MUNI | RECETOX

Bi2003 Ecotoxicology

Ecotoxicological bioassays

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Content

- Introduction – what, why, how, concept
- Types of bioassays
- **Ecotoxicological bioassays' design and results**
- Aquatic bioassays - examples
- Soil bioassays – examples
- Use of bioassays in praxis

Bioassays' general design

General scheme of bioassay

1) Prepare the organism

Culture media, standardized numbers, age, etc.

0) culture of organisms

2) Prepare the sample

Dilution series

water/culture media – direct organism exposure

Include BLANK (medium only)

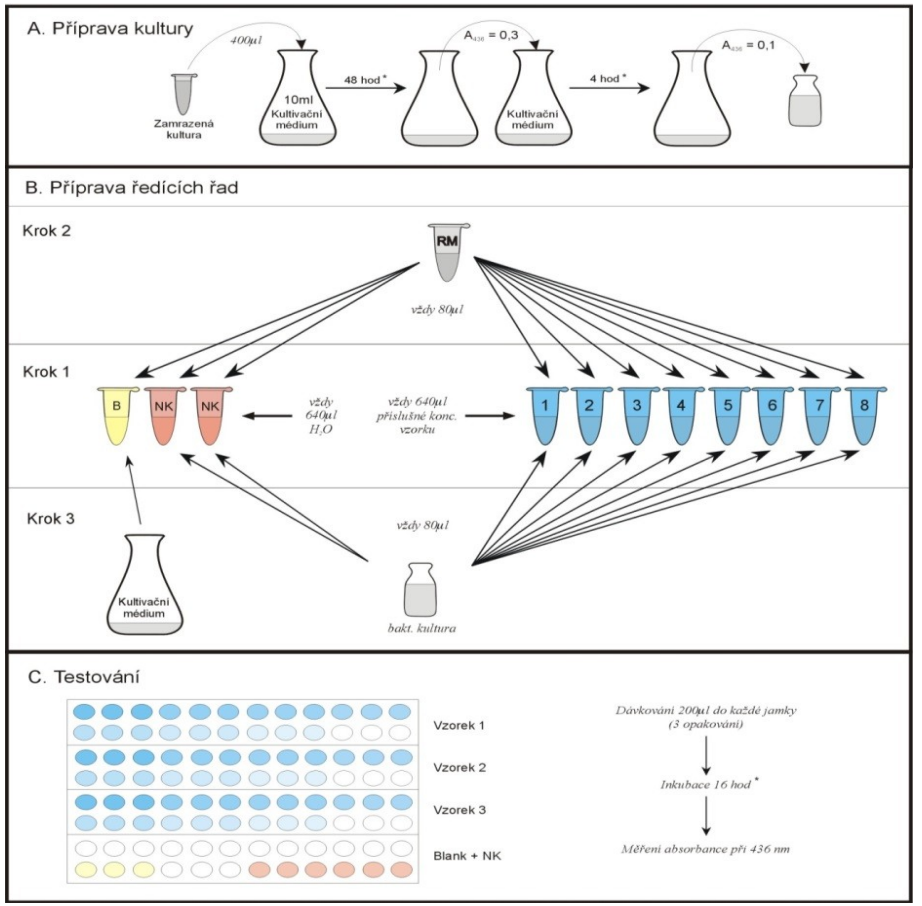
solvent for organic compounds – minimum to be added

Include SOLVENT CONTROL

specifics for the SOLID MATRICES

3) Expose of organisms

… for appropriate time, number of repetitions, under specified conditions



4) Evaluate and report results

measure the endpoint / count organisms

validity criteria

statistical evaluation (means, ANOVA, dose-response …)

Organisms

1) Prepare the organism
Culture media, standardized numbers, age, etc.

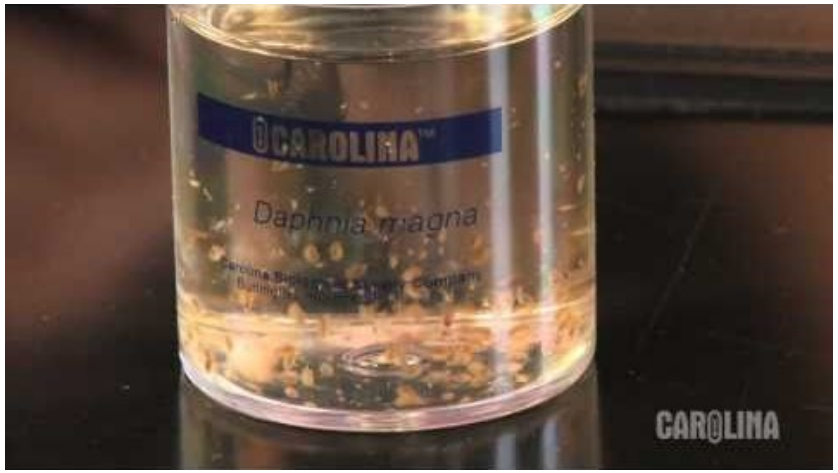
← **0) culture of organisms**

Ideally

- good availability (laboratory cultures, commercial availability ...)
- easy storage and breeding in laboratory conditions in sufficient quantities for experiments
- the biology of the species and the genetics of the respective culture are characterized
- the relative sensitivities of the species / culture to different classes of toxic substances are studied
- the susceptibility of the species should be a good representative of the relevant group of organisms (Daphnia - crustaceans, Danio rerio - freshwater fish)

Organisms

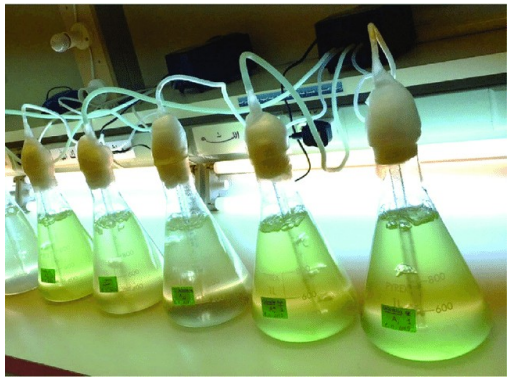
Cultures !!!



1) Prepare the organism

Culture media, standardized numbers, age, etc.

0) culture of organisms



Organisms

- the result of toxicity determination and interpretation is influenced by a number of other biological parameters
  - genetically determined sensitivity of the respective culture / clone / variety ...
  - size and age of individuals
  - sex
  - developmental stage (eggs, embryos, larvae, adults ...)
  - physiological conditions - optimum (diseases, food - antioxidants ...)

- in general, the organisms must be in optimal status before the test ➔ this is checked by the **test validity criteria** and testing **reference substances**

VALIDITY OF THE TEST

8. For a test to be valid, the following performance criteria should be met in the control(s):

- the mortality of the parent animals (female *Daphnia*) does not exceed 20% at the end of the test;
- the mean number of living offspring produced per parent animal surviving at the end of the test is ≥ 60.

