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Use of LC_{50} in aquatic regulatory toxicology-Disharmony in global harmonization of hazard classification of chemicals

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Abstract

In regulatory aquatic toxicology, acute toxicity studies with chemicals are conducted with a species of fish, crustacea, and or alga. The LC_{50}/EC_{50} obtained from these studies is used for the hazard classification and labeling of the chemicals. The methods like probit or logit analysis and Litchfield and Wilcoxon method are prescribed in the OECD guidelines to determine the LC_{50}. In the present study, LC_{50}s were calculated using probit analysis, Litchfield & Wilcoxon method, and also using the method by Trevan (the inventor of median lethal dose) using three sets of concentration-mortality data of fish acute toxicity tests. The slopes of the concentration-mortality curves, fiducial limits (95% confidence interval) of LC_{50}s, and 'mode' of the concentration-mortality curves were compared. Though the methods used in the study resulted in more or less similar LC_{50}s, the LC_{10} and LC_{90}, slopes and 'mode' differed considerably, indicating that LC_{50} does not reveal the exact toxicity profile of a chemical. The LC_{50} calculated using Finney's probit analysis provides better information on the toxicity profile of a chemical than the LC_{50}calculated by Litchfield & Wilcoxon method. While interpreting LC_{50}, the mortality occurred below 16 % (eg., LC_{10}) and above 84 % (eg.,LC_{90}), slope and 'mode' of the concentration-mortality curve may also be considered. It is worth having a relook at the current practice of hazard classification and labeling of the chemicals based only on LC_{50} in regulatory aquatic toxicology.

Keywords: LC_{50}, GHS, Trevan, concentration-mortality curve, probit analysis, Litchfield and Wilcoxon, Finney

INTRODUCTION

In regulatory aquatic acute toxicity studies with chemicals, LC_{50}/EC_{50} isusually determined in a fish species (96 h LC_{50}) (OECD, 2019), a crustacean species (48 h EC_{50}) (OECD, 2004), and/or an alga (72/96 h EC_{50}) (OECD, 2011). Based on the LC_{50}/EC_{50}, the chemicals are classified into a hazard category (Walum, 1998). For example, Globally Harmonized System (GHS) classifies a chemical into hazard category I, if the 96 h LC_{50} of this chemical to fish is ≤ 1 mg $L^{-1}$and into hazard category II if the 96 h LC_{50} is >1 - ≤10 mg L^{-1} (GHS, 2019).

In the OECD guideline of fish acute toxicity test (OECD, 2019), probit or logit analysis is prescribed to determine LC_{50}, and the slope of the concentration-response curve (Finney, 1978), if the data are suitable, otherwise the LC_{50} can be determined using other statistical tools such as Spearman-Karber method (Stephan, 1977), the binomial method (USEPA, 2002), the moving average method (ISO, 1996), or the graphical method (USEPA, 2002). In an acute toxicity study, if the lowest mortality obtained is close to16% and the highest mortality obtained is close to 84%, most of the methods mentioned above will give a similar LC_{50} value and 95% fiducial limits (95% confidence interval) (Pillai *et al*., 2021). The LC_{50} determined

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from a mortality range covering 16-84% will be more reliable in the statistical analysis point of view, as the concentration-mortality curve in this mortality range shows a monotonic linear relationship, and using such LC_{50} for GSH classification of a chemical may have more credibility. But, one disadvantage of calculating LC_{50} using mortality data of 16-84% range is that it does not consider the mortality below 16 and above 84%, which also constitutes parts of a typical concentration-response curve. The LC_{50}s calculated with one or two partial mortalities, as mentioned in the OECD (2019) guideline, may not be used for GSH classification of chemicals, as these LC_{50}s do not provide information on the toxicity profile (all phases of the concentration-mortality curve) of the chemical. Other issues with the LC_{50} are that they vary in a wide range from one species to the other (Geyer et al., 1993) and many times are irreproducible within the same species (Peres & Pihan, 1991), as the physico-chemical parameters of the dilution water play a crucial role in LC_{50} experiments. For example, the 96 h LC_{50} for rainbow trout ranged from 0.24 to 12.20 mgL^{-1}, depending on the chloride content of the dilution water (chloride content ranged from 0.35 to 40.9 mg L^{-1}) (Lewis & Morris, 1986). More than 750 tests conducted on fathead minnow with 644 chemicals revealed that a steady state of 96 h LC_{50} is not met (Mc Carty, 2012).

