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EU regulatory framework (REACH/CLP)

End points and testing methods

Toxicological properties- Human Health and Environment

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Note 1: endpoints/testing methods

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modification brought by Directive 2006/121/ CEE removes the Annex V (testing methods) of the Directive 67/548/CEE

DIRECTIVE 2006/121/EC of 18 December 2006 amending Directive 67/548/EEC
"*Article 3 - Testing and assessment of the properties of substances*"
Tests on substances carried out within the framework of this Directive shall be conducted according to the requirements of Article 13 of Regulation (EC) No 1907/2006 (REACH)

10) Annex V shall be deleted;

REACH Article 13 General requirements for generation of information on intrinsic properties of substances
3. Where tests on substances are required to generate information on intrinsic properties of substances, they shall be conducted in accordance with the test methods laid down in a Commission Regulation or in accordance with other international test methods recognised by the Commission or the Agency as being appropriate

Council Regulation (EC) No 440/2008 of 30 May 2008 laying down test methods pursuant to Regulation (EC) No 1907/2006 of the European Parliament and of the Council on the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH), whereas
(3) The test methods contained in Annex V to Directive 67/548/EEC should be incorporated into this Regulation

How are dangerous substances classified – Health hazards

DSD/DPD hazard classes	CLP hazard classes
Very toxic T+	Acute toxicity
Toxic T	Skin corrosion/irritation
Corrosive C	Serious eye damage/eye irritation
Harmful Xn	Respiratory or skin sensitisation
Irritant Xi	Germ cell mutagenicity
Sensitising R42 and/or R43	Carcinogenicity
Carcinogenic Carc. Cat.	Reproductive toxicity plus additional category for effects on or via lactation
Mutagenic Muta. Cat	Specific target organ toxicity (STOT) – single exposure
Toxic for reproduction Repr. Cat	Specific target organ toxicity (STOT) – repeated exposure
	Aspiration hazard

How are dangerous substances classified – Environmental Effects

DSD/DPD hazard classes	CLP hazard classes
Dangerous for the environment: N or/and R52, R53, R59	Hazardous to aquatic environment
	Hazardous to the ozon layer

DSD/DPD hazard classes	CLP hazard classes
explosive: E	Explosives
oxidising: O	Flammable gasses
extremely flammable: F+	Flammable aerosols
highly flammable: F	Oxidising gases
flammable: R10	Gases under pressure
	Flammable liquids
	Flammable solids
	Self-reactive substances and mixtures
	Pyrophoric liquids
	Pyrophoric solids
	Self-heating substances and mixtures
	Substances and mixtures which in contact with water emit flammable gases
	Oxidising liquids
	Oxidising solids
	Organic peroxides
	Corrosive to metals

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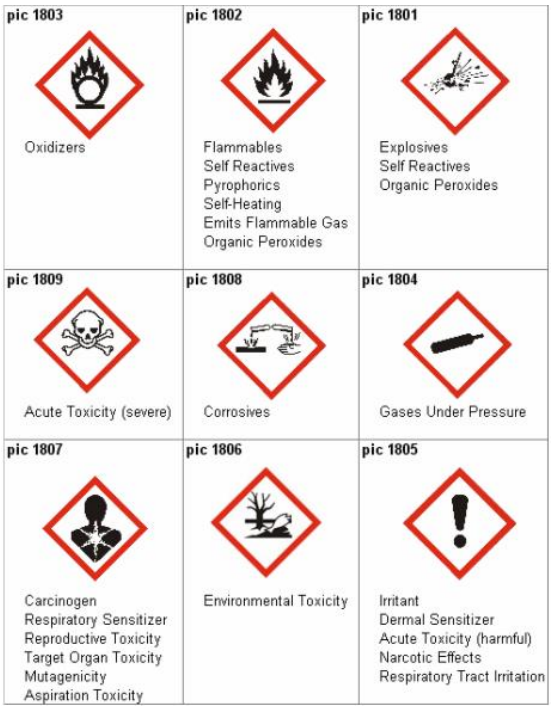
Danger Symbols



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Obligation to Classify

The obligation to classify is based on the CLP Regulation and the REACH Regulation:

- Classification triggered by **CLP** (CLP Article 4(1)).

If you are a manufacturer, importer or downstream user of chemical substances or mixtures to be placed on the market, you must classify these substances or mixtures before placing them on the market, regardless of the tonnage.

- Classification triggered by **REACH** (CLP Article 4(2)).

If you are a manufacturer or importer, you must also classify substances which you do not place on the market if they are subject to registration or notification in line with Articles 6, 9, 17 or 18 of REACH.
This includes the classification of monomers, on-site isolated intermediates, transported intermediates as well as substances used for product and process-orientated research and development (PPORD).