REFERENCE SUBSTANCES

5. A reference substance may be tested for EC_{50} as a means of assuring that the test conditions are reliable. Toxicants used in international ring-tests (1)(5) are recommended for this purpose[1]. Test(s) with a reference substance should be done preferably every month and at least twice a year.

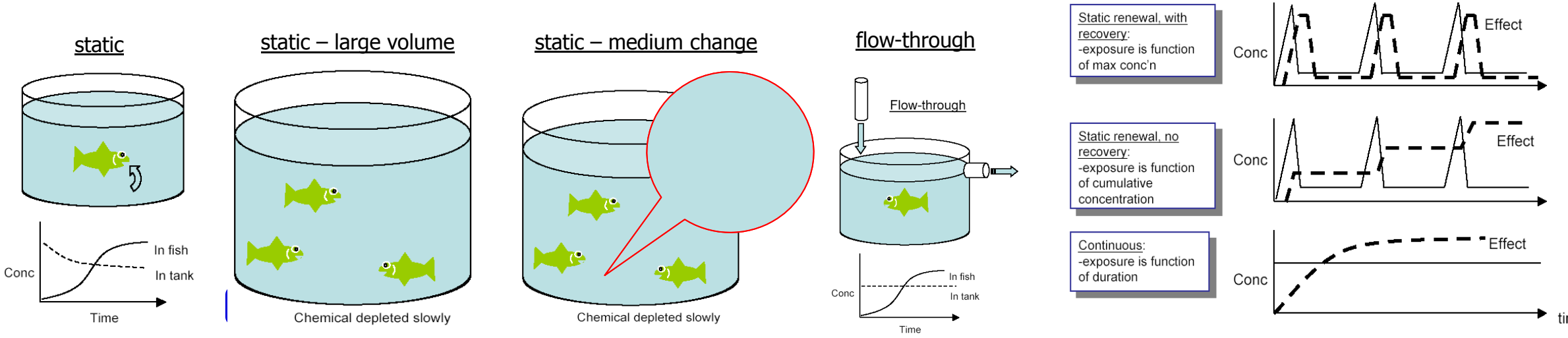
[1] The results of these inter laboratory tests and a Technical Corrigendum to ISO 6341 give an EC_{50}-24 h of the potassium dichromate ($K_2Cr_2O_7$) within the range 0.6 mg/l to 2.1 mg/l

Exposure in aquatic bioassays

- Usually exposure of whole organisms (intake by body surface area, respiratory system, food) less often: single injections (fish, input and dose are not affected by the environment)

Distribution according to the arrangement of the exposure

- static (without exchange of solutions - possible changes of concentrations, oxygen)
- static with medium change/renewal (change at defined times, ~24 h)
- recirculation (medium recirculation, more technically demanding ...)
- flow-through (continuous maintenance of concentrations, technically demanding ...)



Exposure of vertebrate animals in bioassays

- vertebrates - laboratory rodents, birds

- like in "classical" toxicology:
  - injection intramuscular (IM), intraperitoneal (IP), intravenous (IV), subcutaneous (SC)
  - oral - dosing in food, application of gauze (tube directly into the stomach) ...
  - respiration - air contamination - closed containers / cells, inhalation ...

Exposure in soil bioassays

- soil, sediments - bacteria, invertebrates - contact with the whole surface (direct contact tests - solid phase tests)
  - real soil / sediment
  - artificial soil / sediment

- plants - roots - contact with solid or liquid medium, exposure to gaseous pollutants from the air

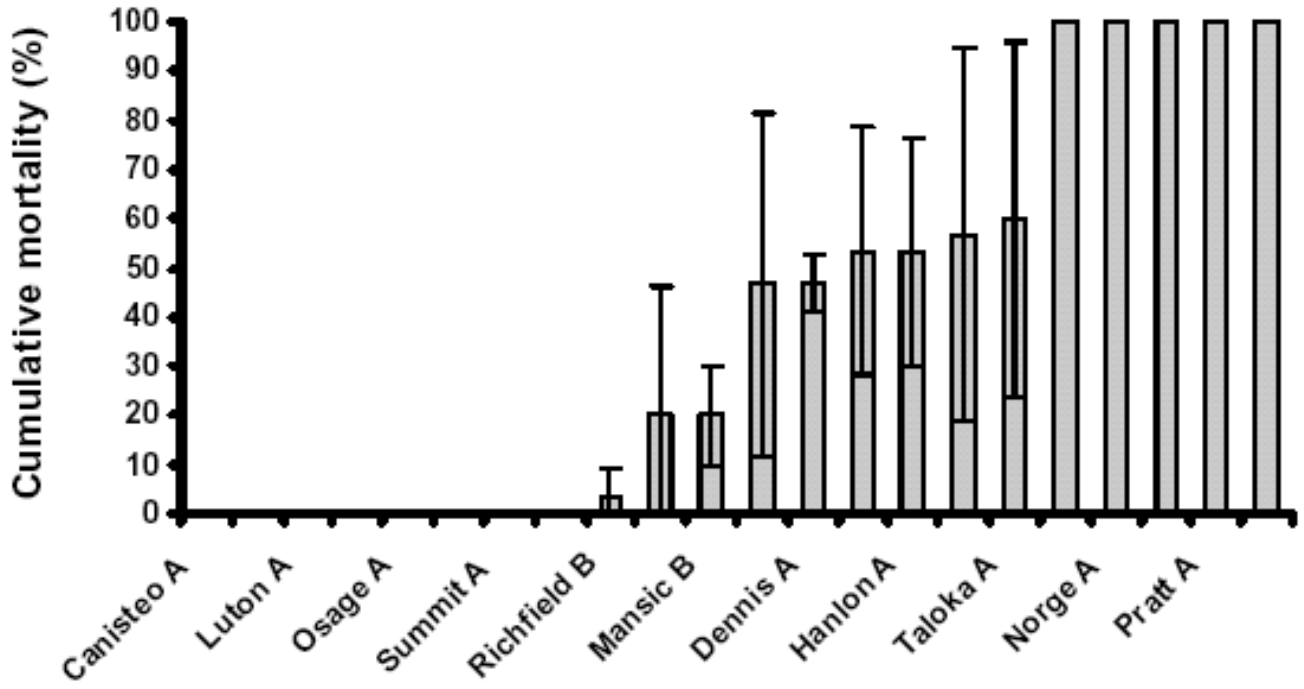
- often several exposure routes can be realistically assumed at the same time

Exposure in soil bioassays

it is specific:

- the fate of the contaminant in the soil environment, the influence on the real **bioavailability** for soil organisms comes into play significantly