The purpose of the present study was to compare the performance of the classical maximum likelihood methods such as the Litchfield & Wilcoxon (1949) method and Probit analysis (Finney, 1978) for determining LC_x (LC_{50}, LC_{10}, and LC_{90}) and the associated statistical parameters of the concentration-mortality curve. LC_{50} determined using the method described by Trevan (1927) in his original publication was also included for comparison. The 'mode', which was explained by Trevan (1927) as the dose at which the inflexion occurs in a typical concentration-mortality curve is also discussed. The worthiness of LC_{50} for GSH classification of chemicals is discussed in light of the results obtained in the study.

MATERIALS AND METHODS

A simulation study was conducted using three different configurations of concentration of chemicals *vs* mortality data in fish (Table 1). The number of test concentrations (5) and the number of fish exposed to each test concentration (7), and the factor for spacing the two successive concentrations (by a factor of 2) are as per the OECD test guideline No. 203 (TG 203) for acute toxicity test in fish (OECD,2019). Based on a survey conducted at contract research organizations, Burden *et al.* (2017) reported that TG203 is the most commonly used guideline in vertebrate ecotoxicology studies. In the present simulation study, in configurations A and C, the concentrations ranged from 0.4 to 6.4 mg L^{-1}, whereas in configuration B, it ranged from 0.2 to 3.2 mg L^{-1}. The above ranges of the concentration were selected to provide a similar LC_{50}, but different toxicity profiles (phases of the concentration-mortality curve). The LC_x and 95% fiducial limits of it (Finney, 1978), and LC_{50} and 'mode' (Trevan, 1927) were calculated manually. LC_x determination by the Litchfield & Wilcoxon (1949) method was done using software (Adams *et al*., 2016), whereas the 95% confidence interval of LC_x was calculated manually. The parallelism of the slopes was examined using the method of Finney (1978).

RESULTS

In configurations A and C, the concentrations to which the fish exposed were the same, and the mortality occurred in 3 concentrations, *viz*., 0.8, 1.6 and 3.2 mg L^{-1} were also the same (Table 1). The LC_{50}s and fiducial limits (95% confidence interval) determined using the methods of Litchfield & Wilcoxon (1949) and Finney (1978) were very close to each other for the configurations A and C. The difference in mortality in configurations C, compared to configurations A, in 0.40 and 6.4 mg L^{-1}did not affect the LC_{50} values and fiducial limits (95% confidence interval), except the fiducial limits determined by probit analysis. But, the difference in mortality markedly affected the slope of the concentration-mortality curves, LC_{10} and LC_{90} and fiducial limits of LC_{10} and LC_{90} of these configurations (Table 2).

No heterogeneity of the points about the regression line established between probit and log concentration was found as evidenced by non-significant χ^2 values (heterogeneity was calculated as given by Finney (1978). The 'modes' of configurations A and C are similar but high in configuration B (Table 2).

DISCUSSION

In acute toxicity experiments with aquatic organisms, it is a common practice to determine median lethal/effective

Table 1. Different configurations (configurations A, B & C) of concentration of chemicals *vs* mortality data in fish -Simulation study.

Configuration-A		Configuration -B		Configuration -C	
Concentration (mg L^{-1})	[1]Mortality	Concentration (mg L^{-1})	[1]Mortality	Concentration (mg L^{-1})	[1]Mortality
0.4	1/7	0.2	1/7	0.4	0/7
0.8	3/7	0.4	1/7	0.8	3/7
1.6	4/7	0.8	3/7	1.6	4/7
3.2	6/7	1.6	5/7	3.2	6/7
6.4	7/7	3.2	6/7	6.4	6/7

Note: [1]Number of fish died/number of fish exposed.

Table 2. The slopes, *b* (Litchfield & Wilcoxon, 1949 and Finney, 1978), intercept, *a* (Finney, 1978) and 'mode' (Trevan, 1927) of the concentration *vs* mortality curve, 96 h LC_{50}, 96 h LC_{10} and 96 h LC_{90}, and χ^2 (heterogeneity) (Finney, 1978)

