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ACUTE TOXICITY TEST IN AQUACULTURE: A REVIEW

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Abstract. Acute toxicity test involves estimation of LC_{50} or LD_{50} which is the concentration or dose which proved to be lethal causing death to 50% of the tested organisms. This article reviews the methods utilized for the determination of Probit and LC_{50} and also explain the use of Minitab 14 statistical package and Microsoft office excel 2007 for accurate, speedy and qualitative presentation of toxicity results. We also present a brief review on the relationship between uptake route of toxicants and their toxic effects.

***Keywords*:** Bioassay, LC_{50}, probit, computation, Minitab 14.

Introduction

The types of toxicity tests which are routinely performed by pharmaceutical manufacturers in investigation of a new drug involve acute, sub–acute and chronic toxicity.

Determination of acute toxicity is usually an initial step in assessment and evaluation of the toxic characteristics of a compound using a bioassay test, hence, providing information on health hazards likely to arise from short–term exposure to chemical [AKHILA *et al.*, 2007; FINNEY, 1978] defined a biological assay as "an experiment for estimating nature, constitution, or potency of a material (or of a process), by means of reaction that follows its application to living matter". Therefore, whenever an investigator administers a chemical substance to a biological system, different types of interactions occurs leading to series of responses [AKHILA *et al.*, 2007].

Acute toxicity test procedure for Aquaculture research

Generally, strength or potency of an agent or stimulus (toxicant) is determined by a response (death) of a subject/organism.

Hence, a researcher prior to performance of a toxicity experiment must have decided which chemical effect is to be determined and what species, strain and sex of fish to use. Considerable research has been made on various chemical effects on clariid fishes in sub–Saharan Africa. In most studies using same toxicant and species of organism, variations in toxicity values are usually observed, most authors suggest that observed differences where due to differences in environmental conditions, water quality, weight and age of fish [AYUBA and OFOJEKWU, 2002; ONUSIRIUKA, 2002; OKOMODA and ATAGUBA, 2011; OKOMODA *et al.*, 2013].

However in most of these experiments, the sex of species is not considered. In an official manual describing test policy for toxicity test by FDA (1988), it was stated clearly that "*Only one sex is studied in an acute toxicity test; generally, the female is assumed to be more sensitive to the acute toxic effects of chemicals than the male*".

Hence most variation recorded in study may be due to sex ratio of organisms as this may be difficult to separate at fry and fingerlings level. Preliminary investigations are first carried out to determine concentration and dose range of chemical to be tested (Please refer to [SOLBE, 1995], for a description on how to perform preliminary test). The toxicity range determined is then used to evaluate acute toxicity of toxicant.

Probit analysis

Probit analysis is a specialized

regression model of binomial response variables. It is used to analyze many kinds of dose–response or binomial response experiments in a variety of fields.

Probit analysis is commonly used in toxicology to determine relative toxicity of chemicals to living organisms. This is done by testing response of an organism under various concentrations of each of chemicals in question and then comparing concentrations at which one encounters a response.

The response is always binomial (e.g. death/no death) and relationship between response and various concentrations is always sigmoid [DEMICHELA *et al.*, 2013].

Probit analysis acts as a transformation from sigmoid to linear and then runs a regression on relationship. Once a regression is run, researcher can use the output of probit analysis to determine concentration or dose of test chemical required to create a response in test organism [KIM, 2008]. There are many endpoints used to compare toxicities of chemicals, but LC_{50} (liquids) or LD_{50} (solids) are most widely used outcomes of modern dose–response experiments.

Hence in toxicity study of aquaculture, LC_{50}/LD_{50} represent the concentration (LC_{50}) or dose (LD_{50}) at which 50% of the population will die. Today, probit analysis is still the preferred statistical method in understanding dose–response relationships.

Determination of Probit and evaluation of LC_{50}

Step 1: Convert % mortality to probits

Method A: Determine probits by looking up those corresponding % Death in Finney's table [FINNEY, 1952] presented below:

Table 1.