Pb: 2 g/kg in soil
mortality of earthworms

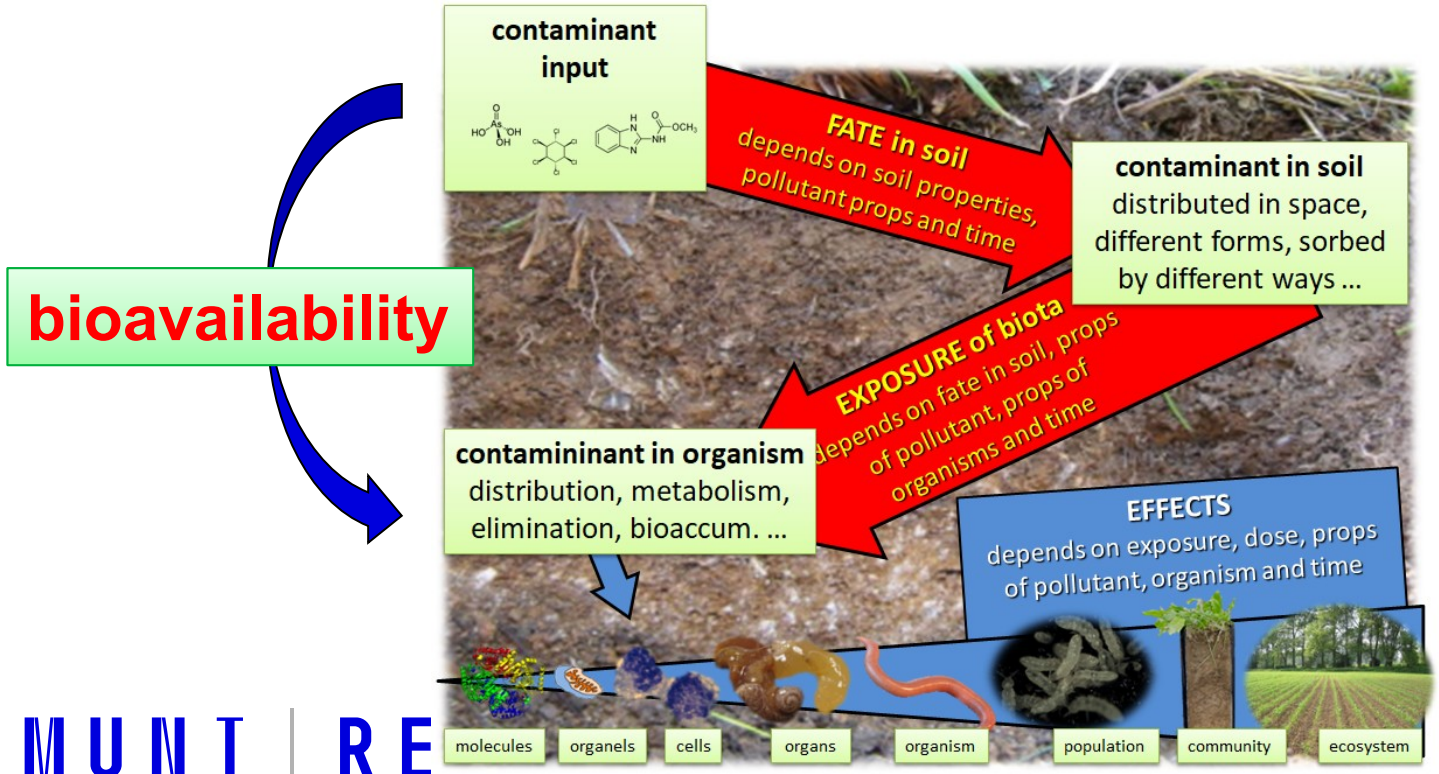


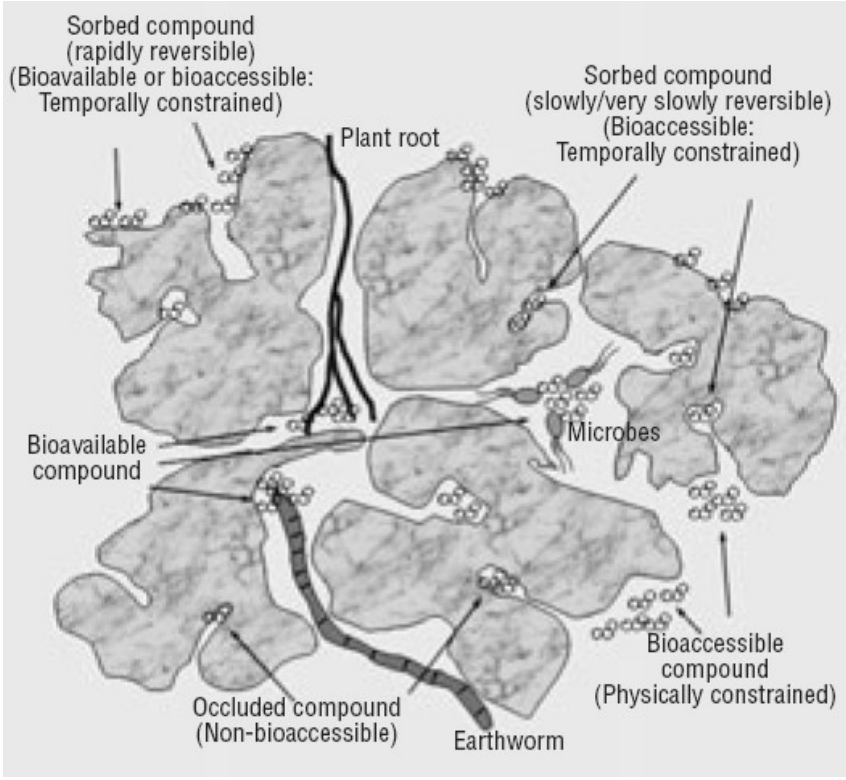
Lanno et al. (2004) Ecotoxicology and Environmental Safety 57, 39-47

Exposure in soil bioassays

Bioavailability

- soil is heterogenous and there is lot of places available for **sorption** or **sequestration** of the pollutants ➔ **fate, beahviour, distribution affected** ➔ **exposure, toxicity, risks affected**





Exposure in soil bioassays

Ingestion and oral

- food and soil particles - organisms consume mineral and organic matter - an important route of exposure for sorbed chemicals
- contaminants can be biomagnified - for example in fungi that are consumed by sprintails
- important path for arthropods

Dermal

- from the soil or soil solution - especially organisms drilling in the soil (earthworms and enchytraeids), which have a thin cuticle and are in contact with the soil and pore water
- it is also possible to model the results of tests in an aquatic environment by supplementing the model of the distribution of the substance between the soil solution and the sorption on particles = the so-called **Equilibrium partitioning theory (EqP)**