Configuration	Analysis	*b*	*a*	Variance	Mode	LC_{50} (mg L^{-1})	LC_{10} (mg L^{-1})	LC_{90} (mg L^{-1})	X^2
A	Litchfield & Wilcoxon	2.68	-	-	-	1.12 (0.54-2.33)	0.31 (0.09-1.14)	4.01 (1.01-14.60)	-
	Probit	2.22	4.88	0.0137	-	1.13 (0.52-2.46)	0.30 (0.02-1.51)	4.26 (±1.08)	0.25
	Trevan	-	-	-	1.20	1.20	-	-	-
B	Litchfield & Wilcoxon	3.29	-	-	-	0.91 (0.44-1.89)	0.19 (0.05-0.85)	4.28 (0.99-8.58)	-
	Probit	1.99	5.05	0.0175	-	0.95 (±0.29)	0.22 (0.02-.43)	4.09 (±1.25)	0.55
	Trevan	-	-	-	1.66	1.00	-	-	-
C	Litchfield& Wilcoxon	3.63	-	-	-	1.25 (0.64-2.44)	0.24 (0.06-0.99)	6.49 (1.56-27.00)	-
	Probit	1.45	4.99	0.0315	-	1.00 (±0.41)	0.10 (± 0.04)	13.42 (±5.48)	0.02
	Trevan	-	-	-	1.02	1.23	-	-	-

Note- Values given in the parentheses in the LC_{50}, LC_{10} and LC_{90} columns are 95% fiducial limits (95% confidence interval); values with the ± sign are standard errors (SE) calculated as given by Finney (1978), the reason being the 95% fiducial limits exploded in a meaninglessly wider range, when calculated using the equation (maximum likelihood estimation) given by Finney (1978).

concentration (LC_{50}/EC_{50}) (Hodson *et al*, 1984; Zagatto *et al*., 2012). Labeling for environmental hazards (acute toxicity categories) is based on LC_{50}/EC_{50} determined in acute toxicity studies conducted with fish, crustacean and or alga (Scholz *et al.*, 2014). It is a requirement of United Nations Global Harmonisation System that the chemicals for distribution require appropriate labeling of environmental hazards. The European Chemicals Agency (ECHA, 2017) uses LC_{50} determined in fish as per the Test Guideline 203 of OECD (OECD, 2019) for environmental classification of a chemical according to the GHS of Classification, Labeling and Packaging of Chemicals (Paparella *et al.*, 2021). But, LC_{50}-based environmental classification of a chemical has a disadvantage, since such classification does not consider the slope of the concentration-mortality curve, the reason being chemicals may show similar LC_{50} but with different slopes of the concentration-mortality curve. Another issue is that the statistical methods prescribed in various guidelines such as USEPA (1996; 2002) and OECD (2019) for analyzing concentration-mortality data result in different LC_x and 95 % confidence interval of the LC_x. For calculation of the slope of a concentration-mortality curve, the probit analysis of Finney (1978) has an advantage over the method of Litchfield & Wilcoxon (1949), as it considers all concentration-mortality data points (except 0 and 100% mortality) in the calculation procedure. On the other hand, Litchfield & Wilcoxon (1949) method calculates the slope of a concentration-mortality curve using the values of LC_{16}, LC_{50}, and LC_{84}. In the present study, the concentrations used in configuration B were in a lower range than that of the other configurations. The LC_{50}s determined using the above two methods varied in a narrow range (0.91-0.99 mg L^{-1}) in this configuration. Based on the results obtained in the present study, as per the GHS (2019), configuration B chemical qualifies to be classified into hazard category I, since the 96 h LC_{50} of it is ≤ 1 mg L^{-1}, though the LC_{50} calculated by the method of Trevan (1927) is 1 mg L^{-1}, whereas configurations A and C chemicals are classified into the category, II as the 96 h LC_{50} of these chemicals is >1 - ≤10 mg L^{-1}. Infact the 96 h LC_{50}s of configuration A, B, and C chemicals are close to each other. A simple method to compare the LC_{50}s is to examine the fiducial limits of LC_{50}s; no significant difference exists if the fiducial limits (confidence interval) overlap (Wheeler *et al*., 2006). The fiducial limits (confidence interval) of 96 h LC_{50}s of chemicals of configurations A, B, and C overlap, hence the 96 h LC_{50}s cannot be considered different. This means that the chemicals A, B, and C have equal opportunities to be classified into the hazard category I or II. Having similar LC_{50}s does not mean that the chemicals possess a similar toxicity profile (Pillai *et al.*, 2021). It is evident from Table 2 that the toxicity profile of chemicals of configurations A, B, and C are different as the LC_{10} of these chemicals differed in the range, 0.10-0.30 mg L^{-1}. In the case of LC_{90}, the chemicals of configurations A and B showed more or less similar values, whereas the chemical of configuration C showed a higher value. The Pesticide Manual published by British Crop Production Council (BCPC, 1972), a widely referred publication by the mammalian and environmental toxicologists provides LC_{50} values for pesticides and related chemicals for fishes, but without mentioning 95% confidence interval and statistical analysis used to calculate