Determine probits by looking up those corresponding % Death in Finney's

%	0	1	2	3	4	5	6	7	8	9
0	–	2.67	2.95	3.12	3.25	3.36	3.45	3.52	3.59	3.66
10	3.72	3.77	3.82	3.87	3.92	3.96	4.01	4.05	4.08	4.12
20	4.16	4.19	4.23	4.26	4.29	4.33	4.36	4.39	4.42	4.45
30	4.48	4.50	4.53	4.56	4.59	4.61	4.64	4.67	4.69	4.72
40	4.75	4.77	4.80	4.82	4.85	4.87	4.90	4.92	4.95	4.97
50	5.00	5.03	5.05	5.08	5.10	5.13	5.15	5.18	5.20	5.23
60	5.25	5.28	5.31	5.33	5.36	5.39	5.41	5.44	5.47	5.5
70	5.52	5.55	5.58	5.61	5.64	5.67	5.71	5.74	5.77	5.81
80	5.84	5.88	5.92	5.95	5.99	6.04	6.08	6.13	6.18	6.23
90	6.28	6.34	6.41	6.48	6.55	6.64	6.75	6.88	7.05	7.33
–	0.0	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9
99	7.33	7.37	7.41	7.46	7.51	7.58	7.65	7.75	7.88	8.09

For example, for a 20% Death, the corresponding probit would be 4.16

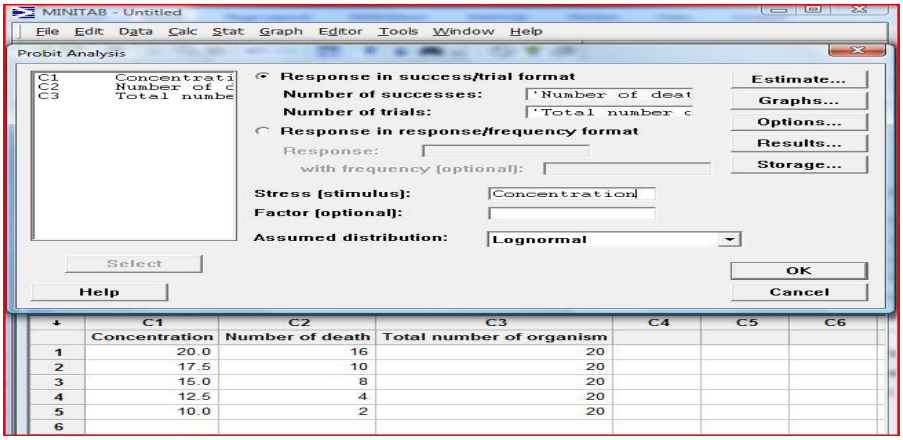

Figure 1. Determine probits by looking up those corresponding % Death in Finney's

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Method B: Hand calculations [FINNEY and STEVENS, 1948]:

The probit Y, of the proportion P is defined by:

$$P = \frac{1}{\sqrt{(2\pi)}} \int_{-\infty}^{Y-5} e^{-\frac{1}{2}u^2} du$$

The standard method of analysis makes use of maximum and minimum working probits:

$$Y_{max} = Y + \frac{\overline{Z}}{P}$$

$$Y_{min.} = Y - \frac{\overline{P}}{Z}$$

And the range 1/Z where

$$Z = \frac{1}{\sqrt{(2\pi)}} e^{-\frac{1}{2}(Y-5)^2}$$

Method C: Computer software determines % mortality and converts percentage to probits automatically.

Step 2: Take log of concentrations

This can either be done by hand if doing hand calculations, or specify this action in computer program of choice for auto computation.

Step 3: Graph probits versus log of concentrations and fit a line of regression.

Method A: Hand fit the line by eye that minimizes space between line and data (i.e. least squares).

Method B: Using a computer program, this estimates linear regression automatically.

