STAR

(Contract Number: Fission-2010-3.5.1-269672)

DELIVERABLE (D-N°5.1)

Plans for laboratory radiation effects studies

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Reporting period: 01/02/11 – 31/10/11

Date of issue of this report: **31/10/2011**

Start date of project: **01/02/2011**   Duration: 54 Months

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Executive Summary

This report describes in details the basic concepts, needs and data treatment for the approaches we will implement under WP-5 dedicated to "ecologically-relevant low doses effects to non-human species". It gives the preliminary list of the potential sets of selected species and endpoints, and the method and tools for establishment of dose (rate) - responses relationships for life traits and for DEBTox parameterisation. A preliminary state-of-the-art is also established to explain the reasoning we are proposing for understanding mechanisms underlying molecular mode of action of radiation exposures, together with a brief review of the various omic tools available for gaining insights into modes of actions. Common guidance for scaling any of our experiments is illustrated through the presentation of the pilot study we decided in order to test our ability to share experiments among partners (hypothesis - choice of species & endpoint – experimental design – scaling the range and space of dose rates - data treatments).

Within this global framework of WP-5, our experimental work will be done on the basis of a limited set of experiments designed to test umbrella hypotheses as follows:

1. Chronic irradiation by internal alpha- or external gamma emitter leads to physiological/metabolic changes at the individual level caused by:

    (i) increase in maintenance costs,

    (ii) decrease in assimilation,

    (iii) increase in energetic costs for somatic growth

    (iv) increase in energetic costs for reproduction,

    (v) direct hazard to embryos.

This umbrella hypothesis will drive Task 5.3 devoted to DEBTox and its consequences at the population level.

2. Differences in radiosensitivity between species are correlated with (sub)cellular properties and processes (*e.g.,* DNA quantity, repair mechanisms).

3. Due to actions at different sites (microdosimetry), the Relative Biological Effectiveness (RBEs) derived for alpha emitters for human cancer risks will not be applicable for ecologically relevant endpoints

These umbrella hypotheses will drive Task 5.2 devoted to the understanding of mechanisms of radiation effects at the individual level.

The next step viewed as "go-no go" actions will take place in our next meeting in January 2012 where we will discuss:

(i)   the main conclusions from our sensitivity analysis outcoming from the theoretical approach where we combined radiosensitivity information from FREDERICA and Leslie matrices for a wide range of species (task 5.1): this discussion will help to decide whether we need to implement experiments (or not) to obtain more robust conclusions about the propagation of effects observed at the individual level to the population level;

(ii)   the results and lessons learnt from the pilot study: this discussion will help to refine the experimental design if needed, to conclude on the feasibility of DEBTox development (task 5.3);

(iii)   the hypotheses we would test to progress in the understanding of the mechanistic modes of actions at the (sub)cellular level (task 5.2).

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1 Introduction and general objectives

The main objective of this document is to present the common approach decided among STAR partners to ensure the consistency of used protocols for performing experiments in different laboratories, and as a consequence to ensure the data collected among STAR WP-4 and WP-5 is comparable. Our willingness is to demonstrate the integration efficiency through the adoption of common protocols and methods for data generation and interpretation. Rationale for selection of hypotheses, exposure conditions, biological models, endpoints, along with principles for experimental design and description of models, tools and statistics for data interpretation are successively presented within this report.

WP-4 and WP-5 have selected the following research issues to be investigated on selected species for each of two radiation types (external γ and internal α) and co-contaminants to be defined within WP-4. For WP-5, we have decided to have three experimental sets, one per task, all of them being strongly related. The three tasks are reminded in Table 1 also with their starting period as written in STAR proposal.

Table 1: Brief description of the main lines of the experiments associated to each task for WP5

WP-Task	Objective of the experimental part	Start of experiments
WP-5 Task 1	On the basis of the theoretical part of Task 5.1, quantify dose-effect relationships on individual life history traits for species according to (i) the major data gaps highlighted by the theoretical part of this task and/or (ii) the most influencing extrapolation assumption used during the theoretical part that could be tested in order to reduce uncertainty	From January 2012
WP-5 Task 2	From a state-of-the-art review on the molecular, cellular and individual parameters influencing radiosensitivity, design experiments using a combination of conventional biomarker and omic tools to test hypothesis about the mode of action of radiological exposures	From May 2012
WP-5 Task 3	DEBTox implementation on two models (one plant, one animal) and combination with population modelling	From January 2013

Experimental design will have to be appropriate for the models & tools we decided to use preferentially to address our research lines:
- **a traditional toxicologically based approach to build dose(rates)-effects relationships for individual life history traits within a species.** This knowledge is then used for predicting population consequences on the basis of individual adverse outcomes (theoretical work (potentially completed by experiments under laboratory conditions) done under Task 5.1 by combining Dose (rate)-effect relationship and Leslie Matrices);
- a physiologically based approach completed by inter-relational links between life history traits with "Dynamic Energy Budget" concepts. Ecological risk assessment has been marked over the last decade by an increasing interest in biology-based models, such as DEBtox (Dynamic Energy Budget applied to toxicity data) models, based on the DEB theory. This theory provides a conceptual framework which specifies how energy is taken from food and allocated to growth and reproduction. For the selected species, the DEBtox concept will be used to test whether chronic exposure to different

stressors (alpha, gamma and a simple mixture) will involve contrasted metabolic mode of actions that drive the consequences in terms of individual fitness.

- **a mechanistically-based approach** to gain knowledge of parameters governing radiosensitivity between and within species. For the selected species chronically exposed to alpha or gamma irradiation, and to a simple mixture of radiation and a chemical, "omic" tools will be used with the aim of advancing the mechanistic understanding of radiation impact at the molecular level, including differences between the relative biological effectiveness (RBE) of alpha and gamma exposures.

- **an approach for integration of all the knowledge acquired**. This will be done at the very end of the project to synthesise all lessons learnt (Task 4.). We expect new knowledge to provide insights into:

- understanding of how radiosensitivity at the molecular and individual levels mechanistically link to impacts on individuals and populations, and
- understanding how dose characteristics (*e.g.,* radiation type, targeted organs) influence the biological efficiency of radiological damage, by identifying the metabolic pathways that produce individual history trait disturbances;
- the ability to deal with extrapolation between species.

Within this global framework, our experimental work will be done on the basis of a limited set of biological models, endpoints and approaches which choices will be justified in due time during the course of the project. The umbrella hypotheses to be tested will be:

1. Chronic irradiation by internal alpha- or external gamma emitter leads to physiological/metabolic changes at the individual level caused by:

(i) increase in maintenance costs,

(ii) decrease in assimilation,

(iii) increase in energetic costs for somatic growth

(iv) increase in energetic costs for reproduction,

(v) direct hazard to embryos.

This umbrella hypothesis will drive Task 5.3 devoted to DEBTox and its consequences at the population level.

2. Differences in radiosensitivity between species are correlated with (sub)cellular properties and processes *(e.g.,* DNA quantity, repair mechanisms).

3. Due to actions at different sites (microdosimetry), the Relative Biological Effectiveness (RBEs) derived for alpha emitters for human cancer risks will not be applicable for ecologically relevant endpoints.

These umbrella hypotheses will drive Task 5.2 devoted to the understanding of mechanisms of radiation effects at the individual level.

This report firstly describes the basic concepts, needs and data treatment for the approaches we will implement. It gives the preliminary list of the potential sets of selected biological species and endpoints (chapter 2), then develops the method and tools for establishment of dose-responses relationships for life traits (chapter 3) and for DEBTox parameterisation (chapter 4). Then chapter 5 establishes the main lines of the state of the art to explain the reasoning we are proposing for understanding mechanisms underlying molecular mode of action of radiation exposures, together with a brief review of the various omic tools available for gaining insights into modes of actions. Before

conclusion (chapter 7), common guidance for scaling any of our experiments is illustrated in chapter 6 through the presentation of the pilot study we decided in order to test our ability to share experiments among partners (hypothesis - choice of species and endpoint – experimental design – scaling the range and space of dose rates - data treatments).

2 Sets of selection

2.1 List of candidate species

Multiple criteria need to be considered for selection of appropriate experimental organisms. These were discussed at a workshop in May 2011 (WP-4/5 workshop in Mol – see Minutes), and the following groups of criteria were drawn up. These were then used as a basis for assessing the suitability of various candidate organisms (summarised in Table 2). The order or the list does not reflect the criteria's importance.

Availability of biomarkers / molecular tools *(column 2 Tab.2)***:**
> Availability of range of biomarkers – and lab experience
> Availability of genetically modified organisms (GMOs) for advanced mechanistic studies
> Availability of genome maps and microarrays

Ecological relevance *(column 3 Tab.2)***:**
> Diversity of species, reproduction mechanisms (sexual, clonal, hermaphrodite, etc), life history stages, radiosensitivity
> Functional role or position in the food web
> Possibility of field study sampling
> (Economic relevance – food for humans – important link to WP3 – but on an ecosystems approach all organisms have indirect relevance)

Assessment relevance *(column 3 Tab.2)***:**
> Representativeness as a test species (*e.g.,* standardized bioassays for chemicals) or as a species used to test human effects (*e.g., C. elegans, D. rerio*)
> Reference Animal or Plant for radiation (RAP)

Population dynamics *(column 4 Tab.2)***:**
> Ease of performing experimental studies of reproduction endpoints
> Reproduction mechanisms (sexual, clonal, hermaphrodite, etc), life history stages
> Availability of multi-generation studies or models

Other contaminant / multiple stressor data *(column 5 Tab.2)***:**
> Ease of getting the toxicant (or alpha emitter) into the organism
> Availability of data on toxicant effects
> Likelihood of exposure to multiple stressors (including alpha/beta/gamma mixtures) at sites of interest
> Bioaccumulation or biomagnification
> Possibility of biokinetic studies (organ sampling)
> Availability of Biotic Ligand Models (BLM)

Knowledge status *(column 6 Tab.2)***:**
> Availability of data – necessary for complex modelling

Lack of data – new data would advance the field and provide data needed for benchmark derivation (SSD)

Dynamic Energy Budget (DEB) *(column 7 Tab.2)***:**

Availability of models

Possibility of generating relevant data in the laboratory

Specific Experimental Considerations *(column 8 Tab.2)***:**

Ease of maintenance / husbandry

Issues of waste generation

Ease of performing chronic/multigeneration experiments

Cost

Ability of STAR Partners to perform experiments on the organisms

Table 2: Candidate organisms for experimental study and their suitability according to the criteria

1. Organism	2. Biomarkers/ toolbox	3. Ecological /assessment relevance	4. Population dynamics	5. Other contaminants/multiple stressor	6. Knowledge status	7. DEB	8. Experimental considerations
Phytoplankton Marine or freshwater species	-All (for some species), -Genome sequenced for *Anabaena* and *Thalassiosira*.	- First trophic level, base of most aquatic food webs. - High species diversity with range of life histories, habitats - Very short generation time. - Standard ecotox species exist.	-Many species have simple asexual reproduction (cell division), -Multigenerational studies easy.	-Relatively easy -BLM -Lots of ecotox data available. -Likely to be found in multi-contaminant sites (*e.g.* lakes)	-Nearly no radiation effect data	-No but very helpful to rapidly conceive a DEB for single celled organism model	-Easy to keep, short generation time. -SU, IRSN
Plants *Lemna* *Arabidopsis*	-All up to microarray -GMOs *Arabidopsis* -Genome map For *Lemna*: genome sequence was going to be released december 2009 but is not yet available	- Ecological relevance variable (*Arabidopsis* is a weed). Higher for *Lemna* then *Arabidopsis*. *Lemna* is a common freshwater macrophyte and crucial in the food chain. - *Lemna* also has economic value (grown as source for protein rich feed) - overall plants have high ecological relevance (biomass) - both are model test organisms	- Reproduction and multi-generation experiments	-Relatively easy -Many data available for *Lemna* not for *Arabidopsis*. -Likely to be found in multi-contaminant sites (*e.g.*, ponds (*Lemna*), soils (*Arabidopsis*))	-Gaps for SSD (But lots of acute data – and some field data) - Large variation in radiosensitivity - "Hormesis" and adaptation	-Feasible starting from scratch. But will be novel in the end. Need ca. 12 months for DEB conception and calibration and 12 months for the DEBtox version	-SCK (both) -UMB & IRSN (*Arabidopsis*)
Nematod *C. elegans*	- All up to microarray - GMOs (lots and cheap) - Genome map	- Soil microorganisms - Different reproduction strategies - Large and ecologically diverse and important phyla - Representative ecotox (and human tox) organism	-Easy multi-generational model	-Relatively easy -Lots of metal (and other chemical) data. -Likely to be found in multi-contaminant sites (*e.g.*, soils/sediments)	- Widely used in human radiation biology – but for other effects and endpoints.	-Available soon at IRSN	-Easy Automated "worm counters" available -IRSN, UMB
Crustacean *D. magna*	-All up to microarray	- Different reproduction strategies - Representative aquatic ecotox organism	-Multigenerational model -Multi cohort models	-Relatively easy. -Likely to be found in multi-contaminant sites (*e.g.*, ponds)	-Effects data available for asexual reproduction – sexual repro under study at IRSN	-Available and operational	-Easy -IRSN, UMB (partners - no own research)

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Bivalve							
Bivalve Blue mussel (etc)	-As for zebrafish (but no microarray)	- Filter feeder, - Structure forming - Major food source (humans and wildlife) - Wide distribution (marine and freshwater) - Long life cycle - Standard chemical tox representative organism	-Multigenerational -Early spawning -Longish lifespan -Broadcast spawning of larvae	-Extensive biomonitoring data) -Biokinetics and organ distribution possible. -Kinetic uptake curves easy. -Likely to be found in multi-contaminant sites (*e.g.* coastal areas, lakes)	-Not a standard organism, so compared to fish less data for radiation effects. -Uptake and speciation studies	-Operational	-Easier than fish – lower maintenance, ethical constraints, size etc.. -SU
Annelid Terrestrial *E. fetida* (lab) *L. rubellus* (field) Aquatic Polychaeta	-All up to microarray -Some GMOs -Genome map close	- Decomposer, important food web component - RAP - Standard procedures from chemical tox	-Reproduction and multi-generation experiments - some polyploid species Different reproductive strategies can be studied	-Lots of data on metals -Biokinetics possible -BLM. -Likely to be found in multi-contaminant sites (*e.g.*, soils)	-Soil RAP representative of the "decomposers" – - Large gaps in general for invertebrates -Variation in radiosensitivity between species (and within whole invertebrate group)	-"DEBish" model available for *L. rubellus* (Klok and de Roos)	-Low maintenance, chronic exposures easy (internal and external) -Metal speciation in soils, soil disposal, cocoon counting time consuming; -UMB ; IRSN
Insect Bee		- Long and complex life cycle - RAP				No	-Large research group based at UMB (but not STAR participants)
Fish Zebrafish Medaka Salmon	All up to microarray GMOs (zebrafish and medaka) Genome map (zebrafish	-Ecological and economic relevance (salmon – human food source) -RAP - Standard procedures from chem. (zebrafish) -Bio-magnification (e.g. Po) - Representative eco-& human tox model (vertebrates)	Reproduction experiments (zebrafish) Sensitive life history; fertilisation experiments (salmon) Models available from fisheries and other stressor assessments	-Data on metals -Some data on radiation+ metal (antagonism) Biokinetics/organ doses -BLM -PBPK model soon available. Likely to be found in multi-contaminant sites (*e.g.* lakes)	-Lots of acute data; less chronic -Lots of data on chemicals (mixture studies)	-Soon operational at IRSN (zebrafish)	-Standard procedures from chem. (zebrafish) -Easy husbandry -UMB, IRSN
Bird Chicken/ duck model organisms available		- Long life cycle - RAP		-Hard to implement in lab	-Not a standard organism, so compared to fish less data for radiation effects.	-No	-Field investigations NRPA

2.2 Radioactive stressors and exposure guidelines

Experimental design will have to be appropriate for the two research lines described above, *i.e.* to build dose(rate)-effects relationships for individual life history traits within a species, and to acquire the data needed for the parameterization of physiologically-based model, such as DEBtox. Both research lines will deal with chronic exposure conditions, involving designs allowing continuous exposure of organisms during a significant length of their life cycle, or at least crucial stages.

A first specific point that will drive the choice of radioactive stressors and exposure conditions is the need for building dose(rates)-effects relationships for the chosen endpoints (see chapter 3). The second point is the selection of the route and duration of the exposure. In order to test the first umbrella hypothesis described above, chronic irradiation by internal alpha and external gamma emitters will need to be implemented to assess physiological/metabolic changes at the individual level and to identify the DEBtox modes of action.

A generic screening value of 10 µGy/h (incremental dose rate) was recently derived, and proposed as the screening benchmark for ecological risk assessment. For this work, the primary source of effects data from radiation on non-human species was the FREDERICA database (www.frederica-online.org, Copplestone *et al*., 2008), which is the most comprehensive compilation of raw biological effects data from the scientific literature. A meta-analysis of this database was first processed by Garnier-Laplace *et al*. (2006), enlarged during the PROTECT program (http://www.ceh.ac.uk/protect/), resulting in a large and consistent set of chronic critical radiotoxicity values (EDR_{10}, dose rate giving a 10% change in observed effect). However, this work has pointed out that there are still large knowledge gaps for radiation effects on non-human biota, mainly linked to our poor understanding of (1) chronic, low-level exposure experiments linked to ecologically-relevant endpoints, and (2) the way effects of external gamma irradiations can be extrapolated to internal irradiations with high-LET particles.

This is why the acquisition of chronic effect data under continuous irradiation nearby the 10 µGy/h benchmark value (*i.e.* from background level of <1 µGy/h up to 100 mGy/h) is the main line that will drive the choice of exposure conditions during STAR.

External gamma irradiation requires a specific irradiation facility under controlled conditions (with a gamma source, *e.g.,* Co-60 or Cs-137 source). Dose(rates) are chosen after a first step of dose calculation (*e.g.,* MCNP) for the identification of the exact location of any experimental unit in the irradiation chamber to obtain the same dose-rate (the chosen dose-rate is an exponential-decreasing function of the distance to the irradiation source). Then, a pre-calibration of external dose rates is needed, using adequate dosimeter calibration tools (*e.g.,* ionisation chambers, thermoluminescence detectors …). A specific point in regard to the choice of a series of external dose(rates) is the need to avoid as much as possible the intra-experimental unit variation of dose(rates), which is highly dependent on the geometry of the experimental unit. UMB (Norway), SCK (Belgium) and IRSN (France) are equipped with adequate tools to specify the external irradiation designs from *ca.* 0.04 to 40 mGy/h.

Criteria for the choice of alpha radionuclide are much more critical. The main criteria that will lead the choice of internal alpha emitter are:

- Concentration factor of the selected species from the exposure medium is favourable to rapid accumulation / low depuration;

- The target cell/organ/tissue(s) is(are) related to the studied endpoint (*e.g.,* digestive system for assimilation endpoints ; gonads for reproductive endpoints ; cell DNA for genotoxicity…), or is homogeneously distributed;

- The exposure route is manageable and reproducible: for direct route (high solubility and bioavailability, low adsorption…) and/or trophic route (feasibility of food contamination, high bioconcentration factor…);

- The needed activity to manipulate is consistent with radioprotection constraints;

- The budget for source providing and waste treatment is not prohibitive;

- The chemical form of the source is compatible with exposure conditions (*e.g.,* element in highly acidic media ; presence of carriers ; purity level);

- The analytical methods allow a precise characterisation of exposure media and internal activities (if possible *in vivo*) and if possible without complex radiochemical separation;

- A short period alpha emitter is favoured;

- The toxicity is due mainly to alpha radiation and not other emissions from the radionuclide and/or its daughters;

- The chemical toxicity of the radionuclide and/or its daughters is negligible.

Combining all of those criteria, the choice of americium (Am-241) as one of the preferred candidate for alpha internal irradiations is viewed as a good compromise.

The thermodynamic database on the speciation of americium(III) is quite well referenced and allows to calculate the chemical speciation and solubility of the radioelement in water (Bion *et al.,* 2005) although experimental evidence would always be needed. This radioelement gives also a realism to actual chronic contaminations: trace concentrations of Am-241 are found worldwide in aquatic ecosystems (10^{-6} to 10^{-5} Bq/L) as a consequence of former atmospheric nuclear weapon testing and accidental releases from nuclear reactors including Chernobyl fallouts, with regional higher levels of 10^{-5} to 10^{-2} Bq/L in freshwaters (Matsunaga *et al.*, 1998; Choppin, 2006). Contaminated soils (10^{-2} to 10 Bq/kg on average, peaking locally up to 10 to 10^{4} Bq/kg) are a potential source of Am-241 for surface water and groundwater (Pourcelot *et al.*, 2003; Agapkina *et al.*, 1995), while the highest concentrations are found in the sediments of continental shelves (up to 105 Bq kg^{-1}). Furthermore, in the future Am-241 will become one of the dominant pollutants in the Chernobyl affected areas due to ingrowth from Pu-241 (Muravitsky *et al.*, 2005). Therefore, knowledge on biological effects of chronic exposure to alpha-emitting radionuclides is urgently needed both for post-accidental situations and for the long-term management of radioactive nuclear waste disposals.

Experimentally, the precise quantification of internal alpha irradiation of organisms will need to quantify the bioaccumulation of Am-241 in relation to concentration and the corresponding calculation of internal alpha dose rates. Several steps will be needed:

- Am-241 concentrations measurement in the medium, and in the organisms at different time (a full biokinetic study would be ideally performed) and life stages. After a proper mineralization, Am-241 activities in samples can be precisely determined alpha liquid scintillation counting using a low background spectrometer (*e.g.,* Quantulus 1220,Wallac Oy, Turku, Finland, the detection limit is *ca.* 0.03 Bq);

- Dose conversion coefficients (DCC, expressed in mGy/h / Bq/ml) needs to be calculated by an adequate method (*e.g.,* based on Monte Carlo calculations with MCNP software). This calculation takes into account alpha particles (5.5 MeV) and beta($-$) particles (from 4.6 keV to 1.0 MeV) emitted by Am-241 that propagate over distances from 2 to 400 µm in the medium and in the contaminated tissues. By this way, Am-241 activities (Bq/ml) measured in each compartment (medium, tissues of the organism…) can be converted to dose rates delivered to daphnids (mGy/h) using a simple calculation. Estimation of DCC needs to take into account the changing body shape and volume in growing organisms. Generally, volumes are calculated assuming that animals are ellipsoids growing

Such an experimental design for the study of Am-241 effects was already implemented on *D magna* for the study of the effects of chronic internal alpha irradiation on physiology, growth and reproductive success endpoints (Alonzo *et al.*, 2006; 2008). As an illustration, Am-241 was used in this study from 0.4 to 40 Bq/ml in the medium, with a bioaccumulation from 0.4 to 28 Bq/daphnid, and subsequent average calculated dose rates from 0.02 to 1 mGy/h (>99% attributable to internal alpha radiation from Am-241 bioaccumulation in tissues).

3 Basic concepts and methods for establishing dose-response relationship for chronic exposure situations to a single stressor

3.1 Test design

3.1.1 Selecting doses

Concerning the number of doses, OECD (2006) advises the use of at least four different doses (control included). In addition, for monotonic dose-response relationships, Environment Canada (2005) recommends that observations encompass the three phases of the relationship (first phase with low effect, second phase with an increasing effect, and third phase with an asymptotic effect). Obviously, these recommendations are mainly theoretical. In order to obtain a good fit for the regression, experimental data points have to be well distributed all along the dose(rate)-response curve. A pilot study can be done previously to help.

For hormetic relationships, there is no specific advise but the same pattern could be used, *i.e.* observations spanning each of the three phases (first stimulatory phase at low doses, second phase with an high increasing (or decreasing) effect, third phase with an asymptotic effect at high doses).

Environment Canada (2005) advises to use a geometric (or logarithm) series of concentrations (or dose (rate) for radionuclides), but here is no statistical reason behind this recommendation. Some specific tests (*e.g.,* OECD guidelines for *Lemna minor* or for Daphnids) suggest first to do a range finding

experiment in dilutions of 10 in order to find out the rough shape of the dose response curve and subsequently to do two-fold diluted experiment within the correct range.

3.1.2 Number of replicates

Here, the terminology given by Environment Canada (2005) is used:

"A replicate (as a noun) is a single test chamber containing a prescribed number of organisms (= *sampling units*), either on one concentration (= *treatment*) of test material, or in a control. In a toxicity test with five test concentrations and a control, using three replicates, 18 test chambers would be used, *i.e.* three chambers for each treatment. A replicate must be an independent test unit, and therefore the test material in a chamber must no have a connection to the test material in another chamber."

From a theoretical point of view, there is no need of replicates in order to estimate an ECp or EDp% and its confidence interval. In other words, replicates are not necessary to obtain an estimation of the curve's parameters or their standard errors. They also represent a security in case of accidental damage. However, it is clear that replicates improve the estimation of the parameters values and their standard error. Moreover, estimation of the residual sum of square used to assess the fit of a model is only possible if replicates are present. It is said that only two replicates are needed to estimate the residual sum of squares, but Environment Canada (2005) recommends at least three replicates per treatment if afterwards want to apply a regression fit to the data. It is also advised that it is preferable to increase the number of concentration or dose(rate)s rather than replicates. It means that in order to have a good estimation of the regression parameter, it is better to have more tested doses all long the curve than 3 doses with a lot of replicates.

3.1.3 Randomization and blind

When toxicity experiment data are analyzed and modelled by regression analysis, procedures of randomization are needed in order to avoid consistent bias in the ECp or EDp% estimate (Davies *et al.*, 1998).

According to Environment Canada (2005), procedures of randomization should include:

- randomization of containers used for the different groups (control and treatment) ;

- randomization of containers' placement within the incubator or room ;

- randomization of sub-sample (organisms) into the containers.

These kinds of procedures require a random numbers table and are well described in the annex E Environment Canada (2005).

When possible, a completely randomised design should be used. That means that the experimental units can be assigned at random to a treatment group; experiment is performed at one time in one location or it can be measured that time and location have negligible effects on the experimental material. Ranges of formal experimental design are well described in many statistical textbooks e.g. (Box *et al.* , 1978; Festing, 2003; Festing and Altman, 2002). In any case, the experimenters will describe the used experimental design and the associated statistical methods.

However, especially when implementing external gamma irradiation experiments, randomization could be a difficult stage to accomplish. Actually, since the dose or dose rate delivered to any experimental unit depends on the location from the gamma source, all replicates within each

dose(rate) treatment will need to be placed together (at a particular distance from the source, or in a particular incubator). Any effect of dose will thus be difficult to separate from any potential effect of position. This can be controlled by measuring relevant parameters such as light and/or temperature in order to be able to exclude this as a confounding factor.

Moreover, (eco)toxicity test should be "blind" tests for the experimenter, meaning that the containers must be identified by a code rather than by a dose level.

3.1.4   Controls

The aim of the control is to provide a baseline of the observed effect. Thus, it has to be identical to the other treatments for all, except for the stressor (*i.e.* a null dose).

3.2 Dose-response curve modelling with R software and 'drc' add-on package

3.2.1   Introduction about R software and 'drc' add-on package

R is a language and environment for statistical computing and graphics. It provides a wide variety of statistical methods (linear and nonlinear modelling, classical statistical tests, time-series analysis, classification, clustering,…) and graphical techniques, and is highly extensible. R is available as free Software and provides an Open Source route. R functions and datasets are stored in packages, which are:

- base packages (part of the R source code, down-loaded with R);
- or add-on packages (have to be specifically down-loaded).

R and packages can be down-loaded at : http://cran.r-project.org/

For more information see the FAQs at:http://cran.r-project.org/

'drc' (for Dose-Response Curve) is an R add-on package. This library provides a wide range of functionalities for dose-response relationships modelling and analysis. A lot of common models for dose-response curves are integrated into the package for convenient use (*i.e.* log-logistic model, Weibull model, Mikaelis-Menten model (see *e.g.,* Scholze *et al.,* 2001).These models are translated into general equations with associated parameters. It is easy to use sub-models of them (i.e. specific equations for instance with one or more parameter fixed). Estimation of critical ecotoxicological values (*i.e.* EDp% or EDRp%) and their standard deviation are facilitated, as the use of hypothesis tests. '*drc*' package uses non linear regression to model dose-response relationship.

Procedures to download R and 'drc' are described in section 8.1; and procedures to import data sets in section 8.2.

3.2.2   Monotonic dose-response curve modelling: the log-logistic models

Several models can be used in order to fit monotonic dose-response data. Nevertheless the log-logistic one appears as the most convenient because of:

- its parameters have a biological meaning;

- it could be employed with continuous or quantal (binary) data;

- its use is very spread in ecotoxicity area;

- the mathematical models used in '*drc*' to fit hormetic dose-response data are all modifications of the 4-parameters log-logistic model.

In '*drc*' the 4-parameters log-logistic model is described with the following parameterization :

$$y = c + \frac{(d-c)}{[1 + \exp(b(\log(x) - \log(e)))]}$$ Equation 1

Where:

- *b* is the slope at the inflexion point (EC50, or parameter 'e')

- *c* is the lower limit of the response (asymptote limit)

- *d* is the upper limit of the response (asymptote limit)

- *e* is the the dose reducing the response 50%(ED50) between *d* and *c*, i.e. point of inflexion

- *x* is the dose

- *y* is the biological response (i.e., root length).