the LC_{50} values. On the contrary, LC_{50}s and their 95% confidence intervals of more than 100 chemicals for rainbow trout were discussed in the Handbook of Acute Toxicity of Chemicals to Fish and Aquatic Invertebrates published by the US Department of the Interior Fish and Wildlife (Waynon & Finley, 1980). In this publication, a minimum of 10 fish each was exposed to a minimum of 6 test concentrations of each chemical, and the LC_{50}s and their 95% confidence intervals were calculated using the method of Litchfield & Wilcoxon (1949). For the majority of the chemicals, the confidence intervals of LC_{50}s varied in a narrow range. The reason for this is that in Litchfield & Wilcoxon (1949) method, for calculation of the slope of the concentration-mortality curve, LC_{16}, LC_{50}, and LC_{84} values from the concentration-mortality curve are considered (usually these values fall linearly). The slope thus calculated is used for the calculation of 95% confidence interval of LC_{50}. For the calculation of the slope, Litchfield & Wilcoxon (1949) method does not consider mortality data <16 % and >84 %, which are also parts of a typical concentration-mortality curve. This can be further explained using an example given in Table 3 and Figure 2. The LC_{50} and its 95% confidence interval/fiducial limits of Chemical A, where the mortality ranged from 10 to 90 %, calculated using Litchfield & Wilcoxon (1949) method and the method of Finney (1978) are more or less the same. For Chemicals B and C, where the mortality ranged from 30 to 80 % and 30 to 70%, respectively (100 % mortality is not considered for the calculation of LC_{50} by both the methods), LC_{50}s calculated by Litchfield & Wilcoxon (1949) method are very close to each other and their 95% confidence interval is also more or less similar, whereas LC_{50}s calculated by the method of Finney (1978) differed and their 95% fiducial limits exploded in a wider range. The slopes of the concentration-mortality curves of Chemicals A, B, and C are given in Figure 2. From the Figure, it is evident that to induce minimum mortality, the concentration required for Chemical B is less, compared to Chemicals A and C (the LC_1s

Table 3. Mortality data, LC_{50}, slope of the concentration-mortality curve and 95% confidence interval of LC_{50} of fish exposed to 5 concentrations of three chemicals.

Concentration (mg L^{-1})	2	4	8	16	32	64	LC_{50} (95% confidence interval) (mg L^{-1})			
	Number of mortality[a]						L&F	Slope[b]	Finney	Slope[c]
Chemical A	1	2	4	6	9	-	9.96 (5.60-17.71)	3.12	10.09 (6.22-17.98)	2.04
Chemical B	-	3	4	7	8	10	8.96 (5.08-16.06)	3.77	7.85 ± 0.41(±SE)	1.33
Chemical C	-	3	4	7	10	10	8.12 (4.78-13.70)	2.79	8.97 ± 0.36 (±SE)	1.73

[a]Number of fish exposed to each concentration is 10; L&F – LC_{50} and (95% confidence interval of LC_{50}) calculated using the method of Litchfield & Wilcoxon (1949); [b] calculated using LC_{16}, LC_{50} and LC_{84} (Litchfield & Wilcoxon,1949); Finney- LC_{50} and 95% fiducial limits to LC_{50} (maximum likelihood estimation) calculated using the method as given by Finney (1978). Where the 95% fiducial limits to LC_{50} exploded in a meaninglessly wider range (Chemicals B and C), standard error of LC_{50} is given.

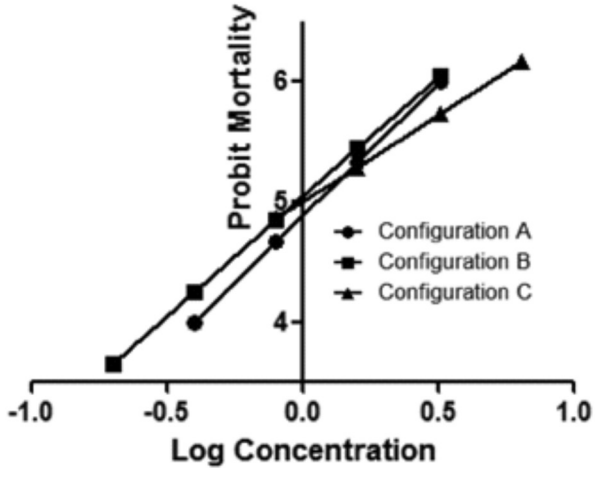

Figure 1. Regressions lines established for probit mortality *vs* log concentration for chemicals of Configurations A, B and C (Probit analysis).