Step 4: Find the LC_{50}

Method A: Using your hand drawn graph, either created by eye or by calculating the regression by hand, you can trace probit of 5 in y–axis down to x–axis and find log of concentration associated with it. Then take inverse of log and voila! You have LC_{50}.

Method B: Using regression equation to determine LC_{50} by substituting for Y=5 and finding equivalent values of X in regression equation.

Method C: Using computer software, this is displayed automatically.

Step 5: Determine 95% confidence intervals (Upper and lower confidence interval):

Method A: This can be hand calculated using following (Stated by [MATSUMURA, 1985].

Determine LC_{84}, LC_{16}, and LC_{50}. Using inverse prediction from the graph as described in "step 4 methods A" or using the regression equation as describe in "step 4 method B".

Calculate S and Log_{10} (S) as:

$$S = \frac{\frac{LC_{84}}{LC_{50}} + \frac{LC_{50}}{LC_{16}}}{2}$$

Determine total number of individuals (N) tested between ranges of dosages from LC_{16} to LC_{84}, then calculate log10(f) and f.

$$f = \text{antilog} \frac{2.77 \log (S)}{\sqrt{N}}$$

Calculating the Upper and Lower 95% Confidence Limits will therefore be gotten by multiplying or dividing conc/dose at LC_{50} by f:

Upper Limit = LC_{50} x f
Lower Limit = LC_{50} / f

Method B: Using computer programs calculates this automatically

Notes of Interest for Probit Analysis

- A raw plot of concentration versus mortality gives a sigmoid curve [DEMICHELA *et al.*, 2013] but probit transformation applies to linear portion on this curve. The calculation of probit for values like 100% mortality and 0% does not make sense. According to [EVANS and SHAPIRO, 1997] sigmoid nature of response curve indicates that extremes of mortality near 0% and 100% provide little information on how population as a whole is responding;
- Probit analysis assumes that relationship between number of organisms responding (not percent response) and concentration is normally distributed. If data are not normally distributed, log it is preferred. Logit is another form of transforming binomial data into linearity and is very similar to probit. Logit functions by taking log of odds:

  logit(P) = log P/ (1–P).

  Yet, relationship between logit and probit is almost indistinguishable: Logit ≈ (π/√3) x probit [FINNEY, 1952; HAHN and SOYER, 2005]. If there is more than 10% mortality in control Data must be corrected. One method is to use [SCHNEIDER–ORELLI'S, 1947] formula:

$$Correction = \frac{\%\ Responded - \%\ Responded\ in\ Control}{100 - Responded\ \%\ in\ Control} \times 100$$

Using minitab 14 for probit analysis

In using Minitab 14 (Minitab Inc., State College, Pennsylvania, USA), you need three columns; Concentrations, number of deaths and total number of organisms

Run the software; enter the values of the specified columns stated above.

In carrying out the analysis, distribution is assumed to be a log–normal distribution provided effect of concentration on survival of test organisms is normally distributed otherwise Logit will be used. (Figure 1 shows the window detailing the inputs).

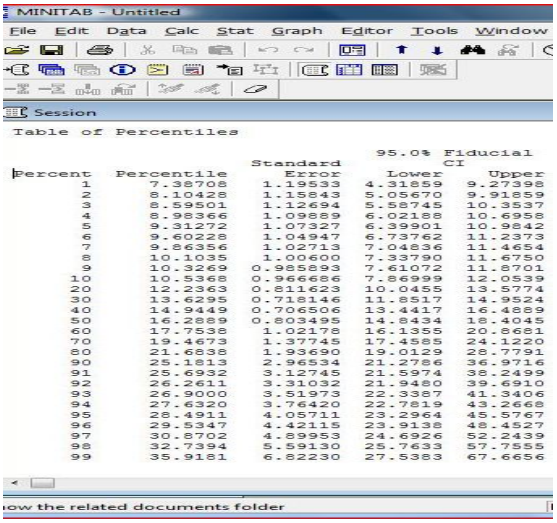

Figure 2. Table of Percentile

Once you are done, output will contain the LC values as "Table of Percentile" with their lower and upper confident limits (P<0.05). Hence LC_{50} will be displaced as 50th percentile. For hypothetical values above it is16.29 $\pm$ 0.80 (Figure 2)

Graphing Probits versus log of concentrations with aid of Microsoft excel 2007.

