tolerance mechanism, by the exposure to the herbicides (Francois and Robinson 1990). Responses such as the synthesis of thylakoid components are considered to be a general adaptive response to situations in which the electron transport rate is strongly limited for photosynthesis (Behra et al. 1999). An increase in protein content in *L. minor* and *C. vulgaris* at lower concentrations of pyridine, α-picoline and β-picoline could also be related with aforesaid detoxification mechanism. For instance, other solvents and herbicides have been shown to be detoxified by various microalgal species and subsequent binding to a protein (Kruglov 1970). Thus, our results of the pyridine, α- and β-picoline toxicity tests with *L. minor* and *C. vulgaris* might be used to compare the toxicities of different materials and be useful for studying biological availability of, and structure-activity relationship between test materials.

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