$Y = a + b \log X$, where Y = Probit mortality; X = Concentration (mg L^{-1}) a = Intercept, b = Slope. For Configuration A, $Y = 4.88 + 2.22 \log X$; For Configuration B, $Y = 5.05 + 1.99 \log X$; For Configuration C, $Y = 4.99 + 1.45 \log X$.

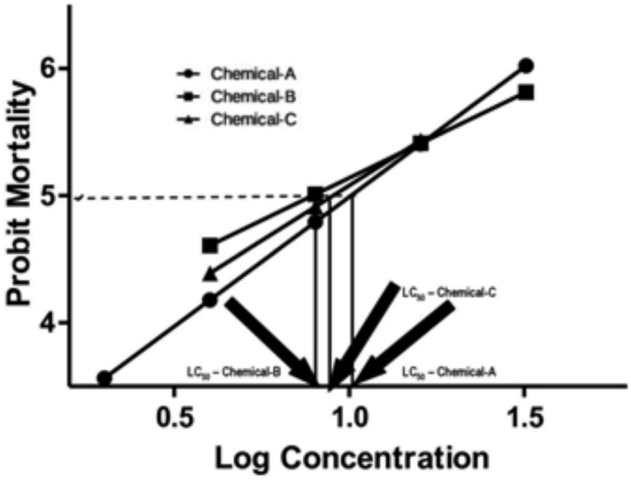

Figure 2. Regressions lines established for probit mortality *vs* log concentration for Chemicals A, B and C (Probit analysis).

Note: $Y = a + b \log X$, where Y = Probit values for mortality, a = Intercept, b = Slope, X = Concentration (mg L^{-1}). For Chemical A, $Y = 2.9505 + 2.0412 \log X$; For Chemical B, $Y = 3.8065 + 1.3338 \log X$; For Chemical C, $Y = 3.3474 + 1.7340 \log X$. Probit 5 corresponds to 50 % mortality.

for Chemicals A, B, and C were calculated as 0.70, 0.40, and 0.69 mg L^{-1}, respectively by Litchfield & Wilcoxon (1949) method, and 0.73, 0.14 and 0.41 mg L^{-1}, respectively by the method of Finney (1978). The LC_{50}s calculated by Litchfield & Wilcoxon (1949) method indicate that Chemical C is more toxic to fish followed by Chemicals B and C, whereas the LC_{50}s calculated by the method of Finney (1978), indicate that Chemical B is more toxic to fish, followed by Chemicals C and A. LC_1 calculated by the method of Finney (1978) provides similar trend of toxicity to that of LC_{50} (Chemical B > Chemical C > Chemical A). When calculated by Litchfield & Wilcoxon (1949) method, the trend of toxicity based on LC_1 changed from that of LC_{50} (for LC_{50} the trend was: Chemical C > Chemical B > Chemical A; for LC_1 the trend was: Chemical B > Chemical C= Chemical A).

The OECD (2019) guideline prescribes to use of a minimum number of 7 fish at each test concentration, and in Table 1, the number of fish exposed to each test concentration is 7. The probable mortality that can occur in 7 numbers fish is 0/7 (0%), 1/7 (14%), 2/7 (29%), 3/7 (43%), 4/7 (57%), 5/7 (71%), 6/7 (86%), or 7/7 (100%). Since 0 and 100% mortality are generally not used for determination of LC_{50} using probit analysis (no probit values corresponding to 0 and 100% mortality), in the methods of Litchfield & Wilcoxon (1949) and Finney (1978), only 6 values (14, 29, 43, 57, 71 and 86%) are useful for LC_{50} determination. Though these 6 values are adequate to calculate a statistically reliable LC_{50} and associated statistical parameters, rarely mortality data does spread out covering all phases of the concentration mortality curve in an acute toxicity test. However, some commercially available software for calculation of LC_{50} by probit analysis accepts the input data of 0 and 100% mortality. The software assigns a value close to 0 (for example 0.1) for 0% mortality and a value close to 100 (for example 99.9) for 100% mortality. The corresponding probit values for 0.1 and 99.9% mortality will be included in the calculation procedure. But, 0.1 and 99.9% mortality may not contribute much to the LC_{50} value calculated by Litchfield & Wilcoxon (1949) method, if the concentration-mortality curve covers 16 and 84% mortality (Pillai *et al*., 2021). An experiment the purpose of which is only to determine LC_{50}, and if the mortality resulted in the experiment is close to 16 and 84%, Litchfield & Wilcoxon (1949) method could be the preferred method, as it is easy to perform. For establishing a concentration *vs* mortality relationship, covering all the data points (excluding 0 and 100% mortality), probit analysis by Finney (1978) is more ideal.