- In '*drc*' this model is named LL.4.
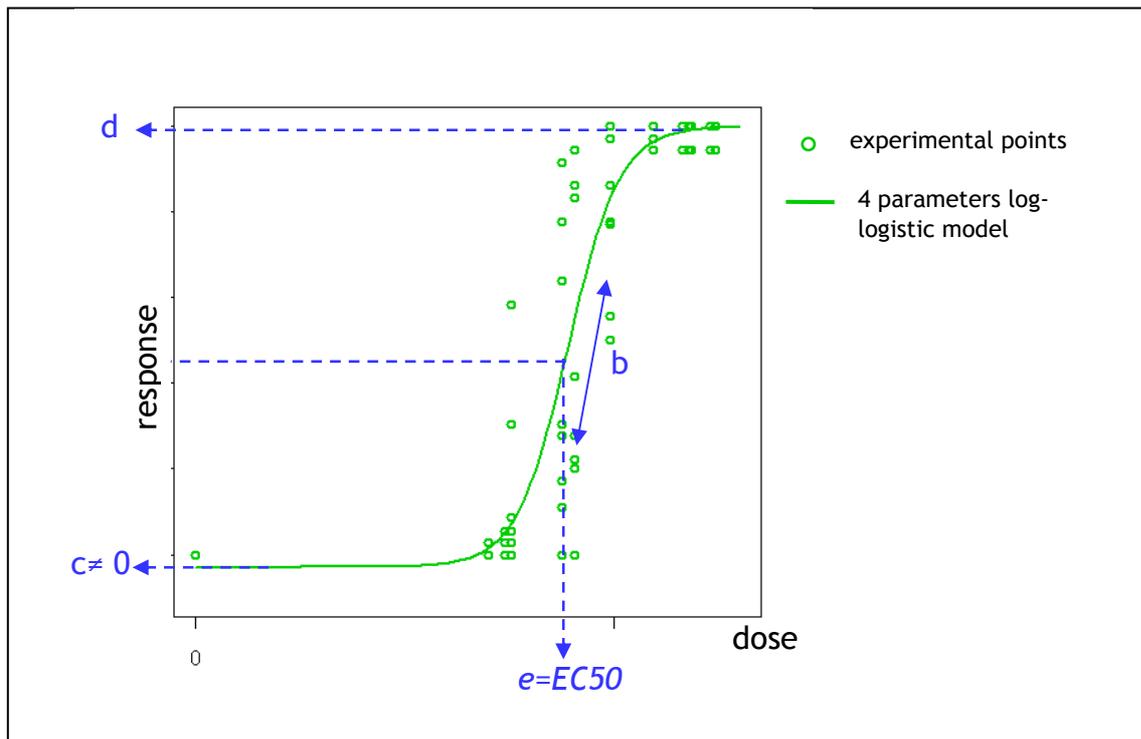
Figure 3.1: example of monotonic dose-response curve modelling with a 4 parameters log-logistic model.

According the direction of the curve (increasing or decreasing) and the type of the observed response (continues or quantal) several simplifications of the 4parameters log-logistic model can be considered. Three sub models are available is '*drc*':

- a model, named LL.3, which contains only 3 parameters since the lower limit ('c' parameter) = 0 ; its equation is:

$$y = 0 + \frac{(d-0)}{1+\exp[b(\log(x)-\log(e))]} \quad \text{Equation 2}$$

- a model, named LL.3U, which also contains only 3 parameters since the upper limit ('d' parameter) = 1. This model can only be used with quantal data; its equation is:

$$y = c + \frac{(1-c)}{1+\exp[b(\log(x)-\log(e))]} \quad \text{Equation 3}$$

- a model, named LL.2, which also contains only 2 parameters since the lower limit (c parameter) = 0 and the upper limit (d parameter) = 1. This model can only be used with quantal data ; its equation is:

$$y = 0 + \frac{(1-0)}{1+\exp[b(\log(x)-\log(e))]} \quad \text{Equation 4}$$

Examples of different monotonic dose-response relationships and their corresponding log-logistic models are given in section 8.3 for both continuous data and quantal data.

3.2.3   Hormetic dose-response curve modelling

Definition and type of curves

Hormesis effect is defined by a biphasic response with a stimulatory effect at the low dose levels. Hormetic dose-response curves can have two shapes :

- an 'inverted U' shape ; it's the case for example, when the observed response is the length of plant leaves (at the control level leaves have a certain length, this one increases at the low dose levels and then falls gradually when strong doses of substances are administered), (Fig.3.2A).

- a 'U' shape; it's the case for example, when the observed response is e.g., the mortality of a species (at the control level there is a certain mortality ratio, this one decreases at the low doses level and then increases gradually when strong doses of substances are administered) (Fig.3.2B).
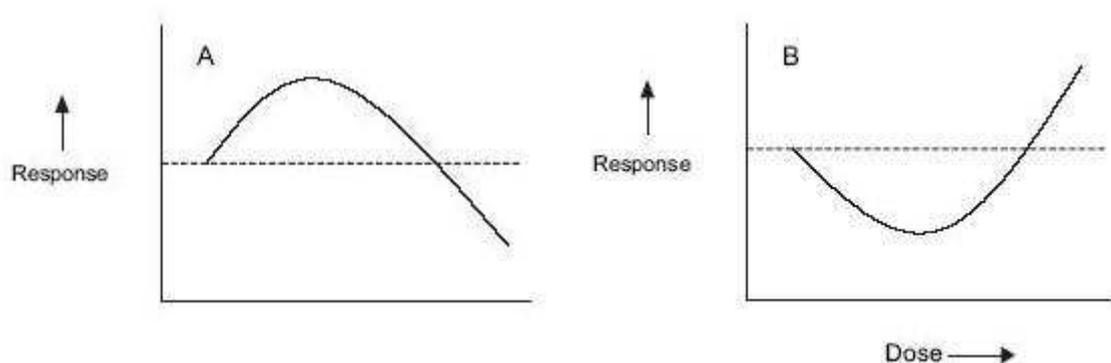
Figure 3.2: Hormetic dose-response curves, inverted U shaped curve (A) and U shaped curve (B).

(from Calabrese *et al.,* 2002).

Mathematical models used to describe hormesis effect

Two types of mathematical models are integrated into 'drc' to describe the hormesis effect. Both models come from the log-logistic model, with which they are nested.

The Brain-Cousens's model (Brain and Cousens, 1989) contains 5 parameters, its equation is:

$$y = c + \frac{d - c + fx}{1 + \exp[b(\log(x) - \log(e))]} \quad \text{Equation 5}$$

Where:

- b has no direct meaning, it reflects "the slope" (but is not strickly the "slope")

- c is the lower limit of the response (asymptote limit)

- d is the upper limit of the response (asymptote limit)

- e has no direct meaning

- f measures the rate of growth stimulation at doses close to zero (f should be positive, and hormetic effect increases with increasing values of f)

- x is the dose

- y is the biological response (i.e., root length).

An example of a 5 parameters Brain-Cousens's fit is shown in the following figure.
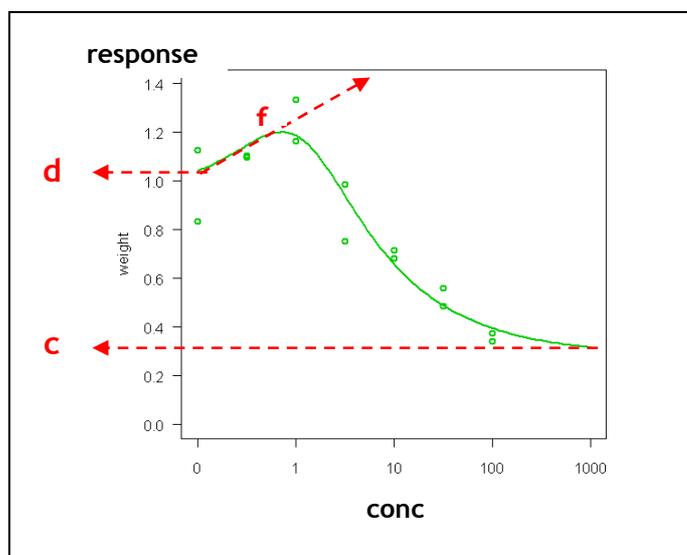
Figure 3.3: Example of a 5 parameters Brains-Cousens model's fit.

This model is named BC.5 in 'drc'.

It is important to note that this model is useful only to model 'inverted U' shaped dose-response relationship.

The other hormetic model available in 'drc' is the Cedergreen-Ritz-Streibig's model (Cedergreen *et al.,* 2005). This model contains 5 parameters too, and its equation is different according the shape of the curve to be modelled.

For inverted U shaped curve, its equation is:

$$y = c + \frac{d - c + f \exp(-1/x^{\alpha})}{1 + \exp[b(\log(x) - \log(e))]} \quad \text{Equation 6}$$

Six parameters are present in the equation. Nevertheless, alpha parameter is, in reality, fixed according 3 values of choice. In 'drc', Cedergreen-Ritz-Streibig's models used to describe inverted U shaped curves are named CRS.5a when alpha=1, CRS.5b when alpha=0.5 and CRS.5c when alpha=0.25.

When the curve has a U shape, equation of the full Cedergreen-Ritz-Streibig's model is:

$$y = (c + d) - \frac{(d - c) + f \exp(-1/x^{\alpha})}{1 + \exp[b(\log(x) - \log(e))]} \quad \text{Equation 7}$$

In 'drc' these models are called UCRS.5a when alpha = 1, UCRS.5b when alpha = 0.5, UCRS.5c when alpha=0.025.

Whatever the shape of the curve, meanings of the Cedergreen-Ritz-Streibig model's parameters and of the variables are:

- b : has no direct meaning
- c : is the lower limit of the response (asymptote limit)
- d : is the upper limit of the response (asymptote limit)
- e : has no direct meaning
- f : measure the hormesis effect (f should be positive, and hormetic effect increases with increasing values of f)
- α : reflecting the steepness of the hormesis peak (fixed at 1 (a), 0.5 (b) or 0.25 (c) levels)
- x is the dose
- y is the biological response (i.e., root length).

An example of a 5 parameters Cedergreen-Ritz-Streibig model's fit, when dose-response relationship has an inverted U shape, is shown in figure 3.4.
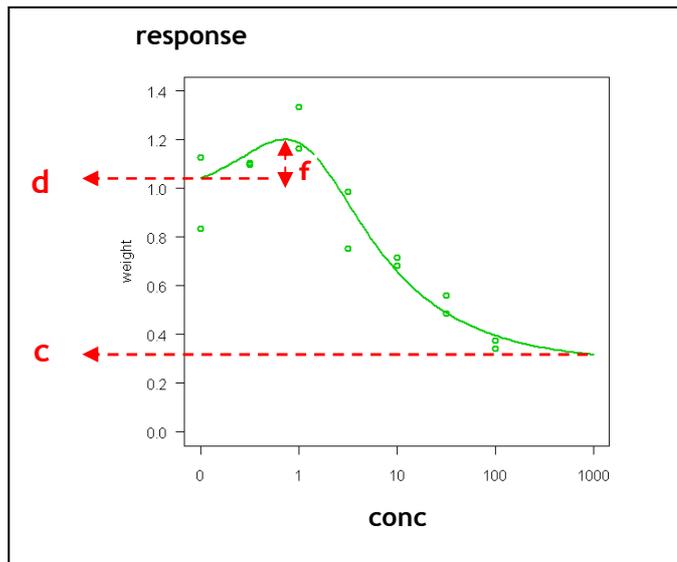
Figure 3.4: Example of a 5 parameters Cedergreen-Ritz-Streibig model's fit, when the dose-response relationship has an inverted U shape.

An example of a 5 parameters Cedergreen-Ritz-Streibig model's fit when dose-response relationship has a U shape is shown in figure 3.5.
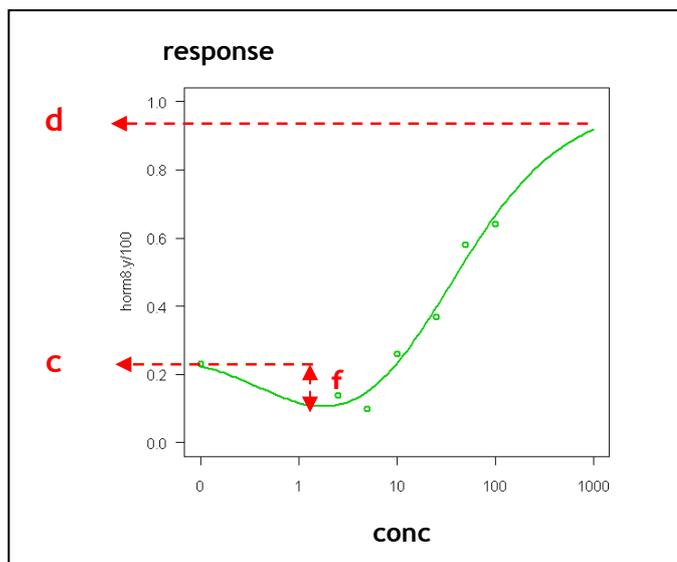

Figure 3.5: Example of a 5 parameters Cedergreen-Ritz-Streibig model's fit, when the dose-response relationship has a U shape.

Mathematical sub-models used to describe hormesis effect

When the dose-response relationship has an inverted U shape, models of Cedergreen-Ritz-Streibig with 4 parameters, specifying that the lower limit of the curve ('c' parameter) =0 are directly available in 'drc'; they are named CRS.4a, CRS.4b and CRS.4c. Their equation is:

$$y = 0 + \frac{(d-0) + f \exp(-1/x^{\alpha})}{1 + \exp[b(\log(x) - \log(e))]} \quad \text{Equation 8}$$

It's the same with the Brain-Cousens' model, its equation is:

$$y = 0 + \frac{(d - 0 + fx)}{1 + \exp[b(\log(x) - \log(e))]} \quad \text{Equation 9}$$

It could be noted that when the dose-response relationship has a U shape, models of Cedergreen-Ritz-Streibig with 4 parameters, specifying that the lower limit of the curve ('c' parameter) =0, are also directly available in 'drc'. They are named UCRS.4a, UCRS.4b and UCRS.4c. Their equation is:

$$y = (0 + d) - \frac{(d - 0) + f \exp(-1/x^{\alpha})}{1 + \exp[b(\log(x) - \log(e))]} \quad \text{Equation 10}$$

Nevertheless, the use of such models seems quite infrequent in ecotoxicology.

On the other hand, when dose-response relationship has a U shape, there is no sub model of Cedergreen-Ritz Streibig's model directly available in 'drc' specifying that the upper limit ('d' parameter) is fixed to1. This type of model could be useful when observations are quantal data (cf. Figure in annexe 8.4) Nevertheless, this type of model could be fitted using the "ucedergreen" general function.

Examples of different hormesis dose-response relationships and their corresponding Brain-Cousens and Cedergreen-Ritz-Streibig models are given in annexe 8.4.

Nested models

Since Brain-Cousens models and Cedergreen-Ritz-Streibig models are modifications of the log-logistic models, it can be established that:

- BC.5 and LL.4 models are nested since BC.5 model with 'f' parameter =0. is a LL.4 model.

- CRS.5a, CRS.5b, CRS.5c and LL.4 models are nested, for the same previous reasons.

- UCRS.5a, UCRS.5b, UCRS.5c and LL.4 models are nested, for the same previous reasons (even if it's less obvious to demonstrate).

- BC.4 and LL.3 models are nested, since BC.4 model with 'f' parameter = 0 is a LL.3 model.

- CRS.4a, CRS.4b, CRS.4c and LL.3 models are nested, for the same previous reasons.

- BC.5 and BC.4 models are nested since BC.5 with 'c' parameter = 0 is a BC.4 model.

- CRS.5a, CRS5b, CRS5c and respectively CRS.4a, CRS.4b, CRS.4c are nested since the first ones with 'c' parameter=0 equal the second ones.

Examples of different hormetic dose-response relationships and their corresponding models are given in section 8.4 for both continuous data and quantal data.

An example of a complete analysis and modelling of a 'hormetic' dose-response relationship with quantal observations, **in absence and in presence of overdispersion**, is provided in section 8.4.5.

4 Specific data needs for physiologically based modelling using DEBtox

4.1 Background and principles

The DEB theory provides a conceptual framework which specifies how energy is taken from food and allocated to growth and reproduction (Fig. 4.1).
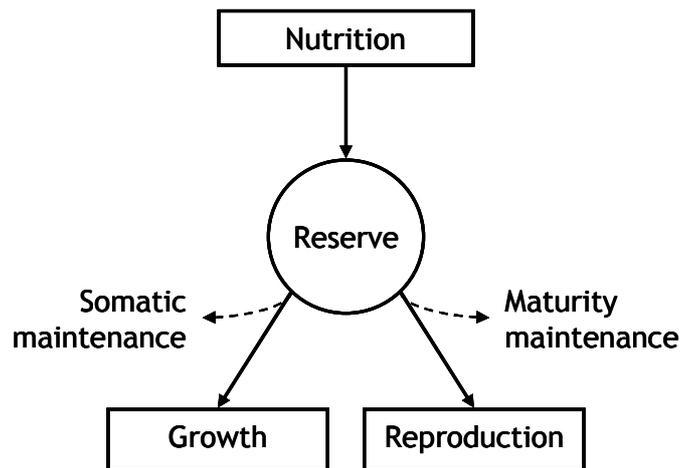
Figure 4.1: Diagram of the individual-based energy allocation model. Note that with a plant model photosynthesis needs to be taken into account for energy input

DEBtox models describe how toxicants accumulate over time in exposed organisms and alter one or many parameters of the DEB by coupling toxicokinetics and effects models. The approach has many advantages, as opposed to standard ecotoxicological approaches involving classical exposure-response relationships :

➔ **First, DEBtox approach gives the possibility to analyse jointly the effects observed on various endpoints (growth and reproduction and eventually survival), as a result of exposure to one or several toxicants.**

Five different primary modes of action are proposed in the standard DEBtox model to explain effects on reproduction (Table 3). Those include direct effects on reproduction such as increased energy cost

of egg production or increased egg mortality during oogenesis (referred below as "Reproduction" and "Hazard" effects). Energy allocation being dependent on size and energy reserve, reductions in both or in any of these two parameters – due to reduced energy assimilation, increased energy cost of maintenance, or increased cost of growth - cause indirect effects on reproduction.

Table 3: Sets of DEBtox equations describing growth and reproduction as a function of time and toxicant concentration. *Daphnia case as an illustration.*

Mode of action	Growth	Reproduction
Assimilation	$\dfrac{d\ell}{dt} = \gamma \dfrac{f+g}{f(1-\sigma(c))+g}(f(1-\sigma(c))-\ell)$	$R(\ell) = \dfrac{R_M}{1-\ell_p^3}\left((1-\sigma(c)\ell^2\,\dfrac{g+\ell}{g+f-\sigma(c)} - \ell_p^3\right)$
Growth	$\dfrac{d\ell}{dt} = \gamma \dfrac{f+g}{f+g(1+\sigma(c))}(f-\ell)$	$R(\ell) = \dfrac{R_M}{1-\ell_p^3}\left(f\ell^2(\dfrac{g(1+\sigma(c))+\ell}{g(1+\sigma(c))+f}) - \ell_p^3\right)$
Maintenance	$\dfrac{d\ell}{dt} = \gamma(f - \ell(1+\sigma(c)))$	$R(\ell) = \dfrac{R_M}{1-\ell_p^3}(1+\sigma(c))\left(f\ell^2(\dfrac{g(1+\sigma(c))^{-1}+\ell}{g+f}) - \ell_p^3\right)$
Reproduction	$\dfrac{d\ell}{dt} = \gamma(f - \ell)$	$R(\ell) = \dfrac{R_M}{1-\ell_p^3}\left(f\ell^2\,\dfrac{g+\ell}{g+f} - \ell_p^3\right)(1+\sigma(c))^{-1}$
Hazard	$\dfrac{d\ell}{dt} = \gamma(f - \ell)$	$R(\ell) = \dfrac{R_M}{1-\ell_p^3}\left(f\ell^2(\dfrac{g+\ell}{g+f}) - \ell_p^3\right)e^{-\sigma(c)}$

Symbols: f nutrition functional response, g energy investment ratio, ℓ scaled body length = L/L_{max}, ℓ_p scaled body length at puberty, γ Von Bertalanffy growth rate, $R(\ell)$ and reproduction rate as a function of ℓ, R_M maximum reproduction rate, et c internal or exposure concentration. $\sigma(c)$ is the stress function expressing the intensity of toxic effect depending on exposure level.

➔ **Second, estimated toxicokinetic parameters do not depend on exposure time.**

1) <u>Time is taken into account through simple one-compartment kinetics models:</u>

In fact, stress functions which relate effect intensity of a toxic compound to internal concentration c_{int} in the organism is given at time t by:

$$\frac{dc_{int}}{dt} = \frac{k_e c_{ext}}{\ell} - c_{int}(\frac{k_e}{\ell} + \frac{d}{dt}\ln\ell^3)$$

where $c_{int} = \dfrac{k_a}{k_e} c_{ext}$ with k_a and k_e the elimination rates (in time^{-1}) and c_{ext} the exposure concentration.

2) <u>Effects are linked to exposure through stress functions $\sigma(c)$:</u>

Classically, DEBtox hypotheses assume that organisms are disturbed by the chemically toxic compound when its internal concentration exceeds a threshold concentration called the no effect

concentration (NEC)(Fig. 4.2). Therefore at toxicant concentrations below the *NEC* no difference is observed between exposed and control organisms. Once the *NEC* is exceeded, effects on one of the DEB parameters (as assumed from the selected mode of action) are proportional to the difference between exposure concentration and *NEC*.

$$\begin{cases} \sigma(c) = 0 & \text{if } c < NEC \\ \sigma(c) = b \cdot (c - NEC) & \text{if } c \geq NEC \end{cases}$$
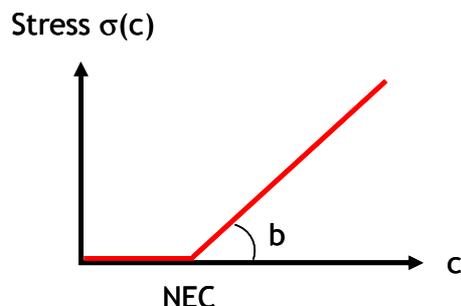
Figure 4.2: Stress function $\sigma(c)$ (dimensionless) affecting energy budget of exposed organisms, as a linear function of internal or exposure concentration c.

3) Particular cases:

In some cases, toxicants might affect a superficial tissue such as the digestive epithelium (with reduction in assimilation as a DEBtox mode of action) and start causing toxicity as soon as they are ingested without further requirement to be internalised. In such cases, effects might be induced immediately upon exposure and toxic stress might better correlate to external concentration or internal concentration ruled by a high k_e value reflecting rapid kinetics. In practice, the stress function $\sigma(c)$ is either related to c_{int} the internal concentration ruled by the kinetics equation or c_{ext} the exposure concentration and both assumptions are evaluated on the basis of compared goodness of fits.

In the case of ionising radiation, one can hypothesize that toxic stress is related to external gamma or internal alpha dose rate, cumulated dose etc. The stress function might write:

$$\begin{cases} \sigma(x) = 0 & \text{if } x < NEX \\ \sigma(x) = b \cdot (x - NEX) & \text{if } x \geq NEX \end{cases}$$

with *NEX* the no-effect exposure level and where x the exposure level is either:
- the dose rate dr translated from external gamma or internal alpha radiation using size-dependent dose conversion coefficients (*DCC*),
- the total received dose cumulated over time,
- an index (*D*) of cumulated damage subjected to some repairing process and ruled by a kinetic equation:

$$\frac{dD}{dt} = dr - k_r \cdot D$$

where k_r is a repairing rate.

➔ **Third, insights on the physiological mode of action (Table 3) can be provided when it is possible to identify the DEB parameter which is altered.** Identifying the mode of action of toxic contaminants is essential to develop biomarkers, presume potential additivity or independence of

actions between several toxicants in mixture, to predict effects on organisms and extrapolate consequences for the population. When equation fits fail pointing which DEBtox parameter is altered, other methods may be used to evidence the altered process(es), including complementary physiological measurements (carbon assimilation in *Daphnia* exposed to uranium in Massarin *et al.,* 2011), histological observations (alteration of midgut epithelium in *Daphnia* exposed to uranium in Massarin *et al.,* 2011), gene expression (*Caenorhabditis* with various chemicals in Swain *et al.*, 2010)…

➔ **Moreover, complicated exposure patterns can be accounted for, including unsuitable range of testing conditions or time-varying concentrations.**

4.2 Parameterisation (assumptions, tools)

Parameterisation techniques:

In some cases, DEBtox equations can be parameterised separately for physiological parameters (namely g the energy investment ratio, ℓ_p the scaled body length at puberty, γ the Von Bertalanffy growth rate and R_M the maximum reproduction rate which describe how unexposed organisms behave physiologically), based on the "control" dataset and toxico-kinetics parameters (namely NEC the no-effect concentration, b the slope of the stress function and k_e the elimination rate of the kinetics equation), based on "exposed" data once the physiological parameters are estimated. In other cases, physiological and toxicokinetics parameters can be fitted conjointly considering the whole (control and exposed altogether) dataset.

The parameterisation is performed using a maximum likelihood method or least squares criterion. In agreement with Jager *et al.* (2004), growth and reproduction equations must be estimated simultaneously to take account of the close relationship between both endpoints. This is commonly achieved using a weighted sum of squares ssq_i for each endpoint i (i being either growth or reproduction) calculated as:

$$ssq_i = \sum_{t=1}^{n_i} \left(\frac{1}{n_i} \cdot \sum_j \left(\frac{i_{sim}(t) - i_j(t)}{\sigma(i(t))} \right)^2 \right)$$

with $i_{sim}(t)$ the simulated value of i at time t, $i_j(t)$ one of the j replicate observations of i at time t, and $\sigma(i(t))$ the variance between the observed $i_j(t)$ values and n_i the number of observations of I when the different endpoints are not measured the same number of times.

If the weighted sum of squares ssq_i is greater for one endpoint i than for the other, their relative contribution to the global sum of squares ssq_{tot} can be balanced using weighting factors $ssq_{i,max}$:

$$ssq_{tot} = \sum_i \frac{ssq_i}{ssq_{i,max}}$$

with $ssq_{i,max}$ the maximum value of ssq_i estimated as:

$$ssq_{i,max} = \sum_{t=1}^{n_i} \left(\frac{1}{n_i} \cdot \sum_j \left(\frac{i_{sim}(t) - \bar{i}}{\sigma(i(t))} \right)^2 \right)$$

where $\bar{i}$ is the average value of all observations of i.

Confidence intervals for the different parameters can be built using a bootstrap technique. To do so, datasets were simulated by randomly sampling from each concentration and time the same number of values (among the measured values) as the observed dataset (with replacement *e.g.,* possibility to select the same value several times). On each sampled datasets, model was adjusted to determine the parameters as described previously. This procedure was performed 10,000 times.

Experimental design:

Properly parameterisation of the NEC requires that the value is included in the range of tested exposure conditions, *e.g.* that the lowest tested concentration lies below the NEC value (with no effect observed on reproduction and growth at that concentration). The tested range should include several exposure concentrations above the NEC, one close to the NEC (with only slight effects observed) and others with significant effects, to maximise chances of identifying NEC and b values. The parameter ke is best explored with several measurements made shortly after exposure induction.

Recommendations:

1) In exposed organisms: reduced number of replicates per treatments (minimum = 3 for bootstrap requirements) to increase number of time points and concentration conditions.

2) In control organisms: higher number of replicates (5 per time points for example) for parameterisation of "physiological" parameters.

4.3 Type of parameters to measure throughout the experiments for DEBtox parameterisation

Parameterising DEBtox equations requires that reproduction and growth is monitored at several exposure conditions (at least 4 different exposure concentrations plus a control) over the life time of tested organisms. The choice of the laboratory species is taking this necessity into account (*e.g.,* species with relatively fast growth and short generation time and that are easy to maintain in the laboratory (*e.g., C. elegans* or *D. magna*). If several organisms are tested, these should be from different trophic levels.
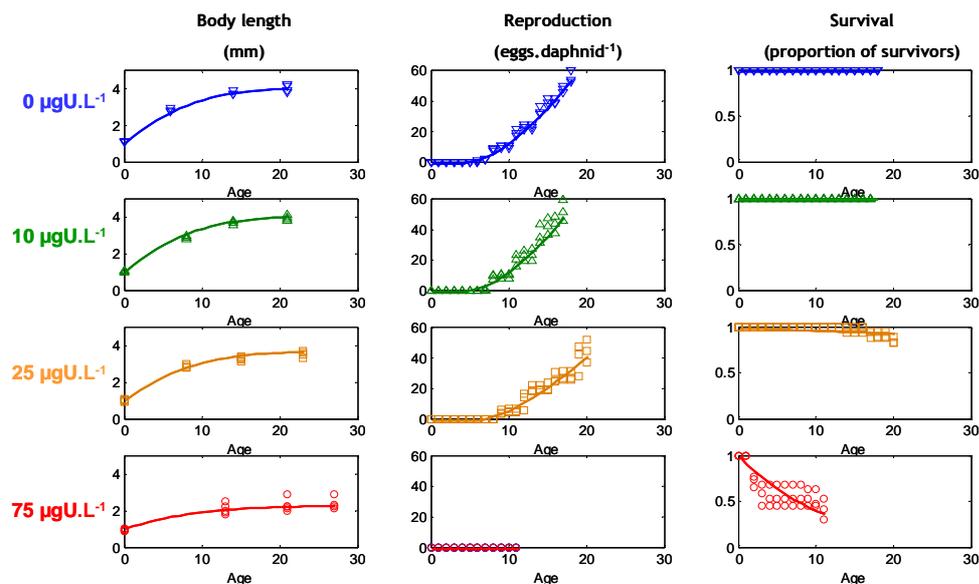
Figure 4.2: Example of fitted DEBtox equations (with effect on assimilation- equations in first row of table 3, as confirmed by assimilation measurements and histological damages observed on the epithelial wall) to growth and reproduction and survival equation for *Daphnia magna* exposed to different concentrations of depleted uranium (based on experimental results by Massarin *et al.* 2010). Stress functions were correlated to exposure concentration. NEC value for uranium is between 0 and 10 µg.L^{-1}. With exposure concentrations ranging from 10 to 75.L^{-1}, the exact value was not identifiable based on the dataset and observed increase in effect severity across generations yielded differences in NEC which were not significant.

