Bull. Environ. Contam. Toxicol. (2005) 74:886–893
© 2005 Springer Science+Business Media, Inc.
DOI: 10.1007/s00128-005-0664-5

Bulletin of Environmental Contamination and Toxicology

pH Dependent Toxicity of Pyridine Raffinate to a Common Duckweed, *Lemna minor* L.

R. Chandra, B. B. Singh

Environmental Microbiology Section, Industrial Toxicology Research Center, M. G. Marg, Post Box No. 80, Lucknow-226 001 (U.P.), India

Received: 20 August 2004/Accepted: 22 February 2005

Industrial effluents have complex mixture of chemicals, displaying an extremely diversified toxic activity. Among the factors that may affect results in an aquatic environment are temperature, pH, buffering capacity, hardness and salinity. For complete evaluation of the environmental impact of any complex industrial waste, both physico-chemical and toxicological bioassay should be performed (Weber et al. 1989).

Effluent from pyridine manufacturing industries is collectively known as pyridine raffinate. It is a dark brown, complex mixture of formaldehyde, phenolics, residual pyridine and particulate matters having high alkalinity (pH 12.0) and water solubility. These constituents of pyridine raffinate cause human health hazards (Verschueren 1983). Worldwide, pyridine raffinate is either directly disposed or incinerated in environment at high temperature (650–1600^{0}C) (HSDB 1989). Thus, it is a major threat to the environment. Pyridine is widely used as an industrial solvent, a food additive and in dyes, explosives, pharmaceuticals and pesticides (Goe 1978). The change in pH also affects the toxicity of pyridine raffinate in the aquatic ecosystem due to interconversion of its constituents. However, the extent of toxicity at different pH values in aquatic organisms is not known. Therefore, the differing pH values, i.e. 5.0, 7.0, 9.0 and 12.0 (original) were chosen to evaluate the toxicity of pyridine raffinate in the aquatic environment. The common duckweed *Lemna minor* is a free-floating, fast growing and wide spread vascular plant (Hillman and Culley 1978). The advantages of using duckweed instead of an alga is that test solutions can be renewed daily in the former. It is difficult to test coloured effluent for toxicity by using algal assays without filtering, which decreases sample integrity. Many studies have shown that duckweed is an excellent candidate for toxicity tests (Taraldsen and Norberg-King 1990; OECD 2000).

MATERIALS AND METHODS

The effluent was collected from the pyridine plant of M/s Jubilant Organosys, Gajraula, India (APHA 1998). Samples were analyzed for various physico-chemical parameters following standard methods (APHA 1998). Parameters analyzed were biochemical oxygen demand (BOD, 5-day test), chemical oxygen demand (COD, open reflux, titrimetric method), total nitrogen (Macro-Kjeldahl

Correspondence to: R. Chandra

method), nitrate (Orion-960 ion meter), total phenols (detection limit- 0.001-0.25 mg/L; chloroform extraction method), ammonia (Orion-960 ion meter), sulphate (gravimetric method with drying of residue), sodium (Orion-960 ion meter), potassium (Orion-960 ion meter), colour (Co-Pt method), total solids (TS), total suspended solids (TSS) and total dissolved solids (TDS) as per the methods specified by APHA (1998). Pyridine content was measured by HPLC (detection limit- 0.02-1.00 mg/L; Metrohm, Micro Devices, Switzerland) method (Liu and Kuo 1997). Formaldehyde content was measured by the colorimetric method (detection limit- 0.001-0.8 mg/L; Nash 1953). Heavy metals (detection limit: Cr-0.007-50 mg/L; Cu-0.006-50 mg/L; Fe-0.007-100 mg/L; Zn-0.002-100 mg/L) were analyzed by inductively coupled plasma (ICP) spectrometer (model- 8440, Plasma Lab, Australia).

The duckweed culture was collected from a pollution free water body. Water samples were taken to confirm that the water body was 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 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 duckweed toxicity tests were performed in 100 mL test solution in disposable petri dish (120 x 15 mm). The stock solution of culture media of *Lemna minor* was prepared following by OECD (2000) guideline.

Triplicte test plants were grown in culture medium (without EDTA) under conditions as summarized in table 1 with different concentrations of effluent (0.0, 2.0, 5.0, 10.0, 25.0, 50.0, 75.0 and 100 % (v/v) prepared by dilution of stock solution of effluent using tap water as diluent. The pH of raffinate was adjusted with 5.66 N hydrochloric acid. Control plants were grown in culture medium without effluent under aforesaid control conditions. Harvesting of plants was done after 96 hr. Total chlorophyll content was determined by acetone (80%, v/v) extraction (Arnon 1949). Protein content was determined in accordance with the method described Lowry et al. (1951). Biomass was determined by the dry weight method (APHA 1998).