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WHERE TO FIND DATA REQUIRED FOR CLASSIFICATION AND LABELLING

- Step 1: is your substance on the harmonised classification list?
  - If no, go to step 2
  - If yes, classify accordingly (or justify new classifcation based on new evidence and submit Annex XV (REACH))

Note: Already Labelled (harmonized-ATP)
Annex 1 of Directive 67/548/EEC
(Annex 1 of 67/548/EEC has been replaced by part 3 of Annex VI of CLP Regulation 1272/2008 EC)
Search harmonized classification (via http://echa.europa.eu/information-on-chemicals/cl-inventory-database)

- Step 2: you have data and self classification under old system
  - If yes: use translation tables
  - If no: go to step 3

- Step 3: conduct base testing under REACH/CLP and gather additional relevant information and classify accordingly (CLP Title II)

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Endpoints acute oral toxicity

DSD	T+ R28		T R25		Xn	
Oral – LD50 (mg/kg bw)	≤ 5	5-25	25-50	50-200	200-300	300-2000
CLP	Cat. 1	Category 2		Category 3		Category 4

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Example 7: 2,3-Dichloropropene

Application	Discrimination from STOT-SE		
	Test Data	Classification	Rationale
Available information	Animal data: - Oral LD_{50}, rat 250-320 mg/kg bw (assumption: results from different tests; lowest LD_{50} is valid) - Inhalation LC_{50} rat 2.3 mg/l/4h (vapour) Observations: extensive liver and kidney damage following oral and inhalation exposure to lethal doses (insufficient information)	Category 3 oral and Category 3 inhalation	Classification according to criteria for acute inhalation and oral toxicity in CLP Annex I, Table 3.1.1.
Remarks	The substance is classified for acute toxicity and not for STOT-SE, since the observed organ toxicity is clearly the cause of the lethality.		

http://echa.europa.eu/documents/10162/13562/clp_en.pdf

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Endpoints acute dermal toxicity

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DSD	T+ R27	T R24	Xn R21

Dermal, rat or rabbit – LD50 (mg/kg bw)	≤ 50	50-200	200-400	400-1000	1000-2000

CLP	Cat. 1	Cat. 2	Category 3	Category 4

Application: 10% of total body surface
Exposure period: 24-hours
Observation period: 14 days minimum

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Endpoints acute toxicity inhalation

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DSD Aerosols & particulates	T+ R26	T R23	Xn R20

Inhalation, rat – LC50 (mg/l)	≤ 0.05	0.05-0.25	0.25-0.5	0.5-1	1-5

CLP Dust & Mist	Cat. 1	Category 2	Cat. 3	Category 4

Application: oro-nasal, head only or whole body
Exposure period: 4-hours
Observation period: 14 days minimum

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Endpoints acute toxicity inhalation

ECRAN

DSD Vapours	T+ R26	T R23	Xn R20	
Inhalation, rat – LC50 (mg/l)	≤ 0.5	0.5-2	2-10	10-20
CLP (gases)	Cat. 1	Cat. 2	Category 3	Cat. 4
Inhalation, rat – LC50 (ppm V)	≤ 100	100-500	500-2500	2500-20000

Application: oro-nasal, head only or whole body
Exposure period: 4-hours
Observation period: 14 days minimum

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Specific Target Organ Systemic Toxicity – Single exposure

ECRAN

DSD/DPD	T+,R39	T,R39	Xn R68	R37 R67
oral, rat (mg/kg bw/day)	< 25	25-200	200-2000	R37 Irritating to respiratory system R67 Vapours may cause drowsiness and dizziness.
dermal, rat or rabbit (mg/kg bw/day)	< 50	50-400	400-2000	
inhalation (gas, vapour), rat (mg/l/4hr)	< 0.5	0.5-2	2-20	
inhalation (aerosols or particulates), rat (mg/l/4hr)	< 0.25	0.25-1	1-5	

CLP	Cat. 1	Cat. 2	Cat. 3
oral, rat (mg/kg bw/day)	< 300	300-2000	Transient target organ effects.
dermal, rat or rabbit (mg/kg bw/day)	< 1000	1000-2000	
inhalation (gas), rat (ppm)	< 2500	2500-5000	
Inhalation (vapour), rat (mg/l)	10	10 -< 20	
inhalation (dust/mist/fume), rat (mg/l/4hr)	< 1	1- < 5	

small changes: body/organ weight, haematology
adaptive responses: liver hypertrophy

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Liver Hypertrophy

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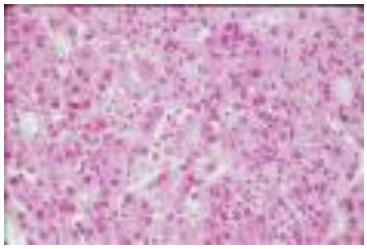
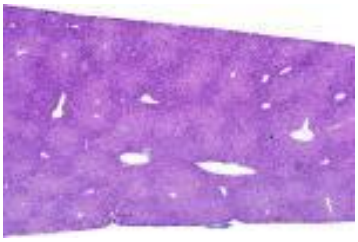