5 Approaches to understand mechanisms underlying the mode of action of radiation exposures

Compared to many other environmental stressors, the underlying mechanisms and modes of action (MoA) of ionising radiation toxicity are rather well defined, even though the majority of work has been driven by a focus on human effects, and especially cancer.

This section will provide a brief review of the state-of the-art regarding our understanding of the biological effects and mode of action of ionising radiation specifically focussing on differences in radiosensitivity between and within species, and the contribution that toxicogenomics can play in gaining insight into modes of action. Based on a literature search a preliminary overview on possible biomarkers for chronic radiation or radiosensitivity was produced (Table 4). This table will be used as a starting point to develop a common experimental approach.

5.1 Cellular and biological features driving radiosensitivity

Radiosensitivity of organisms is influenced by a number of different factors, and is known to vary for different endpoints (*e.g.,* reproduction versus mortality); life stage (embryos, larvae, and juveniles stages are the most sensitive), individuals and species. Mammals are generally considered to be among the most radiosensitive species (at least based on LD$_{50,}$ with LD$_{50/30}$ being *ca*. 6-10 Gy for small

mammals and 1.5-2.5 Gy for larger animals and domestic livestock; UNSCEAR, 1996) whereas some bacteria from the *Deincoccus-Thermus* group are extremely radioresistant and can survive more than 12 000 Gy (UNSCEAR, 1996; Daly, 2009). The exact mechanisms/reasons for this huge variation in radiosensitivity are not completely understood.

Some general parameters known to determine the sensitivity of an organism to radiation are: the DNA content (*i.e.* mean chromosome volume) of the cell; the efficiency and types of DNA repair/pathways; the cell repopulation capacity; and the ability of tissue and organs to regenerate (reviewed in Harrison and Anderson, 1996). It is also recognised that prior or combined exposure to other stressors can also influence an organism's radiosensitivity. These issues will be further discussed in WP-4. Already in 1961 Sparrow and co-workers reviewed nuclear parameters determining the sensitivity of plants to radiation. They recognised that it correlated with a larger nucleus or chromosome, more acrocentric chromosomes and with lower chromosome or endoploidy. Also plants that mainly reproduced by sexual reproduction or had longer intermitotic time showed higher sensitivity (Sparrow and Miksche, 1961).

5.1.1 Initial infliction of DNA damage

Generally, it appears that species with high DNA content are more radiosensitive (Hall and Giaccia, 2006; Harrison and Anderson, 1996; UNSCEAR, 1996). This is because the linear density of DNA double strand breaks inflicted per Mbp (0.004-0.01) is similar for different organisms following irradiation, and therefore cells with small genomes suffer fewer double strand breaks (DSBs) than cells with large genomes (discussed by Daly, 2009). Increasing ploidy to more than two, on the other hand, would be expected to reduce radiosensitivity. However, the sensitivity can vary substantially for the same chromosome volume, so other factors are obviously also important (UNSCEAR, 1996).

In different cell types the number of radiation-induced DNA lesions might vary to some extent depending on the levels of low-molecular-mass scavengers (reviewed in Okunieff *et al.*, 2008), the level of oxygen (anoxia, hypoxia) (Hall and Giaccia, 2006), and physical protection afforded by different degree of packaging of DNA (*e.g.,* histones *versus* proteamines etc). For example, the high radioresistance of mature spermatozoa has been attributed to the extremely condensed sperm chromatin (*e.g.,* Haines *et al.*, 1998).

5.1.2 Checkpoint control mechanisms and DNA repair

The consequence of the induced DNA damage depends on whether the lesions are repaired, and if the mode of repair is error-free or error-prone. First, the damage has to be recognised, leading to a block in cell cycle before the cells enters into S-phase (G1-block) or into mitosis (G2-block). These checkpoints apparently give the cells additional time for DNA repair. The various types of DNA damage are repaired by different repair pathways, which differ with respect to the speed and fidelity. It is clear that inherent radiosensitivity relates to the efficiency of checkpoint control mechanisms and the repair capability of the cells (reviewed by UNSCEAR, 2000). The factors that influence the efficiency and fidelity of DNA repair are fundamental in determining cell and organism sensitivity to ionising radiation and this relates in particular to DSBs repair. This is because when both strands of the DNA are damaged in the same location no template strand is available for repair. Homologous recombination repair (HR) and non-homologous endjoining (NHEJ) are the two pathways for DSB repair, of which the former is more complex and considered to be less error prone than the latter (reviewed in UNSCEAR, 2000).

The extreme radioresistance of *D. radiodurans* has been ascribed to a highly efficient homologous recombination, although the underlying mechanism for this is not understood (discussed by Daly, 2009). Interestingly these bacteria do not contain higher concentrations of DNA repair enzymes, but have compared to more radiosensitive microorganisms high [Mn]/[Fe] ratios. Daly (2009) proposed that the accumulation of near millimolar concentrations of Mn^{2+} in the cells makes manganese complexes that prevent the production of iron-dependent reactive oxygen species. This would prevent protein oxidation during irradiation, with the result that sufficient repair enzymes survive radiation damage and allow subsequent DNA repair.

5.1.3   Induction of cell death

Exposure to ionising radiation can lead to different types of cell death (*i.e.* necrosis, apoptosis and mitotic death). Apoptosis (programmed cell death) can be viewed as a complementary way of getting rid of cells with DNA damage (UNSCEAR, 2000). At low doses this is probably the most efficient response since it removes damaged cells from the population and reduces the probability of misrepair. Cells that are seriously damaged by ionising radiation (*e.g.,* containing asymmetric exchange type chromosome aberrations) will undergo mitotic death (genetic death), leading to loss of proliferation capacity. It is well known that rapidly dividing cells are more radiosensitive than non-dividing cells and that the sensitivity varies within the cell cycle (UNSCEAR, 1996). When it comes to mitotic death, the highest and lowest sensitivity is apparent at mitosis (and late G2 phase) and S phase, respectively, whereas the opposite pattern applies to apoptosis. Furthermore, the mitotic death shows a substantial dose rate effect, whereas the induction of apoptosis for a given dose appears to be independent of dose rate and dose fractionation (Hall and Giaccia, 2006; UNSCEAR, 2000). In response to radiation, the relative importance of the two mechanisms varies with dose and dose rate, as well as with the cell type and its developmental stage (UNSCEAR, 2000).

5.1.4   Tissue regeneration

For higher, multicellular organisms, the ability and capacity of cells to repopulate themselves, to replace cells damaged or killed by radiation and to orchestrate tissue and organ regeneration can influence both organ and species radiosensitivity. Many primitive organisms tend to show more efficient tissue and organ regeneration, several of which appear to be relatively radioresistant as adults (Harrison and Anderson, 1996).

5.1.5   Cell cycle sensitivity

Cells are generally found to be most sensitive to radiation at the beginning of the mitosis and least sensitive during S-phase. The varying sensitivity of cells in different phases of the cell cycle is not fully understood but several correlations have been found. The varying amount (duplication during S-phase) or form (relaxed versus condensed) of DNA during a cell cycle might influence the sensitivity. There is also a correlation between radiosensitivity and the levels of naturally occurring sulfhydryl compounds in the cell through the cell cycle. They act as free-radical scavengers, and can facilitate direct chemical repair at sites of DNA damage by hydrogen atom donation (Hall and Giacca, 2006). The efficiencies of different repair processes in different phases of the cell cycle can also impact radiosensitivity.

5.1.6   Life Stage

Reproduction is known to be one of the most radiosensitive life stages, and it might be impaired at doses corresponding to less than 10% of the dose causing mortality. This can in part be explained by the fact that actively dividing cells are most sensitive, and consequently the highest radiosensitivity is

likely to be found in cell systems undergoing rapid cell division for either renewal (*e.g.,* spermatogonia) or growth (*e.g.,* the developing embryo) (UNSCEAR, 1996). It is well known that irradiation leads to apoptosis of cells in the early stages of spermatogenesis, thereby reducing the production of spermatozoa. Furthermore, the successful transfer of the genome to the offspring may be disturbed by gene mutations induced in the germ cells. Induction of recessive and dominant lethal mutations in male and female germ cells may lead to an early death of the developing embryo. Other more "subtle" mutations may lead to development of abnormalities influencing the fitness and fertility of the offspring in the next and subsequent generations. Understanding the underlying mechanisms for differences in sensitivity of germ cells (reproductive organs) and embryonic tissues between different species would therefore be of high relevance since these factors would also be of importance for reproductive strategy (*e.g.,* sexual *versus* non-sexual reproduction, parthenogenesis).

5.2 Application of biomarkers and toxicogenomics in mechanistic studies

A biomarker can be defined as a biological parameter that can be measured in a given subject and is in some way related to a biological effect (Durante, 2007). Brooks (1999) distinguished three different classes of biomarkers: exposure, sensitivity and disease. For exposure biomarkers a dose-response relationship can be established. Biomarkers of sensitivity are genetic markers associated with an increase in individual susceptibility towards e.g. radiation. Finally, biomarkers of disease are those biological events that can be used to anticipate the diagnosis of a specific illness. The latter class of biomarkers is in our objective not relevant. Examples of biomarker of both exposure and sensitivity that can be utilized within both human and ecological toxicology to identify the response to ionising radiation, ranging from molecular, cellular and organism levels are given in table 4. We have classified the biomarkers according to whether they test for DNA damage and repair, oxidative stress or general stress responses. This table does not profess to be complete, but will be used within WP-5 as a working instrument to design a common experimental approach to test for hypotheses within task 5.2.

Table 4: Overview of biomarkers of both exposure and sensitivity that can be utilized within human and ecological toxicology to identify the response to ionising radiation.

Biomarker	Method/assay	Tested endpoint	Correlation with dose or radiation sensitivity	Species tested	reference
		DNA damage and repair mechanisms			
Antibody against Gamma-H2AX	Fluorescence imaging, Western blot, 2-D gel electrophoresis, flow cytometry ELISA High-throuput	DNA damage (Double strand breaks)	Sensitive to and correlating with degree of damage	Human gamma-H2AX phosphorylation site has been shown to be highly conserved throughout eucaryotes	(Redon *et al.*, 2011) (Kuo and Yang, 2008)
Cytogenic biomarker	Chromosomal abberations	Genotoxicity	Validated correlation with long-term morbidity endpoints like risk to induce cancer	Human blood	(Durante, 2007)
Mitochondrial DNA mutation frequency	Sequence analysis	DNA mutation	Not sensitive enough for environmental relevant concentrations	Compost worm (Eisenia fetida)	(Wilding *et al.*, 2006)
Oxidation of DNA	7,8-Dihydro-8-oxo-guanine (8-OHgua) (HPLC-analysis, GC-MS, modified Comet-assay)	DNA damage and repair	Not clear whether this is a good biomarker (Collins *et al.*, 1996) due to high background	Numerous?? Including Compost worm (*Eisenia fetida*)	(Collins *et al.*, 1996; Hertel-Aas *et al.,* 2011)
Oxidation of DNA	8-OHgua detection with antibodies) (HPLC-analysis, GC-MS) Comet-assay	DNA damage and repair	Linear relationship with gamma-irradiation dose and sensitive		(Bruskov *et al.,* 1999)

Repair capacity of blood cells	Comet assay	DNA damage and repair	Correlate with chronic exposure	Numerous Including blood cells of chronic (Chernobyl) exposed people	(Plappert *et al.*, 1997) (Hertel-Aas *et al.*, 2011)
Cytogenic endpoints		DNA repair		Human	(Abdel-Rahman and El-Zein, 2011)-
Methylation status of DNA specifically of	Bisulphite sequencing, RT-PCR Methylation specific-PCR Western blotting	Reduced transcription through gene silencing of protein involved in DNA-repair	Relation between radiosensitivity and methylation status of ERCC1 (excision repair cross complementing protein 1) promotor	Human gliomas	(Liu *et al.,* 2009)
Oxidative stress					
Antioxidants and antioxidant enzymes	Spectrophotometric assays of enzyme activities (POD, SOD, catalase) Carotenoids levels	Oxidative stress	No correlation between oxidative stress tolerance and gamma radiation resistance Nb Anti-inflammatory agents (corticosteroid) reduce the oxidative stress (NFkB activity) in macrophage cells (personal data, UMB)	Bacteria	(Shashidhar *et al.*, 2011)
Fe/Mn ratio	Atomeric Absorption Spectrometry	Protection of proteins and DNA for oxidative damage	Inverse correlation between [Mn]/[Fe] ratio and level of protein oxidation (Confalonieri and Sommer, 2011) No direct correlation with radiation resistance (Shashidhar *et al.*, 2011)	Bacteria: *Deinococcus, Thermophyllus*	(Confalonieri and Sommer, 2011); (Shashidhar *et al.*, 2011)
General stress responses					
Heat Shock Proteins	Antibody detection: Western blot	Stress induced proteins			(Lewis *et al.*, 1999)
Transcriptomic changes	Microarray etc,..	Changed gene expression	Acute exposure was comparable to other abiotic stressors whereas chronic exposure revealed a complete distinct gene expression profile (Kovalchuk *et al.*, 2007)	*Arabidopsis* vegetative (Kovalchuk *et al.,* 2007), *Arabidopsis*	(Kim *et al.*, 2007; Kovalchuk *et al.*, 2007)

[STAR] 32

			Down regulation of growth/rhythm responses and up-regulation of defence/stress regulation in post irradiation reproduction state (KIM07)	during flowering (Kim *et al.,* 2007)	
Radiation metabolomics	GS-MS QTOFMS	Changed metabolite abundance Some could be linked to food deprivation and starvation (Johnson *et al.,* 2011)	Dose and time dependent, cross-species (Johnson *et al.,* 2011)	Rat, cell and mouse	(Coy *et al.,* 2011 Johnson *et al.,* 2011 Lanz *et al.,* 2009)

Developments in molecular biology have the potential to improve the mechanistic understanding of the effects of stressors and underlying processes (NRC, 2007; Ankley *et al.*, 2009). Improved understanding of the underlying modes of action (MOA) of toxicity for different types of pollutants can aid in the development of methods for assessing exposure and effects, thereby reducing uncertainties related to extrapolation across species, endpoints and chemical groups with similar function or structure (Ankley *et al.*, 2009). In recent years, the "omics" approaches, transcriptomics, proteomics, and metabolomics, have been adopted as high-throughput and high density analysis to study the transcriptome (the complete makeup of RNA transcripts), proteome (the complete makeup of proteins), and metabolome (the complete makeup of metabolites), respectively (Boverhof and Gollapudi, 2011).

A key promise of the omic technologies is their potential to offer a more holistic understanding of interactions and the responses of biological pathways and networks to chemical perturbations (Boverhof and Gollapudi, 2011). Unlike targeted approaches (*e.g.,* investigating a small subset of genes, proteins or metabolites), the omic tools are open, non-targeted techniques that do not require *a priori* knowledge about the pathways or systems that may be affected by exposure to the stressors. The techniques examine multiple expression changes and, consequently, may reveal new genes, proteins or metabolites involved in toxicological responses that have not been described previously (Figure 5.1.). This is an obvious advantage when possible interactions of chemical mixtures are to be studied, including those involving ionising radiation. (see WP-4 for more details). Because of this non-targeted approach, omic experiments are often hypothesis generating rather than hypothesis testing, in that they can provide the basis for further investigations of possible modes of action using other techniques (*e.g.,* Boverhof and Gollapudi, 2011).
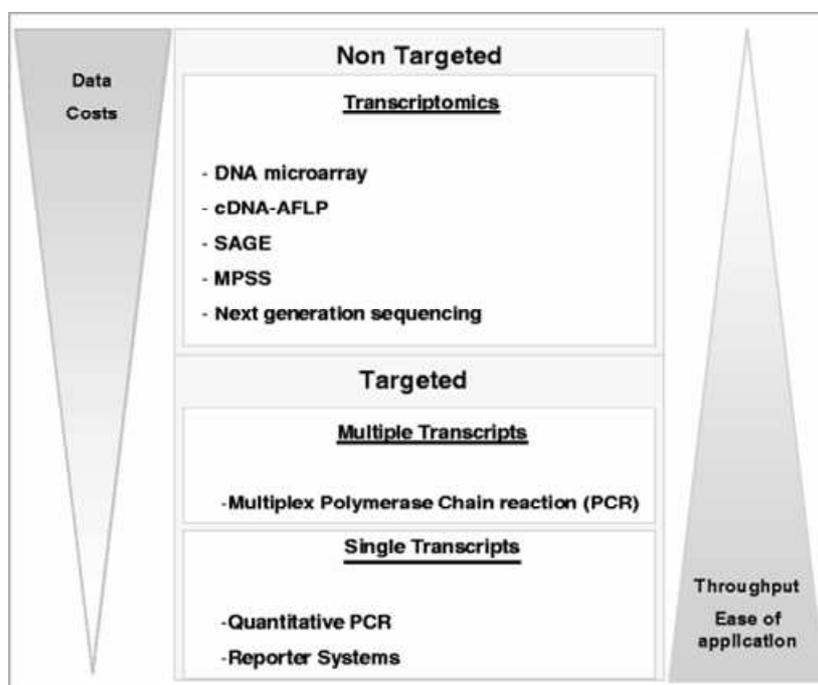

Figure 5.1: Overview of single and multiple transcript and transcriptomic techniques......

Although the promise is great, there are some general and specific limitations related to the different omic tools that need to be considered. Omic-technologies acquire hundreds to thousands of variables for a very limited number of repeated observations. Thus, bias and confounding factors can make it difficult to distinguish actual differences in expression levels associated with the toxicant exposure from the background fluctuations (Monsinjon and Knigge, 2007). Furthermore, it can be challenging to organize the data and interpret them in a biological meaningful way. The interpretation of omic data is therefore highly reliant on advanced computational and statistical methods (*i.e.* bioinformatic tools), many of which are still being developed (van Aggelen *et al.*, 2010). A full review of the omic tools and their application in radiation effect studies (including combined exposures) will be provided in a later STAR deliverable. In table 5, an overview is given of the pros and cons specific to the different omic techniques.

Table 5: Overview of advantages and drawbacks of different toxicogenomic approaches.

	Techniques	Advantages	limitations
Transcriptomics	DNA microarray cDNA amplified fragment length polymorphism (AFLP) Serial analysis of gene expression (SAGE) Massive parallel signature sequencing (MPS) Next generation sequencing	Measure changes at the level of gene expression Limited number of genes compared to proteins and metabolites For next generation sequencing, no need for the genome map	Limited to few model species with known genome Expression needs to be combined to functional gene ontology Gene expression profile varies with both dose and time after exposure (expression of adaptive, compensatory and adversity genes) From all omic approaches greatest difference seen between gene expression and eventual phenotype
Proteomics	Electrophoresis combined to Mass Spectrometry	Protein expression profiling sometimes specifically Possibility to focus on post-translational changes e.g. phosphorylations	Currently no techniques that can handle complete proteome Huge difference in concentration levels between abundant housekeeping proteins and proteins interest Successful identification and annotation of proteins are also dependent on sequenced and annotated genome
Metabolomics	NMR LC-MS, GC-MS or DIMS	Measures changes in metabolites Most closely linked to phenotype No need for genome sequencing as metabolites are universal Possibility to analyse non-invasive samples such as blood/urine	Large amount of different metabolites Metabolites are often transient and are rapidly biotransformed

An international consortium on fish toxicogenomics (Van Aggelen *et al.*, 2010) concluded that variability in omics data is an ongoing concern. They gave an overview of the sources of technical and biological variations that need to be considered when using the different omic tools, and provided

recommendations on how these should be managed in terms of experimental design. (Van Aggelen *et al.,* 2010, supplemental material, doi:10.1289/ehp.0900985.S1) claimed that technical variability and artefacts can for example arise from study design, inadequate sample numbers, and methods of sample acquisition, preparation, storage, processing, and analysis. They concluded that the major sources of variability were methods of normalization and statistical interpretation. Inter-individual biological variability can be classified into genotypic and phenotypic variation. The general recommendations are that experiments should be designed to minimise both technical and intra-class variation, thereby maximising inter-class differences that can be explored using data mining techniques (Van Aggelen *et al.*, 2010 and supplemental material).

At this point we can conclude that the development and increased application of biomarker and omics techniques are ongoing and their potential to provide new insight related to mode of action is promising. However, expertise within several disciplines (*e.g.,* genetics, analytical chemistry, bioinformatics) is needed to utilize the information obtained in an ecological relevant context. Due to the large numbers of factors impacting on radiosensitivity, and the enormous number of species and life stages that could be tested, it will obviously only be possible to perform some few targeted studies to gain further insight into mechanisms. Given the requirement for any laboratory omics study to include conventional toxicological endpoints such as pathological changes, reproductive output and growth (Denslow *et al.*, 2007), it should be clear that the use of these techniques would be most fruitful in combination with the studies carried out in task 5.2.1. A good example of an integrated approach for endocrine disruptors in fish is summarised in Ankley *et al.* (2009). They used a combination of different omic tools, bioinformatics and standard toxicity testing and modeling approaches, performed in different experimental phases, to develop response linkages across biological levels of organization. Furthermore, links between transciptomics, metabolomics and DEBtox models have also been tested in recent studies on *C elegans*, (Swain *et al.*, 2010). Both approaches could make a useful guideline for how the STAR participants could plan and proceed with the experimentation during different phases.

The overall approach would be to use the omic studies (and preferably transcriptomics or metabolomics) as hypothesis generating rather than hypothesis testing experiments, hence more concrete hypothesis will be postulated after selection of organisms. Since the types of organisms for which full omic capabilities exist (*e.g., C elegans, Arabidopsis*, and zebrafish), are rather radioresistant (although there are admittedly large differences in the radiosensitivity between the species), the application of omics on these species would be limited to studies that can give a broader insight into mode of action, including differences in RBE. There are also quite extensive omics data on *C elegans, Arabidopsis* and zebrafish, including studies of radiation on reproduction and life-history studies. One aim of the omics investigations could also be to enable more targeted biomarker and effect studies in other more sensitive and ecologically relevant species, or life stages. This point still needs to be discussed further among partners.

To conclude, the different methods will be thoroughly reviewed within STAR in order to select those that are practically feasible within the consortium and that can best provide answers to our scientific questions.

6 The pilot study

6.1 Background

6.1.1 Objectives

The aim of this study is to use and to develop a bioenergetics approach based on the DEB theory (Dynamic Energy Budget) developed by Kooijman (2000) to increase knowledge about links between assimilation disruptions, growth, reproduction, and life span fluctuations in exposed organisms to ionizing radiation.

This study will be conducted using a model organism, *Caenorhabditis elegans* (nematode). The *C. elegans* model can be summarised by a short life cycle, a small size and a great ease to handle and cultivate in various devices (Brenner, 1974). These characteristics make it a suitable model to conduct this type of study. In addition, a first experiment was already done at IRSN to investigate the effects of external gamma radiation on the growth and reproduction of C. *elegans*. In the pilot study, the objective is to obtain accurate data to implement a DEBtox model applied to gamma radiation. The ultimate target is to assess potential consequences on the population.

6.1.2 Biological model: *Caenorhabditis elegans*

Nematodes are phytophages, bacteriophages, fungivore and/or predator. They can be defined as cosmopolitan species, easily reared in labs. They can be found in soil at high density depending on the level of organic matter. Nematodes presence in soils is highly important for the ecosystem and particularly for the mineralization process (Jager *et al.*, 2004).

Caenorhabditis elegans is an ubiquitous free nematode living in soils discovered by Maupas (1990). This bacteriophage organism is found at the same time in warm and cold earth areas. *C. elegans* measure 250 µm long at hatching and up to 1.6 mm at adult stage. It cultivates easily at 15, 20 and 25°C although effect have been showed on eggs at 15 and 25°C. At its reference temperature (20°C), it breeds in 3 days and lives up to 21 days. *C. elegans* integument is transparent so its development and effects of stressors on the organism are observable. It reproduces by androdioecy. Hermaphrodites can fertilize themselves and the presence of male is optional. In the N2 strain, males occur at a very low density (<0.1%).

C. elegans development is well known. Hermaphrodites have 959 cells at adult stage, 2n=12 (10 autosomes + XX) and produce male gamete before female gamete. As a consequence, the egg laying size depends on the number of sperm cell.

C. elegans development, presented in figure 6.1 and table 6, is made in two steps: embryonic and post-embryonic. This development is invariant and the lineage as well as the cell genealogy of *C. elegans* are determined. In the hermaphrodite, 1090 cells are produced including 131 for apoptosis.
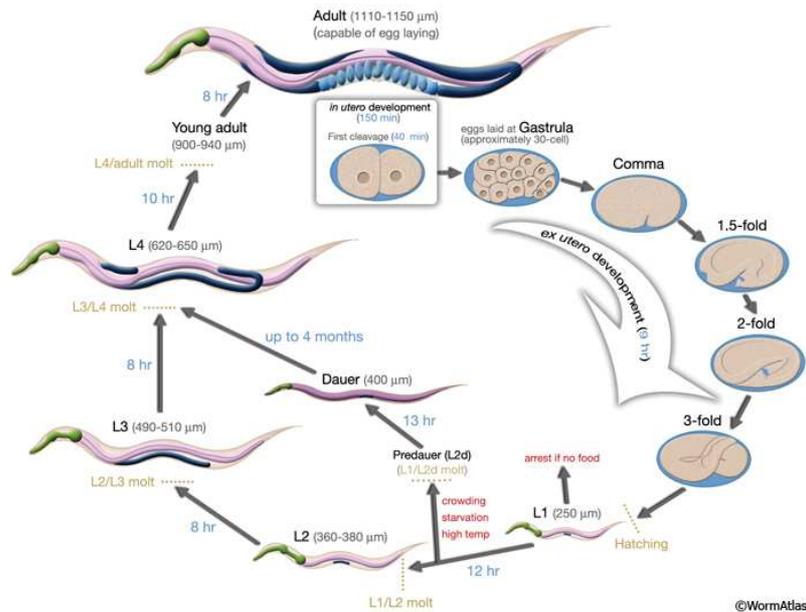
Figure 6.1: *C. elegans* life cycle at 22°C (Altun and Hall, 2009).

Table 6: Development of *C. elegans* at different growth temperatures (based on Byerly *et al.*, 1976)

	16°C ± 0.3	20°C ± 0.5	25°C ± 0.2
Egg laid	0 h	0 h	0 h
Egg hatches	16-18 h	10-12 h	8-9 h
First molt	36.5 h	26 h	18 h
Second molt	48 h	34.5 h	25.5 h
Third molt	60 h	43.5 h	31 h
Fourth molt	75 h	56 h	39 h
Egg–laying begins	~90 h	~65 h	~47 h
Egg-laying maximal	~140 h	~96 h	~62 h
Egg-laying ends	~180 h	~128 h	~88 h
Length at first molt	360 µm	370 µm	380 µm
Length at second molt	490 µm	480 µm	510 µm
Length at third molt	650 µm	640 µm	620 µm
Length at fourth molt	900 µm	850 µm	940 µm
Length at egg-laying onset	1150 µm	1060 µm	1110 µm
Maximal egg-laying rate	5.4 h^{-1}	9.1 h^{-1}	8.1 h^{-1}
Total eggs laid	275	280	170

- ■ *Embryo*

Embryogenesis is done in two stages in *C. elegans*. Initially, the first growth consists in a cell division until reaching 550 cells essentially undifferentiated. At the end of this first phase, the embryo is spheroid in shape and is composed of three germ layers (ectoderm, mesoderm and endoderm). The second phase of embryogenesis is the phase of organogenesis and morphogenesis. At the end of this phase, the larva begins to move within the egg (Altun and Hall, 2009).

- *Larval stages*

The post-embryonic development takes place in four stages separated by molts during which the nematode enters into lethargy (Araiz *et al.*, 2008, Alda Álvarez *et al.*, 2005, Altun and Hall, 2009, Jager *et al.*, 2005). Table 6 shows *C. elegans* length for each molt at different growth temperatures. *C. elegans* body size increases between molts unlike its buccal cavity which increases only during moulting (Alda Álvarez *et al.*, 2005, Jager *et al.*, 2005, Knight *et al.*, 2002).