Table 1. Summary of the growth conditions.

1.	Test type	Static
2.	Temperature	25 ± 2^0C
3.	Light quality	Cool, white and fluorescent of 4,300 lux
4.	Photoperiod	16hr light/ 8hr dark
5.	Test specimen/ petri dish	50 colonies
6.	Dilutions/ sample	8
7.	Test end point	Net frond increase

RESULTS AND DISCUSSION

Physico-chemical analysis revealed pyridine raffinate had high COD, BOD, TS, total phenols, formaldehyde, total nitrogen and heavy metals (Table 2). In general, the effluent sample was characterized as highly complex and any alteration in the

pH of the effluent sample also altered composition due to interconversion. The total nitrogen (84280 mg/L), nitrate (502 mg/L), ammonium (367 mg/L), total phenol (1098 mg/L), COD (524000 mg/L) and BOD (262000 mg/L) were very high at original pH (pH 12.0). The raffinate samples having pH 5.0 contained elevated level of potassium (1960 mg/L), sodium (280 mg/L), formaldehyde (0.72 mg/L), chloride (1135 mg/L), sulphate (10183 mg/L), total solids (319841 mg/L) and colour (13500 Co-Pt unit). There were no substantial differences in pyridine and heavy metals contents with differing pH values. Hence, phytotoxicity tests showed considerable differences as discussed below among samples with differing pH.

Toxicity assessment of pyridine raffinate with *Lemna minor* was determined in terms of total chlorophyll, protein and biomass. In undiluted samples of pyridine raffinate, 100% duckweed mortality was observed on 96 hr exposure. The plant showed chlorosis (yellowing of frond tissue) although the colony structure remained intact in the original pH (pH 12.0). At altered pH, duckweed specimens also exhibited visible signs of injuries from 48-96 hr including chlorosis and lesion (localized dead frond tissue). At 100% concentration duckweed showed a decrease of 98% in total chlorophyll (0.023 mg/g) at the original pH (pH 12.0). The decreases in protein (2.15 mg/g) and biomass were 90%, whereas growth was totally inhibited at raffinate concentrations of 50% and above. The decrease in chlorophyll (Fig. 1), protein (Fig. 2) and biomass (Fig. 3) was considerably less at pH 9.0 and pH 5.0. Growth in these cases was inhibited at 75% and 100%, respectively. At pH 9.0 and 5.0, the IC_{50} was below 5%. At neutral pH (pH 7.0) however the raffinate supported growth at a very low concentration (2%, v/v) and the IC_{50} was 25%. Further, among all parameters, chlorophyll was found most sensitive as it showed chlorosis followed by tissue necrosis even at a low (2%, v/v) level of pyridine raffinate at the original pH (pH 12.0). The chlorophyll content dropped to 11% in comparison to the control (Table 3a). High toxicity was noted for all tested physiological parameters at alkaline pH (pH 9.0) (Table 3b) and acidic pH (pH 5.0) (Table 3d). The least toxicity was observed at the neutral pH (pH 7.0) simply because it is the suitable growth pH range for all biological samples (Table 3c). The EC_{50} values of pyridine raffinate at differing pH values are shown in table 5. The sensitivity pattern of *L. minor* at pH 5.0 and 9.0 was as follows: chlorophyll>protein>biomass. While at pH 7.0 and 12.0, the sensitivity pattern was in following order: chlorophyll>biomass>protein (Table 5).

Since pyridine, a major component of raffinate is a very stable aromatic compound at ambient temperature, it requires high temperature for any possible nucleophillic as well as electrophillic substitution reactions (Giri and Misra 1982). Thus, pH does not alter the quantity of pyridine (Table 2) in raffinate significantly at ambient temperature (Table 1). Hence, any increase or decrease in toxicity of raffinate in comparison to toxicity at neutral pH, is not associated with pyridine level. Earlier workers clearly indicated that phenolics and formaldehyde are very toxic (Babich and Stotzky 1985; Qu and Bhattacharya 1997). High levels of phenolics, COD and BOD may enhance the toxicity of pyridine raffinate at original pH (pH 12.0). However, the role of formaldehyde in toxicity enhancement is doubtful because, at higher pH (pH 12.0), two molecules of

Table 2. Physico-chemical analysis of pyridine raffinate at different pHs.