Normal liver tissue



Hypertrophic Liver tissue

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Hyperplastic response

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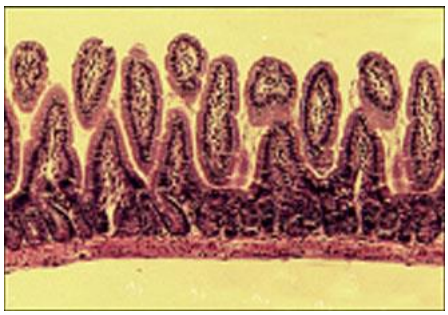
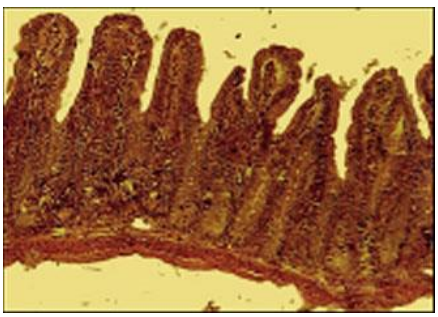


Normal epithelium



Hyperplastic epithelium

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Classification Carcinogenic, Mutagenic, Reprotoxic (CMR)

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General under CLP
Category 1a: the substance is ***known to be CMR*** for man (epidemiological)

Category 1b: the substance ***should be regarded as if*** they are CMR in man (evidence in more than 1 other animal species)

Category 2 the substance ***cause concern*** for man (evidence one species, results variable etc.)

NOTE classification somewhat different from eg. IARC classification

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Steps in the Development of Tumours

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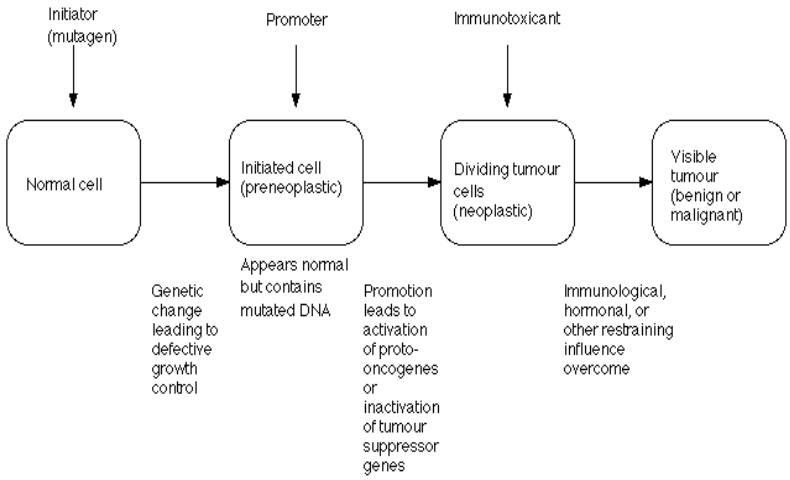

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Carcinogenic substances

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DSD	Cat. 1 T R45 & T R49T+ R26	Cat.2 T R45 & T R49	Cat.3 Xn R40
Criteria	Substances known to be carcinogenic to man	Substances which should be regarded as if they are carcinogenic to man	Substances which cause concern for man owing to possible carcinogenic effects

CLP	Category 1		Category 2
	Category 1A	Category 1B	
Criteria	Chemicals knows to have carcinogenic potential for humans	Chemicals presumed to have carcinogenic potential for humans	Suspected human carcinogens

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Example genotoxicity

Sister Chromatide exchange

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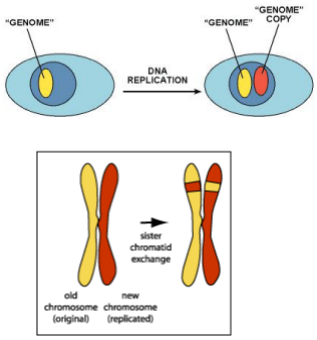

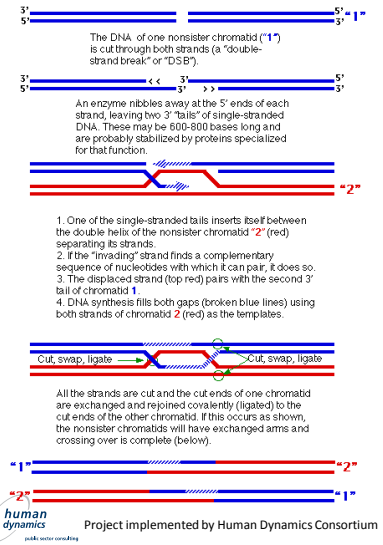

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Example Genotoxicity

Sister Chromatide exchange

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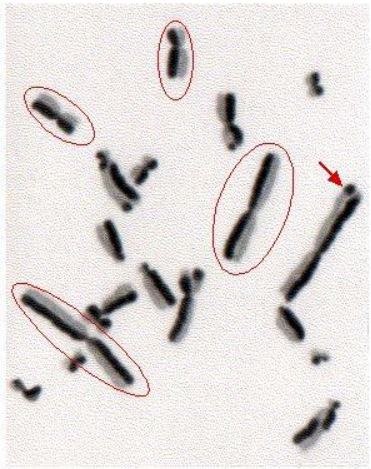