L1 stage: After hatching, the larva is in L1 stage. It measures about 250 µm long, has 558 cells and begins to feed. The post-embryonic development is more affected by organogenesis events and cell mass modifications than by mitosis (Araiz *et al.*, 2008).

Environmental conditions can lead L1 larvae to three situations:
- Stopping the growth and death
- Switching to resistance stage
- Transition to L2 stage

L2 stage:According to Altun and Hall (2009), few cell divisions occur during the L2 stage. This author suggests that germ cells are among the few cells to divide during this stage and that they will quadruple their number.

L3 stage: According to Alda Alvarez *et al.* (2005), the end of the third larval stage is characterised by a shift in gamete production of the hermaphrodite. Thus, the male gametes located in the proximal gonad become mature. This causes a change in the type of matured gamete in the distal part of the gonad. Now, only oocytes are matured.

L4 stage: L4 stage is characterized by the end of the gonadogenesis process and by the generation of the terminal cells of the vulva and of the uterus (Altun and Hall, 2009).

- **Adult stage**

At about 48 h (at 22°C) after hatching, a mature hermaphrodite begins to lay its first eggs (Byerly *et al.*, 1976). The adult hermaphrodite will lay up to depletion of its sperm supply (about four days). It will then survive for 10 to 15 days. Hermaphrodite can be fertilized by males. In this case, egg laying may continue until oocytes stock is depleted (Alda Álvarez *et al.*, 2005).

6.1.3   Dynamic Energy Budget

The DEB theory (Kooijman, 2000) provides a conceptual framework which explains mechanistically how organisms acquire their energy from food and allocate it to survival, growth and reproduction. On this basis, DEBtox models describes how toxicants accumulate in exposed organisms and alter main DEB processes (Kooijman and Bedaux, 1996). A specific DEBtox models was recently developed for *C. elegans* (Jager *et al.* 2005) and was used to identify possible primary modes of action of various chemical compounds, together with complementary transcriptional analyses (Swain *et al.* 2010; Wren *et al.* 2011). *C. elegans* DEBtox model or its revised version (as part of a PhD project) will be used to analyse conjointly effect data of gamma radiation on survival, somatic growth and offspring production (if these are available from experiments). Required data should at least include size at birth

and puberty and maximum adult size and reproduction rate. Experiments must be carried out at constant, well defined temperature and *ad libitum* feeding condition.

6.2 Experimental protocol for external gamma irradiation

6.2.1 Organism husbandry

The wild-type N2 nematode (ie. the most classically used strain) is maintained on the nematode growth medium (NGM) plates seeded with *Escherichia coli* strain OP50, at 20 °C (Brenner, 1974; Stiernagle, 2006). Gravid worms are selected from the stock population and placed on a Petri dish at t = 0h. After 2 hours, laid embryos are considered to be age-synchronized.

Eggs are individually placed into petri plate containing NGM seeded with OP50 *E. coli* (for each treatment). Worms are cultured at 20°C and 80% RH in the dark.

6.2.2 External gamma irradiation

For the purpose of the study we will use 5 nominal dose rates of: 0, 1, 10, 100, 1000 mGy/day delivered by for Cs-137 sources (see photos 1 to 4 at the end of section 6). Each source is installed in independent incubator and experimental units are placed around the source. During the test, temperature and moisture are continuously measured using data logger.

The choice was made on the use of liquid and solid sources of Cs-137 of various activities. The experimental units (e.u) of small size containing the samples to be irradiated are laid out in circle around the source of caesium. Table 7 gives the activities of the various sources used in the device of irradiation.

Table 7: Activities of 137-Cs sources used in the irradiation facility

	Bq	Type of source
S1	1.6E+09	solid
S2	2.16E+08	liquid
S3	2.30E+07	liquid
S4	2.12E+06	liquid

To allow daily handling of the experimental units, a device allowing the lowering of a lead protection is dimensioned for the experiments. The support can accomodate two models different of protection:
• a cap thickness 20 mm, which could be used with the liquid sources of weak activity,
• a cap thickness 30 mm, intended for the handling of the liquid sources of strong activity as well as the solid sources. Figure 6.2 presents a diagrammatic view of the system of irradiation.
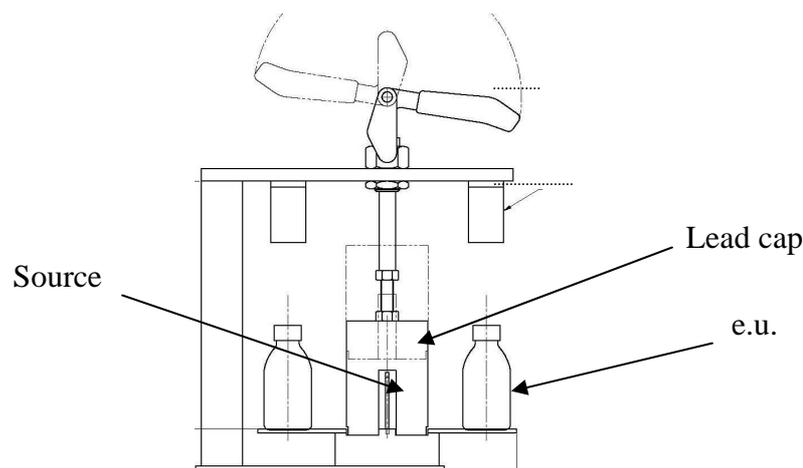
Figure 6.2: Scheme of the Irradiation facility

In order to be able to implement within an experiment a sufficient number of u.e, the adopted solution is to lay out several e.u. rings some around the source. Figure 6.2 presents the configuration retained in the most restrictive case of the study. The characteristics of the geometry used as a e.u. are given on figure 6.3.
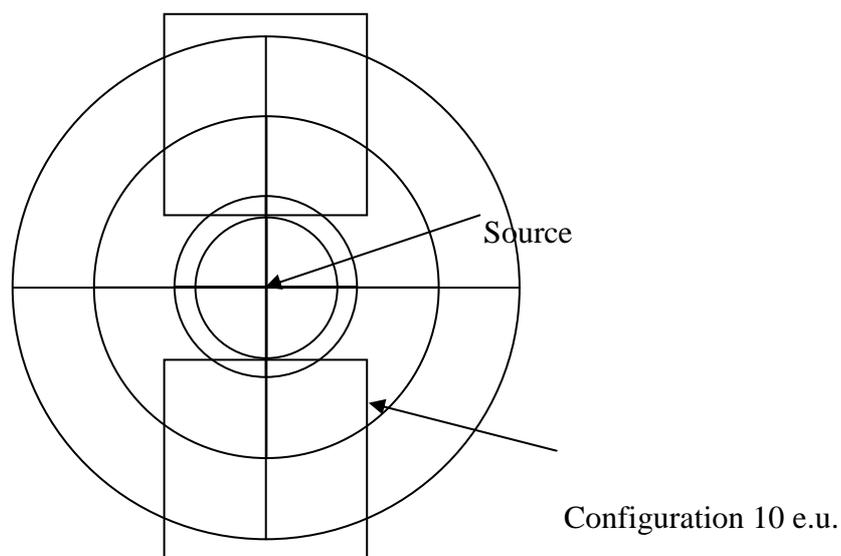

Figure 6.3: Configuration for irradiation (10 e.u.) / viewed from the top

Thermoluminescent dosimeters (TLD) will be laid out along the lower, median and higher lines of the u.e for the various configurations given and the whole of the sources available. The flows of Kerma were also calculated by digital simulation within the TLD in order to compare the calculation and results of measurement, and thus to validate the data on the activity of the sources. In complement, the isotropy of the radiation field on the level of the e.u. for the solid source was studied using TLD. Moreover, the absorbed dose rates inside the geometry of the e.u. were determined by calculation

(modelling the transport and the interactions of the particles charged or neutral in the air. Only, the transport of the principal line of cesium 137 to 661.6 keV was taken into account).

Measurements will be realized with lithium fluoride powder doped Mg, Cu, P (GR207) conditioned in polypropylene tubes. For each configuration of irradiation and all the sources used, a series of dosimeters will be placed at the center of the e.u. on the various stages of the e.u. A number of e.u. identical to the use in routine was used so taking into account the radiation diffused by the adjacent samples of the site of the dosimeters.

6.2.3 Endpoints measurement
- ***Brood size***

To assay brood size we will daily transferred worms into new individual plate containing NGM from t=72h (beginning of lay) till the end of spawn. Hatched progeny will be counted twice, the day following each transfer (Swain *et al.*, 2004).

- ***Growth***

Growth will be measured (using a stereomicroscope with a connected camera) twice a day from hatching to maturity then once a day until maximal size.

- ***Lifespan***

Lifespan will be studied by daily counting dead worm. The death of a nematode will be recorded upon failure to respond to repeated touch stimulation of the posterior end.

6.2.4 Statistics and data treatment

The endpoints will be compared among the treatments using generalized mixed-effect models and Tukey's multiple comparisons. 'Growth' endpoint will be analyzed using a normal distribution, 'Brood Size' using a Poisson distribution, and 'lifespan' using a binomial distribution. Depending on the number of censored data, 'lifespan' endpoint could also be studied using a survival analysis.

Prior to the experiment, power analyses will be used to figure out what sample size (number of worms per condition) would be needed to observe a given treatment effect *(e.g.,* 5% change in fecundity or growth). These power analyses will be done simulating data based on the variance structure estimated from a preliminary study (Browne et al, 2009 and Job, 2009).

(see the following links:

http://seis.bris.ac.uk/~frwjb/esrc/MLPOWSIMmanual.pdf

http://www.unc.edu/~toddjobe/blog/2009/09/power-analysis-for-mixed-effect-models.html)

6.2.5 DEBtox modelling
- **Specificities related to the nematode DEBtox model**

C. elegans DEBtox model (Kooijman and Bedaux1996; Jager *et al.* 2005; Swain *et al.* 2010) will be used to analyse toxic effects of gamma irradiation on survival, somatic growth and offspring production. Different sets of equations describing changes in survivorship, body size (length or mass) and reproduction rate with age (assuming *ad libitum* feeding) are available each specifically corresponding to a possible mode of action for toxicants proposed by the standard DEBtox model among:

1) increased cost of maintenance,

2) reduced assimilation,

3) increased cost of growth,

4) increased cost of egg production,

5) increased mortality during oogenesis.

Further effects of toxicants have been introduced to take account of nematode specificities, including:

6) reduced size at puberty

7) decreased maximum reproductive rate

8) decreased toxicant intake with feeding conditions (not appliable in the present study)

▪ **Specificities related to the experimental setup design**

Fitting procedure implies the underlying assumption that observed values of survival, reproduction and size are independent e.g. values at different time points should be measured on separate individuals. For practical reasons, individual nematodes are monitored over their entire life in the pilot study. Thus, DEBtox equations will be fitted in a preliminary procedure, in order to check that model parameters do not differ significantly among individuals. This is achieved graphically based on the comparison of parameter confidence intervals among nematodes.

▪ **Screening of DEBtox stress factors and modes of action**

The different modes of action have differential consequences for maximum adult size, age at puberty, reproductive outcome, allowing a first screening round. In order to identify possible modes of action for gamma radiation, sets of equations are fitted to data obtained on the range of dose rates and most probable modes are selected using a weighed least square criterion as an estimate of goodness of fit (Jager *et al.* 2004). Different hypotheses will be also compared by linking the toxic stress function to the dose rate, the cumulated dose or an index of cumulated damage level subjected to a repairing process. Our overall objectives will be to estimate values of No Effect Dose Rate (NEDR), cumulated Dose (NED) or damage Level (NEL) and to point potential metabolic modes of action for gamma radiation.

6.3 Expected outcomes and alternative options

As presented above, the expected outcome of this pilot study is to build a set of effects data of external gamma radiation on the life history traits of C. *elegans* and to obtain accurate data to implement a DEBtox model applied to gamma radiation. This study will help the different teams involved in STAR WP5 to implement, for the first time a DEBtox approach on gamma irradiation (see chapter 4.1). This will be the firs step towards a full DEBtox parameterization.

The generated dataset will also be used to implement a Power analysis (chapter 6.2.4), in order to refine the test design for future studies (chapter 3.1): the observed variation in the measured endpoints, and difference between control and irradiated conditions will help to define the needed number of treatments, replicates per treatments, accuracy of endpoints measurements…

There is a risk for the planned study to fail in generating such a dataset, mainly due to the test design actual limitation (*e.g.,* up to a maximum of 30 replicates par condition, fixed dose-rate range…). This could result in a lack of sensitivity of the measured endpoints to gamma irradiation, under those specific conditions. In this case, other options could be:

- to change the *C. elegans* strain to a potentially more sensitive strain (or even to change the species used to a more sensitive species, *e.g.*, daphnid, earthworm…);
- to increase the tested dose-rates and/or to increase the number of tested replicates: this would need to use a larger irradiation facility (*e.g.,* irradiation facility at UMB, Norway);
- to examine other sensitive endpoints to be measured;
- to enhance the accuracy of endpoints measurements in order to decrease the variation due to individual measurements (*e.g.,* to evolve towards a liquid medium exposure design);
- …

Illustrations of the Pilot study: Photos 1 to 4 by JM Bonzom/IRSN

Photo 1: Experimental Units around the irradiation source (liquid)
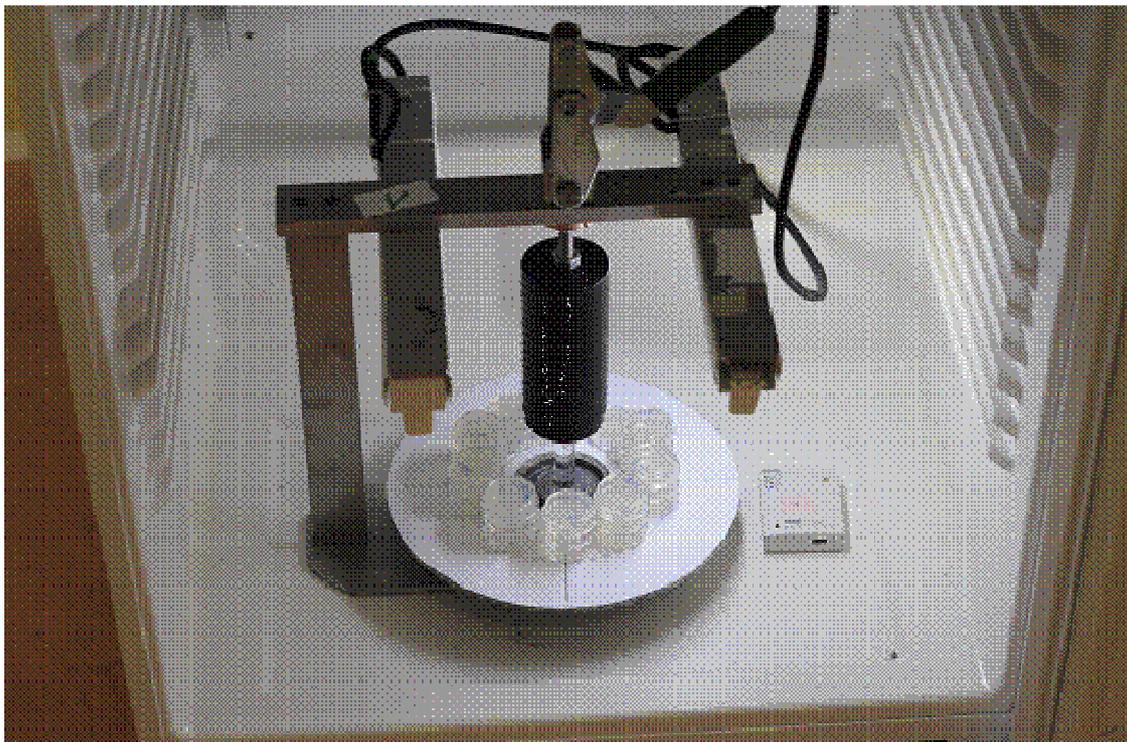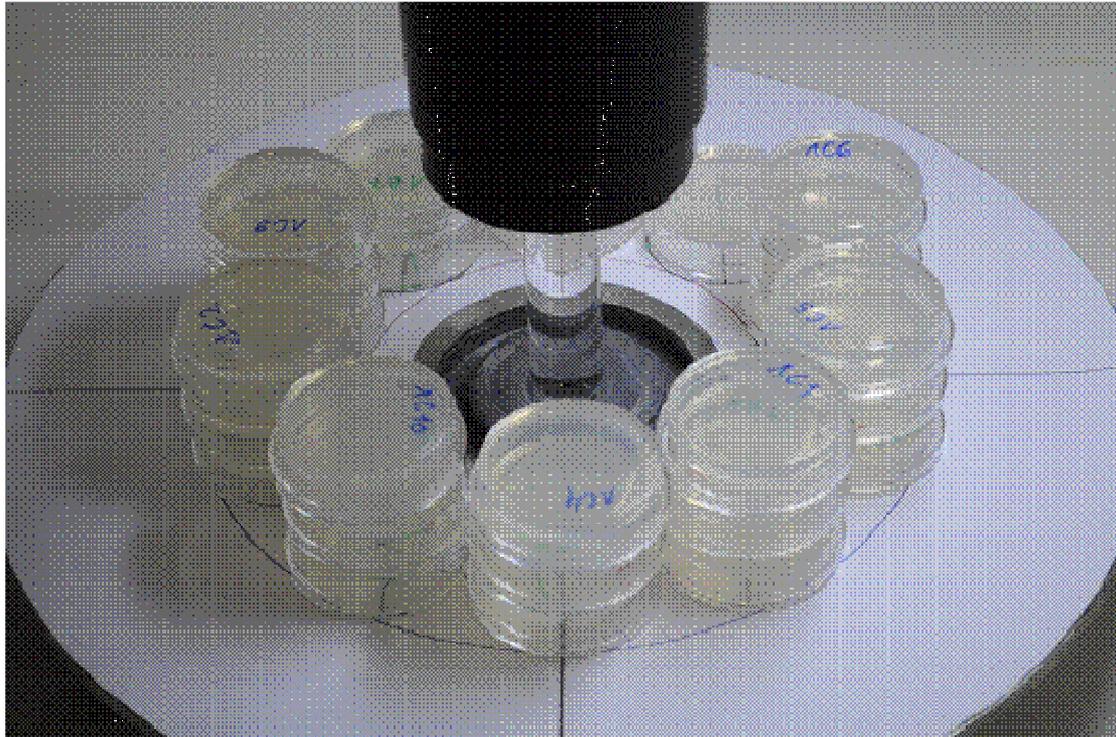
Photo 2: 5 independent incubators with irradiation facilities

(1, 10, 100, 750 and 1000 mGy/d)
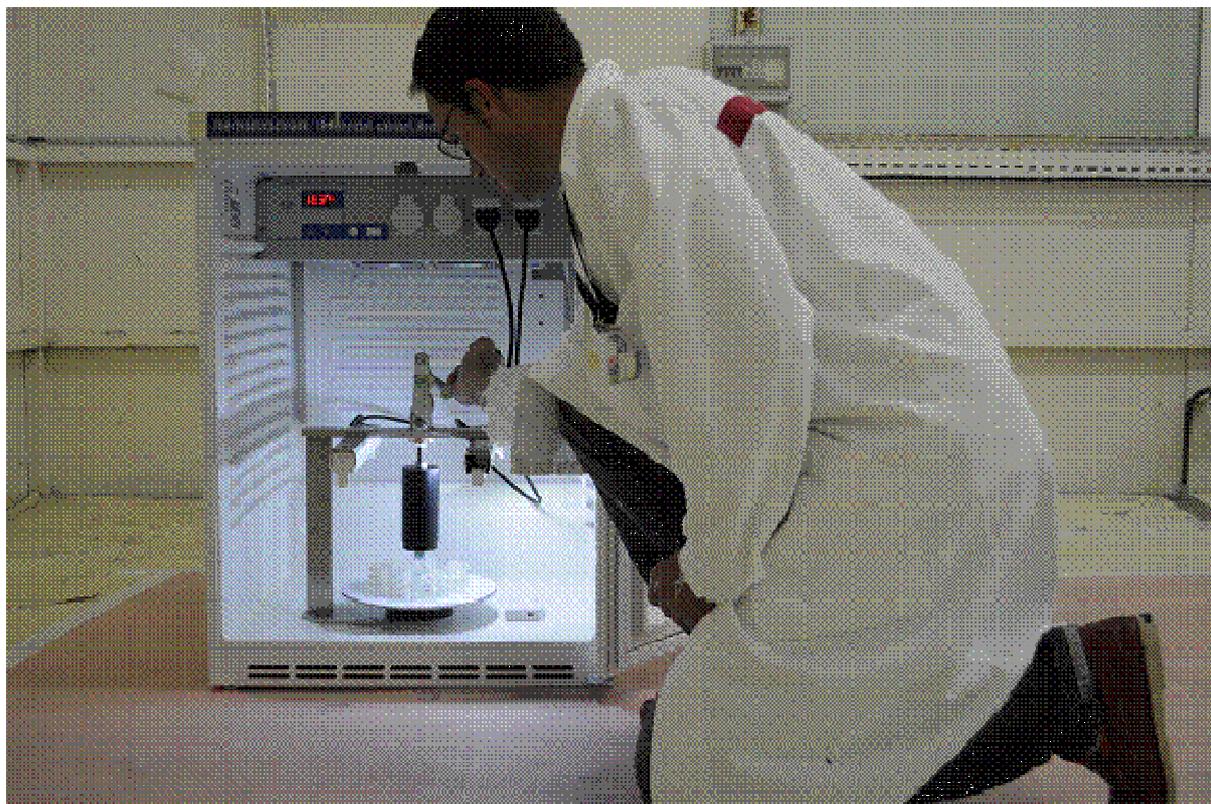
Photo 3: Counting and photography of nematodes under a binocular
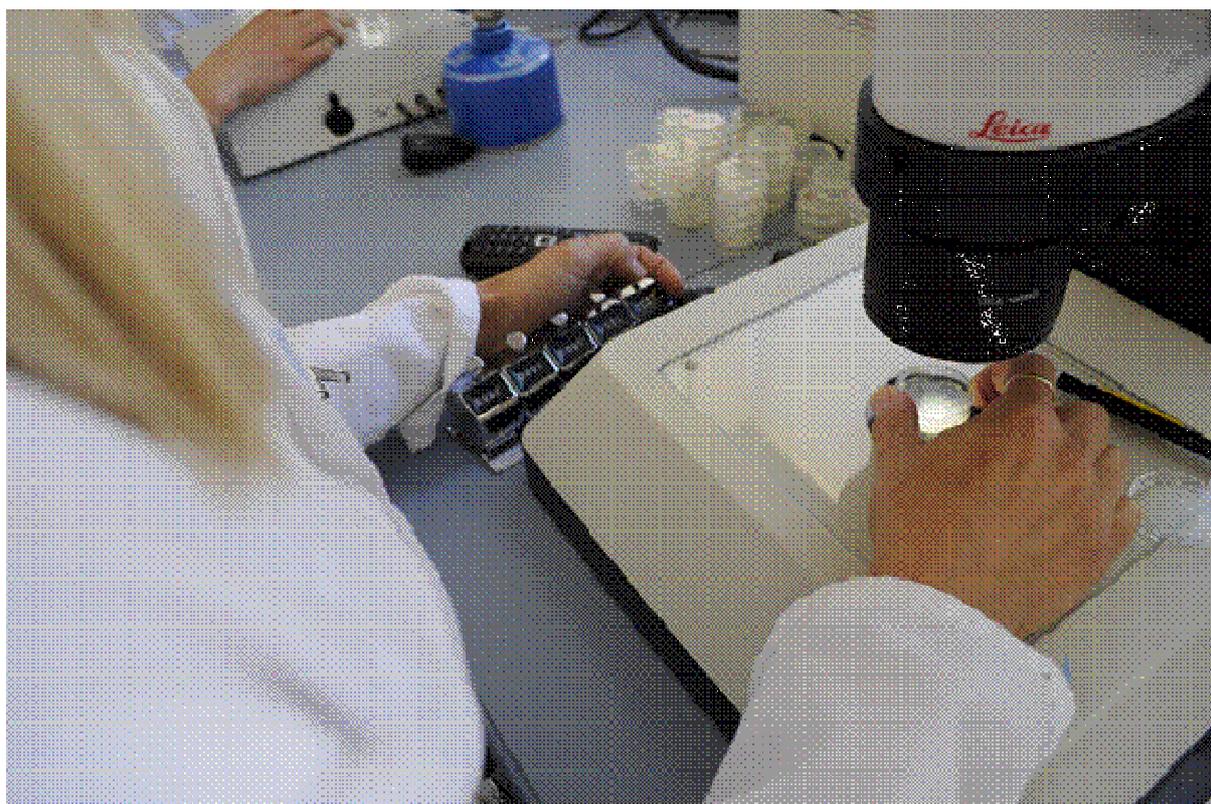
Photo 4: Size of nematode from day 1 to day 10
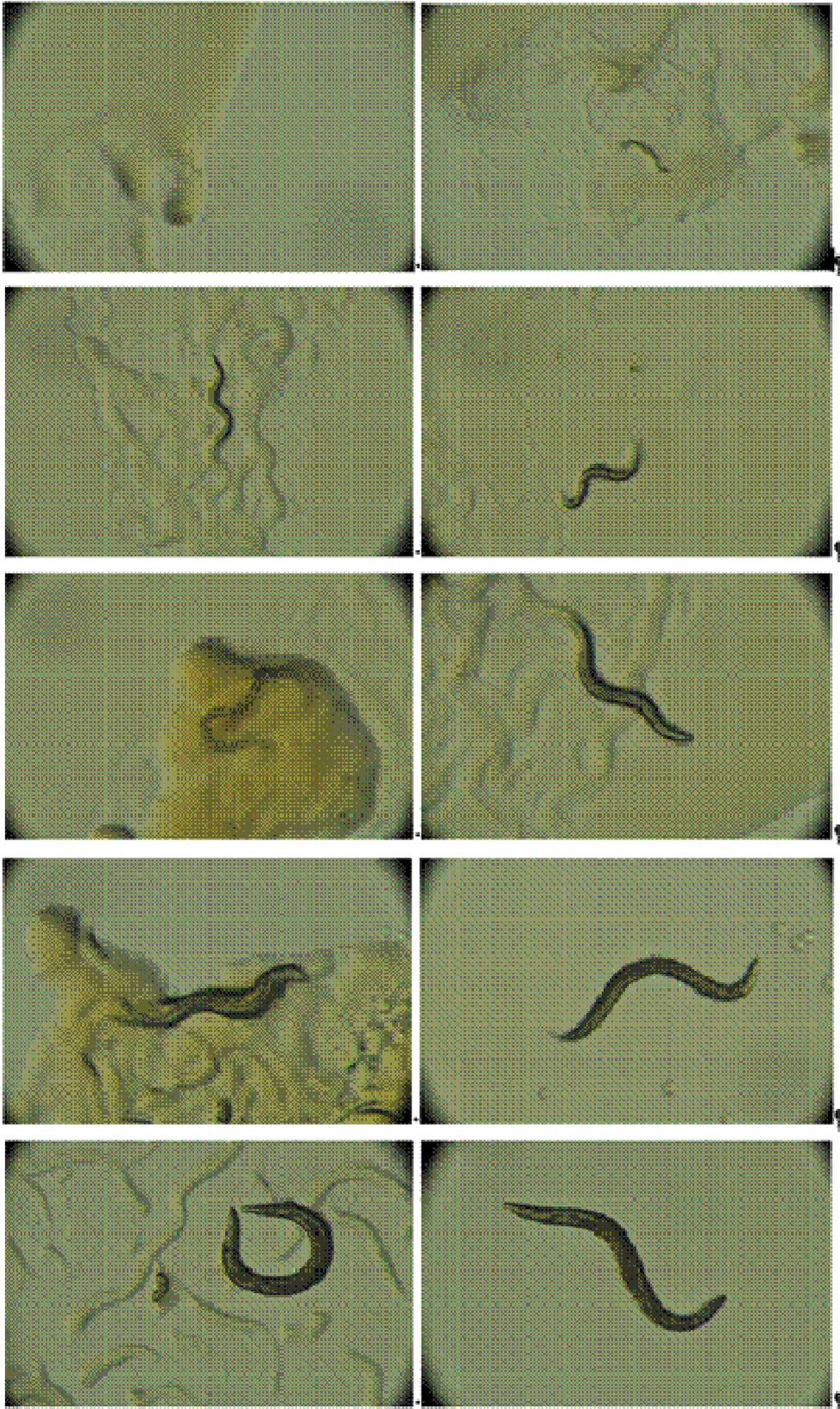
7 Perspectives

This report justifies our first choices for the set of experiments that will support our research development under WP-5. Two biological models have been selected first to support both dose rate – response relationship on life history traits under external gamma and internal alpha irradiation exposure: one plant model (*L. minor*) and one animal model (*C. elegans*). Both of them will also be used for DEBTox parameterisation. External gamma irradiation exposure will use irradiation facilities that allow to cover a range of dose rates from background up to 100 mGy/h if needed. The selected alpha-emitting radionuclide is Am-241.