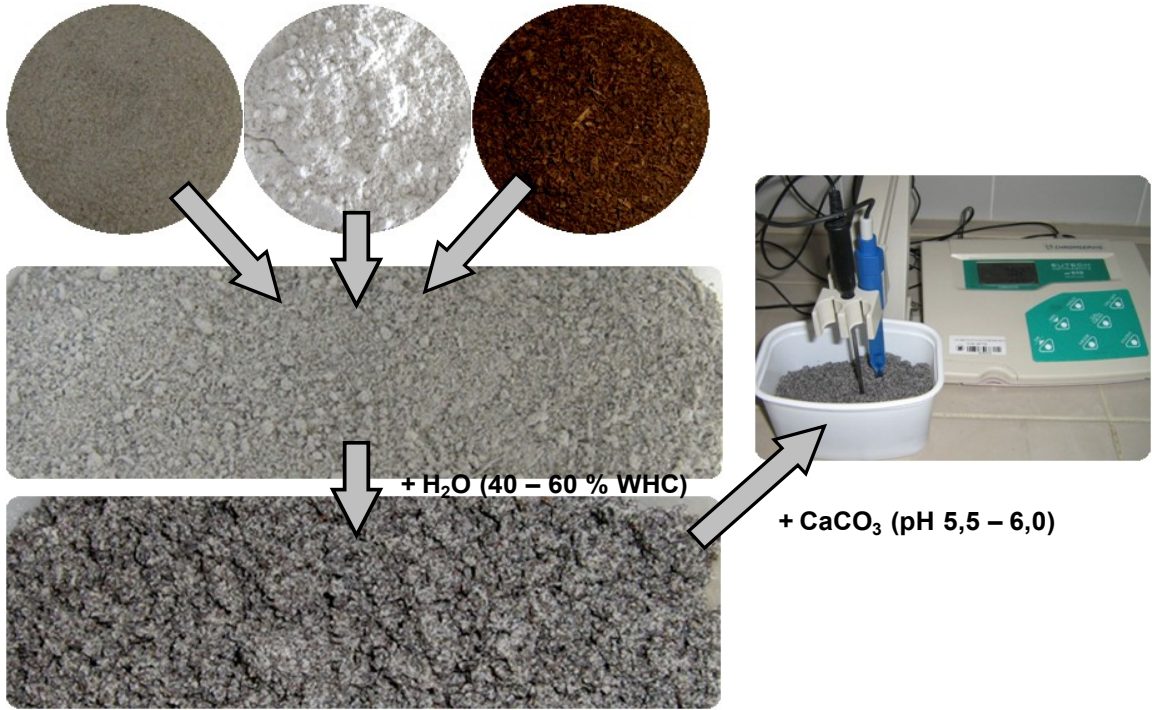
Breathing

almost no data

Exposure in soil bioassays

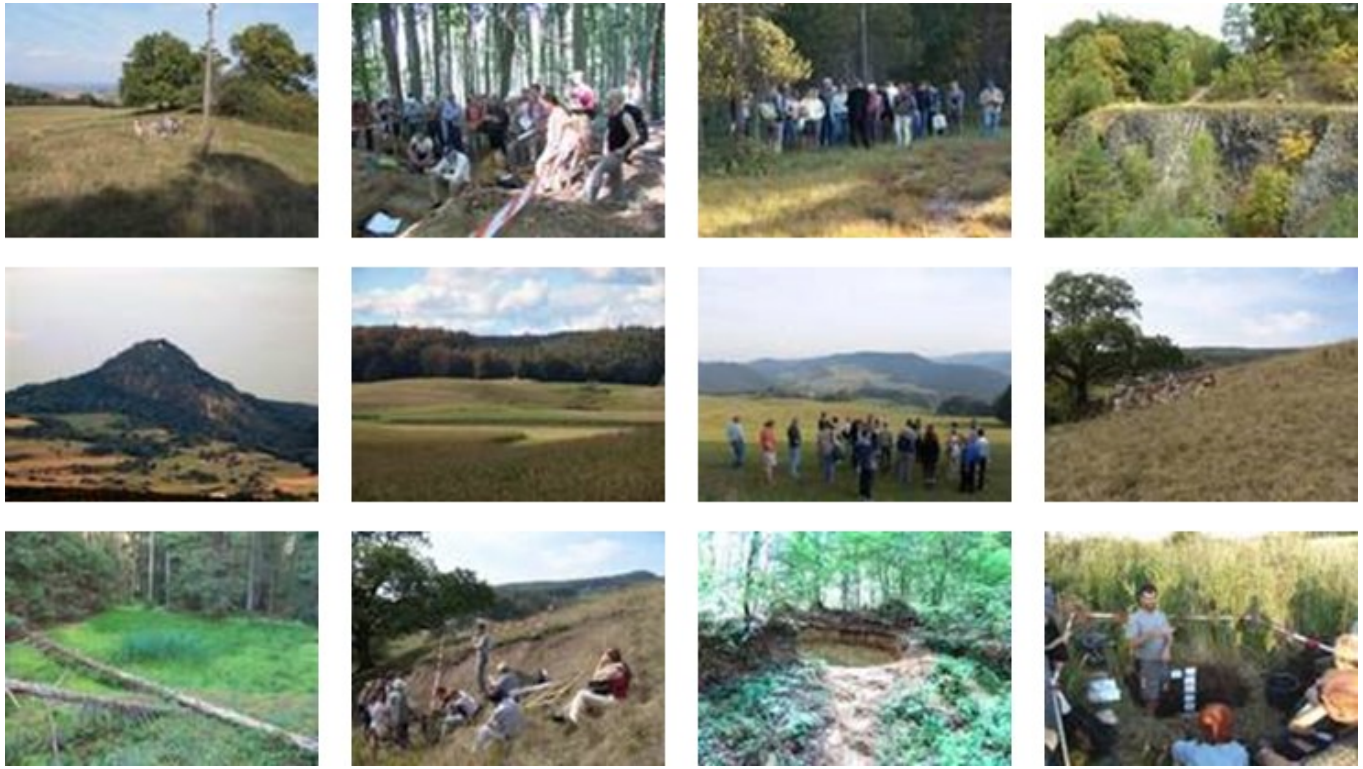
ARTIFICIAL SOIL

- 10% dry fine peat
- 20% caoline clay, min 30% calolinite
- 70% quartz sand fine min. 50% of size 0.05 – 0.2 mm
- 0.3-1% calcium carbonate → pH of 6 ± 0.5