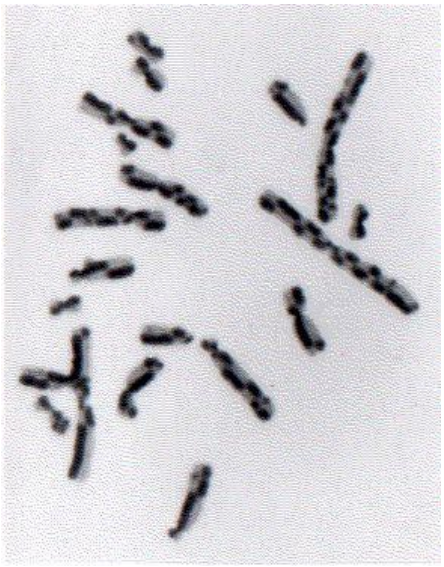

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Genotoxicty and mutagenicity

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DSD	Cat. 1 T R46	Cat.2 T R46	Cat.3 Xn R68
Criteria	Substances known to be mutagenic to man	Substances which should be regarded as if they are mutagenic to man	Substances which cause concern for man owing to possible mutagenic effects
CLP	Category 1		Category 2
	Category 1A	Category 1B	
Criteria	Chemicals knows to induce heritable mutations in germ cells of humans	Chemicals which should be regarded as if they induce heritable mutations in germ cells of humans	Chemicals which cause concern for man owing to the possibility that they may induce heritable mutations in germ cells of humans

Genotoxicity: direct and indirect effects on genetic material (broader incl. for example sister chromatide exchange mechanisms)

Mutagenicity: induction of permanent transmissible change in amount or structure of genetic material

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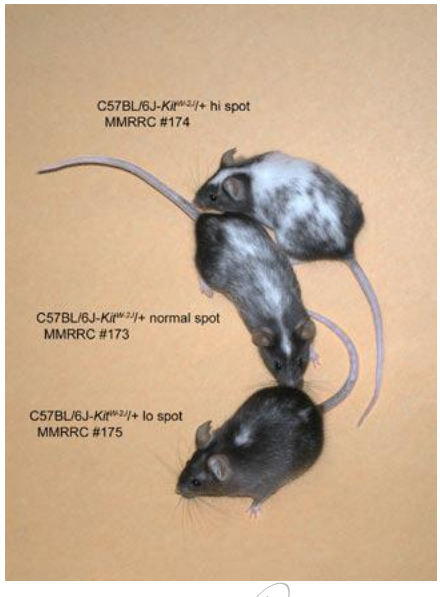
Specific locus mutation test (visible)



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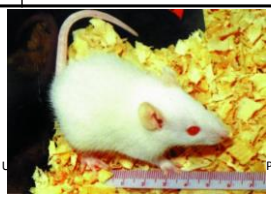
Reprotoxic substances

DSD	Cat. 1 T R60 & T R61	Cat.2 T R60 & T R61	Cat.3 T R62 & R63
Criteria	Substances known to impair fertility in humans or to cause developmental toxicity in humans	Substances which should be regarded as if they impair fertility to humans or cause developmental toxicity in humans	Substances which cause concern for human fertility or to possible developmental toxic effects
CLP	Category 1		Category 2
	Category 1A	Category 1B	
Criteria	Chemicals knows human reproductive toxicant	Chemicals presumed human reproductive toxicant	Chemicals suspected human reproductive toxicant



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End points and testing methods: ecotoxicological properties

Reference:

- Martin Murín, MSc. Ekotoxikologické centrum Bratislavas.r.o.
- Annex V to Directive 67/548/EEC

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Test methods

- The legally binding EU standardised Testing Methods to determine the hazardous properties of chemicals are contained in Annex V of Dir 67/548/EEC on the Classification, Packaging and Labelling of Dangerous Substances. They play a central role in the EU policy on chemicals control and they are referred in many other pieces of EU legislation (e.g. those related to dangerous preparations, pesticides, cosmetics and biocides also refer to these methods)

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Eco-toxicological studies

according to volume

				additional studies birds
			bioaccumulation	bioaccumulation
			prolonged fish	prolonged fish
			earthworms	earthworms
			higher plants	higher plants
			Daphnia, 21-days	Daphnia, 21-days
		algal growth	algal growth	algal growth
		acute Daphnia	acute Daphnia	acute Daphnia
		acute fish	acute fish	acute fish
		bacterial inhibition	bacterial inhibition	bacterial inhibition
		adsorption/desorption	adsorption/desorption	adsorption/desorption
	biodegradation	biodegradation	biodegradation	biodegradation
none	**0.1**	**1**	**10 / 100**	**1000** **t/a**
0.01				

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ANNEX V*

Annex V to Dir 67/548/EEC is divided in three parts which contain Testing Methods for chemicals that address all areas of concern:

- Part A contains methods for the determination of PHYSICO-CHEMICAL properties (e.g. melting and boiling point, density, flash point, flammability, explosivity, oxidizing power, etc...).
- Part B contains methods for the determination of effects on HUMAN HEALTH (e.g. acute or chronic toxicity, skin sensitisation, irritancy, corrosivity, carcinogenicity, neurotoxicity, etc..., they include also in vitro or alternative methods).
- Part C contains methos for **ENVIRONMENTAL EFFECTS**, ecotoxicity and environmental fate (e.g. toxicity to fish, daphnia or algae, bioconcentration, biodegradability, etc..).