Parameters (mg/L)	pH							
	5.0		7.0		9.0		12.0	
Total nitrogen	53480	±640	81480	±921	80030	±854	84280	±985
Nitrate	312	±25	369	±36	425	±56	502	±62
Ammonium	352	±46	302	±21	350	±32	367	±23
Potassium	1960	±26	1645	±35	980	±18	86	±11
Sodium	280	±15	210	±18	50	±9	30	±5
Chloride	1135	±27	1110	±22	976	±17	1350	±26
Total phenol	290	±14	556	±36	435	±24	1098	±54
Sulphate	10183	±125	9261	±145	7889	±98	2630	±102
Formaldehyde	0.72	±0.04	0.84	±0.03	0.50	±0.02	0.33	±0.01
Pyridine	0.76	±0.05	0.78	±0.05	0.75	±0.07	0.80	±0.07
TS	319841	±1005	184190	±988	154650	±1254	21640	±655
TDS	205120	±2565	179210	±1364	137880	±2154	14024	±365
TSS	114721	±2987	4980	±254	16770	±965	7616	±254
COD	384000	±3254	428000	±6892	468000	±7521	524000	±5436
BOD	199000	±2456	214000	±3654	230000	±6541	262000	±8534
Fe	6.63	±0.05	6.25	±0.07	6.17	±0.08	6.55	±0.05
Cr	0.10	±0.04	0.11	±0.04	0.11	±0.03	0.12	±0.03
Zn	0.10	±0.03	0.09	±0.04	0.08	±0.03	0.11	±0.03
Cu	0.09	±0.03	0.07	±0.03	0.09	±0.01	0.11	±0.03
Cd, Ni and Pb	ND		ND		ND		ND	
Colour*	13500	±265	12600	±221	10500	±245	6000	±165

*Co-Pt unit, ND-not detectable, All values are mean (n=3)±S.D.

formaldehyde react in such a way that one molecule is oxidized while the other is reduced. The net result is the production of methanol (a reduction product) and a formate (a oxidation product). These products are less toxic than formaldehyde. In weak alkali solution (pH 9.0), formaldehyde polymerizes to sugars. The course of polymerization probably involves a series of aldol condensation (Giri and Misra 1982). The same type of reaction might be happening in green leaves where the photochemical reaction between CO_2 and water initially forms formaldehyde. This formaldehyde might then be changing into sugar (Salisbury and Ross 1986). The elevated level of potassium, sodium, sulphate and total solids seem to be involved in toxicity reduction of pyridine raffinate at the lowest (pH 5.0) as well as the neutral pH (pH 7.0). Toxicity assessment of pyridine raffinate with *L. minor* revealed that there was a significant reduction (ANOVA, $p<0.05$,Table 4)

Table 3. Effect of pH on total chlorophyll, protein and biomass content (in%) of *L. minor* treated with different concentrations of pyridine raffinate.

	Effluent conc. (%)	Total chlorophyll	Protein	Biomass
(a) At pH 12.0	0	100.00	100.00	100.00
(original)	2	11.74±0.42	87.88±0.19	89.88±0.53
	5	7.86±0.38	59.09±0.33	49.41±0.44
	10	4.31±0.15	50.09±0.22	37.65±0.23
	25	4.25±0.16	39.39±0.15	22.35±0.15
	50	3.81±0.12	30.32±0.12	21.18±0.18
	75	2.72±0.15	24.24±0.18	11.76±0.15
	100	2.28±0.10	10.67±0.12	10.22±0.10
(b) At pH 9.0	0	100.00	100.00	100.00
(alkaline)	2	23.32±0.14	95.56±0.54	93.72±0.66
	5	17.12±0.12	94.44±0.55	87.64±0.64
	10	15.13±0.10	88.87±0.25	84.27±0.43
	25	14.07±0.12	53.33±0.28	68.54±0.12
	50	12.86±0.14	40.21±0.21	57.42±0.36
	75	10.29±0.26	24.44±0.12	55.03±0.15
	100	3.27±0.10	11.11±0.12	44.21±0.15
(c) At pH 7.0	0	100.00	100.00	100.00
(neutral)	2	35.23±0.12	97.88±1.65	96.97±1.54
	5	33.04±0.15	95.88±1.25	87.88±1.05
	10	32.24±0.15	94.44±1.58	78.79±1.25
	25	29.24±0.22	88.87±1.05	71.21±0.88
	50	24.61±0.46	79.72±0.56	62.12±0.45
	75	22.61±0.15	69.42±0.15	53.03±0.25
	100	14.91±0.10	43.31±0.15	46.97±0.18
(d) At pH 5.0	0	100.00	100.00	100.00
(acidic)	2	26.33±0.12	89.58±0.74	95.29±1.98
	5	25.67±0.13	85.42±1.25	88.48±2.25
	10	13.09±0.15	83.33±1.75	76.39±1.05
	25	11.01±0.15	60.42±1.12	73.25±0.72
	50	8.08±0.25	33.33±0.15	61.67±1.15
	75	3.85±0.15	26.67±0.12	53.82±0.15
	100	2.21±0.20	14.17±0.54	39.11±0.25