Exposure in soil bioassays

- artificial soil is NOT real soil



Exposure in soil bioassays

- artificial soil is NOT real soil

http://lufa-speyer.de

	LUFA 2.1	LUFA 2.2	LUFA 2.3	LUFA 5M	LUFA 6S
organic carbon (%)	0.81 ± 0.21	2.16 ± 0.40	0.98 ± 0.05	1.29 ± 0.20	1.75 ± 0.11
particles < 0.02 mm (%)	8.2 ± 0.9	13.9 ± 1.1	22.7 ± 1.1	25.3 ± 1.8	65.1 ± 2.7
pH (0.01M $CaCl_2$)	5.1± 0.4	5.4 ± 0.1	6.4 ± 0.6	7.2 ± 0.1	7.2 ± 0.1
cation exchange capacity (meq/100g)	4± 1	10 ± 1	8 ± 2	15 ± 3	22 ± 6
water holding capacity (g/100g)	33.2 ± 1	48.2 ± 5	34.4 ± 2	42.1 ± 4	40.7 ± 5
weight per volume (g/1000ml)	1404 ± 46	1197 ± 60	1291 ± 30	1212 ± 56	1264 ± 90
Particle size (mm) distribution according to German DIN (in %):					
<0.002	3.0 ± 0.9	6.4 ± 0.9	9.4 ± 0.9	10.8 ± 1.3	42.1 ± 1.8
0.002 - 0.006	2.2 ± 0.7	3.5 ± 0.7	4.2 ± 0.8	5.4 ± 0.3	10.8 ± 0.7
0.006 - 0.02	2.9 ± 0.7	3.8 ± 0.7	9.1 ± 0.5	9.1 ± 0.5	12.1 ± 1.3
0.02 - 0.063	5.3 ± 1.8	5.4 ± 1.2	18.6 ± 2.3	19.5 ± 1.3	14.1 ± 2.5
0.063 - 0.2	27.0 ± 3.1	35.4 ± 2.3	29.3 ± 3.4	38.9 ± 1.0	8.7 ± 0.9
0.2 - 0.63	57.2 ± 4.3	44.8 ± 2.7	26.9 ± 0.7	14.9 ± 1.0	9.0 ± 0.3
0.63 - 2.0	2.4 ± 0.6	0.7 ± 0.1	2.5 ± 0.8	1.4 ± 0.1	3.2 ± 0.7
soil type	sand (S)	loamy sand (lS)	loamy sand (lS)	silty sand (uS)	clayey loam (tL)
Particle size (mm) distribution according to USDA (in %)					
<0.002	3.0 ± 0.9	6.4 ± 0.9	9.4 ± 0.9	10.8 ± 1.3	42.1 ± 1.8
0.002 - 0.05	8.8 ± 1.8	12.2 ± 0.6	29.8 ± 3.0	27.5 ± 2.2	36.0 ±2.3
0.05 - 2.0	88.2 ± 1.2	81.4 ± 1.2	60.8 ± 2.6	61.7 ± 3.2	21.9 ± 1.6
soil type	sand	loamy sand	sandy loam	sandy loam	clay

Exposure in soil bioassays

- the goal is **HOMOGENITY of exposure** to the test substance

water soluble chemicals

- in water which is also used to adjust soil moisture

insoluble in water

- using carrier - non-toxic, water soluble/miscible (acetone, ethanol)
- using carrier - non-toxic volatile organic solvent and evaporated rapidly
- in both cases, solution can be added to:
  - small amount (1-10%) of fine quartz sand; after evaporation of solvent, this is added to soil and mixed
  - directly into soil (dry or wet) followed by evaporation and mixing
- in all carrier cases, it is necessary to include a **control for the carrier/solvent**

insoluble in water or solvent

- mixed directly with quartz sand or whole soil

Factors / conditions of the assay

- CRUCIAL !!! they affect both the organisms and the tested chemical, they significantly affect the exposure and the final results
- **must be standardized !!!**
  - temperature
  - light / photoperiod
  - oxygen (aquatic consumers)
  - pH
  - water hardness
  - clay and organic matter content in soil
  - food added/non-added
  - etc.

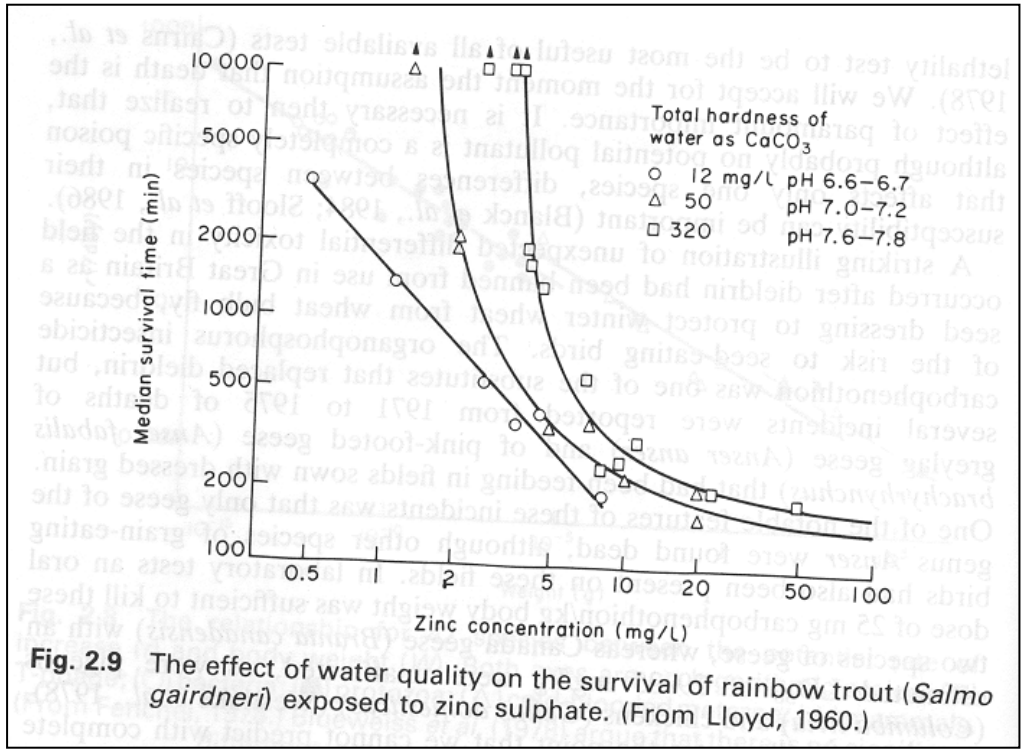


Fig. 2.9 The effect of water quality on the survival of rainbow trout (*Salmo gairdneri*) exposed to zinc sulphate. (From Lloyd, 1960.)

Results of the bioassays

Parameters of evaluation - endpoints

- **effects ~ response** = result of the exposure to toxic chemical (stressor)
  - higher/lower with increasing stressor intenzity (except hormesis)
- **endpoint** = measured (measurable) response / effect
  - original units (numbers, weight, enzyme activity etc.) or relative (% of control)

- acute effects
  - animals – letality/mortality, imobilization in case of Daphnia
  - plants – algae: growth, chlorofyl (fluorescnence); vacular: emergence, growth
  - destruents – bacteria: growth, activity ...
- chronic/sublethal effects
  - animals –growth, malformations, reproduction, behavior
  - plants – growth, reproduction...