The next step viewed as "go-no go" actions will take place in our next meeting in January 2012 where we will discuss:

(iv)   the main conclusions from our sensitivity analysis outcoming from the theoretical approach where we combined radiosensitivity information from FREDERICA and Leslie matrices for a wide range of species (task 5.1): this discussion will help to decide whether we need to implement experiments (or not) to obtain more robust conclusions about the propagation of effects observed at the individual level to the population level;

(v)   the results and lessons learnt from the pilot study: this discussion will help to refine the experimental design if needed, to conclude on the feasibility of DEBTox development (task 5.3);

(vi)   the hypotheses we would test to progress in the understanding of the mechanistic modes of actions at the (sub)cellular level (task 5.2).

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8   Annex

8.1   How to download and install R and 'drc' add-on package

8.1.1   R software

R software can be freely downloaded at the address: http://cran.r-project.org/. Then in the 'Download and Install R' windows it's necessary to choose and double click on its own exploitation system.
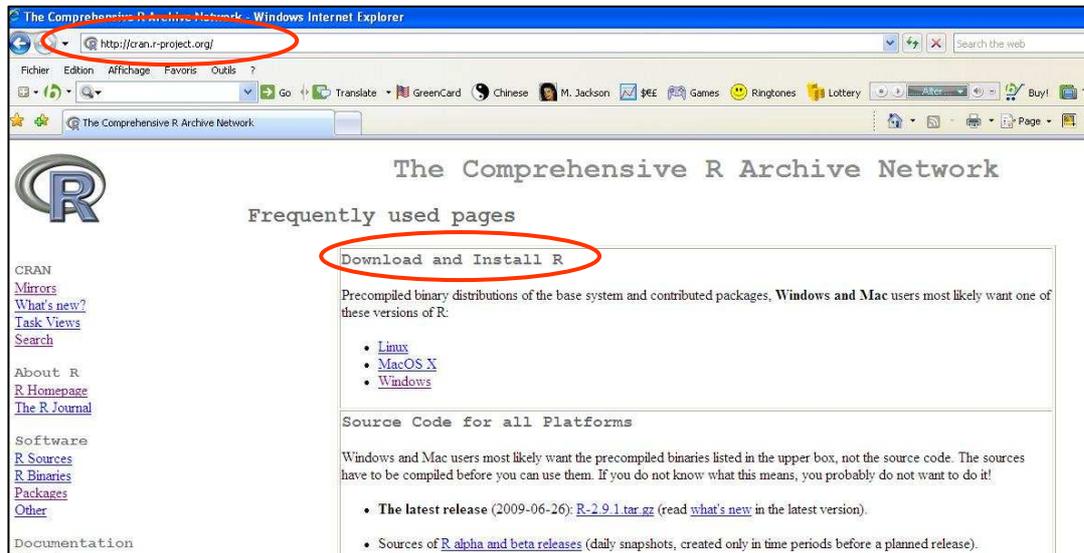
A new window, names 'R for windows' for example, is opening. It's necessary to double click on the 'base' option; it permits to import base packages in the same time of the download of R.
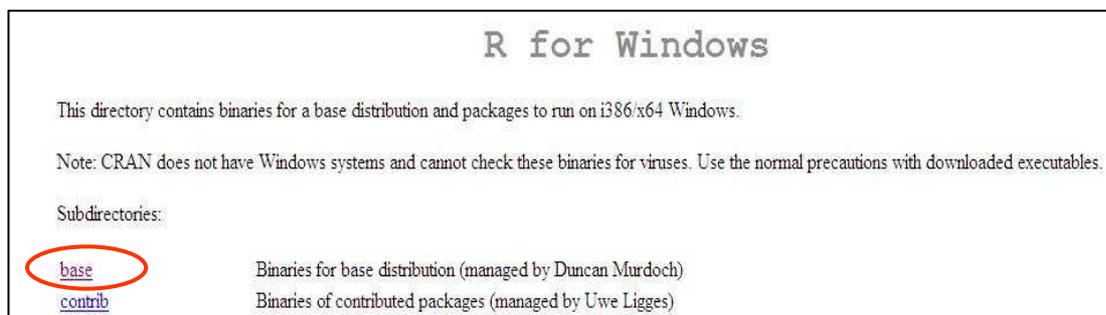
The latest version of R is available by double clicking on 'download' as shown in the following window.

Then, it's necessary to save and execute the file.
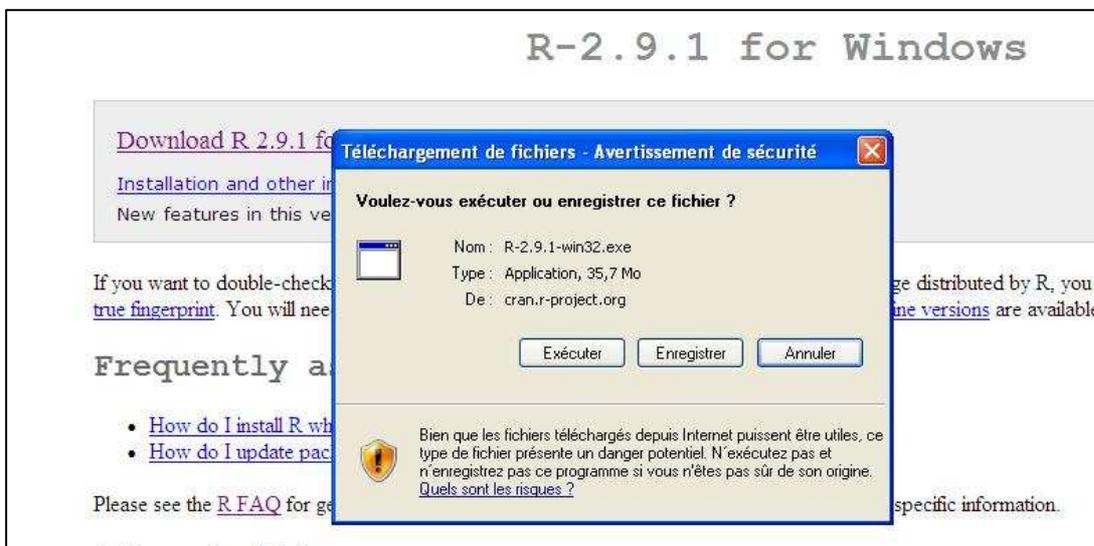
A short cut appears on the desktop.

Double-clicking on the shortcut permits to open the R console where commands lines will be used.
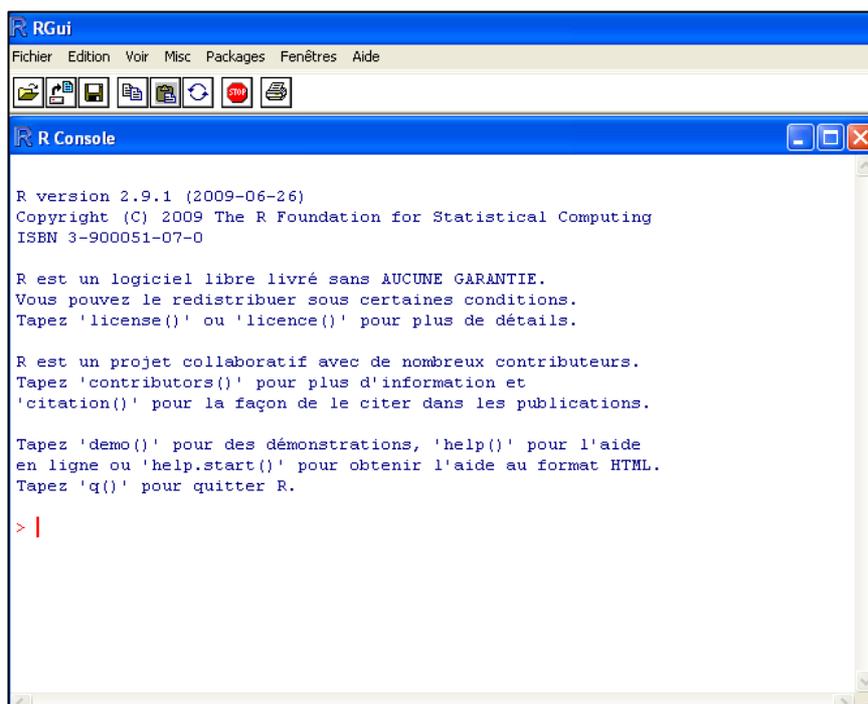
8.1.2 'drc' add-on package :

The easiest way to download and install '*drc*' and all the other add-on packages needed by '*drc*' is to use the following commands in the R console.

(D-N°:5.1) – Experimental plan
Dissemination level: PU
Date of issue of this report: **31/10/2011**
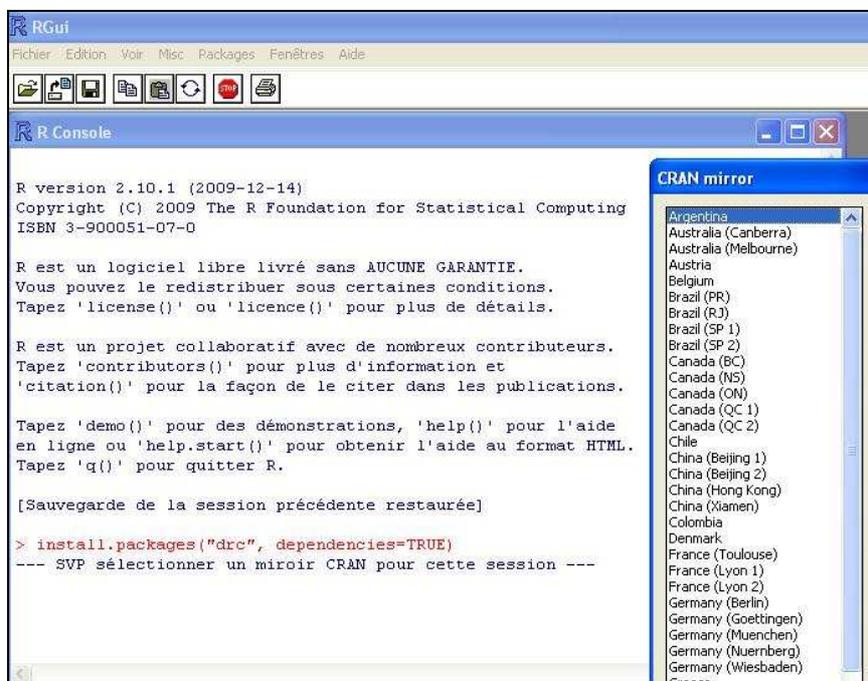
Next a 'Cran mirror' windows opens and a site has to be chosen. Then '*drc*' and all others needed packages are download automatically.

To finish, in order to use all the functions of the package '*drc*', it's necessary to enter the following commands: library(drc).

8.2 Import of a dataset

One of the more easy ways to import a dataset in R is to use an Excel File. First, the decimal separator must be the comma symbol. Then the file has to be saved in a 'csv' format (semicolon delimited file). Then the import in R is done using the following command: `read.csv2(file.choose())`which permits to open a window for dataset selection.

8.3 Log-logistic models

8.3.1 Strategy of analysis and modelling of monotonic dose-response relationships with continuous observations

When observations are continuous data, '*drc*' library uses least square non linear fitting to estimate the model's parameters, and uses parametric methods to estimate the parameters' uncertainty. These parametric methods need the validity of three assumptions: independence of data, normality of the residuals, and homogeneity of the residuals. These same assumptions are also needed for the validity of the *'Lack of fit'* test and the one of the '*F*' test for nested models, both tests used in dose-response relationships analysis.

In case of continuous response, dose-response relationship analysis and modelling contains 5 levels:

1) Use of the full log-logistic model (e.g. the 4parameters log-logistic model) to fit the data.

2) Assessment of residual normality and homogeneity assumptions (with use of Box-Cox transformation if needed).

3) Assessment of the quality of the model's fit by a *'Lack of fit'* test

4) Reduction, if possible, of the full model (e.g. the 4 parameters log-logistic model). That means assessment of the equality to 0 of the lower limit of the curve ('c' parameter), by the way of an '*F*' test for nested model. The simplest model is kept according the Parsimony rule.

5) Estimation of the EDp% and its uncertainty.

The strategy of analysis and modelling of dose-response relationship with continuous observation is summarized in Fig.8.1.
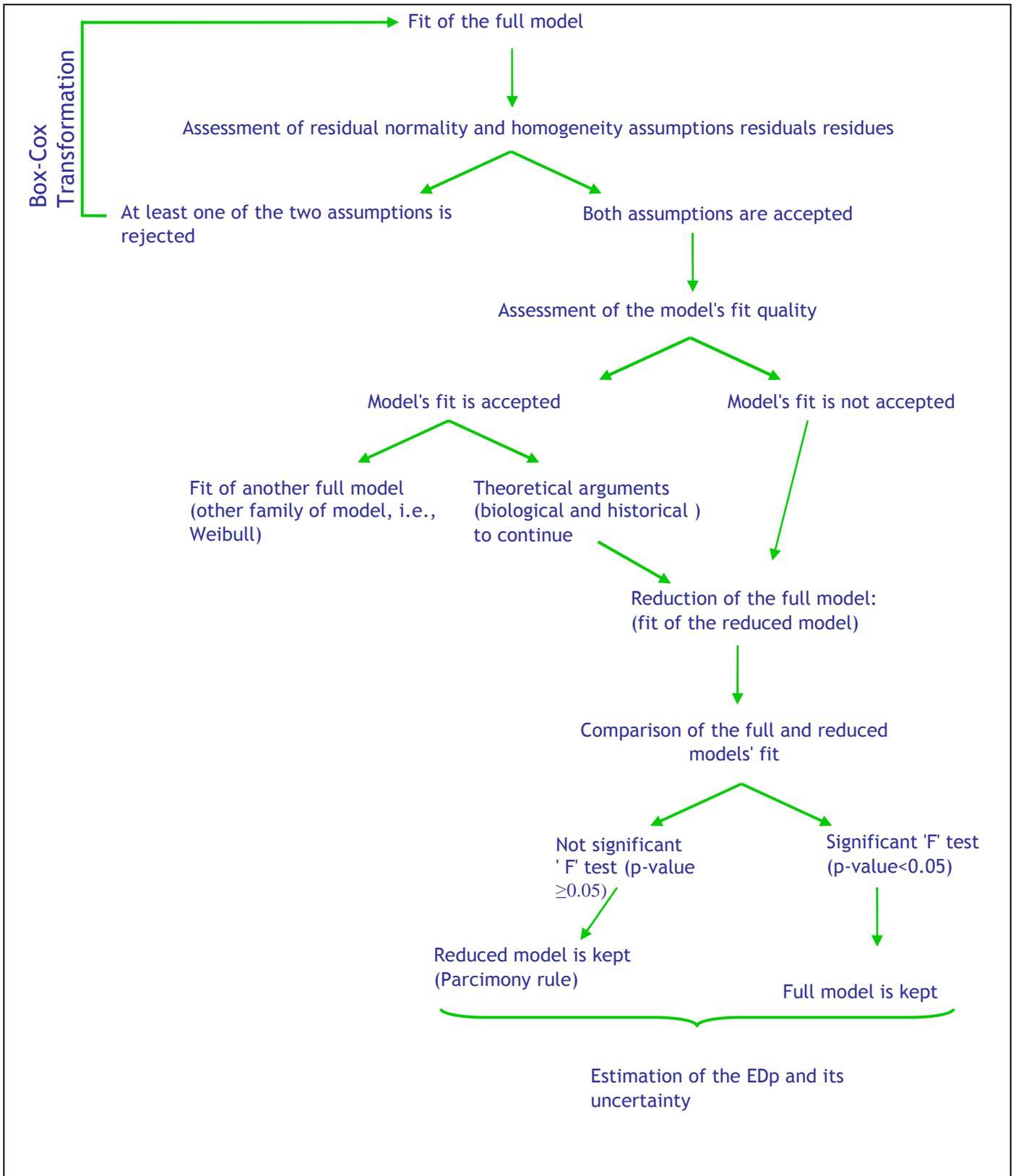
Figure 8.1: Strategy of analysis and modelling of monotonic dose-response relationship with continuous data.

Residual normality assumption is usually assessed visually, by the way of a 'quantile-quantile' plot, where points have to approximately follow a straight line. Residual normality assumption can also be assessed by a statistical test as the Shapiro-Wilk's test (Ritz *et al.*, 2008).

As the same, residual homogeneity assumption is generally assessed visually, by the way of a 'fitted values vs. standardized residuals' plot, where points have to be distributed without any pattern. Residuals homogeneity assumption can also be assessed by statistical tests as Levene's test or Bartlett's test.

Independence assumption of the data is also needed, but usually it's only validated on the experimental design basis.

When at least one of the homogeneity and normality assumptions is rejected, a Box-Cox transformation (both side type in order to conserve the relation between the response and the doses) can be used. Nevertheless there is no warranty about the subsequent validity of the assumptions. Box-Cox transformation is empiric power transformation, defined by:

$$h_\lambda(y) = \begin{cases} \dfrac{y^\lambda - 1}{\lambda} & \lambda \neq 0 \\ \log(y) & \lambda = 0 \end{cases} \qquad \text{Equation 11}$$

If the use of the Box-Cox transformation doesn't improve the residual normality and/or homogeneity assumption, a weighted non linear regression can be considered.

Concerning the quality of the model's fit, it is generally assessed graphically, and then completed by the way of a '*Lack of fit*' test. For the visual assessment, 2 plots can be done:

- the first one displays both the experimental points and the fitted model ; proximity between them is needed in order to consider the fit as satisfactory

- the seconds one is a 'residuals vs. fitted' plot. For each dose level, a uniform distribution of the residuals on both sides of a 0 X-axis line is needed in order to consider the fit as satisfactory.

The principle is to compare the fit of the 4 parameters log-logistic model with the fit of a more general model (considered as a reference model) which is the one-way ANOVA model. The statistic of the test is:

$$F = \frac{(RSS_{LL4} - RSS_{ANOVA})/(n - p - df_{ANOVA})}{RSS_{ANOVA} / df_{ANOVA}} \qquad \text{Equation 12}$$

Where:

- *RSS* means 'residuals sum of squares'

- *df* means 'degrees of freedom'

- *n* means number of data (total)

- *p* means number of parameters of the model (here 4 because of the LL.4 model).

'F' statistic follows an F distribution with (n-p-df$_{ANOVA}$, df$_{ANOVA}$) degrees of freedom. If the p-value of the F test is not significant (p-value $\geq$0.05), then the fit of 4 parameters log-logistic model is considered as satisfactory than the one of the reference model ; and so the model is accepted On the contrary, if the p-value is significant (e.g., p-value<0.05) , it means that the fit of the 4 parameters log-logistic model is not as satisfactory than the one of the ANOVA model, and then it's rejected.

Concerning the reduction of the full model, in case of continues data and when the dose-response relationship is decreasing, a 3 parameters log-logistic model can be considered (is the reduced model). The 4 parameters log-logistic and the 3 parameters log-logistic models are nested since the 3 parameters model is the 4 parameters one with 'c' parameter =0. Then the fits of these two models can be assessed by an 'F' test (similar to the F '*Lack of fit test*'), the statistic is:

$$F = \frac{(RSS_{Re\,duced} - RSS_{Full})/(df_{Re\,duced} - df_{Full})}{RSS_{Full} / dfFull} \qquad \text{Equation 13}$$

'F' statistic follows an F distribution with (df$_{Reduced}$ - df$_{Full}$, df$_{Full}$) degrees of freedom. If the p-value of the 'F' test is not significant (p-value $\geq$0.05), then the fit of 3 parameters log-logistic model is considered as satisfactory as the one of the 4 parameters model. In other words, it means that the lower limit is not significantly different from 0, and that the 3 parameters model is kept. On the contrary, if the p-value is significant (e.g., p-value<0.05), it means that the fit of the 3 parameters log-logistic model is not as satisfactory as the one of the 4 parameters model. Thus, the 3 parameters model is rejected and the 4 parameters model is kept.

Once the final model is obtained, the EDp(%) value can be estimated based on this kept model, using the following equation:

$$EDp = e\left[\frac{p}{(100-p)}\right]^{1/b} \qquad \text{Equation 14}$$

Meanings of e and b parameters have been given previously. '*drc*' use the delta method to estimate the uncertainty of the EDp.

8.3.2 Strategy of analysis and modelling of monotonic dos-response relationship when observed response are quantal data

Observations are quantal (or binary) data when they concern an event which can take only two levels E^+ or E^- for each individual (dead or alive for example).

Table 8.1: example of observed responses of quantal type, with different replicate's size.

Dose (i)	Repliquat (j)	Individual (l)	Response (R_{ijk})	Number of individuals in the replicat (N_{ij})	$Y_{ij\,obs}$	$P_{ij\,obs}$
0	1		0	6	1	0.17
			0			
		N°3	0			
		N°4	1			
		N°5	0			
		N°6	0			
0	2	N°1	0	4	1	0.25
		N°2	1			
		N°3	0			
		N°4	0			
...	...	...	...	...	...	
...	...	...	...	...	...	
5	4	N°1	1	5	4	0.8
		N°2	1			
		N°3	1			
		N°4	1			
		N°5	0			

When observations are quantal (or binary) data, the modelled response variable is the percentage of event (p_{ijobs}). '*drc*' use the maximum likelihood method to estimate the model's parameters and the model's parameters uncertainty. Estimation of the standard error of the model's parameters is based on a Binomial distribution and requires the data independence. When observed data are quantal data, it's necessary to check the absence of an overdispersion, e.g. that the observed variance is not higher than those calculated according the binomial distribution. When an overdispersion is highlighted, estimated standard errors of the model's parameters have to be corrected in the increasing direction. This could be easily done with '*drc*' using the `type="binomial"` argument in the `drm` function and using the `od=T` argument in the `summary` function. Moreover, in case of overdispersion, the result of the *'Goodness of fit'* test (used to assess the quality of the model), and the result of the *'likelihood ratio'* test (used to compare the fit of two nested models), are not valid.

In case of quantal response, dose-response curve modelling contains 5 levels too:

1) Fit of the full 4 parameters log-logistic model

2) Assessment of the model quality fit

3) Assessment of an eventual overdispersion

4) If possible, reduction of the full model, e.g. test the equality to 0 of the lower limit ('c' parameter) and or test the equality to 1 of the upper limit ('d' parameter). The simplest model is kept according the Parsimony rule.

5) Estimation of the EDp and its uncertainty.

Management of the steps 4 and 5 will be different if a ?overdispersion is detected or not.

The strategy of analysis and modelling of dose-response relationships, when observations are quantal data and when **no overdispersion was highlighted**, is summarized in the Figure 8.2.
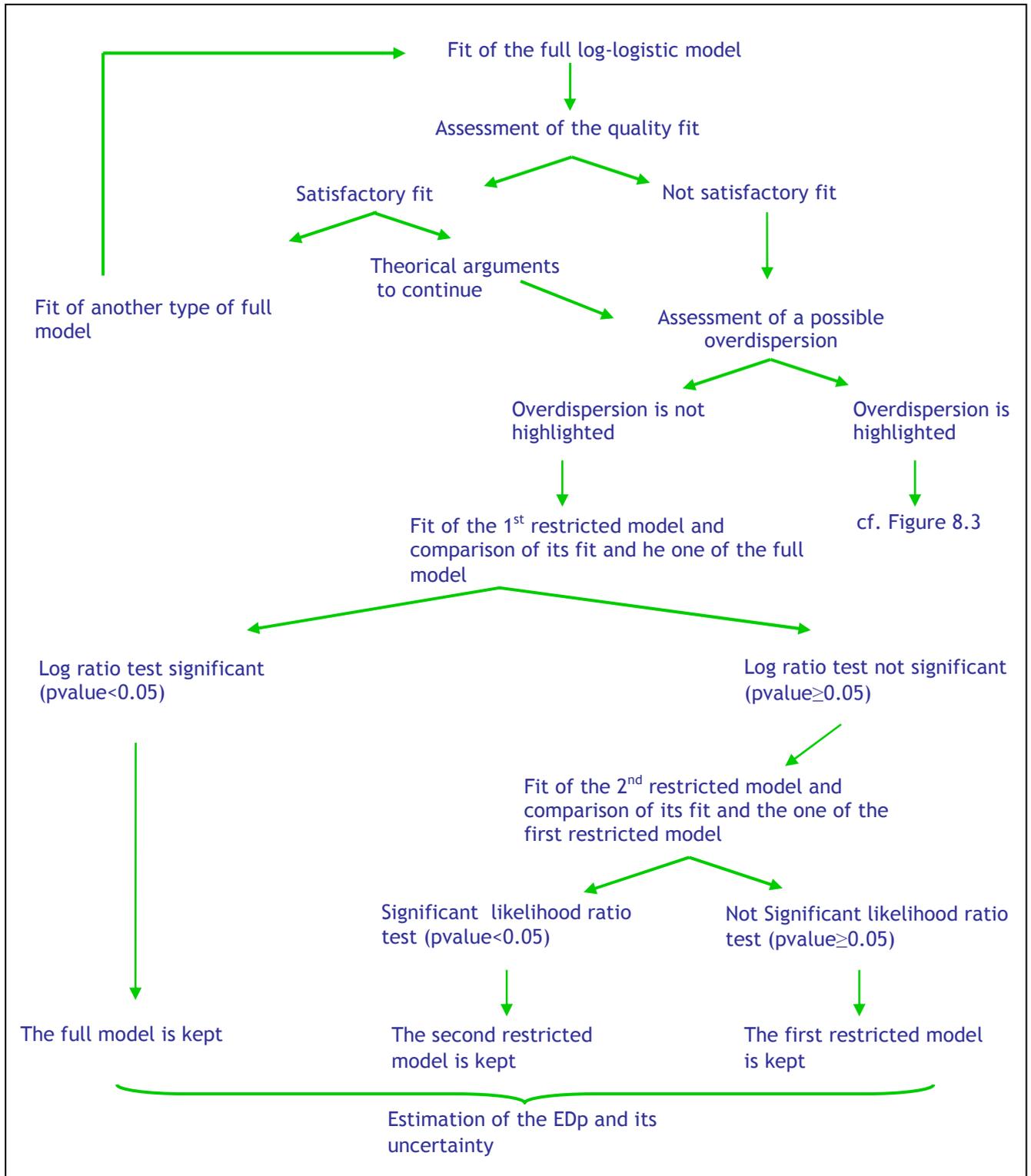
Figure 8.2 : Strategy of analysis and modelling of monotonic dose-response relationships with quantal data, when no overdispersion has been highlighted.

In case of quantal (binomial) observations, independence hypothesis is accepted or rejected only on the basis of the experimental design. When observations are quantal data, it is needed to precise their type using the type="binomial" argument in the drm function. It is also necessary to give the total number of observations by the way of the weight=n argument. 'drc' package needs both information in order to give an adequate estimation of the parameters, and above all of their standard error.

Concerning the assessment of fit's quality, it is generally done graphically and completed by the use of a statistical test. For the visual assessment, 2 plots can be done:

- the first one displays both the experimental points and the fitted model ; proximity between them is needed in order to consider the fit as satisfactory.

- The seconds one is a 'residuals vs fitted' plot. For each dose level, a uniform distribution of the residuals on both sides of a 0 X-axis line is needed in order to consider the fit as satisfactory.

When observations are quantal data, the test used to assess the quality of the fit is called 'Goodness of fit' test. Its principle is different of those of the 'Lack of fit' test, since here there is no reference model (one-way ANOVA for the 'Lack of fit' test), and since no replicates are needed. The aim of the 'Goodness of fit' is to assess if the sum of the standardized squares between the observed number of events (Y_{ijobs}) and the number of events predicted by the model (Y_{ijcal}) is not too big. These squares are called 'standardized' because they are reported to the residual variance. The statistic of the test is:

$$Q_{(n-p)ddl} = \sum\sum\left[\frac{\left(Y_{ij\,obs} - Y_{ij\,calc}\right)^2}{Var(Y_{ij})}\right] \quad \text{Equation 15}$$

Theoretically, numbers of observed events (Y_{ijobs}) are distributed according a Binomial distribution with parameters N_{ij} and p_i (theoretical probability that an event appears for a given dose level (x_i). Numbers of events predicted by the model (Y_{ijcal}) are also distributed according a Binomial distribution; percentages of predicted events $(\hat{p}_i)$ are then depending of the parameters model.

The variance $(Var(Y_{i\,j}))$ is the one predicted by the Binomial distribution:

$Var(Y_{ij}) = n_{ij} \times \hat{p}_i \times (1 - \hat{p}_i)$ Equation 16

With:

- i : index of the doses
- j : index of the replicates
- $\hat{p}_i$: percentages of individuals presenting the event E^+ at each level of i.

The Q statistic of the 'Goodness of fit' test is based on the Pearson's statistic which follows a Chi-2 distribution with $n - p$ degrees of freedom. If the p-value of the test is not significant (p-value ≥0.05),

then the fit of 4 parameters log-logistic model is considered as satisfactory. On the contrary, if the p-value is significant (e.g., p-value<0.05), it means that the fit of the 4 parameters log-logistic model is not satisfactory.

In case of overdispersion, result of the test is not valid.

A very simple method can be used in order to assess the presence of an overdispersion. It consists in comparing the ratio (ϕ) of the Q statistic over its degrees of freedom, to the value 1.

$$\phi = \frac{Q}{n-p} \qquad \text{Equation 17}$$

If the ratio equals *ca.* 1 then there is no overdispersion. If the ratio is higher than 1, then an overdispersion is highlighted.