*Council Regulation (EC) No 440/2008 of 30 May 2008 laying down test methods pursuant to Regulation (EC) No 1907/2006 of the European Parliament and of the Council on the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH), whereas (3) The test methods contained in **Annex V to Directive 67/548/EEC** should be incorporated into this Regulation

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Part C: Methods for the determination of

Environmental effects

C.1 ACUTE TOXICITY FOR FISH
C.2 ACUTE TOXICITY FOR DAPHNIA
C.3 ALGAL INHIBITION TEST
C.4 BIODEGRADATION: DETERMINATION OF THE "READY" BIODEGRADABILITY

C.4-A DISSOLVED ORGANIC CARBON (DOC) DIE-AWAY TEST
C.4-B MODIFIED OECD SCREENING TEST
C.4-C CARBON DIOXIDE EVOLUTION TEST
C.4-D MANOMETRIC RESPIROMETRY TEST
C.4-E CLOSED BOTTLE TEST
C.4-F MITI TEST

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Environmental effects

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C.5 DEGRADATION : BIOCHEMICAL OXYGEN DEMAND
C.6 DEGRADATION: CHEMICAL OXYGEN DEMAND
C.7 DEGRADATION: ABIOTIC DEGRADATION: HYDROLYSIS AS A FUNCTION OF PH
C.8 TOXICITY FOR EARTHWORMS : ARTIFICIAL SOIL TEST
C.9 BIODEGRADATION: ZAHN - WELLENS TEST
C.10 BIODEGRADATION: ACTIVATED SLUDGE SIMULATION TEST
C.11 BIODEGRADATION: ACTIVATED SLUDGE RESPIRATION INHIBITION TEST
C.12 BIODEGRADATION: MODIFIED SCAS TEST
C.13 BIOCONCENTRATION: FLOW-THROUGH FISH TEST
C.14 FISH JUVENILE GROWTH TEST
C.15 FISH, SHORT-TERM TOXICITY TEST ON EMBRYO AND SAC-FRY STAGES
C.16 HONEYBEES - ACUTE ORAL TOXICITY TEST
C.17 HONEYBEES - ACUTE CONTACT TOXICITY TEST
C.18 ADSORPTION/DESORPTION USING A BATCH EQUILIBRIUM METHOD
C.19 ESTIMATION OF THE ADSORPTION COEFFICIENT (KOC) ON SOIL AND ON SEWAGE SLUDGE USING HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)
C.20 DAPHNIA MAGNA REPRODUCTION TEST
C.21 SOIL MICROORGANISMS: NITROGEN TRANSFORMATION TEST
C.22 SOIL MICROORGANISMS: CARBON TRANSFORMATION TEST
C.23 AEROBIC AND ANAEROBIC TRANSFORMATION IN SOIL
C.24 AEROBIC AND ANAEROBIC TRANSFORMATION IN AQUATIC SEDIMENT SYSTEMS

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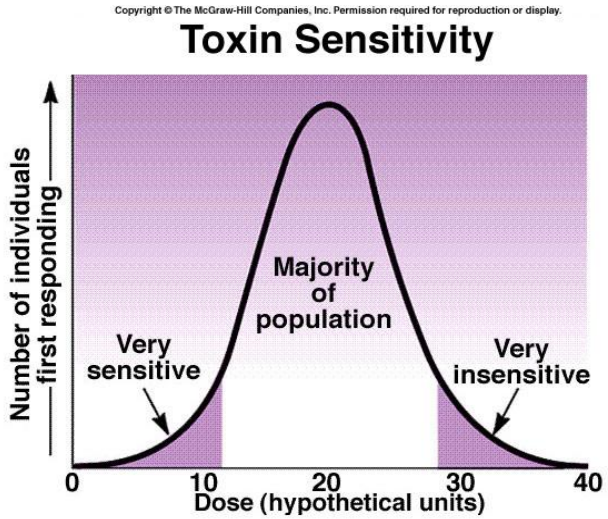
CHEMICALS



Acute Toxicity

- **Acute toxicity:** It involves lethal concentrations and short-term exposures
- Acute effects of a toxin appear immediately after exposure.
- The end point is usually death (lethal), hence it is used to derive $LD_{50,}$ LC_{50}
- An LD_{50} / LC_{50} is a dose / concentration of a toxic chemical that kills half of the population.
- LD_{50} is obtained by plotting, for a given dose the proportion of the population that responded to that dose and all lower doses
- Other end-points:

  EC = effective concentration IC = inhibitory concentration

CHEMICALS: Major Types of Toxicity

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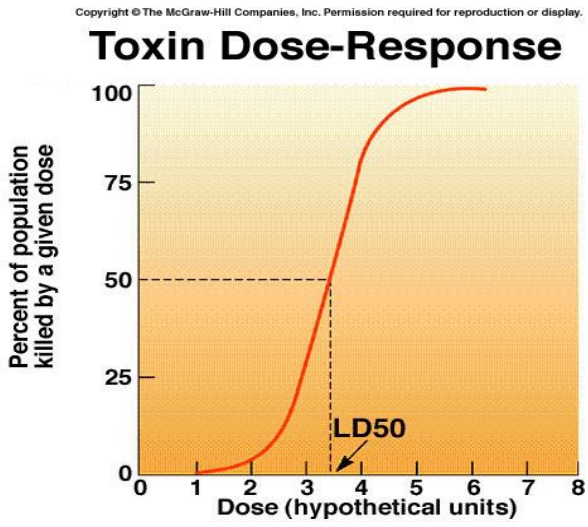

Other parameters

- Biodegradation
  - **Ready** **C.4 BIODEGRADATION: DETERMINATION OF THE "READY" BIODEGRADABILITY**

  or Inherent
- Bioaccumulation
  - **C.13 BIOCONCENTRATION: FLOW-THROUGH FISH TEST**

  - **Octanol/water partition coefficient (Kow)**

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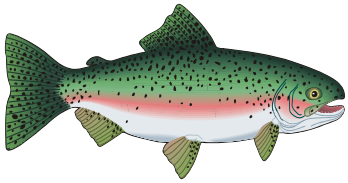
C.1. ACUTE TOXICITY FOR FISH

- LC = lethal concentration

acute lethal toxicity of a substance to fish in fresh water



- acute toxicity is expressed as the median lethal concentration (LC50), that is the concentration in water which kills 50% of a test batch of fish within a continuous period of exposure which must be stated.

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C.1. Information need

- water solubility, vapour pressure, chemical stability, dissociation constants and biodegradability of the substance to help in the selection of the most appropriate test method

  (static, semi-static or flow-through)
- Additional information (for instance structural formula, degree of purity, nature and percentage ofsignificant impurities, presence and amounts of additives, and n-octanol/water partition coefficient) should be taken into consideration in both the planning of the test and interpretation of the results.

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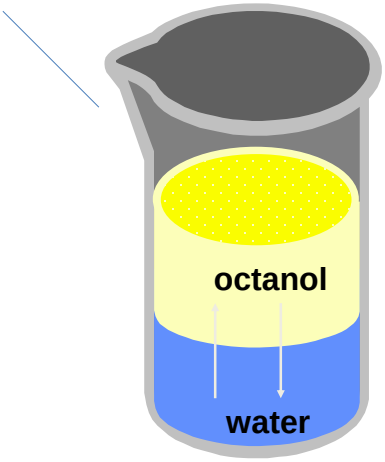
Basic properties

Octanol/water partition coefficient : K_{ow}

$$K_{ow} = \frac{[\text{Octanol}]}{[\text{water}]}$$



Used for describing :

- sorption of chemicals onto particles
- bioconcentration of chemicals in organisms

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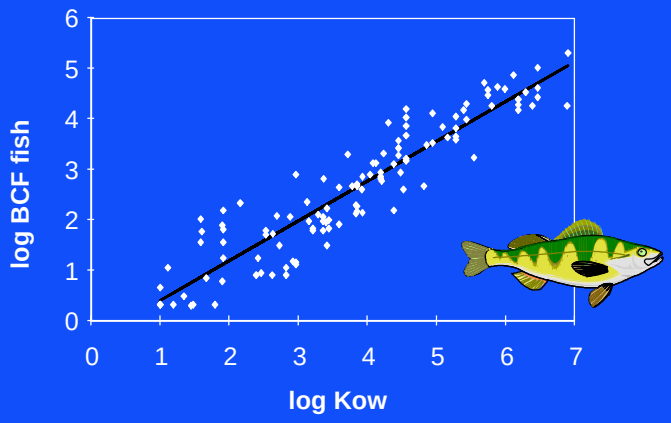
Basic properties

Quantitative Structure-Activity Relationship: QSAR

Relating a “cheap” parameter to an “expensive” one



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C.1. Test principle

- A limit test may be performed at 100 mg per litre in order to demonstrate that the LC50 is greater than this concentration.
- The fish are exposed to the test substance added to water at a range of concentrations for a period of 96 hours.
- Mortalities are recorded at least at 24-hour intervals, and the concentrations killing 50% of the fish (LC50) at each observation time are calculated where possible.

