Bull. Environ. Contam. Toxicol. (2004) 72:873–880

DOI: 10.1007/s00128-004-0325-0

Bulletin of Environmental Contamination and Toxicology

Use of the Up-and-Down Acute Toxicity Test Procedure to Generate LC50 Data for Fish

R. M. Sunderam, R. W. Patra, M. Julli, M. St. J. Warne*

Ecotoxicology and Water Science Section, Department of Environment and Conservation (DEC), New South Wales and Center for Ecotoxicology, 480 Weeroona Road, Lidcombe, NSW 2141, Australia

Received: 30 April 2003/Accepted: 9 March 2004

The up-and-down procedure, described originally by Dixon and Mood (1948), to test the sensitivity of explosives, was adapted for acute toxicity testing of rats (Bruce, 1985). This later procedure was adapted by ASTM in 1987 and revised in 1990 (ASTM, 1991). The OECD is also developing a similar guideline (OECD, 2000). In this study this method is modified to generate LC50 data for fish.

Ecotoxicity tests are used to assess the potential of chemicals, effluents, spills and illegal discharges to cause environmental harm. Fish tests form an integral component of such assessments. Quality assurance and quality control procedures for such tests require the assessment of the relative sensitivity of batches of the test animals to a reference toxicant. This provides information on whether the test animals are abnormally sensitive or tolerant compared to historical values and therefore provides information on the acceptability of each batch of test animals as well as the test results (USEPA, 1993). The use of animal welfare and ethics legislation in many states and countries, including Australia require the refinement, reduction and replacement ("3Rs") of animals in research. This apparent dilemma could be resolved by using the up-and-down procedure to generate data on reference toxicants using a smaller number of fish than is required using the traditional method.

The aim of this study was to assess the applicability of the up-and-down method for quality assurance purposes by determining the variability of up-and-down LC50 values and by comparing these LC50 values to those generated by the traditional method.

MATERIALS AND METHODS

Prior to conducting any up-and-down experiments, the utility of the method was assessed by simulating LC50 values using historical data. Data on the toxicity of endosulfan to silver perch (*Bidyanus bidyanus*) (Patra, 1999) and phenol to eastern rainbow fish (*Melanotaenia duboulayi* Castelnau) (Johnston *et al*, 1990)

*Present address: CSIRO Land and Water, PMB 2, Glen Osmond, SA 5064, Australia
Correspondence to: R. M. Sunderam

were used in this simulation. The lowest concentration showing 100% mortality and the highest concentration showing no mortality along with all concentrations in between were selected. These data were re-tabulated and one of these concentrations was selected at random, as the starting point for the simulation. An animal from this concentration was selected, at random, and its fate recorded as either alive (A) or dead (D). The simulation was repeated twelve times for each set of concentration-response data and the resulting data used in the maximum likelihood estimation procedure to determine twelve LC50 values.

Analytical reagent (>99.8%) grade Phenol (Cas No.108-95-2) was used in all tests. A stock solution of phenol was prepared in polished reverse osmosis water. These were stored in a refrigerator and used within three months of preparation. All toxicity tests were conducted in dechlorinated Sydney mains water under static conditions. This water, had a mean pH of 7.5 and total alkalinity and total hardness of 25 and 45 mg/L as $CaCO_3$ respectively.

Eastern rainbow fish was the test species because they are native to Australia, are a common resident of freshwater systems of the East Coast of Australia (Merrick and Schmida, 1984) and because it has been widely used in toxicity tests (eg: Sunderam *et al*, 1992; Patra, 1999). Rainbow fish were reared in the laboratory. Four different age classes (1, 2-3, 4-5 week old and over one year old [45-61 mm length]) of *M. duboulayi* were used. The fish were maintained and tested at 25°C with a 16:8h light: dark cycle.

Both traditional and up-and-down toxicity tests were conducted in parallel, in the same test facility, and using a common batch of *M. duboulayi*. These tests were conducted under Royal North Shore Hospital/University of Technology, Sydney ethics approval number 9809-045A, following Ministerial approval.

Traditional LC50 testing generally followed the OECD guideline No. 203 (OECD, 1981). Twenty four hour tests were carried out under static conditions in glass beakers. For the three youngest age classes of fish, 250mL beakers containing 200mL of test solution were used. For the fish greater than one year old, 1.2L beakers containing one litre of test solution were used. Each experiment contained six duplicated treatments, which consisted of a control and five logarithmically arranged concentrations of phenol. Five fish were randomly assigned to each beaker that were randomly arranged inside an incubator.