The probit–log concentration graph is an excellent way of toxicity result presentation.

Using the Microsoft excel 2007 makes this easier. However the value of mortality has to be transformed by looking up corresponding values in probit table as describe in "Step 1 method A" above, and concentration auto converted to Log_{10} of concentration using formula function.

The graphing function can then be used to display a regression graph while trend line, coefficient of determination (r^2) and regression equation can then be added appropriately.

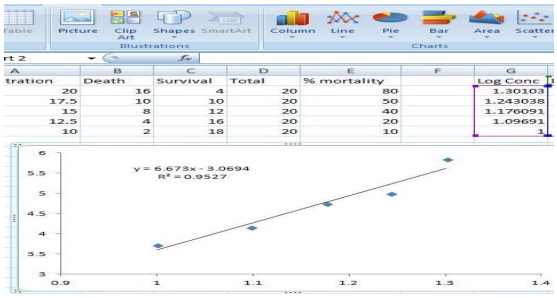

Figure 3. Regression graph, coefficient of determination (r^2) and regression equation

The window displaced below gives a typical view of process (Figure 3).

Uptake route and toxicity

Accidents, which involve chemicals reaching lakes and rivers, are ones with most serious ecological consequences [BOURDEAU *et al.*, 1989]. Scientific studies of toxicity tests are based on static water conditions which are created in laboratory to mimic real world conditions.

However, dynamics of chemical exposure in real world may be different from the laboratory.

According to [JEZIERSKA and WITESKA, 2006], accumulation of toxicants in organisms depends on concentration, time of exposure, uptake route, environmental conditions, and intrinsic factors (fish age, feeding habits). [UNO *et al.*, 2010], detected significantly higher concentrations of all alkylated Polycyclic Aromatic Hydrocarbons (PAH) homologues in shellfish than in fish and attributed differences to uptake routes and/or their metabolizing abilities.

For fish, the most important routes of uptake are via gills and digestive tract while whole body surface is route for invertebrates [BOURDEAU *et al.*, 1989; CHEIKYULA, 2012] reported that accumulation of PAH in red sea bream under water–borne exposure increased with exposure duration, but decreased with exposure period in dietary exposures (accumulating all PAH's) and thus concluded

main PAH uptake route in red sea bream to be water–borne route for LMW PAHs and dietary route for HMW PAHs.

The relative importance of direct uptake from water and uptake from food will depend on characteristics of chemical [BOURDEAU *et al.*, 1989].

Hydrophilic excretion does not get rid of PAHs, rather they are biotransformed into hydrophilic metabolites and will still persist in fish [CHEIKYULA, 2012].

If chemical is persistent, and particularly if it is also lipophilic [BOURDEAU *et al.*, 1989], then food chain effects can be expected to predominate.

Conclusion and Recommendation

Outcome of most study of toxicity using same toxicant and test organism differ in LC_{50} values. Majority of the assumption associated with disparity in values have been discussed earlier.

However, differences in method of determination of LC_{50} could also lead to variance in LC_{50}.

Reliability of the use of spread sheets to determine accurate values is presumed higher compared to other conventional methods, hence further studies are suggested to confirm this hypothesis, more so, comparison of the outcome of values using different statistical packages also need investigation.

Most statistical packages used for analyzing fisheries and aquaculture oriented research are social science and general statistical software, it is recommended that more statistical packages that are fisheries inclined be developed to resolve the numerous challenges of data analysis encountered in diverse area of Aquaculture and Fisheries.

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