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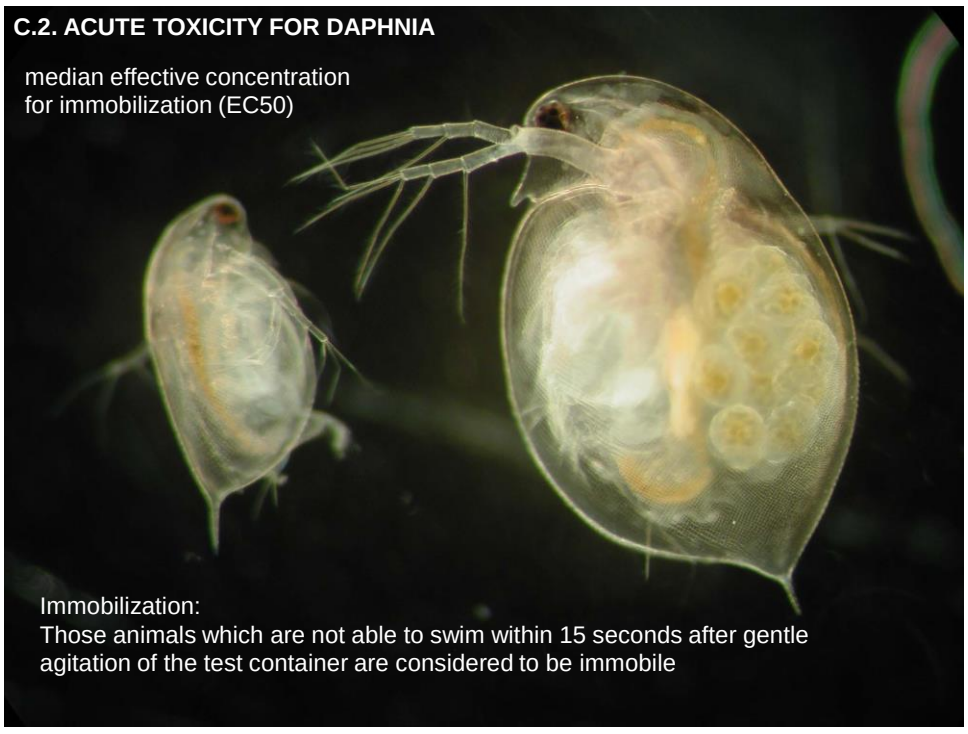

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C.2. Information need

- water solubility, vapour pressure, chemical stability , dissociation constants and biodegradability of the substance before starting the test.
- Additional information (for instance structural formula, degree of purity, nature and percentage ofsignificant impurities, presence and amounts of additives, and n-octanol/water partition coefficient) should be taken into consideration in both the planning of the test and interpretation of the results.

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C.2. Test principle

- A limit test may be performed at 100 mg per litre in order to demonstrate that the EC50 is greater than
- this concentration.
- The *Daphnia* are exposed to the test substance added to water at a range of concentrations for 48 hours. If a shorter test is used, justification should be given in the test report.
- Under otherwise identical test conditions, and an adequate range of test substance concentrations, different concentrations of a test substance exert different average degrees of effect on the swimming ability of *Daphnia* . Different concentrations result in different percentages of *Daphnia* being no longer capable of swimming at the end of the test. The concentrations causing zero or 100 % immobilization are derived directly from the test observations whereas the 48-hour EC50 is determined by calculation if possible.

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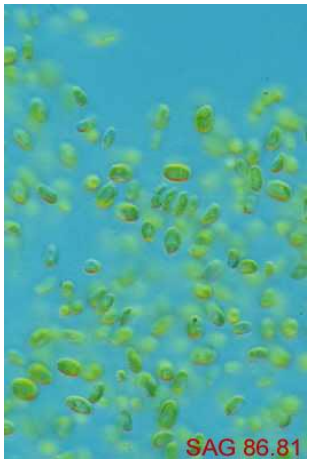
C.3. Algal Inhibition Test



- End-point – inhibition of growth
- effects of a substance on the growth of a unicellular green algal species.

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C.3. Information need

- water solubility, vapour pressure, chemical stability , dissociation constants and biodegradability of the substance before starting the test.
- Additional information (for instance structural formula, degree of purity, nature and percentage ofsignificant impurities, presence and amounts of additives, and n-octanol/water partition coefficient) should be taken into consideration in both the planning of the test and interpretation of the results.

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C.3. Test principle

- A limit test may be performed at 100 mg per litre in order to demonstrate that the EC50 is greater than this concentration.
- Exponentially-growing cultures of selected green algae are exposed to various concentrations of the test substance over several generations under defined conditions.
- The test solutions are incubated for a period of 72 hours, during which the cell density in each solution is measured at least every 24 hours. The inhibition of growth in relation to a control culture is determined.