Measures of exposure

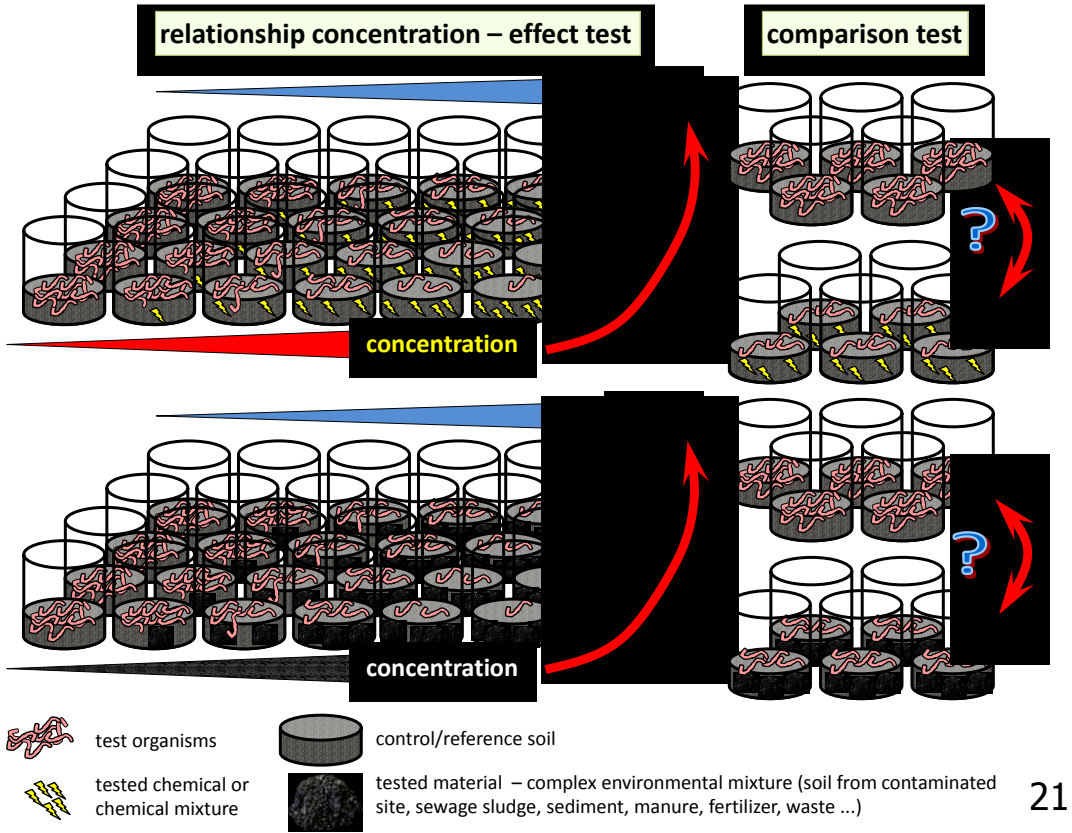
- DOSE versus CONCENTRATION
  - toxicology – dose - mg/kg b.w. - body weight, mg/kg b.w./day
  - ecotoxicology – usually the concentration in the medium - mg/L, mg/kg$_{soil}$ etc

Concentration and Dose

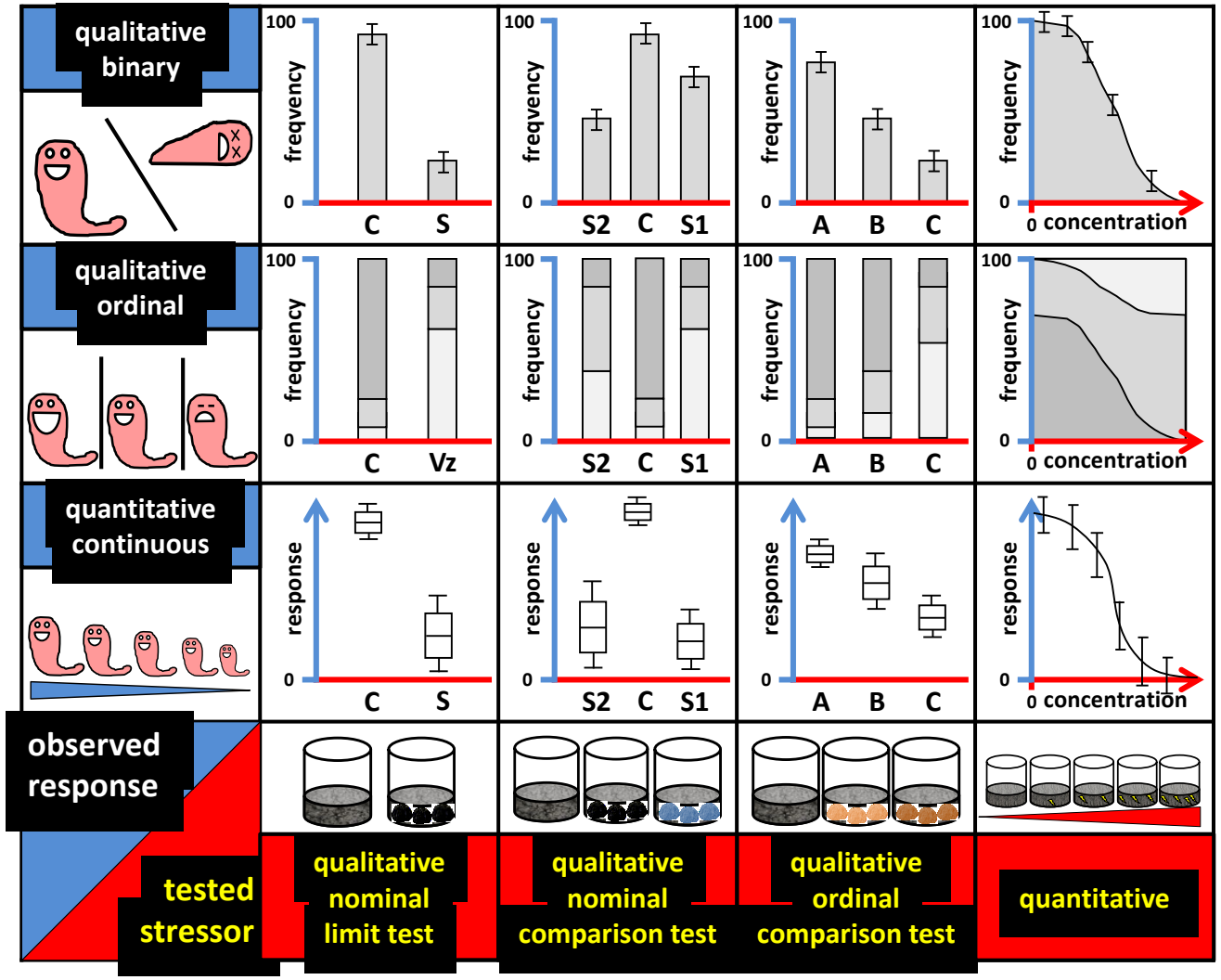
Concentration and dose both refer to the amount of test material to which the test organism is subjected. Concentrations are used to describe the amount of test material in the testing environment (e.g., mg/L in water, mg/kg in soil or mg/kg in food). Doses are used to describe the amount of test material administered to a subject (e.g., mg/kg-bodyweight in an avian bolus study). Statistical methods for both types of studies are identical; however, interpretations are different. Although "concentration" is used throughout this document, all the statistical methods presented here also apply to studies in which a dose is used.