All values are mean (n=3)±S.D.

in toxicity at neutral pH. It was also found that the alteration in toxicity due to pH of raffinate was not pyridine dependent. There were phenolics that might be responsible for pyridine raffinate toxicity at different pH levels. At differing pH values, pyridine raffinate samples showed no substantial differences in heavy metals content (Table 2). All heavy metals (Fe, Cr, Zn and Cu) in pyridine raffinate were found to be above water quality standards (APHA 1998) and

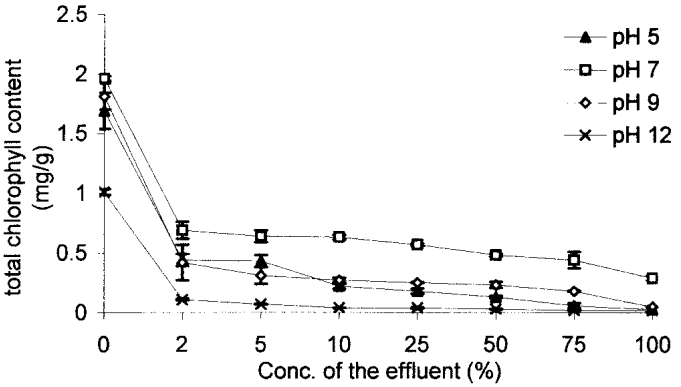

Fig. 1. Effect of pH on total chlorophyll content (mg/g) of *L. minor* treated with different concentrations of pyridine raffinate after 96 hr.

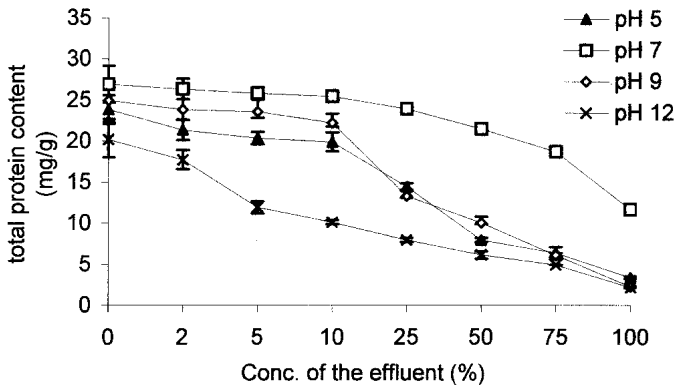

Fig. 2. Effect of pH on total protein content (mg/g) of *L. minor* treated with different concentrations of pyridine raffinate after 96 hr.

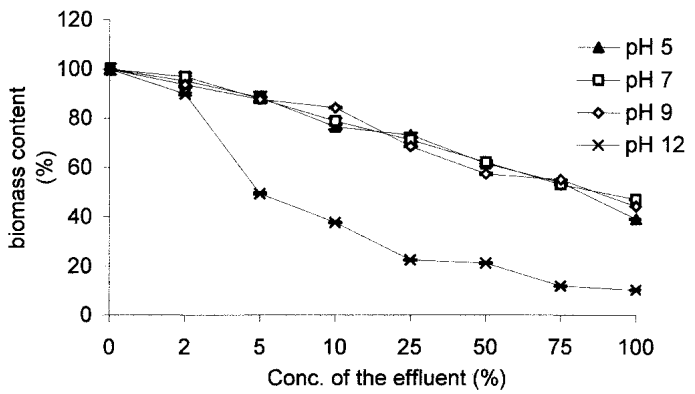

Fig. 3. Effect of pH on biomass content (%) of *L. minor* treated with different concentrations of pyridine raffinate after 96 hr.

Table 4. Difference in toxicity level at different concentrations of pyridine raffinate observed at different pHs.