Concerning the reduction of the full 4 parameters model, since the quantal data are bracketed by the values 0 and 1, three sub-models can be considered:

- the 3 parameters log-logistic model (LL.3), with lower limit ('c' parameter) =0 and higher limit ('d' parameter) <1;

- the second 3 parameters log logistic model (LL.3u), with lower limit ('c' parameter) > 0 and higher limit ('d" parameter) =1;

- the 2 parameters log-logistic model (LL.2), with lower limit ('c' parameter) = 0, and higher limit ('d' parameter) = 1.

In absence of overdispersion, the reduction of the full 4 parameters log-logistic model needs the following several steps:

- fit a restricted model with 3 parameters (LL.3 or LL.3u according the situations) ;

- compare its fit with the one of the full (4 parameters) model by a 'likelihood ratio' test ;

- keep the restricted model if its fit is satisfactory (p-value of the test ≥ 0.05), or in controversy keep the full model if the fit of the restricted model is not satisfactory (p-value>0.05) ;

- if the restricted model is kept, do again the process with the restricted 2 parameters log-logistic model (LL.2).

All the log-logistic models available are described in Figure 8.7.

The statistic of the 'likelihood ratio' test used to compare the fits of two nested models is:

$$R = 2Ln\left(\frac{V_{Complet}}{V_{Re\,streint}}\right) = 2\left[Ln(V_{Complet}) - Ln(V_{Re\,streint})\right] \qquad \text{Equation 18}$$

The R statistic follows a Chi-2 distribution with ($df_{Re\,stricted} - df_{Full}$) degrees of freedom. With:

- $df_{Full} = n - p_{Full}$;

- $df_{\text{Re}\,stricetd} = n - p_{\text{Re}\,stricted}$;

- n , the number of data ;

- p_{Full} : the number of parameters of the full log-logistic model;

- $p_{\text{Re}\,stricted}$: The number of parameters of the restricted model.

If the p-value of the '*likelihood ratio*' test is not significant (p-value ≥0.05), then the fit of the restricted model is considered as satisfactory as the one of the full model. On the contrary, if the p-value is significant (e.g., p-value<0.05), it means that the fit of the restricted model is not as satisfactory as the one of the full model. Thus, the restricted model is rejected and the full model kept.

Once the final model is obtained, the EDp value can be estimated based on this kept model (cf. paragraph 8.3.1)

An example of complete analysis and modelling of a monotonic dose-response relationship with quantal observations and when no overdispersion has been highlighted is given in annexe 8.3.5

The strategy of analysis and modelling of monotonic dose-response relationship with quantal observation and when **an overdispersion is highlighted** is summarized in the Figure 8.3.

Fit of the full model

↓

Assessment of the quality of the fit

Not satisfactory fit   Satisfactory fit

Fit of another type of full model

Theoric arguments to continue

Assessment of a possible overdispersion

Overdispersion not highlighted   Overdispersion highlighted

cf. Figure 8.2

Choice of the final model assessing both:

- The possibility to fix the lower limit to 0 looking at the 't' test of equality to 0 of the 'c' parameter, given by the `summary` function with the `od=T` argument.

- The possibility to fix the higher limit to 1 by :

    - comparing, visually, the fit of a model with 'd' =1 and another one with 'd'<1.

    - Having a look on the 'd' parameter estimate of the model fixing 'd' parameter <1.

↓

Estimation of the EDp from the final kept model using the `od=TRUE` argument in the `ED` function

Figure 8.3: Strategy of analysis and modelling of monotonic dose-response relationships with quantal data, when an overdispersion has been highlighted.

In case of overdispersion, the result of the '*likelihood ratio*' test used to compare the fits of two nested models is not valid. The used strategy is then rougher; it consists in estimating alternately (the order dependent on data):

- The equality of the lower limit to 0, by using the p-value of the '*t*' test concerning the parameter ' c ' which is supplies by the `summary` function. However, it is indispensable to have used the argument `od=T` in this last one.

- The equality of the higher limit to 1, by studying the estimation of the parameter 'd' of in the full previous model, and\or by comparing visually the fits of the models fixing and not fixing the parameter 'd' to 1. In this assessment, only the estimation of the parameter is considered, the use of `od=T` is not thus indispensable. However it seems more careful to always use this argument when an overdispersion was highlighted.

In case of overdispersion, the use of the '*t*' test to assess the possibility to fix the lower limit ('c' parameter) to 0, in other words to reduce the 4 parameters model in a 3 parameters model is presented in figure 8.4.

```
summary(deguelin.ll4, od=T)
model fitted: log-logistic (ed50 as parameter) (4 parms)
parameter estimates:
estimate std. error   t-value     p-value
b:(intercept) -3.46316     6.74243 -0.51364     0.6075
c:(intercept)  0.31003     0.18623  1.66472     0.0960
d:(intercept)  1.01870     0.24968  4.07996 4.504e-05
e:(intercept) 18.50393     3.44660  5.36875 7.929e-08
```

Figure 8.4: Use of the 't' test to estimate, in case of surdispersion, the possibility of fixing the lower limit ('c' parameter) to 0, in other words the possibility of reducing the model of 4 parameters towards a model in 3 parameters.

Here, the '*t*' test concerning the assessment of the equality of the 'c' parameter to 0, is not significant (p-value=0.0960). Thus, the estimate of the 'c' parameter is not significantly different from 0. Hence, the use of the 3 parameters log-logistic model is justified. The possibility of fixing the higher limit of the curve ('d' parameter) to 1 is then studied.

The evaluation of the possibility of fixing the higher limit of the curve ('c' parameter) to 1, by visually comparing the fits of the 3 parameters ('c'=0 and 'd'<1: left graph) and the 2 parameters ('c'=0 and 'd'=1: right graph) log-logistic models is presented in the Figure 8.5 below.
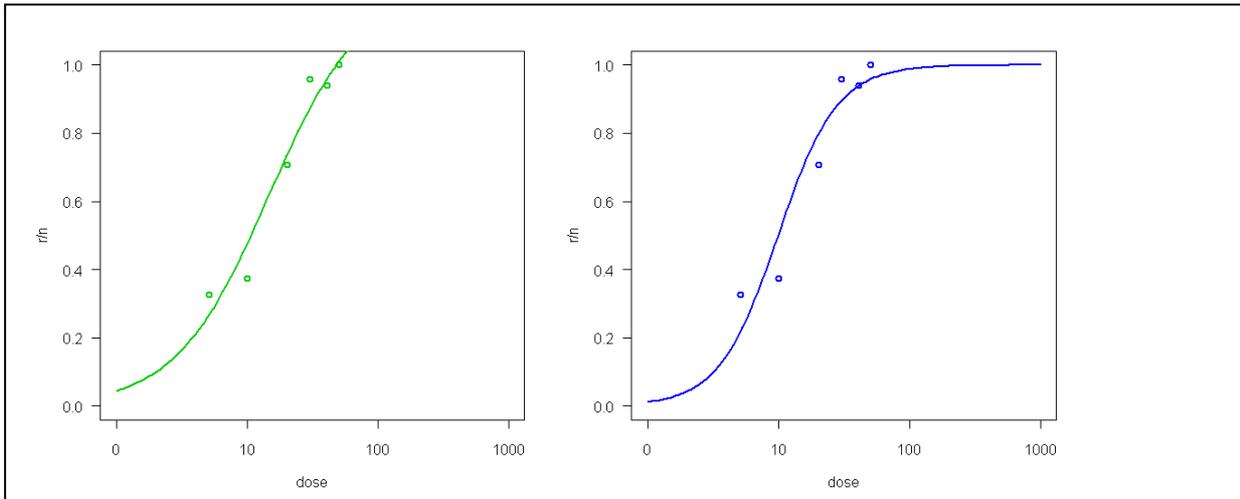
Figure 8.5 : Assessment of the possibility of fixing the higher limit of the curve ('d' parameter) to 1, by comparing the fits of the 3 parameters log-logistic model (c=0 and d< 1: left graph) and of the 2 parameters log-logistic model (c=0 and d=1: right graph).

In that case, it is obvious that the fit of the 3 parameters log-logistic model leads to an over-estimation of the higher limit because the d' parameter's estimate exceeds the value 1. This is confirmed by the outputs of the summary function because the 'c' parameter's estimate equals 1.23 (cf. Figure 8.).

```
summary(deguelin.ll3,od=T)
model fitted: log-logistic (ed50 as parameter) with lower limit
at 0 (3 parms)
parameter estimates:

                estimate std. error   t-value p-value
b:(intercept) -1.22214     0.73312 -1.66704  0.0955
d:(intercept)  1.23503     0.47594  2.59491  0.0095
e:(intercept) 14.72358    11.69516  1.25895  0.2080
```

Figure 8.6 : Visualization of the 3 parameters log-logistic model's estimates fitted to the 'deguelin' dataset.

An example of complete analysis and modelling of a monotonic dose-response relationship with quantal observations and when an overdispersion has been highlighted is given in annexe 8.3.6.

8.3.3 Examples for different situations and corresponding log-logistic models

Named in 'drc'	Continues data		Quantal data	
	Decreasing relation	*Increasing relation*	*Decreasing relation*	*Increasing relation*
LL.4				
LL.3				
LL.3u				
LL.2				
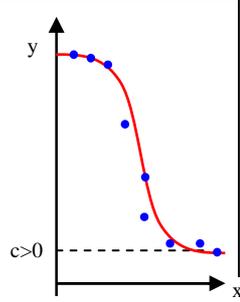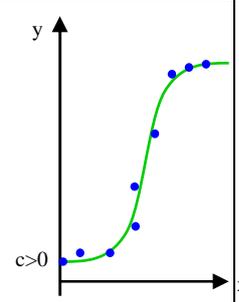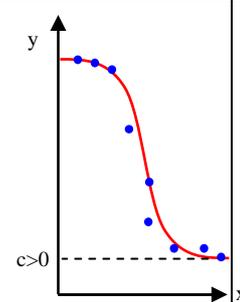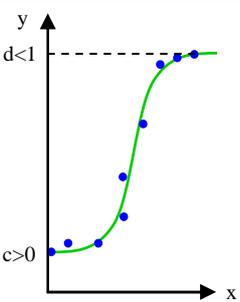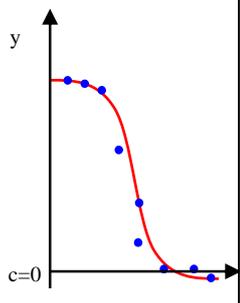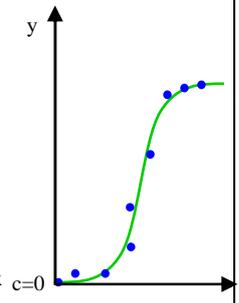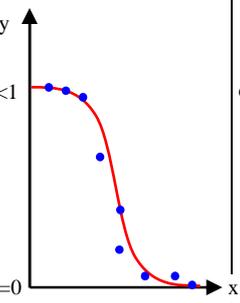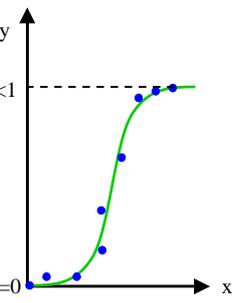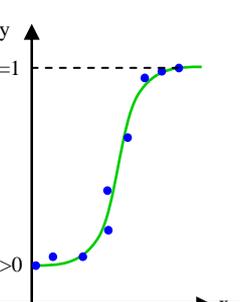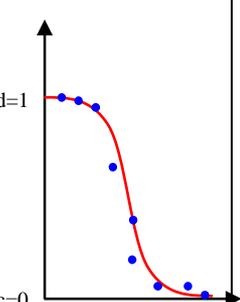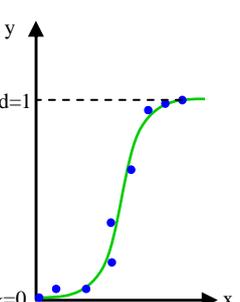
Figure 8.7 : Log-logistic models available in 'drc' to analyse monotonic dose-response relationships according the type of the observations and the shape of the relationship

8.3.4 Illustration for DRC application to the analysis and modelling of a monotonic dose-response relationship with continuous data

Data used in this example are those of the 'ryegrass' dataset included in the '*drc*' add-on package.

In the following pages, commands used with '*drc*' are in red colour and the R outputs are in blue colour. In green and bracketed by # symbols are descriptions of the commands.

```
# loading of the drc package #
library(drc)
#opening of the help page about ryegrass dataset#
?ryegrass
#display of the ryegrass dataset#
ryegrass
        rootl  conc
1  7.5800000  0.00
2  8.0000000  0.00
3  8.3285714  0.00
4  7.2500000  0.00
5  7.3750000  0.00
6  7.9625000  0.00
7  8.3555556  0.94
8  6.9142857  0.94
9  7.7500000  0.94
10 6.8714286  1.88
11 6.4500000  1.88
12 5.9222222  1.88
13 1.9250000  3.75
14 2.8857143  3.75
15 4.2333333  3.75
16 1.1875000  7.50
17 0.8571429  7.50
18 1.0571429  7.50
19 0.6875000 15.00
20 0.5250000 15.00
21 0.8250000 15.00
22 0.2500000 30.00
23 0.2200000 30.00
24 0.4400000 30.00

# visualization of the data in x vs y plot #
plot(ryegrass$conc,ryegrass$rootl,lwd=2,col=3)
```
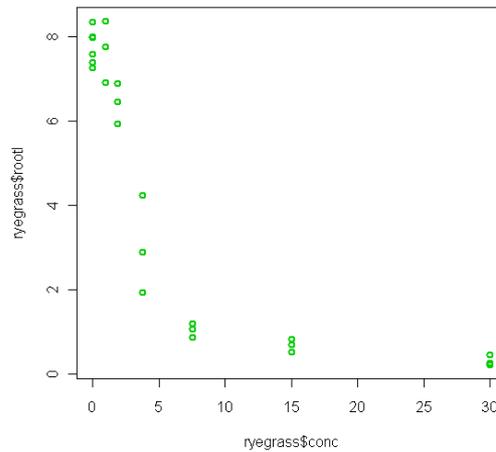
```
# display of the dose variable levels #
levels(as.factor(ryegrass$conc))
[1] "0"     "0.94" "1.88" "3.75" "7.5"   "15"    "30"

# display of the number of data per dose level #
table(as.factor(ryegrass$conc))
   0 0.94 1.88 3.75  7.5   15    30
   6    3    3    3    3    3    3

#fit of the full 4 parameters log-logistic model#
ryegrass.ll4<-drm(rootl~conc, data=ryegrass, fct=ll.4())
# display of the parameters estimates #
summary(ryegrass.ll4)
model fitted: log-logistic (ed50 as parameter) (4 parms)
parameter estimates:
               estimate std. error   t-value     p-value
b:(intercept)   2.98222    0.46506   6.41251 2.960e-06
c:(intercept)   0.48141    0.21219   2.26876    0.0345
d:(intercept)   7.79296    0.18857 41.32722 3.822e-21
e:(intercept)   3.05795    0.18573 16.46440 4.268e-13

residual standard error:
 0.5196256 (20 degrees of freedom)


# visualization of the fit#
plot(ryegrass.ll4,type="all",col=3,lwd=2)
```
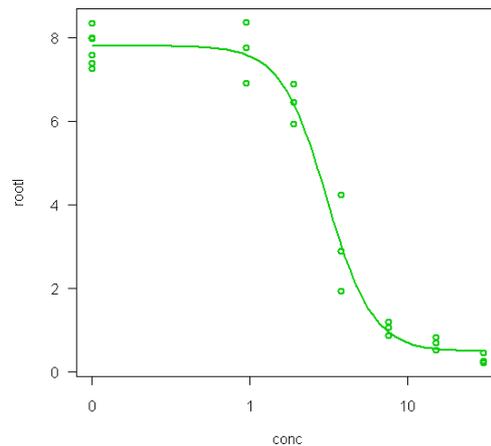
```
# visual assessment of the residuals normality assumption by qqplot #
qqnorm(residuals(ryegrass.ll4))
qqline(residuals(ryegrass.ll4))
```

Normal Q-Q Plot
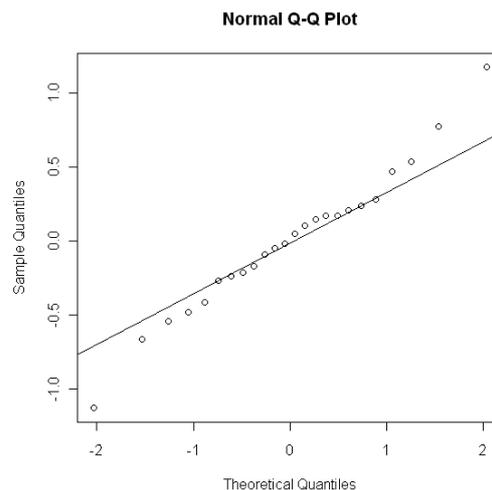
Since the residuals are distributed along the straight line, the normality assumption is accepted.

```
# assessment of the residual normality assumption by a shapiro-wilk's test#
shapiro.test(residuals(ryegrass.ll4))
        shapiro-wilk normality test
data:  residuals(ryegrass.ll4)
w = 0.9823, p-value = 0.9345
```

Since the p-value is higher than 0.05, the hypothesis of residual normality is not rejected.

```
#visual assessment of the residual homogeneity assumption by 'standardized
residuals vs. fitted values' plot #
plot(fitted(ryegrass.ll4),(residuals(ryegrass.ll4,type="standardised")))
abline(h=0,col=2)
```

[STAR]

(D-N°:5.1) – Experimental plan
Dissemination level: PU
Date of issue of this report: **31/10/2011**
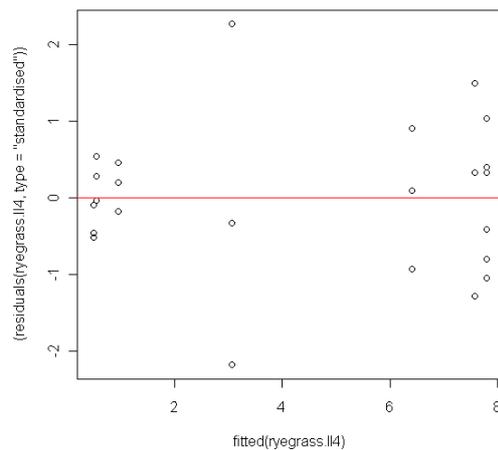
There seems to be an overall increasing trend with increasing fitted values. In others words the variance seems to be an increasing function of the mean. Residuals homogeneity assumption is thus rejected.

```
#assessment of residuals homogeneity assumption by a levene's test#
library(car) #loading of "car" package containing the levene's test#
levene.test(residuals(ryegrass.ll4),as.factor(ryegrass$conc))
levene's test for homogeneity of variance
          df f value pr(>f)
    group  6  1.9266 0.1344
          17
```

The test is not significant (p-value>0.05), thus the homogeneity assumption is not rejected.

```
#assessment of residuals homogeneity assumption by a bartlett's test#
bartlett.test(residuals(ryegrass.ll4),as.factor(ryegrass$conc))
bartlett test of homogeneity of variances
data:  residuals(ryegrass.ll4) and as.factor(ryegrass$conc)
bartlett's k-squared = 13.2162, df = 6, p-value = 0.03973
```

The p-value is significant (e.g. <0.05), thus the homogeneity assumption is rejected. Taking in account this latest results and the pattern of the residuals in the "standardized residuals vs. fitted" plot, residuals homogeneity assumption is finally rejected. A Box-Cox transformation could be used in order to try to improve the homogeneity.

```
# use of a box cox both side transformation #
ryegrass.ll4bc<-boxcox(ryegrass.ll4)
```

```
The latest command permits both to use the box-cox transformation and
to fit the model.
```

```
# display of the estimates obtained with the box cox transformation #
summary(ryegrass.ll4bc)
model fitted: log-logistic (ed50 as parameter) (4 parms)
parameter estimates:

                estimate std. error  t-value    p-value
b:(intercept)   2.61839    0.39151   6.68795 1.649e-06
c:(intercept)   0.39083    0.10429   3.74744    0.0013
d:(intercept)   7.86633    0.29558  26.61364 2.176e-17
e:(intercept)   3.01662    0.21005  14.36124 5.354e-12


residual standard error:
 0.2962958 (20 degrees of freedom)



non-normality/heterogeneity adjustment through optimal box-cox
transformation

estimated lambda: 0.5
confidence interval for lambda: [0.269,0.949]
```

Estimate of the Box Cox lambda parameter

```
# visualization of the fit#
plot(ryegrass.ll4bc,type="all",col=3,lwd=2)
```
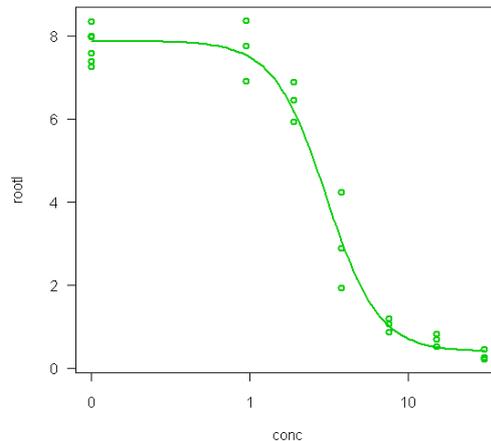

Fit seems to be good since the curve is close to all the observed points.

```
# visual assessment of the residuals normality assumption #
qqnorm(residuals(ryegrass.ll4bc))
qqline(residuals(ryegrass.ll4bc))
```

Normal Q-Q Plot
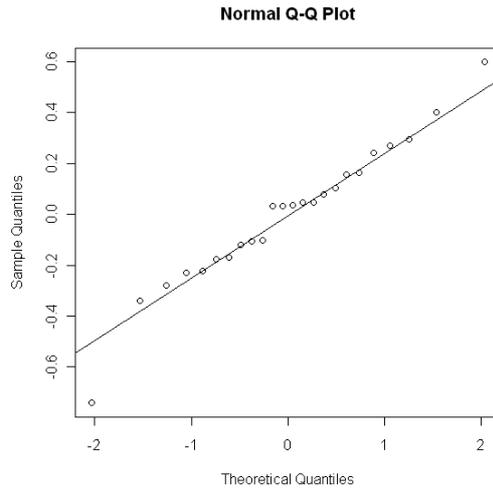
Globally, points are always distributed along the straight line, the normality assumption is accepted.

```
# assessment of the normality residuals assumption by an evaluation de
shapiro-wilk's test#
shapiro.test(residuals(ryegrass.ll4bc))
        shapiro-wilk normality test
data:  residuals(ryegrass.ll4bc)
w = 0.9716, p-value = 0.7066
```

The test is again not significant, thus residuals normality assumption is not rejected.

```
#visual assessment of the residuals homogeneity assumption #
plot(fitted(ryegrass.ll4bc),residuals(ryegrass.ll4bc, type="standardised"))
abline(h=0,col=2)
```
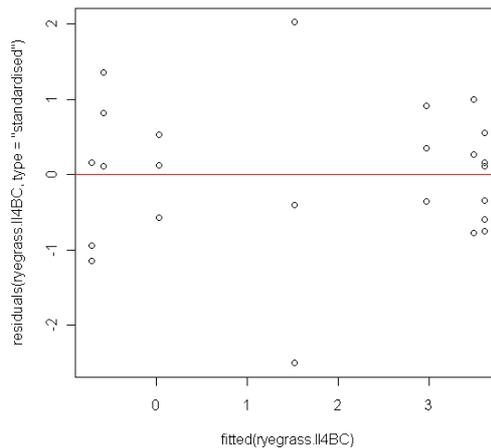

Now, there is no more an overall increasing trend with increasing fitted values. Residuals seem to be distributed on both side of 0 (the red line) for all the fitted values levels. Thus residuals homogeneity assumption is no more rejected.

```
#residuals homogeneity assumption by levene's test#
levene.test(residuals(ryegrass.ll4bc),as.factor(ryegrass$conc))
levene's test for homogeneity of variance
      df f value pr(>f)
group  6   1.529 0.2282
      17


# residuals homogeneity assumption by bartlett's test #
bartlett.test(residuals(ryegrass.ll4bc),as.factor(ryegrass$conc))
      bartlett test of homogeneity of variances
data:  residuals(ryegrass.ll4bc) and as.factor(ryegrass$conc)
bartlett's k-squared = 9.1898, df = 6, p-value = 0.1632
```

With the Box-Cox transformation both tests are not significant. Thus, after Box-Cox transformation, the residuals homogeneity assumption is no more rejected.

```
#visual assessment of the fit of the 4 parameters log-logistic model with
box cox transformation#
plot(ryegrass.ll4bc,type="all",col=3,lwd=2)
```
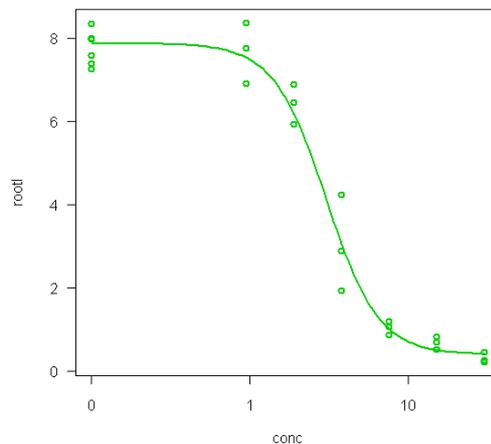

The fit seems to be satisfactory since the curve is closed to all the points.

```
#visual assessment of the fit of the 4 parameters log-logistic model with
box cox transformation by the use of a 'residuals vs. fitted' plot#
plot(fitted(ryegrass.ll4bc),residuals(ryegrass.ll4bc))
abline(h=0,col=2)
```
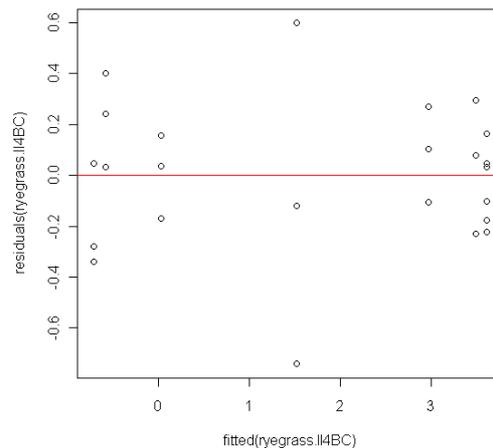
Whatever the fitted value, residual are globally uniformly distributed on both sides of the red line of 0 y-axis. Thus the fit seems satisfactory.

```
#assessment of the fit's quality by 'lack of fit' test #
modelfit(ryegrass.ll4bc)
lack-of-fit test
          modeldf     rss df f value p value
anova           17 1.4292
drc model       20 1.7558  3  1.2949  0.3084
```

Since the p-value is higher than 0.05, the *'Lack of fit'* test is not significant. Thus, the 4 parameters log-logistic model is acceptable. Next, the reduction of the full 4 parameters log-logistic model is going to be undertaken. For this, the 3 parameters log-logistic model (saying that lower limit, e.g. 'c' parameter = 0) is fitted.

```
# fit of the 3 parameters log-logistic model (lower limit, e.g. 'c'
parameter = 0) with box cox transformation #
ryegrass.ll3bc<-drm(rootl~conc,data=ryegrass,bcval=0.5,fct=ll.3())
```

bcVal argument permits to give the Box-Cox lambda value, as it was determined previously when using the boxcox function.