For up-and-down tests the American Society for Testing and Materials (ASTM) method for estimating acute oral toxicity in rats, Designation E 1163 (ASTM, 1991) was adapted for fish. Fish were exposed one at a time, exposing the first animal at an estimate of the LC50. If this animal survived the exposure for 24 hrs then the next fish was exposed to a concentration 1.3 times greater. However, if the first animal died or appeared moribund after 24 hrs exposure, the next fish was exposed to a concentration 1.3 times less. The concentration for each subsequent fish was adjusted up or down using this above method depending upon the

outcome for the previous fish. The exposure was repeated as above, until four fish were exposed after the reversal of the initial outcome.

In both methods the fish were checked for mortality 3 and 24-hours after exposure. Death was defined as the absence of opercula movement, loss of equilibrium and absence of response to external stimuli. Temperature, dissolved oxygen concentration (DO), pH and conductivity were measured at the start and finish of each exposure period.

All calculations were based on nominal phenol concentrations. LC50 values for traditional tests were calculated using the trimmed Spearman-Karber method (Hamilton *et al*, 1977, 1978). The LC50 values for all the up-and-down studies, were calculated using a maximum likelihood method (Dixon, 1965) in a BASIC computer programme described in ASTM (ASTM, 1991). The maximum likelihood estimators use the evidence from the experimental data to determine the concentration that has the greatest likelihood of being the LC50.

To determine the variability in the LC50 values obtained by the up-and-down method, the tests were repeated. With 1 week and 4-5 week old fish the up-and-down test was repeated ten times, while for the other two age groups it was repeated five times due to limited numbers of appropriately aged fish.

The standard error of the difference test (Zar, 1974) was used to determine whether the LC50 values determined by the two methods were significantly different and to compare the LC50 values determined by the up-and-down method.

RESULTS AND DISCUSSION

24-h and 96-h LC50 values for endosulfan to *B. bidyanus* at 15, 20, 25, 30 and 35°C were obtained by the traditional and up-and-down methods using historical data. Comparison of the values obtained by the two different methods showed that they were not significantly ($p>0.05$) different for the 24h values at 30°C and 96h values at 25°C. The 24h values at 20 and 25°C and 96h values at 30 and 35°C showed no significant ($p<0.05$) difference in 92% of the cases. The 24h values at 15 and 35°C and 96h values at 20°C showed no significant ($p<0.05$) difference in 83 percent of cases and the 96h values at 15°C showed no significant ($p<0.05$) difference in 75% of the cases (Fig 1).

Traditional and up-and-down LC50 values for phenol to *M. duboulayi* at three temperatures (Johnston *et al*, 1990) were similarly derived using historical data. Comparison of the values showed that they were not significantly different ($p<0.05$) except for the 24h LC50 values at 15°C where they were not significantly different ($p>0.05$) in 92% of the cases (Fig 2). Thus overall the LC50 values obtained by the two different methods for the two chemicals using