Parameters	Effluent conc. (%)	pH vs pH					
		12 x 9	12 x 7	12 x 5	9 x 7	9 x 5	7 x 5
Total chlorophyll	2	***	***	***	***	***	***
	5	***	***	***	***	***	***
	10	***	***	***	***	***	***
	25	***	***	***	***	***	***
	50	***	***	***	***	***	***
	75	***	***	***	***	***	***
	100	***	#	***	***	***	***
Protein	2	***	***	#	#	***	***
	5	***	***	***	#	***	***
	10	***	***	***	**	**	***
	25	***	***	***	***	***	***
	50	***	***	***	***	***	***
	75	#	***	***	***	***	***
	100	#	***	***	***	***	***
Biomass	2	*	***	**	#	#	#
	5	***	***	***	#	#	#
	10	***	***	***	***	***	*
	25	***	***	***	**	***	*
	50	***	***	***	***	***	#
	75	***	***	***	***	***	#
	100	***	***	***	***	***	***

One-way analysis of variance (ANOVA) with Tukey-Kramer multiple comparisons test, * $p< 0.05$, ** $p<0.01$, *** $p<0.001$, # $p>0.05$

Table 5. Toxicity of pyridine raffinate to *L. minor* at different pHs.

pH	EC_{50} (%)*		
	Chlorophyll	Protein	Biomass
5.0	1.3	34	77
7.0	1.5	93	86
9.0	1.3	26	84
12.0	1.1	10	5

* EC_{50} (%): < 5 highly toxic; <50 toxic; <75 slightly toxic; <100 non toxic
All values are mean (n=3)±S.D.

dissolved readily at acidic pH (pH 5.0) and precipitated at higher pHs (pH 9.0 and 12.0) (Kepert 1973). This is additional evidence that fluctuation in pH-dependent toxicity of pyridine raffinate depends on level of phenolics instead of pyridine, formaldehyde and heavy metals.

The results of toxicity tests with duckweed might be an important consideration while assessing the hazards of materials to aquatic organisms or when deriving water quality criteria for aquatic organisms. It might well be used to compare the toxicities of different materials and to study the effect of various environmental factors.

Acknowledgments We thank the Director, Industrial Toxicology Research Centre, Lucknow for his encouragement and the financial assistance from Department of

Biotechnology, New Delhi is highly acknowledged. The technical assistance of Mr. Satyarth Prakash is also acknowledged.

REFERENCES

American Public Health Association, American Water Works Association and Water Environmental Federation (1998) Standard methods for the examination of water and wastewater. 18th ed, Washington, DC

Arnon DI (1949) Copper enzymes in isolated chloroplast: Polyphenoloxidase in *Beta vulgaris*. Plant Physiol 24:1-15

Babich H, Stotzky G (1985) A microbial assay for determining the influence of physico-chemical environmental factors on the toxicity of phenol. Arch Environ Contam Toxicol 14:409-415

Giri S, Misra TN (1982) Modern organic chemistry-I. United Book Depot Allahabad, India

Goe GL (1978) Pyridine and pyridine derivatives. Encycl Chem Technol 3rd ed, 19:454-483

Hillman WS (1961) The Lamnaceae or duckweed: A review of the descriptive and experimental literature. Bot Rev 27:221-318

Hillman WS, Culley DD (1978) The use of duckweed. Am Scientist 66:442-451

HSDB (1989) Hazardous substances data bank. National Library of Medicine, National Toxicity Information Program, MD

Kepert DL (1973) Comprehensive inorganic chemistry. Pregamon Press, Oxford

Liu SM, Kuo CL (1997) Anaerobic biotransformation of pyridine in estuarine sediments. Chemosphere 35:2250-2268

Lowry OH, Rosenbrough NJ, Farr AL, Randall RJ (1951) Protein measurement with folin phenol reagent. J Biol Chem 193:265-275

Nash T (1953) The colorimetric estimation of formaldehyde by means of the Hantzsch reaction. Biochem J 55:416-421

Organization for Economic Cooperation and Development (2000) *Lemna* sp. growth inhibition test. Test guideline No. 221. OECD guidelines for testing of chemicals, Paris

Qu M, Bhattacharya SK (1997) Toxicity and biodegradation of formaldehyde in anaerobic methenogenic culture. Biotechnol Bioeng 55:727-736

Salisbury FB, Ross CW (1986) Plant physiology. CBS Publication and Distributors, New Delhi

Taraldsen JE, Norberg-King TJ (1990) New method for determining effluent toxicity using duckweed (*Lemna minor*). Environ Toxicol Chem 9:761-767

Verschueren K (1983) Handbook of environmental data on organic chemicals. 2nd ed, Van Nostrand Reinhold Company, New York

Weber CI, Peltier WH, Norberg-King TJ, Horning WB, Kessler F, Menkedick J, Neiheisel TW, Lewis PA, Klemm DJ, Pickering OH, Robinson EL, Lazorchak J, Wymer L, Fryberg R (1989) Short-term methods for estimating the chronic toxicity of effluents and receiving waters to freshwater organisms, 2nd ed. EPA-600/4-89-001 and supplement EPA-600/4-89-001A, Cincinnati, Ohio