- pure chemicals and defined mixtures
  - conc. in media - mg/L, mmol/L (= mM), mg/kg etc.

- environmental samples
  - extracts of the samples and their % dilutions
  - % of the sample in the reference material



Data from ecotoxicity bioassays



Error bars in the graphs indicate that regardless of the type of response, it is measured in several replicates and the resulting data have some variability

Data from ecotoxicity bioassays

Tested factor - qualitative, nominal
Contamination (or other stressor tested) in variants (samples) is not quantified, is not quantifiable, or is quantified, but the aim is not to study the influence of its intensity on the effect. Variants (samples) cannot be (or it is not the goal) arranged in any way. It is a comparative test of several variants (samples) each other and / or against the control (e.g. soils from monitoring from different localities, samples of different soil materials - sludge, sediments, waste). The extreme is „the limit test“ - one tested variant (sample) is compared with the control.
Tested factor - kvalitative, ordinal
Contamination (or other stressor tested) in variants (samples) is not fully quantified, but variants (samples) can be ranked based on some criteria. However, it is not possible to determine how many times the variant is larger or smaller than the previous or next one - the intensity of contamination (stress) cannot be plotted on the axis and no relationship between it and the effect can be modeled. It is a comparative test of several variants (samples) each other and / or against the control (e.g. soils little, medium and very far from the source; soils from little, medium and very damaged ecosystem, etc.).
Tested factor – quantitative
Contamination (stressor) is quantified to the extent that it is possible to say how many times or by how much its intensity is greater or less than in the previous or next variant. The contaminant concentration (stressor intensity) can be plotted on an axis and the experiment arranged and evaluated as a test of the relationship between concentration and effect, this relationship can then be graphically expressed, modeled and ecotoxicity parameters calculated. Whether the tested factor is quantified by discrete (integer) or continuous (even decimal) data is not very relevant.

Data from ecotoxicity bioassays

Observed response – qualitative, binary
The answer is not quantifiable, it is only possible to determine whether it has occurred or not - a typical example is mortality / survival of organisms, occurrence of some signs (lesions, swelling, mutations,), immobilization, escape reaction, etc. Finding on a number of organisms (biological systems), the binary result can be converted to the frequency or affected fraction and expressed as a percentage of the effect (e.g. mortality, lethality, survival, leakage) or as an affected fraction with values from 0 to 1. In this form, this type of data can be evaluated similarly to quantitative continuous data (test, model, regressions, etc.), but statistical methods designed for binomial data should be used correctly, including, for example, another formula for calculating variance.
Observed response – qualitative, ordinal
The response is quantified to the extent that the results can be ranked, eg small, medium and large damage. However, it is not possible to determine between the samples (variants) how many times or by how much the result is larger or smaller, and therefore it is not possible to model the relationship between the effect and the concentration. As with binary data, these results can be converted to fraction or frequency and expressed as a percentage in each category.
Observed response – quantitative, continuous
The response is quantified to the extent that it is possible to say how many times or how much is greater or less in one variant (concentration) than in another (eg weight, size, enzyme activity, production, number of juveniles, biomarker concentration…). The results can be plotted and the relationship between concentration and effect evaluated.

Data from ecotoxicity bioassays

Quantal data

Quantal data arise when a particular property is recorded to be present or absent in each individual (e.g. an individual shows an effect or it does not show an effect). Therefore, these data can exhibit only two states. Typically, quantal data are presented as the number of individuals showing the property (e.g., mortality) out of a total number of individuals observed in each experimental unit. Although this can be expressed as a fraction, it should be noted that the total number of individuals cannot be omitted.

Continuous data

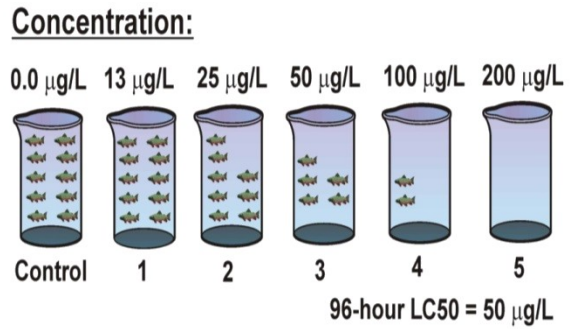
Data are continuous when they can (theoretically) take any value in an open interval, for instance any positive number. Examples include measurements of length, body weight, etc. Due to practical reasons the measured resolution depends on the quality of the measurement device. For example, if test units are observed once per day then 'time to hatch' can only be recorded in whole days; however, the underlying distribution of 'time to hatch' is continuous. Typically, continuous data have a dimension (e.g. grams, moles/litre).

Discrete data

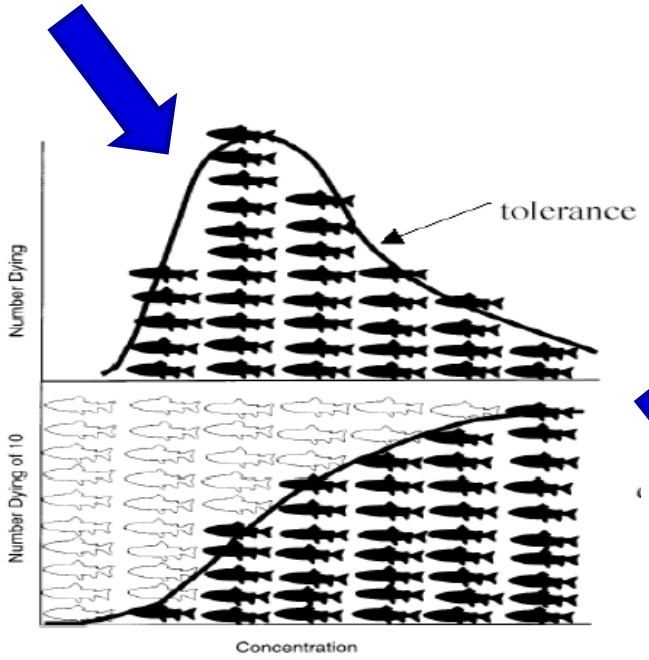
Discrete data are data that have a finite or countable number of values. There are three classes of discrete data: nominal, ordinal and interval. *Nominal data* express qualitative attributes that do not form a natural order (e.g. colours). *Ordinal data* reflect the relative magnitude from low to high (e.g. an individual shows no effect, minimal effect, moderate effect or high effect)These data cannot be interpreted with regard to relative scale (i.e., an ordinal variable with a value of '4' can be interpreted as being higher then the value of '2', but not twice as high). *Interval data* (e.g., number of eggs or offspring per parent) allows the ranking of the items that are measured, and the differences between individuals and groups can be quantified. Often, interval data can be analysed as if the data were continuous. The analyses for interval discrete data are presented in this document; analyses of nominal and ordinal data are not included but will be addressed in a future revision. Ordinal data can often be reduced to quantal data.