The update function can also be used.

```
ryegrass.ll3bcbis<-update(ryegrass.ll4bc, fct=ll.3())
```

```
# visualization of the fit #
plot(ryegrass.ll3bc,col=3,lwd=2,type="all")
```
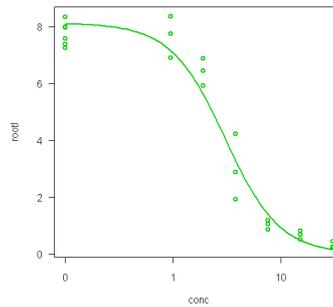
Visually the fit seems to be satisfactory.

```
# comparison of the 4 and 3 parameters log-logistic models' fit by the f
test#
anova(ryegrass.ll4bc,ryegrass.ll3bc)
1st model
 fct:       ll.3()
2nd model
 fct:       ll.4()
anova table

          modeldf     rss df f value p value
2nd model       21 2.8883
1st model       20 1.7558  1 12.9002  0.0018
```

The '*F*' test is significant (p-value < 0.05). The fit of the 3 parameters log-logistic is then significantly less satisfactory than those of the 4 parameters. Thus, it's the 4 parameters log-logistic model which is kept in order to estimate the EDp values.

```
#estimation of the ec10, ec25 and ec50 with their standard error and their
95% confidence interval)
ed(ryegrass.ll4bc,c(10,25,50),interval="delta")
estimated effective doses
(delta method-based confidence interval(s))
   estimate std. error   lower   upper
10  1.30341    0.21405 0.85690 1.7499
25  1.98290    0.21698 1.53029 2.4355
50  3.01662    0.21005 2.57846 3.4548
```

For more details about the ED function and its arguments, see the ED help page using the following command: `?ED`

8.3.5 Example of DRC application to the analysis and modelling of a monotonic dose-response relationship with quantal data in absence of overdispersion

The data used in this example are those of the 'earthworms' dataset included in the *'drc' package*. More derails about this dataset are given in its help page, which can be consulted using `?earthworms` command.

```
# loading of the 'drc' package #
library(drc)

ew<-earthworms # permits to give a shorter name to the dataet #
ew #display of the dataset#
   dose number total
1  0.00      3     5
2  0.00      3     5
3  0.00      3     4
4  0.00      5     8
5  0.00      4     8
6  0.19      4    11
7  0.19      4     9
8  0.19      2     8
9  0.19      3     8
10 0.19      2     4
11 0.38      2     9
12 0.38      0     4
13 0.38      6    11
14 0.38      4    11
15 0.38      4    15
16 0.76      0    11
17 0.76      1     6
18 0.76      1     8
19 0.76      0     9
20 0.76      0     8
21 1.53      0     6
22 1.53      1    10
23 1.53      0    10
24 1.53      0    10
25 1.53      0     8
26 3.05      0     5
27 3.05      0     9
28 3.05      0     7
29 3.05      0     8
30 3.05      1     8
31 6.11      0     3
32 6.11      0     7
33 6.11      0    11
34 6.11      0    10
35 6.11      1     7

# visualization of the data #
plot(ew$dose,ew$number/ew$total)
```
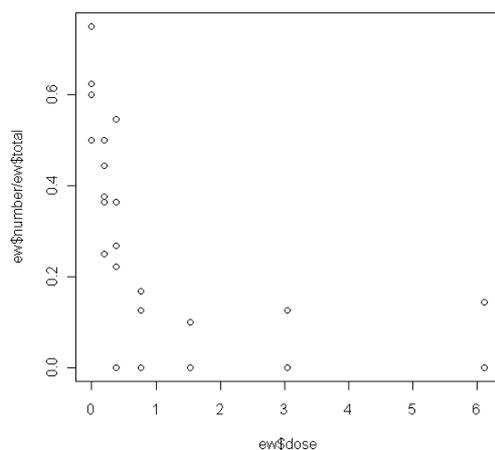
```
#fit of the full 4 parameters log-logistic model#
ew.ll4<-drm(number/total~dose, weight=total, data=ew,
type="binomial",fct=ll.4())

#display of the estimates' parameters#
summary(ew.ll4)
model fitted: log-logistic (ed50 as parameter) (4 parms)
parameter estimates:
                estimate std. error  t-value    p-value
b:(intercept) 4.243855   3.155072 1.345090   0.1786
c:(intercept) 0.023154   0.014404 1.607473   0.1080
d:(intercept) 0.489633   0.083342 5.874965 4.229e-09
e:(intercept) 0.417026   0.081438 5.120804 3.042e-07
```

When observations are quantal data, it is needed to use the `type="binomial"` argument in the `drm` function in order to precise the type of the data. Moreover, it is also needed to precise the number of individual through the `weight=total` argument. This information is used by the likelihood method to estimate the model's parameters and above all their standard error; since a percentage of event observed with 2 individuals is not the same situation than a percentage observed with 200 individuals.

```
# visual assessment of the fit's quality #
plot(ew.ll4,type="all", col=3,lwd=2)
```
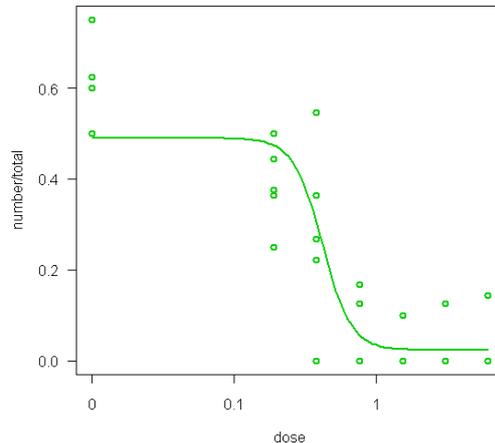

Globally, the fit seems to be good despite a weakness at the control level.

```
# other visual assessment of the model's fit quality by a residuals vs.
predicted values plot#
plot(fitted(ew.ll4),residuals(ew.ll4))
abline(h=0,col=2,lwd=2)
```
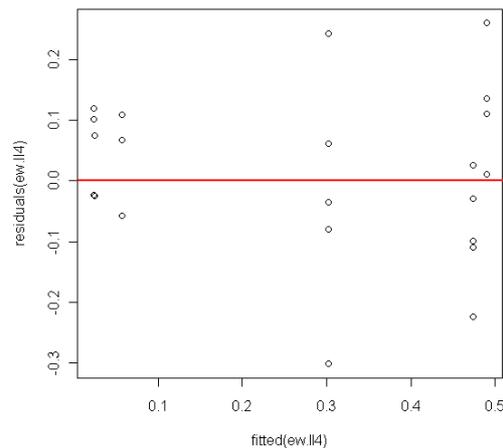
For each level of predicted response (abscissa axis), globally residuals are uniformly spread both side of the red line (0 y axis) ; except those concerning the higher predicted percentage for which all the residuals are higher than 0. This phenomenon was already observed in the previous graph.

```
#assessment of the fit(s quality by a 'goodness of fit' test #
modelfit(ew.ll4)
goodness-of-fit test
          df chisq value p value

drc model   31     26.444  0.6998
```

The *'Goodness of fit'* test is not significant (p-value > 0.05). Taking in account this result and those of the previous graph, the 4 parameters log-logistic model seems satisfactory.

```
# asessement of a possible overdispersion #
```

$R = \dfrac{26.444}{31} \approx 1$ **.** No overdispersion is brought to light. thus the conclusion of the *'goodness of fit'*

test remains valid.

```
# first reduction of the full 4 parameters log-logistic model#
# fit of the first restricted model, i.e., the 3 parameters log-logistic
model for which the lower limit ('c'parameter is fixed to 0) #
ew.ll3<-update(ew.ll4,fct=ll.3()) # the update function permits tofit
very easily a model from a previous' one#


summary(ew.ll3)
model fitted: log-logistic (ed50 as parameter) with lower limit at 0
(3 parms)
parameter estimates:
             estimate std. error  t-value    p-value
b:(intercept) 1.505679   0.338992 4.441641 8.928e-06
d:(intercept) 0.604929   0.085800 7.050498 1.783e-12
```

```
e:(intercept) 0.292428   0.083895 3.485636     5e-04
```

```
# visulaization of the ew.ll3 model's fit#
plot(ew.ll3, col=3,lwd=2,type="all")
```
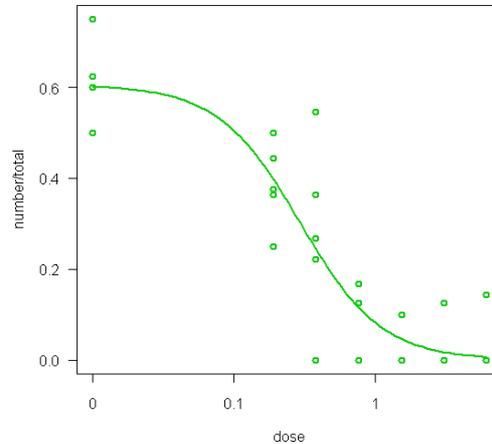

This fit is more satisfactory at the control level (dose=0) than the fit of the full 4 parameters log-logistic model.

Since no overdispersion was highlighted with the full model, fits of the 4 and 3 parameters log-logistic model can be compared by the *'likelihood ratio'* test.

```
# likelihood ratio test#
anova(ew.ll4,ew.ll3)
1st model
 fct:      ll.4()
2nd model
 fct:      ll.3()
anova-like table
          modeldf  loglik df lr value p value
1st model       4 -34.670
2nd model       3 -36.155  1   2.970  0.0848
```

Since the test is not significant (p-value≥0.05) the fit of the 3 parameters log-logistic model is as satisfactory as those of the 4 parameters log-logistic model. According the Parsimony rule, the 3 parameters log-logistic model is kept, and a second reduction is going to be done.

```
# second reduction of the full 4 parameters log-logistic model#
#fit of the 2nd restricted model, i.e., fit of the 2 parameters log logistic
model (e.g. with lower limit (c parameter) = 0 and higher limit (d
parameter) =1 #
ew.ll2<-update(ew.ll4,fct=ll.2())
```

```
# display of the estimates#
summary(ew.ll2)
model fitted: log-logistic (ed50 as parameter) with lower limit at 0
and upper limit at 1 (2 parms)
```

```
parameter estimates:
                estimate std. error  t-value   p-value
b:(intercept) 1.260321   0.246707 5.108564 3.246e-07
e:(intercept) 0.145140   0.036797 3.944389    1e-04


# visualization of the plot #
plot(ew.ll2, col=3,lwd=2,type="all")
```
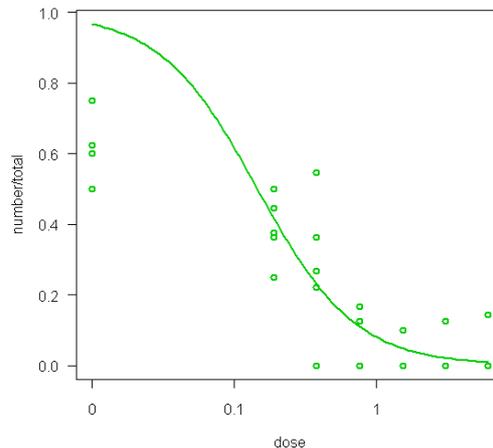

The fit of this model is not satisfactory at the control's level.

```
# comparison of the 3 and 2 parameters log-logistic models #
anova(ew.ll2,ew.ll3)
1st model
 fct:       ll.2()
2nd model
 fct:       ll.3()
anova-like table
          modeldf  loglik df lr value p value
1st model       2 -347.55
2nd model       3  -36.16  1   622.79        0
```

The test is significant (p-value ≈0), then the 2 parameters log logistic model's fit is not as satisfactory as those of the 3 parameters model. Thus, the 2 parameters model is rejected and the 3 parameters model is kept. And then, the 3 parameters model will be used to estimate the EDp parameters.

```
# estimation of the ed5, ed30 et ed85, for example#
ed(ew.ll3,c(5,30,85),interval="delta")
estimated effective doses
(delta method-based confidence interval(s))

     estimate std. error      lower   upper
5   0.041374   0.027118 -0.011776 0.0945
30  0.166581   0.062886  0.043327 0.2898
85  0.925430   0.225137  0.484169 1.3667
```

8.3.6 Example of DRC application to the analysis and modelling of a monotonic dose-response relationship with quantal data in presence of overdispersion

The data used in this example are the ones of the '*deguelin*' dataset included in the '*drc*' package. These data come from a study which aim is to assess the action of the *deguelin* on the *Macrosiphoniella sanborni* species. The predictive variable is the insecticide dose, 6 doses were used. The total number of insects put in touch with the insecticide is reported in the variable 'n'. The number of dead insects is reported in the variable 'r'. The observed and modelled variable is the percentage of dead insects ('n'/ 'r'). For each dose, there are no replicates. Finally the dataset contains 6 couples of dose-response data. In the presence of replicates the approach would be strictly identical to that presented below.

```
library(drc)# loading of the 'drc' package#
deg<-deguelin #permits to give a shorter name to the dataset#
deg #displaying of the dataset#
       dose log10dose  r  n
1  5.128614      0.71 16 49
2 10.000000      1.00 18 48
3 20.417379      1.31 34 48
4 30.199517      1.48 47 49
5 40.738028      1.61 47 50
6 50.118723      1.70 48 48

# visualization of the data #
plot(deg$dose,deg$r/deg$n,lwd=2, col=3)
```
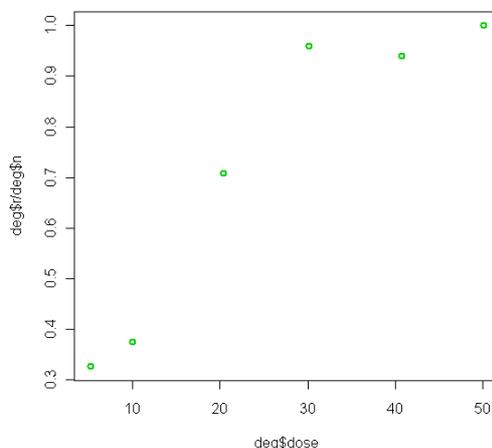


```
# fit of the full 4 parameters log-logistic model #
deg.ll4<-drm(r/n~dose, weight=n, data=deg, type="binomial",fct=ll.4())
summary(deg.ll4)
model fitted: log-logistic (ed50 as parameter) (4 parms)
parameter estimates:
             estimate std. error  t-value    p-value
b:(intercept) -3.46316    4.56542 -0.75856    0.4481
c:(intercept)  0.31003    0.12610  2.45854    0.0140
d:(intercept)  1.01870    0.16907  6.02548 1.686e-09
e:(intercept) 18.50393    2.33375  7.92882 2.216e-15
```

When observations are quantal data, it is necessary to use the `type="binomial"` argument in the `drm` function in order to precise the type of the data. It is also necessary to give the total number of observations by the way of the `weight=n` argument. *'drc'* package needs both information in order to give an adequate estimation of the parameters, and above all of their standard error.

```
# visual assessment of the fit's quality#
plot(deg.ll4,lwd=2,col=3)
```
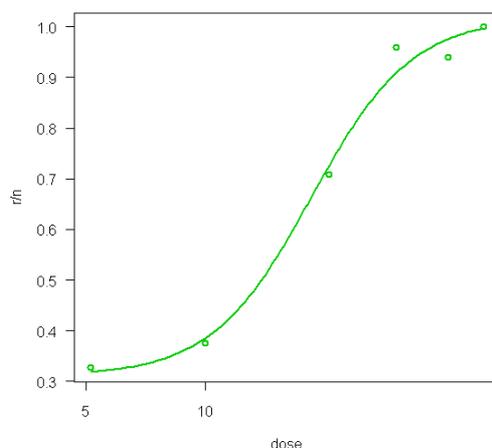

Globally, the fit seems satisfactory.

```
# visual assessment of the fit's quality by a 'residuals vs fitted' plot #
plot(fitted(deg.ll4),residuals(deg.ll4))
abline(h=0,col=2,lwd=2)
```

(D-N°:5.1) – Experimental plan
Dissemination level: PU
Date of issue of this report: **31/10/2011**
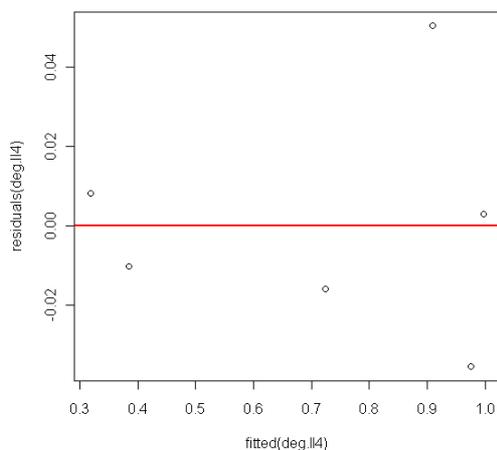
Globally, the residuals are randomly distributed on both sides by of the line of 0 Y-axis, thus the fit seems satisfactory.

```
#assessment of the quality of the fit by a 'goodness of fit' test #
modelfit(deg.ll4)
goodness-of-fit test
          df chisq value p value
drc model    2      4.3622  0.1129
```

The *'Goodness of fit'* test is not significant (p-value≥0.05). Taking in account this result and the previous graphs, the fit of the full 4 parameters log-logistic model is satisfactory.

```
#assessment of the possible overdispersion #
```

$R = \dfrac{4.3622}{2} \approx 2.18$. The ratio is widely higher than 1, thus an overdispersion is highlighted. This has three major consequences:

- the *'Goodness of fit'* test previously done is not valid. Nevertheless, the quality of the fit is always considered as satisfactory considering the visual assessment.

- the *'likelihood ratio'* test permitting to compare the fit of two nested models couldn't be used ;

It will be necessary to specify the presence of the overdispersion in the `summary` and `ED` functions, using the `od=T` argument, in order to apply a correction to the parameters standard error's estimates.

```
#displaying of the parameters'estimates#
summary(deg.ll4, od=t)
model fitted: log-logistic (ed50 as parameter) (4 parms)
parameter estimates:
              estimate std. error  t-value    p-value
b:(intercept) -3.46316   6.74243 -0.51364    0.6075
c:(intercept)  0.31003   0.18623  1.66472    0.0960
d:(intercept)  1.01870   0.24968  4.07996 4.504e-05
e:(intercept) 18.50393   3.44660  5.36875 7.929e-08
```

From these estimations, two remarks can be done. Firstly, since 'd' parameter's estimate is slightly superior to 1, then the higher limit seems to be able to be fixed to 1. Secondly, the lower limit seems to be able to be fixed to 0 because the p-value of the corresponding *'t'* test is not significant (p–value ≥ 0.05).

```
#first reduction of the full model – fit of the 3 parameters log-logistic
model fixing the higher limit ('d' parameter) to 1 #
```

```
deg.ll3u<-update(deg.ll4,fct=ll.3u()) # the update function permits to
easily fit a new model from a previous one#
summary(deg.ll3u, od=t)
model fitted: log-logistic (ed50 as parameter) with upper limit at 1
(3 parms)
parameter estimates:
                estimate std. error   t-value    p-value
b:(intercept) -4.136805   1.154850 -3.582116      3e-04
c:(intercept)  0.323801   0.068429  4.731937 2.224e-06
e:(intercept) 18.603779   2.522874  7.374041 1.655e-13
```

Having fixed the 'd' parameter to 1, the 't' test concerning the parameter 'c' is become significant (p <0.05). Thus, It is not justified to reduce once again the model by fixing the lower limit to 0. The reduction of the full model stops in this stage. The ECp parameters will be estimated from the 3 parameters log-logistic model.

```
# visulization of the ew.ll3's fit #
plot(deg.ll3u, col=3,lwd=2,type="all")
```
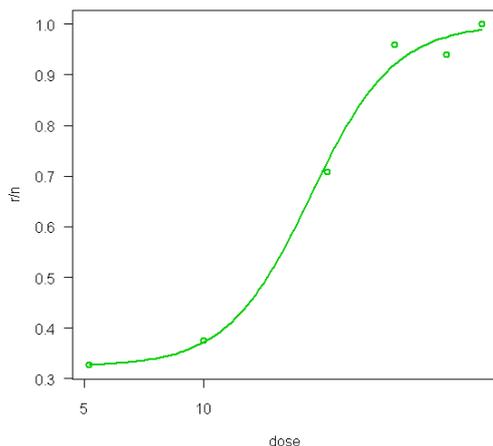

Visually, the fit of the 3 parameters log-logistic model is satisfactory. By the end ECp% are estimated from this 3-parameters log-logistic model.

```
# for example, estimation of the ed5, ed30 and ed85#
ed(deg.ll3u,c(5,30,85), od=t, interval="delta")
estimated effective doses
(delta method-based confidence interval(s))
    estimate std. error    lower  upper
5     9.1303     2.8536   3.5374 14.723
30   15.1583     2.7584   9.7519 20.565
85   28.2947     2.6303 23.1394 33.450
```

8.4 Hormetic models

8.4.1 Strategy of analysis and modelling of hormetic relationship, when observed response are continuous data

The first aim of the hormetic dose-response modelling is generally to assess the significance of the hormesis effect. This assessment is based on the comparison of the fits of a 'hormesis' model and its corresponding log-logistic model. Indeed, these models are nested (cf. 0), and their difference comes only from the added 'f' parameter of the 'hormesis' model, which characterizes this effect.

Hormesis dose-response relationship modelling, in case of continuous observations, contains 7 steps:

1) Fit of all the available full hormesis models (5 parameters models)

2) Selection of the best full hormesis model using model's fit visualization and / or lowest residual variance

3) Assessment of the residual normality and homogeneity assumptions (with use of a Box-Cox transformation if needed)

4) Assessment of the quality of the model

5) If possible, reduction of the best full hormesis model

6) Assessment of the hormesis effect by comparison of the fits of the 'hormesis' and corresponding log-logistic models by 'F test' for nested models

7) Estimation of the EDp and their standard error (see below).

The strategy of analysis and modelling of hormetic dose-response relationship with continuous observations is summarized in Figure 8.8.

Fit of all the available full hormesis models

↓

Selection of the best full hormesis model
(visual assessment, lowest residual variance)

↓

Assessment of the residual normality and homogeneity assumptions

At least one of the two assumptions are rejected ← → Both assumptions are accepted

↓

Assessment of the model's fit quality

Not satisfactory fit ← → Satisfactory fit

Fit of another type of full hormesis model ← → Theoretical arguments to continue

↓

If possible, reduction of the best full 'hormesis' model (fit of restricted models)

↓

Comparison of full and restricted models' fit

Non significant 'F' Test
(p-value ≥ 0.05)
the restricted model is kept

Significant 'F' Test
(p-value < 0.05)
the full model is kept

↓

Fit of the 3 parameters log-logistic model

Fit of the 4 parameters log-logistic model

↓

Comparison of the fits of the kept hormesis and log-logistic models

Non Significant 'F' Test
(p-value ≥ 0.05)
hormesis effect is not significant

Significant 'F' Test '
(p-value < 0.05)
hormesis effect is significant

↓

Estimation of EDp and their standard error from the kept log-logistic model

Estimation of EDp and their standard error from the kept hormesis model

Figure 8.8: Strategy of analysis and modelling of hormesis dose-response relationships with continuous data.

The number of full 'hormesis' models which can be fitted depends on the shape of the dose-response relationship to be modelled (cf. Figure):

- If the relationship has an 'inverted U' shape, then 4 full 'hormesis' models (with 5 parameters) can be fitted:

    - the Brain-Cousens' model (BC.5),

    - the 3 models of Cedergreen-Ritz-Streibig : CRS.5a, CRS.5b, CRS.5c.

- If the relationship has a 'U' shape :

    - only the 3 full Cedergreen-Ritz-Streibig's models: UCRS4a, UCRS4b, UCRS4c.

The use of these models can leads to more or less satisfactory fit, but difficulty predictable. Our approach will be to try them all, and to use the one which is better adapted to the data. Since these models are not nested, their fits can't be directly compared by a statistical test. The kept full 'hormesis' model will be the one for whom the fit will be visually the best. Generally this model has also the lower residual variance. In case of not concordance it's better to choose the model having visually the best fit (cf.annexe 8.4.4)

Assessment of residual normality and homogeneity assumptions and Box-Cox transformation are described in paragraph 8.3.1. It is important to note that after the use of a Box Cox transformation it is needed to visualize the fit of the best full 'hormesis' model in order to check that the hormesis effect is still well fitted (cf.annexe 8.4.4)

Assessment of the quality of the fit is generally done visually and completed by a '*Lack of fit*' test. These approaches are described in details in paragraph 8.3.1.

Reduction of full 'hormesis' model concerns only the 'inverted U' shaped relationship. Firstly, the 4 parameters sub-model (e.g. with 'c' parameter =0) corresponding to the kept 5 parameters 'hormesis' model (the best full model), is fitted. Secondly, the fits of the 5 parameters and 4 parameters hormesis models are compared by an 'F' test for nested models:

- If the test is not significant (p-value ≥ 0.05), then the fit of the 4 parameters model is as satisfactory that the one of the 5 parameters model, and so the restricted model is kept (according to the Parsimony rule).

- On the contrary, if the test is significant, (p-value<0.05), then the fit of the 4 parameters 'hormesis' model is less satisfactory than the one of the 5 parameters model, and so the full model is kept.

It is important to note that it is needed to visualize the fit of the reduced model in order to check that the 'hormesis' effect is still well described.

Assessment of the 'hormesis' effect is based on the comparison of the fits of the 'hormesis' model and its corresponding log-logistic model. Hence, at first it is question of fit the log-logistic model corresponding to the kept 'hormesis' model:

- if the relationship has an 'inverted U' shape, it is :

- the 4 parameters log-logistic model if the kept 'hormesis' model contains 5 parameters.

- the 3 parameters log-logistic model if the kept 'hormesis' model contains 4 parameters (lower limit, e.g. 'c' parameter = 0).

- if the relationship has a 'U' shape, it is obligatorily the 4 parameters log-logistic model.

In a second phase, fits of the kept 'hormesis' model and its corresponding log-logistic model are compared, using an 'F' test for nested models (cf. paragraph 3.2.3).

- if the test 'F' is not significant (p-value ≥ 0.05) then the 'f' parameter of the 'hormesis' effect is not significant. Thus the hypothesis of a 'hormesis' effect is rejected.

- if the test 'F' is significant (p-value <0.05) then the 'f' parameter of the 'hormesis' effect is significant. Thus the hypothesis of a 'hormesis' effect is not rejected.

Estimates of EDp% of the Brain-Cousens model are obtained solving the following equation (Cedergreen en al, 2005):

$$\frac{d - c + fEDp}{1 + \exp[b(\log(EDp) - \log(e))]} = c + \left(1 - \frac{p}{100}\right)(d - c) \text{ Equation 19}$$

Estimates of EDp% of the Cedergreen-Ritz-Streibig models are obtained solving the following equation (Cedergreen en al, 2005):

$$c + \frac{d - c + f \exp(-1/x^{\alpha})}{1 + \exp[b(\log(x) - \log(e))]} = c + \left(1 - \frac{p}{100}\right)d \text{ Equation 20}$$

In both cases, uncertainty of EDp% is estimated using the delta-method (Cedergreen en al, 2005).

An example of a complete analysis and modelling of a 'hormetic' dose-response relationship with continuous observation is given in annexe 8.3.4.

8.4.2 Strategy of analysis and modelling of hormetic relationship, when observed response are quantal data

Definition of quantal data is given in paragraph 8.3.2. A reminder of the estimation of the parameters, their uncertainty and the hypotheses needed for their validity is present in the same paragraph.

The first aim of the of hormetic dose-response modelling is generally to assess the significance of the hormesis effect. This assessment is based on the comparison of the fits of a 'hormesis' model and its corresponding log-logistic model. Indeed, these models are nested (cf. paragraph 3.2.3), and their difference comes only from the added 'f' parameter of the 'hormesis' model which characterizes this effect.

Hormesis dose-response relationship modelling, in case of quantal observations, contains 7 main steps:

1) Fit of all the available full hormesis models (5 parameters) ;

2) Selection of the best full hormesis model using model's fit visualization and / or highest log-likelihood value;

3) Assessment of the quality of the model ;

4) Assessment of a possible overdispersion ;

5) If possible, reduction of the best full hormesis model ;

6) Assessment of the hormesis effect by comparison of the fits of the 'hormesis' and corresponding log-logistic models by 'F test' for nested models ;

7) Estimation of the EDp% and their standard error.

The management of steps 5 ad 6 will be different if an overdispersion is highlighted or not, at the step 4.

The strategy of analysis and modelling of hormetic dose-response relationship with quantal observation and when no overdispersion has been highlighted is summarized in the figure 8.9.

Fit of all the full 'hormesis' model available

Selection of the best full 'hormesis' model
(visual assessment, log likelihood)

Assessment of the quality of the model

Not satisfactory fit

Satisfactory fit

Fit of another type of
full 'hormesis' model

Theoric argument
(biological, historical)
to continue

Assessment of a possible
overdispersion

Overdispersion not
highlighted

Overdispersion is
highlighted

Reduction of the model
(fit of a restricted model corresponding to
the full selected model)

cf. Figure 8.10

Comparison of the full and restricted
models' fits.

Not significant 'likelihood ratio' test
(p-value≥0.05)
The restricted model is kept

Significant 'likelihood ratio' test
(p-value<0.05)
The full model is kept

Fit of the 3 parameters log-
logistic model

Fit of the 4 parameters log-
logistic model

Comparison of the kept hormesis and log-logistic models' fits

Non significant likelihood ratio test
(p-value ≥0.05) hormesis effect is not
significant

Significant likelihood ratio test
(p-value <0.05) hormesis effect is
significant

Estimation of the ECp% and their
uncertainty from the kept log-logistic
model

Estimation of the ECp% and their
uncertainty from the kept hormesis
model

Figure 8.9 : Strategy of analysis and modelling of 'hormetic' dose-response relationships with quantal data, when there is no overdispersion.

The management of steps 5 ad 6 is different if an overdispersion is highlighted or not at the step 4.

First of all, in case of quantal (binary) observations, independence hypothesis is accepted or rejected only on the basis of the experimental design. Then, it is needed to precise the quantal type of the observation using the type="binomial" argument in the drm function. It is also necessary to give the total number of observations by the way of the weight=n argument. 'drc' package needs both information in order to give an adequate estimation of the parameters, and above all of their standard error.

The number of full 'hormesis' models which can be fit depends on the shape of the dose-response relationship to be modelled (cf. Figure):

- If the relationship has an 'inverted U' shape, then 4 full 'hormesis' models (with 5 parameters) can be fitted:

  - the Brain-Cousens' model (BC.5),

  - the 3 models of Cedergreen-Ritz-Streibig: CRS.5a, CRS.5b, CRS.5c.

- If the relationship has a 'U' shape :

  - only the 3 full Cedergreen-Ritz-Streibig's models: UCRS4a, UCRS4b, and UCRS4c.

The use of these models can leads to more or less satisfactory fit, but difficulty predictable. Our approach will be to try them all, and to use the one which is better adapted to the data. Since these models are not nested, their fits can't be directly compared by a statistical test. The kept full 'hormesis' model will be the one for whom the fit will be visually the best. Generally this model has also the higher log-likelihood value. In case of not concordance it's better to choose the model having visually the best fit.

Assessment of the fit's quality is generally done visually. Thus, two plots can be done: the first one showing the observed data with the fitted model, the other being a plot of the residuals vs. the fitted values. This visual assessment can be completed by a '*Goodness of fit*' test (cf. paragraph 8.3 .2).