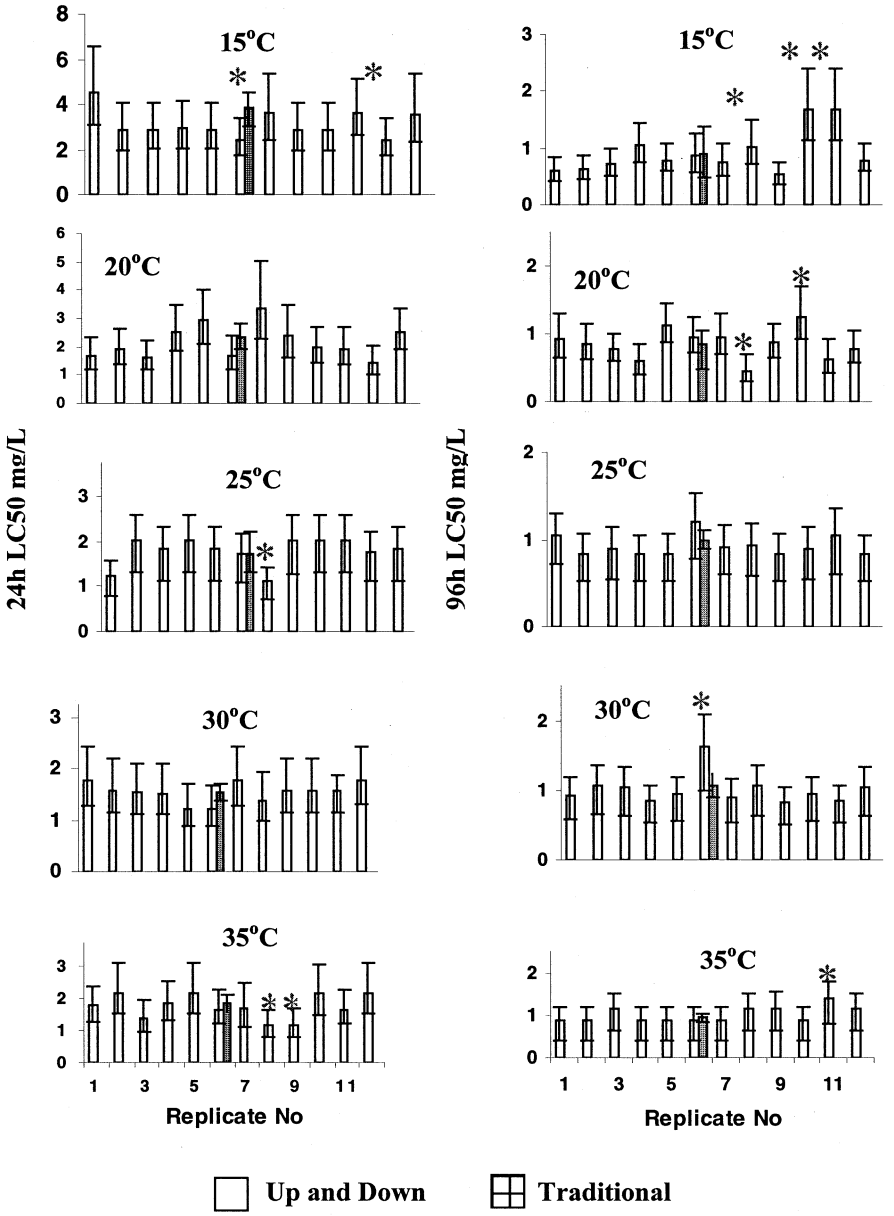

Figure 1. 24h and 96h LC50 values obtained by the traditional and up-and-down method using historical data for endosulfan to silverperch (*Bidyanus bidyanus*) at 15, 20, 25, 30 and 35°C. (*) significantly ($p<0.05$) different from the traditional LC50 value

historical data indicated that they were not significantly different ($p>0.05$) in 89% of the cases. No significant differences ($p<0.05$) were observed among the up-and-down LC50 values of either chemical.