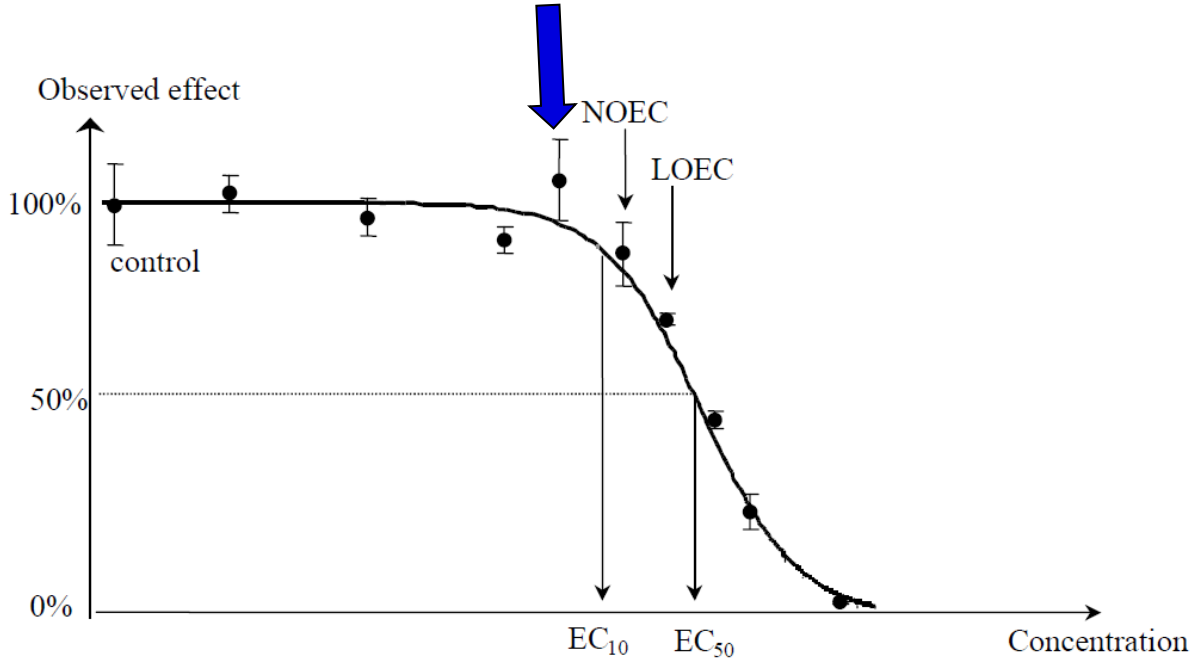
Draft Guidance Document for on the Statistical Analysis of Ecotoxicity Data. OECD Environmental Health and Safety Publications, Series on Testing and Assessment, Environment Directorate, OECD, Paris 2003.

Dose(concentration) - response relationship



each conc./dose is tested in several replicates !!!

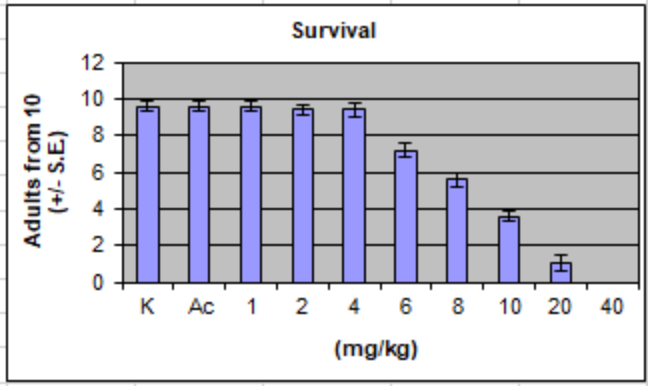




No Observed Effect Concentration (NOEC)
Lowest Observed Effect Concentration (LOEC)
ECx (x % effects concentration)
LCx (x % lethal concentration)

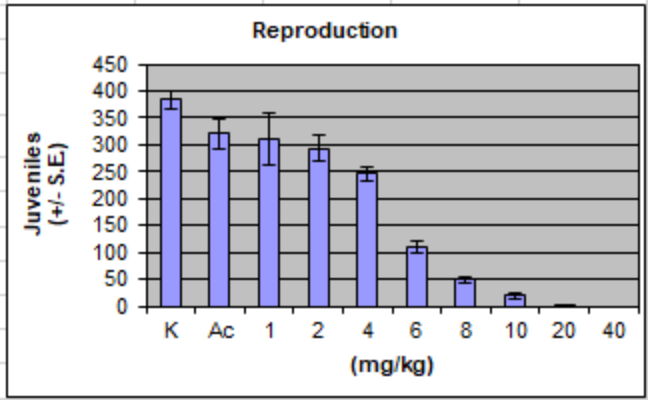
Adults

	K	Ac	1	2	4	6	8	10	20	40
1	10	10	9	10	8	6	7	4	1	
2	9	10	10	9	10	8	5	3	2	0
3	10	10	9	10	9	7	6	4	0	0
4	10	9	10	9	10	7	5	4	2	0
5	9	9	10	9	10	8	5	3	0	0
Mean	9.60	9.60	9.60	9.40	9.40	7.20	5.60	3.60	1.00	0.00
S.D.	0.55	0.55	0.55	0.55	0.89	0.84	0.89	0.55	1.00	0.00
S.E.	0.24	0.24	0.24	0.24	0.40	0.37	0.40	0.24	0.45	0.00
Adjusted to control		100.00	100.00	97.92	97.92	75.00	58.33	37.50	10.42	0.00
c.v.	5.71	5.71	5.71	5.83	9.52	11.62	15.97	15.21	100.00	########
S.D.(x) (binomial)	0.620	0.620	0.620	0.751	0.751	1.420	1.570	1.518	0.949	0.000
p	0.960	0.960	0.960	0.940	0.940	0.720	0.560	0.360	0.100	0.000
S.D.(p)	0.196	0.196	0.196	0.237	0.237	0.449	0.496	0.480	0.300	0.000
		96	96	94	94	72	56	36	10	0



Juveniles

	K	Ac	1	2	4	6	8	10	20	40
1	372	310	227	368	201	87	57	22	5	0
2	395	402	417	319	277	79	35	38	0	0
3	368	295	314	305	247	143	45	12	4	0
4	345	233	174	220	236	123	52	25	0	0
5	442	362	420	256	271	115	53	0	0	0
Mean	384.40	320.40	310.40	293.60	246.40	109.40	48.40	19.40	1.80	0.00
S.D.	36.76	64.77	110.62	57.32	30.46	26.32	8.65	14.28	2.49	0.00
S.E.	16.44	28.97	49.47	25.63	13.62	11.77	3.87	6.38	1.11	0.00
Adjusted to control		100.00	96.88	91.64	76.90	34.14	15.11	6.05	0.56	0.00
c.v.	9.56	20.22	35.64	19.52	12.36	24.06	17.87	73.59	138.33	########



Dose(concentration) - response relationship