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C.4. DETERMINATION OF 'READY' BIODEGRADABILITY

- A solution, or suspension, of the test substance in a mineral medium is inoculated and incubated under aerobic conditions in the dark or in diffuse light. The amount of DOC in the test solution due to the inoculum should be kept as low as possible compared to the amount of DOC due to the test substance.
- Allowance is made for the endogenous activity of the inoculum by running parallel blank tests with inoculum but without test substance, although the endogenous activity of cells in the presence of the substance will not exactly match that in the endogenous control. A reference substance is run in parallel to check the operation of the procedures.

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C.4. DETERMINATION OF 'READY' BIODEGRADABILITY

- In order to select the most appropriate method, information on the chemical's **solubility, vapour pressure** and **adsorption** characteristics is essential.
- The chemical structure or formula should be known in order to calculate theoretical values and/or check measured values of parameters, e.g. ThOD, ThCO2, DOC, TOC, COD (see Annexes I and II).

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C.4. DETERMINATION OF 'READY' BIODEGRADABILITY

Test	Analytical Method	Suitability for substances which are:		
		poorly soluble	volatile	adsorbing
DOC Die-Away	Dissolved organic carbon	—	—	+ / –
Mod. OECD Die-Away	Dissolved organic carbon	—	—	+ / –
CO_2 Evolution	Respirometry: CO_2 evolution	+	—	+
Manometric Respirometry	Manometric respirometry: oxygen consumption	+	+ / –	+
Closed Bottle	Respirometry: dissolved oxygen	+ / –	+	+
MITI	Respirometry: oxygen consumption	+	+ / –	+

Test	DOC Die-Away	CO_2 Evolution	Manometric Respirometry	Modified OECD Screeing	Closed Bottle	MITI (I)
Concentration of Test Substance as mg/l mg DOC/l mg ThOD/l	10-40	10-20	100 50-100	10-40	2-10 5-10	100
Concentration of Inoculum (in cells/l, approximatively)	≤ 30 mg/l SS or ≤ 100 ml effluent/l ($10^7 - 10^8$)			0,5 ml secondary effluent/l (10^5)	≤ 5 ml of effluent/l ($10^4 - 10^6$)	30 mg/l SS ($10^7 - 10^8$)
Concentration of elements in mineral medium (in mg/l) P N Na K Mg Ca Fe	116 1,3 86 122 2,2 9,9 0,05-0,1				11,6 0,13 8,6 12,2 2,2 9,9 0,05-0,1	29 1,3 17,2 36,5 6,6 29,7 0,15
pH	7,4 ± 0,2					preferably 7,0
Temperature	22 ± 2 °C					25 ± 1 °C

DOC = Dissolved organic Carbon ThOD = Theoretical Oxygen Demand SS = Suspended Solids

C.4 Ready biodegradability

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- Normally, the test lasts for **28 days**. Tests however may be ended before 28 days, i.e. as soon as the biodegradation curve has reached a plateau for at least 3 determinations. Tests may also be prolonged beyond 28 days when the curve shows that biodegradation has started but that the plateau has not been reached day 28.
- Inocula may be pre-conditioned to the experimental conditions, but not pre-adapted to the test chemical. Pre-conditioning consists of aerating activated sludge in mineral medium or secondary effluent for 5-7 days at the test temperature. Pre-conditioning sometimes improves the precision of the test methods by reducing blank values. It is considered unnecessary to pre-condition MITI inoculum.

C.4. Test validity

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- A test is considered valid if the difference of extremes of replicate values of the removal of test chemical at the plateau, at the end of the test or at the end of the 10 -day window, as appropriate, is less than 20% and if the percentage degradation of the reference substance has reached the level for ready biodegradability by 14 days.

  <u>If either of these conditions is not met, the test should be repeated.</u>
- Because of the stringency of the methods, low values do not necessarily mean that the test substance is not biodegradable under environmental conditions, but indicates that more work will be necessary to establish biodegradability. If in a toxicity test, containing both the test substance and a reference chemical, less than 35% degradation (based on DOC) or less than 25 % (based on ThOD or ThCO2) occurred in 14 days, the test chemicals can be assumed to be inhibitory (see also Annex IV). The test series should be repeated, if possible using a lower concentration of test chemical and/or a higher concentration of inoculum, but not greater than 30 mg solids/litre.

C. 9. BIODEGRADATION ZAHN -WELLENS TEST

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- The purpose of the method is the evaluation of the potential ultimate biodegradability of water-soluble, non-volatile organic substances when exposed to relatively high concentrations of micro-organisms in a static test.
- The substances to be studied are used in concentrations corresponding to DOC-values in the range of **<u>50 to 400 mg/litre</u>** or COD-values in the range of 100 to 1000 mg/litre (DOC = dissolved organic carbon; COD = chemical oxygen demand). These relatively high concentrations have the advantage of analytical reliability. Compounds with toxic properties may delay or inhibit the degradation process.
- In this method, the measure of the concentration of dissolved organic carbon or the chemical oxygen demand is used to assess the ultimate biodegradability of the test substance.
- Activated sludge in an amount corresponding to 0,2 to 1,0 g/litre dry matter in the final mixture.

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