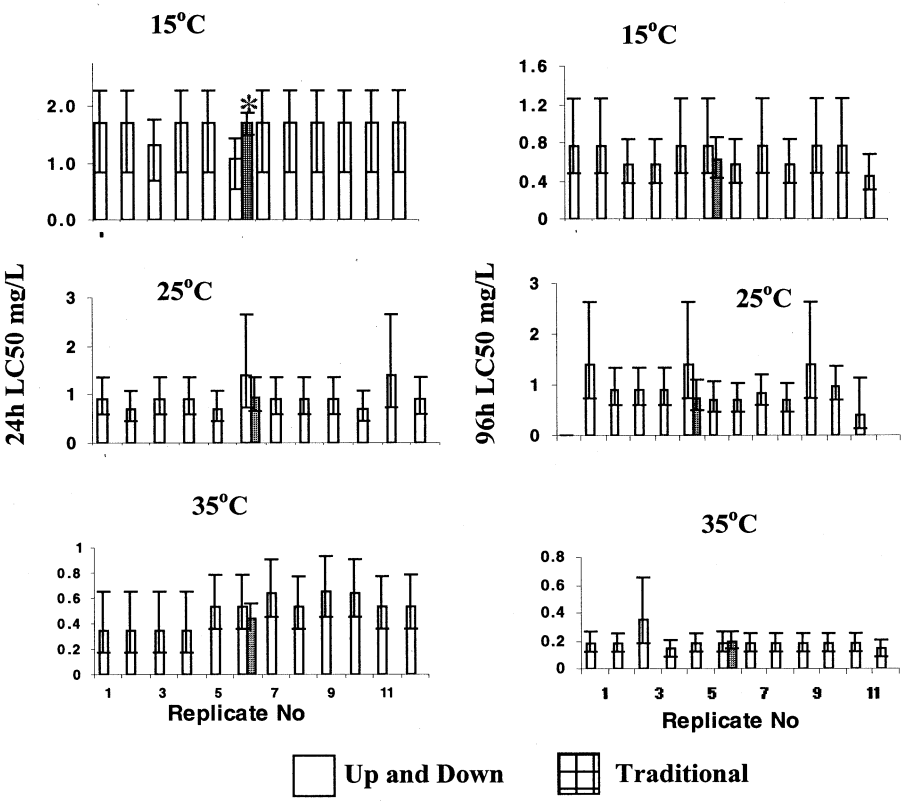

Figure 2. 24h and 96h LC50 values obtained by the traditional and up-and-down method using historical data for phenol to the eastern rainbow fish (*M. duboulayi*) at 15, 25and 35°C. (*) Significantly ($p<0.05$) different from the traditional LC50 value

Table 1 presents the number of animals used, the 24-h LC50 values and the corresponding 95% fiducial intervals for phenol calculated by both methods for the toxicity tests conducted in this study. As with the results discussed above there is general agreement of LC50 values calculated by the two methods, though the agreement is better for 1 week and >1 year old fish. Comparison of the traditional and up-and-down method LC50 values showed they were not significantly ($p>0.05$) different in 80% of the cases. Within an age group the LC50 values obtained by the up-and-down method were not significantly ($p<0.05$) different to each other.

Table 1. Comparison of acute LC50 values of phenol to eastern rainbow fish (*Melanotaenia duboulayi*) determined by traditional and up-and-down tests.

A g e (weeks)	24h LC50 values (mg/L) (95% Fiducial Intervals in parentheses)					
	Traditional method [1]	Up-and-Down method [2]				
1	33 (26-41)	34 (25-48)	35 (26-49)	32 (23-44)	28 (20-39)	34 (25-48)
		33 (24-45)	33 (24-45)	40 (29-56)	33 (24-45)	38 (27-51)
2 - 3	23 (20-26)	32* (24-43)	30 (22-40)	25* (19-34)	30 (22-40)	28 (21-37)
4 - 5	24 (20-29)	33 (24-45)	33 (24-47)	37* (27-50)	32 (23-44)	35 (25-48)
		30[a] (22-41)	33[a] (24-44)	36*[a] (26-49)	28 (20-37)	30 (22-41)
<52	28 (25-30)	29 (21-39)	23 (17-32)	25 (19-34)	30 (22-41)	23 (17-32)

*Significantly ($p<0.05$) different from traditional LC50 value

[1] 60 fish used per test.

[2] Six fish used per test except where indicated by [a] where seven fish were used per test.

The traditional method used 60 fish per test while each up-and-down test required only 6-7 fish. Thus the substantial reduction in animal usage required to determine a 50% lethal concentration reported for rats (Bruce, 1985; 1987) has been confirmed in this study with fish.

The present study was limited to 24-hour exposures. The traditional method provided LC50 values in 24 hours. In contrast, when the ASTM (1991) or OECD (1999) up-and-down methods were used a LC50 determination takes a minimum of six and five days respectively. Thus, the up-and-down method is easiest to apply to materials that produce mortality within one or two days. The method would not be practical to use when considerably delayed mortality can be expected. For fish 96-h LC50 values have typically been used to represent acute toxicity. The up-and-down method could be extended to determine 96-h LC50 values, however using the ASTM (1991) method it will take a minimum of 24 days to complete a single determination. The time needed to obtain a 96-h LC50 by the up-and-down method could be reduced by starting two or three tests simultaneously (OECD, 1999) which could reduce the minimum time to 20 or 16 days respectively. A major limitation to the usefulness of the up-and-down method is the time it takes to derive a LC50 value. Whether this is a problem or not depends on how rapidly a LC50 value is required and on the duration of the exposure period.

The magnitude of a fiducial interval around an LC50 is proportional to the reciprocal of the square root of the sample size. Essentially, in the LC50 determinations, the number of organisms tested at concentrations immediately above and below the LC50 value is important. With the traditional method, used in this study, there were 10 fish either side of LC50 or 20 altogether. With the up-and-down method the number of fish is likely to be 6. Thus it would be expected that the fiducial intervals of LC50 values generated by the up-and-down method would be larger than those generated by the traditional method. This is clearly observed in Table 1. This would mean that larger differences in LC50 values would be required in order for them to be statistically significant ($p<0.05$).