Parallel regression lines of mortality probits on log doses indicate that the mode of actions of chemicals on test organisms are similar (Finney, 1978). That means non-parallel regression lines of mortality probits show that the chemicals possess different mode of actions on that particular organism. In the present study, though the linearity between log concentration and probit mortality was well established without heterogeneity for all three configurations (Tables 2), the regression lines were found to be not parallel ($P<0.05$), when tested for parallelism. The expression mode of action for a concentration-response relationship is not different from the term 'characteristic' coined by Trevan (1927). Mode of actions ('characteristics') of chemicals of different structures are rarely the same for a particular organism, which cannot be judged by LC_{50}. One way to differentiate mode of action from LC_{50} is determining the 'mode' of the concentration-response relationship. The 'mode' is the individual lethal dose which occurs most frequently, and which is the dose at which the inflexion, or steepest portion, occurs in typical concentration-mortality curve (Trevan, 1927). The 'mode' is calculated from mean and median values. For calculating the mean value, initially the mid point (*d*) of each concentration interval is found out. Then, difference of percent mortality (*f*) against each mid point is calculated. For each concentration interval, $f \times d$ is calculated, sum of $f \times d$ is found out, and is divided by sum of *f* to obtain the mean value. The median is the concentration necessary to kill 50% of the fish, that can be calculated from the concentrations which caused >50% (concentration *a*) and < 50 % (concentration *b*) mortality, percentage difference of mortality between 50 and *x* (*x* is the % mortality subsequent low or high to 50% mortality), and percentage difference of mortality between *a* and *b*. The mode is then calculated using the formula, Mode = Mean-3 (Mean-Median). If the mean concentration is nearly equal to LC_{50} (median value), the reliability of the 'mode' determined will be higher. If the concentrations are chosen on the basis of a well designed range finding experiment, such a mean value for the concentrations can be achieved. The OECD (2019) guideline allows to perform a range-finding test before the definitive test for selection of the appropriate concentration range. With the information on the toxicity of a chemical available in the public domain, and also obtained from the range finding study it is practically possible to choose a range of at least 5 concentrations which would cause a partial mortality in a wider range covering all phases of the concentration-mortality curve.

A study conducted on EU Registration, Evaluation, Authorisation, and Restriction of Chemicals (REACH) database indicated that for fish acute toxicity studies, the dominant endpoint is LC_{50} (Saouter *et al*., 2019). As discussed in this paper, LC_{50} is used for GHS classification of chemicals. OECD guidelines normally followed by GLP (Good Laboratory Practice) certified testing laboratories provide various options for the selection of statistical analysis of concentration *vs* mortality data. The LC_{50}s and their 95% confidence limits of a chemical substance for a species calculated using various methods proposed in the OECD guidelines may or may not be comparable. For academic research, the LC_{50}s calculated using any standard method may be acceptable, but for a regulatory purpose, where LC_{50} is used to classify chemicals based on acute toxicity, to avoid ambiguity, the guidelines may clearly indicate the statistical method to be used to calculate LC_{50} and associated statistical parameters. The chemicals that show similar LC_{50} values may show different toxicity patterns at the lower and higher concentrations tested. For example, some chemicals may show high toxicity at lower concentrations and low toxicity at higher concentrations; and *vice versa*. This information

about the expression of toxicity of the chemical is missed out in certain methods of calculation of LC_{50} prescribed in OECD guidelines. From the present study it may be concluded that classifying chemical into a hazard category based only on LC_{50} under-expresses or over-expresses the toxicity profile of the chemical. Hence, hazard classification of chemicals based on a single value like LC_{50} has a disadvantage, and further studies to develop a more integrative approach to classify chemicals are needed.

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