The advantage of the three simultaneous test method of the OECD (1999) is that this will estimate both the LC50 and the slope of the concentration-response curve, which has critical application in the evaluation of new chemicals. The ASTM (1991) method does not permit internal estimation of the slope of the concentration-response curve or its related parameter, σ (reciprocal of slope). This means that the σ used in the maximum likelihood statistics to calculate LC50 values is a value that is based upon historical data. The use of a single σ value for all chemicals assumes that there is essentially no difference in the slope of the concentration-response relationship for all chemicals. Despite this assumption being incorrect Lipnick *et al*; (1995) found that varying σ from 0.05 to 0.25 made very little difference to the resulting LD50 values. Thus the inability of the ASTM (1991) up-and-down method to calculate σ does not appear to be a major limitation.

Many regulatory authorities require, as part of quality assurance and quality control (QA/QC) procedures, that reference toxicant tests be conducted in parallel with toxicity tests. However, in some jurisdictions there is also legislation that controls the use of vertebrates in toxicity tests. In such circumstances the up-and-down method could meet both QA/QC requirements and ethical constraints by providing a reliable means of determining LC50 values but using a minimum number of fish.

Acknowledgments: We thank Dr. John Chapman for providing valuable comments on earlier drafts of this manuscript. We also thank Dr. Anu Kumar and Mr.Ian Shannon for reviewing the manuscript.

REFERENCES

ASTM (1991) Standard test method for estimating acute oral toxicity in rats. Designation E 1163-90. American Society for Testing and Materials, Philadelphia, USA pp741-746.

Bruce RD (1985) An up-and-down procedure for acute toxicity testing. Fundam Appl Toxicol 5:151-157

Bruce RD (1987) A confirmatory study of the up-and-down method for acute toxicity testing. Fundam Appl Toxicol 8:97-100

Dixon WJ (1965) The up-and-down method for small samples. J American Stat Assoc. 60:967-978

Dixon WJ, Mood AM (1948) A method for obtaining and analyzing sensitivity data. J American Stat Assoc 43:109-126

Hamilton MA, Russo RC, Thurston RV (1977) Trimmed Spearman-Karber Method for Estimating Median Lethal Concentrations in Toxicity Bioassays. Environ Sci Technol 11:714-719. Correction: 1978: 12. 417.

Johnston N, Skidmore JF; Thompson G (1990) Applicability of OECD test date to Australian aquatic species. A report to the Advisory Committee on Chemicals in the environment. Australian and New Zealand Environment Council. Sydney, NSW, pp115

Lipnick RL, Cotruvo JA, Hill RN, Bruce RD, Stitzel KA, Walker AP, Chu I, Goddard M, Segal L, Springer JA, Myers RC, (1995) Comparison of the up-and-down, traditional LD50, and fixed-dose acute toxicity trocedures. Fd Chem Toxic 33:223-231

Merrick JR, Schmida GE (1984) Australian Freshwater Fishes. Biology and Management Griffin Press, South Australia 409pp.

OECD (1981) OECD Guidelines No. 203 "Fish acute toxicity test". Organization for Economic Co-operation and Development, Paris, France p. 1-12.

OECD (1999) OECD Guidelines for Testing of Chemicals. Revised Draft Guideline No. 425 "Acute Oral Toxicity: Modified Up-and Down Procedure". Organization for Economic Co-operation and Development, Paris, France p. 1-12.

OECD (2000) The Revised Up-And-Down Procedure: A Test Method For Determining The Acute Oral Toxicity: of Chemicals And Products. Organization for Economic Co-operation and Development, Paris, France.

Patra RWR (1999) Effects of temperature on the toxicity of chemicals to Australian fish and invertebrates. Ph.D. Thesis, University of Technology, Sydney, Australia pp332.

Ruesink RG, Smith LL.Jr, (1975) The relationship of the 96-hour LC50 to the lethal threshold concentration of hexavalent chromium, phenol and sodiumpentachlorophenate for fathead minnows (*Pimephales promelas* Rafinesque). Trans American Fish Soc 105:567-570.

Sunderam RIM., Cheng DMH, Thompson GB (1992) Toxicity of endosulfan to native and introduced fish in Australia. Environ Toxicol Chem 11:1469-1476.

USEPA (1993) Methods for measuring the acute toxicity of effluents and receiving waters to freshwater and marine organisms. Weber CI(Ed.) 4th ed. Environmental Monitoring and Support Laboratory, U.S. Environmental Protection Agency, Cincinnati, Ohio. EPA /600/4-90/027F

Zar JH (1974) Biostatistical Analysis, Prentice Hall Inc., New York