The presence of a possible overdispersion (defined as an observed variance higher than the variance predicted by the Binomial distribution) has to be assessed. Indeed, in case of overdispersion the results of the 'likelihood ratio' test and of the 'Goodness of fit' test are not valid, and the estimation of the parameters' uncertainty are biased. The described approach consists in comparing the ratio (ϕ) of the Q statistic over its degrees of freedom, to 1 (cf Equation 17). If the ratio approximately equals 1, then no overdispersion is highlighted. If the ratio is higher than 1, then an overdispersion is highlighted.

In case of overdispersion, estimation of parameters' uncertainty has to be corrected using the od=T argument in the summary and ED functions.

If there is no overdispersion is highlighted, the reduction of the full model contains the following several steps:

- In a first time, it is question of fitting the restricted 4 parameters model. When observed responses are quantal data, reduction concerns both shapes of curves :

  - when the relationship has a 'inverted U' shape, the restricted model is the one specifying that the lower limit ('c' parameter) =0, (cf. Figure).

  - when the relationship has a 'U' shape, the restricted model is the one specifying that the higher limit ('d' parameter) =1 (cf. Figure).

In a second time, since full and restricted 'hormesis' models are nested, their fits are compared by the 'likelihood ratio' test :

  - if the test is not significant (p-value≥0.05), then the fit of the restricted 4 parameters model is as satisfactory as the fit of the full 5 parameters model. According the Parsimony rule, the restricted 4 parameters model is kept.

  - if the test is significant (p-value<0.05), then the fit of the restricted 4 parameters model is less satisfactory than the fit of the full 5 parameters model. Thus the kept model is the 5 parameters model.

Assessment of the hormesis effect is based on the comparison of the fits of the 'hormesis' model and its corresponding log-logistic model:

- In a first time, it's question of fitting the log-logistic model corresponding to the kept hormesis model. This is :

  - the 4 parameters log-logistic model if the kept 'hormesis' model has 5 parameters;

  - the 3 parameters log-logistic model if the kept 'hormesis' model is a restricted one with 4 parameters. In this case, if the curve has a :

    - 'inverted U' shape, then the lower limit ('c'' parameter) is fixed to 0 (cf.Figure);

    - 'U' shape, then the higher limit ('d' parameter) is fixed to 1 (cf.Figure).

- In a second time, since the 'hormesis' and log-logistic models are nested, their fits are compared by a 'likelihood ratio' test :

  - If the test is not significant (p-value≥0.05), then 'f' parameter of the hormesis effect is not significantly different from 0. In other words the presence of a hormesis effect is rejected.

  - On the contrary, if the test is significant (p-value<0.05), then 'f' parameter of the hormesis effect is significantly different from 0. In other words the presence of a hormesis effect is accepted.

Estimation of the EDp% is described in the paragraph 8.4.1.

The strategy of analysis and modelling of hormetic dose-response relationship with quantal observation and **when an overdispersion has been highlighted** is summarized in the figure 8.10.

Fit of all the available full 'hormesis' models

Selection of the best full 'hormesis' model
(visual assessment + 'log-likelihood test

Assessment of the model's fit quality

Not satisfactory fit   Satisfactory fit

Fit of another full   Theoretical arguments   Assessment of a possible
'hormesis' model   to continue   overdispersion

No overdispersion   overdispersion
highlighted   highlighted
(cf.Figure)

Reduction of the model : fit of the restricted model corresponding to the full selected model:
- 'inverted U' shape : assessment of the possibility to fix the lower limit ('c' parameter) to 0, using the p-value of the t test of the `summary` function used with the `od=T` argument.
- 'U' shape : assessment of the possibility to fix the higher limit ('d' parameter) to 1 by visual comparison of the fits of the full and restricted models + consideration of the higher limit estimate ('d' parameter) in the full model

Reduction done   Reduction not done

Assessment of the hormesis from the 4 parameters model using `od=T` in the `summary` function

Assessment of the hormesis effect from the 5 parameters model using `od=T` in the `summary` function

| 't' test corresponding to the 'f' parameter is non significant (pvalue$\geq$0.05) | 't' test corresponding to the 'f' parameter is significant (pvalue$<$0.05) | 't' test corresponding to the 'f' parameter is non significant (pvalue$\geq$0.05) | 't' test corresponding to the 'f' parameter is significant (pvalue$<$0.05) |

| Not significant hormesis effect | significant hormesis effect | Not significant hormesis effect | significant hormesis effect |

| Estimation of the EDp% from the reduced log-logistic model | Estimation of the EDp% from the reduced hormesis model | Estimation of the EDp% from the full log-logistic model | Estimation of the EDp% from the full hormesis model |

Figure 8.10 : Strategy of analysis and modelling of 'hormetic' dose-response relationship with quantal data, when there is an overdispersion.

When an overdispersion has been highlighted, the result of the 'likelihood ratio' test used to compare the fit of two nested models is no valid anymore. Thus, the strategy used to reduce the full hormesis model is rougher and is different according to the shape of the dose-response relationship to be modelled:

- when the relationship has a 'inverted U' shape, the possibility to fix the lower limit ('c' parameter) will be assessed using the p-value of the 't' test given by the `summary` function, used with the `od=T` argument. If the test is not significant (p-value ≥0.05), then the restricted 4 parameters model is kept. In the inverse case, it's the 5 parameters model which is kept.

- when the relationship has a 'U' shape, the possibility to fix the upper limit ('d' parameter) will be assessed comparing the fits of the 5 and 4 parameters 'hormesis' model. The estimate of the 'd' parameter can also be used. For example, if the estimate is higher than 1, or slightly lower than 1, then the upper limit could be fixed to 1. And the 4 parameters model is kept.

Concerning the assessment of the hormesis effect, when an overdispersion has been previously highlighted, the comparison of the hormesis and log-logistic models' fit can't be done directly by the 'likelihood ratio' test. Thus, in this situation, the hormesis effect can be assessed using the p-value of 't' test corresponding to the 'f' parameter provided by the `summary` function, used with the `od=T` argument :

- If the test is not significant, then the hormesis effect will be considered as non significant, and the restricted model will be is for the estimation of the EDp%.

- In the inverse case, hormesis effect will be considered as significant and the 5 parameters model will be used to estimate the EDp%.

Estimation of the EDp% is described in the paragraph 8.4.1.

8.4.3 Different hormesis situations and corresponding Brain-Cousens and Cedergreen-Ritz-Streibig models

	Inverted U shaped curves	
Name of the models	Continuous data	Quantal data
CRS.5a() CRS.5b() CRS.5c() BC.5()		
CRS.4a() CRS.4b() CRS.4c() BC.4()		
	U shaped curves	
	Continuous data	Quantal data
UCRS.5a() UCRS.5b() UCRS.5c()		
No models directly available		
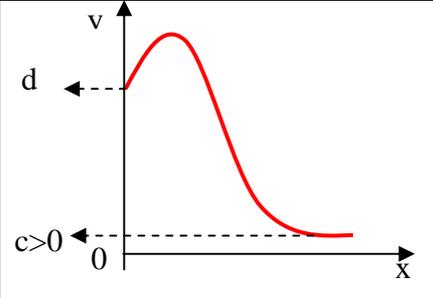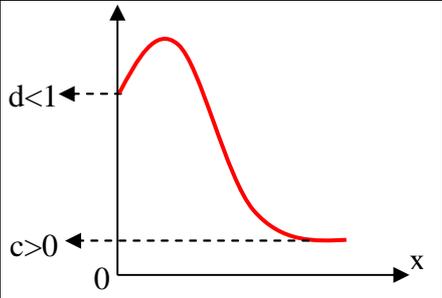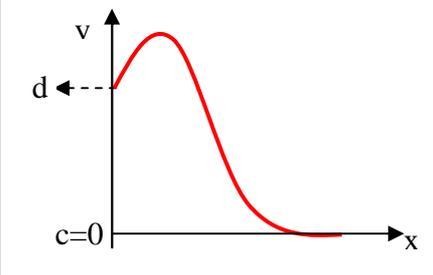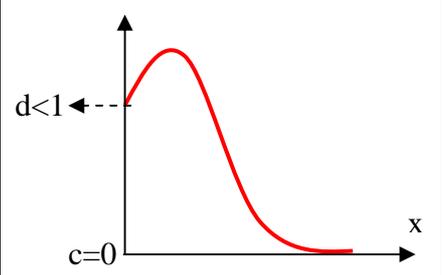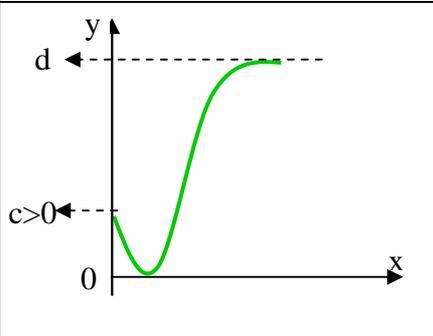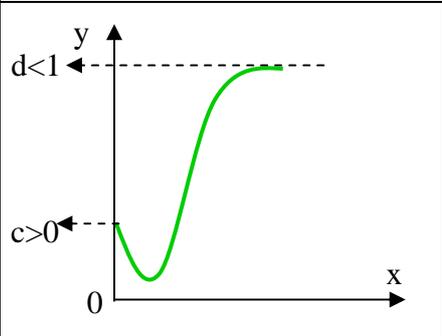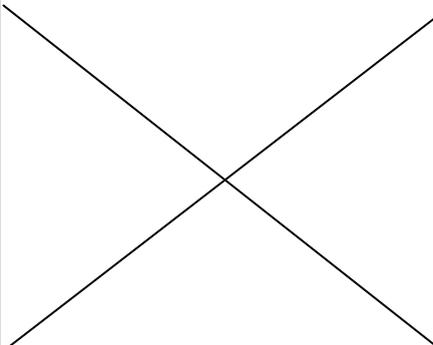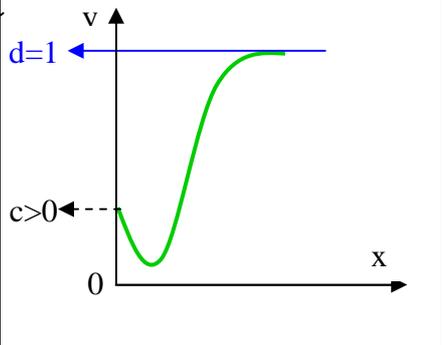
Figure 8.11 : available models in 'drc' to analyse hormetic dose-response relationships according the type of the observations and the shape of the relationship.

8.4.4 Example of DRC application to the analysis and modelling of a hormesis dose-response relationship with continuous data

Data used in this example arte those of the '*lettuce*' dataset included in the '*drc*' package. These data come from an experiment which aim was to assess the inhibitory action of the *isobutanol* (dissolved in a solution of nutriments) on the biomass of lettuces, measured after 21 days of exposure.

The observed variable is the mass of lettuce leaves ('weight' variable). Seven concentrations of *isobutanol* have been tested. Two replicates are reported for each concentration. Finally the dataset is composed of 14 dose-response couple.

```
#opening of the help page of the lettuce dataset#
?lettuce
# display of the dataset#
lettuce
      conc weight
1     0.00  1.126
2     0.00  0.833
3     0.32  1.096
4     0.32  1.106
5     1.00  1.163
6     1.00  1.336
7     3.20  0.985
8     3.20  0.754
9    10.00  0.716
10   10.00  0.683
11   32.00  0.560
12   32.00  0.488
13  100.00  0.375
14  100.00  0.344

# visualization of the experimental points #
plot(lettuce$conc,lettuce$weight,log="x")
```
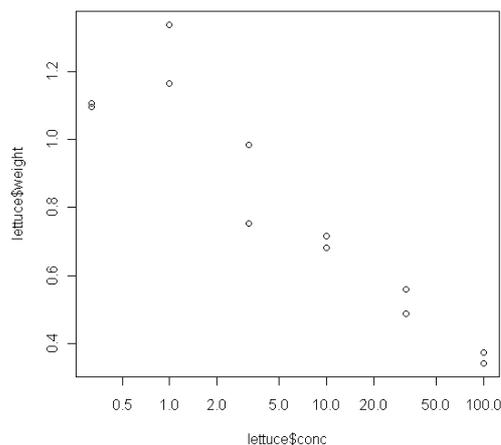


```
#fit of the de 5 parameters brain-cousens' model #
lettuce.bc5<-drm(weight~conc,data=lettuce,fct=bc.5())
```

```
summary(lettuce.bc5)
model fitted: brain-cousens (hormesis) (5 parms)
parameter estimates:

                estimate  std. error   t-value    p-value
b:(intercept)   1.502065   0.352231    4.264439    0.0021
c:(intercept)   0.280173   0.248569    1.127142    0.2888
d:(intercept)   0.963030   0.078186   12.317149  6.164e-07
e:(intercept)   1.120457   0.612908    1.828100    0.1008
f:(intercept)   0.988182   0.776136    1.273207    0.2348

residual standard error:
 0.1149117 (9 degrees of freedom)

# fit of the 5 parameters cedergreen-ritz-streibig's model with alpha=1#

lettuce.crs5a<-drm(weight~conc,data=lettuce,fct=crs.5a())


summary(lettuce.crs5a)
model fitted: cedergreen-ritz-streibig (5 parms)
parameter estimates:

                estimate  std. error   t-value    p-value
b:(intercept)   1.334285   0.358663    3.720169    0.0048
c:(intercept)   0.448019   0.080675    5.553390    0.0004
d:(intercept)   1.035664   0.077329   13.392999  3.006e-07
e:(intercept)   1.336143   1.184243    1.128267    0.2884
f:(intercept)   1.996199   2.021349    0.987558    0.3492

residual standard error:
 0.1305057 (9 degrees of freedom)

# fit of the 5 parameters cedergreen-ritz-streibig's model with alpha=0.5#

lettuce.crs5b<-drm(weight~conc,data=lettuce,fct=crs.5b())
summary(lettuce.crs5b)
model fitted: cedergreen-ritz-streibig (5 parms)
parameter estimates:

                estimate  std. error   t-value    p-value
b:(intercept)   0.834820   0.459691    1.816046    0.1027
c:(intercept)   0.326694   0.170299    1.918358    0.0873
d:(intercept)   0.970761   0.081559   11.902501  8.254e-07
e:(intercept)   0.959902   2.407158    0.398770    0.6994
f:(intercept)   2.927607   5.229475    0.559828    0.5893
residual standard error:
0.1170469 (9 degrees of freedom)

# fit of the 5 parameters cedergreen-ritz-streibig's model with alpha=0.25#

lettuce.crs5c<-drm(weight~conc,data=lettuce,fct=crs.5c())
summary(lettuce.crs5c)
model fitted: cedergreen-ritz-streibig (5 parms)
parameter estimates:
                estimate  std. error   t-value    p-value
b:(intercept)   0.981944   0.559332    1.755565    0.1130
c:(intercept)   0.336670   0.182883    1.840906    0.0988
d:(intercept)   0.969845   0.088261   10.988317  1.624e-06
e:(intercept)   3.883893   2.462305    1.577340    0.1492
f:(intercept)   1.027935   0.766821    1.340516    0.2129
```

```
residual standard error:
0.1256841 (9 degrees of freedom)
```

The lowest residual standard error is the one of the Brain-Cousens' model. Nevertheless, in order to select the best 5 parameters 'hormesis' model, it is needed to visualize the fit of those 4 'hormesis' models.

```
# splitting of the graphical window in 4 parts, 2 by column and 2 by raw #
par(mfrow=c(2,2))
plot(lettuce.bc5,type="all")
plot(lettuce.crs5a, type="all")
plot(lettuce.crs5b, type="all")
plot(lettuce.crs5c, type="all")
```
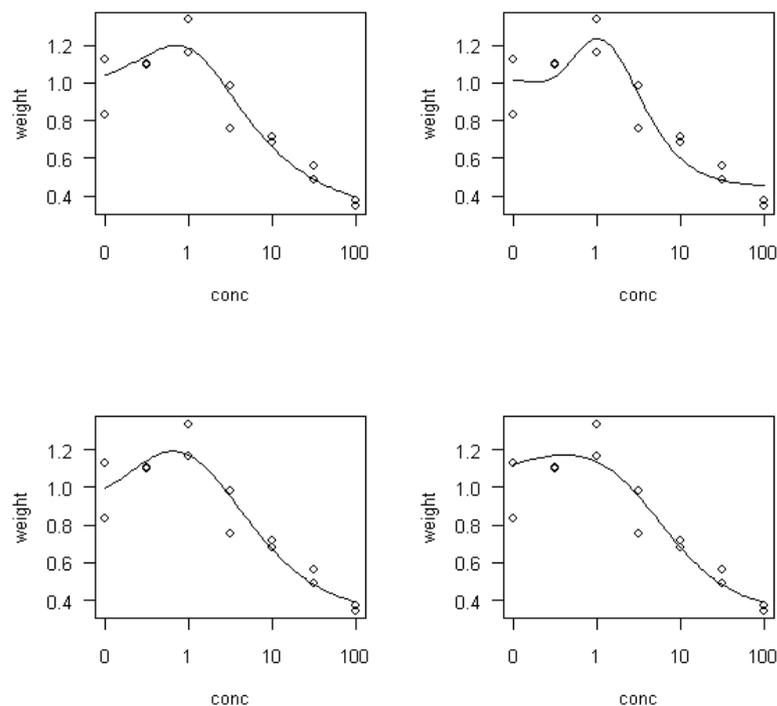

Visually, the CRS.5a model can be considered as the model having the best fit because it is the one which describes best the effect hormesis (at the price of a less good fit at the high dose levels).

On the other hand, if it is the residual error term which is considered, then it is the CRS.5a model which has the biggest one, and so which has the less satisfactory fit. This is because of the distances between the experimental points and the curve at the high dose levels. However, as notified in the paragraph 8.4.1, in case of conflict between the visual evaluation and the residual error, the visual approach must be privileged. Hence, the lettuce.crs5a model will be considered as the best model ' hormesis '.

```
# visual assessment of the normality assumption of the residuals#
qqnorm(residuals(lettuce.crs5a))
qqline(residuals(lettuce.crs5a),col=2)
```

Normal Q-Q Plot
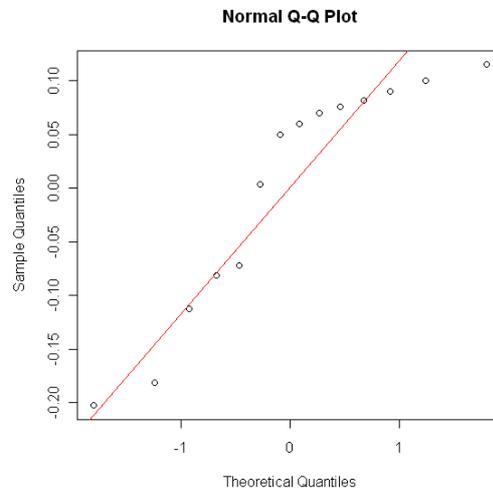
Visually, the normality of the residuals is not satisfactory.

```
# assessment of the normality assumption of the residuals using a shapiro-
wilk's test#
shapiro.test(residuals(lettuce.crs5a))
 shapiro-wilk normality test
data:  residuals(lettuce.crs5a)
w = 0.8596, p-value = 0.03003
```

Shapiro-Wilk's test is significant (p-value <0.05), thus the normality assumption is not satisfactory. By the end the residual normality assumption is rejected. Thus, a Box-Cox transformation is going to be used at this level without waiting the assessment of the homogeneity assumption.

```
# use of the box-cox transformation #
lettuce.crs5abox<-boxcox(lettuce.crs5a)

# visualization of the fit after the use of the box cox transformation#
plot(lettuce.crs5abox, type="all")
```

(D-N°:5.1) – Experimental plan
Dissemination level: PU
Date of issue of this report: **31/10/2011**
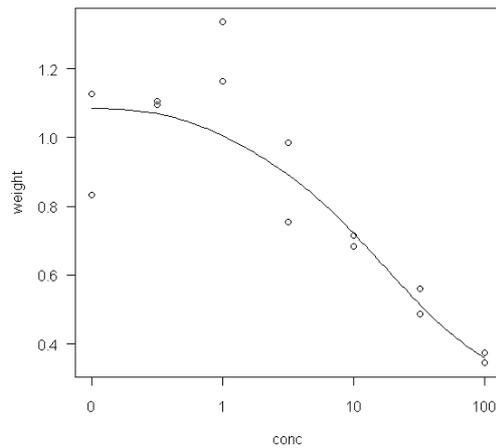
After the Box-Cox transformation the fit of the CRS.5a model is no more satisfactory. Hence it is necessary to start again with another 5 parameters 'hormesis' model. Visually, the model having the best fit after the CRS.5a model is the BC.5 model (high left corner of the previous graph displaying the fit of the 4 hormesis models). The BC.5 model is also the one which has the lowest residual error. At the end, the BC.5 model is considered has the best full 'hormesis' model.

```
# visual assessment of the normality assumption of the residuals#
qqnorm(residuals(lettuce.bc5))
qqline(residuals(lettuce.bc5),col=2)
```
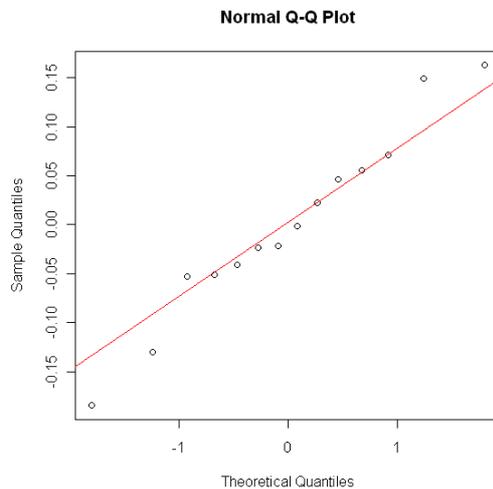

Visually the residual normality assumption is quite satisfactory.

```
# assessment of the normality of the residuals by the use of a shapiro-
wilk's test#
shapiro.test(residuals(lettuce.bc5))
        shapiro-wilk normality test

data:  residuals(lettuce.bc5)
```

```
w = 0.9714, p-value = 0.8942
```

The Shapiro-Wilk's test is not significant (p-value >0.05), thus the normality of the residuals is satisfactory. By the end, the residual normality assumption is accepted.

```
# visual assessment of the homogeneity assumption of the residuals #
plot(fitted(lettuce.bc5),(residuals(lettuce.bc5,type="standardised")))
abline(h=0,col=2)
```
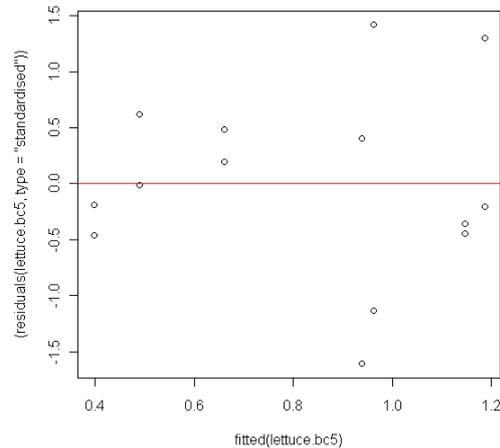

Globally, it seems to be an increase trend of the residuals value with the increase of the fitted values. To have a better view of this phenomenon, the same graph could be done with the absolute value of the residuals.

```
plot(fitted(lettuce.bc5),abs(residuals(lettuce.bc5,type="standardised")))
```
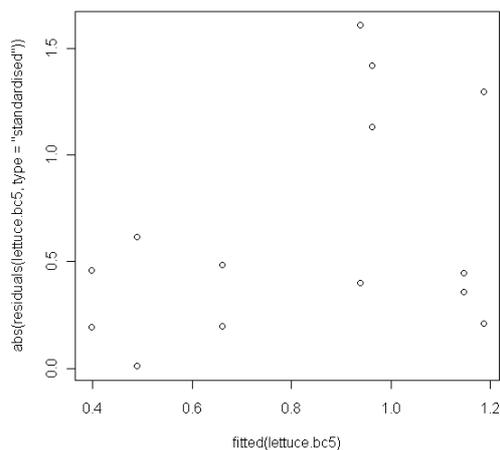

Here the increase trend of the residuals with increase of the fitted values is more visible (but is not very strong). Homogeneity assumption of the residuals seems thus rejected. Since there are only two replicates by concentration levels, Bartlett's and Levene's tests are not reliable and are not used here.

On the other hand, a Box-Cox transformation can be used in order to try to improve the residuals' homogeneity.

```
# use of the box-cox transformation #
lettuce.bc5box<-boxcox(lettuce.bc5)

# visualization of the fit after the box-cox transformation #
plot(lettuce.bc5box, type="all")
```
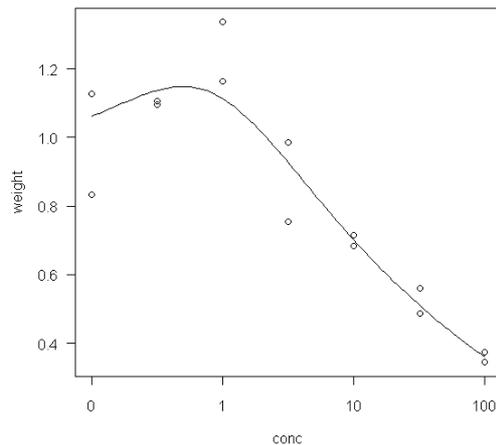

After the Box-Cox transformation, the BC.5 model describes less well the 'hormesis' effect but remains satisfactory.

```
# visual assessment of the normality assumption of the residuals #
qqnorm(residuals(lettuce.bc5box))
qqline(residuals(lettuce.bc5box),col=2)
```
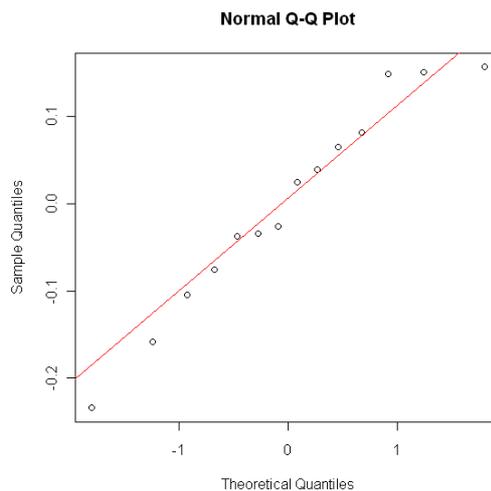

The residuals normality which was already satisfactory before the Box-Cox transformation seems to have still improved.

```
# assessment of the normality assumption of the residuals by the shapiro-
wilk's test#
shapiro.test(residuals(lettuce.bc5box))
shapiro-wilk normality test
data:  residuals(lettuce.bc5box)
w = 0.9572, p-value = 0.6774
```

The Shapiro-Wilk's test is still not significant (p-value >0.05). By the end the normality assumption of the residuals is accepted.

```
# visual assessment of the homogeneity assumption of the residuals #
plot(fitted(lettuce.bc5box),(residuals(lettuce.bc5box,type="standardised"))
)
abline(h=0,col=2)
```
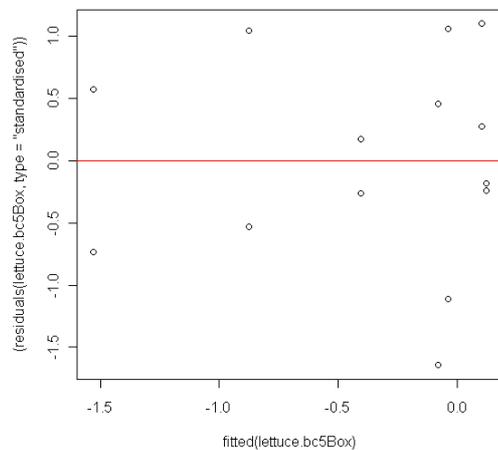

In a general way, after the Box-Cox transformation the residuals' values don't seem to increase when the fitted values increase. A same plot with the absolute values of the residuals can be used to have a better view.

```
# plot with the absolute values of the residuals#
plot(fitted(lettuce.bc5box),(abs(residuals(lettuce.bc5box,type="standardise
d"))))
abline(h=0,col=2)
```
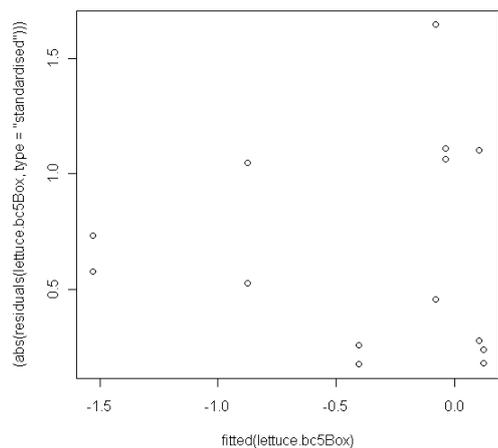
On the last plot it appears that, in a general way, the values of the residuals don't systematically increase when fitted values increase. Indeed, throughout the X axis, the values of residuals are alternately more or less high. By the end, after the Box-Cox transformation the homogeneity assumption of the residuals is accepted. Now, the quality of the lettuce.bc5Box's model must be assessed.

```
# visual assessment of the quality of the lettuce.bc5box's fit by a
residuals vs. predicted values plot#
plot(fitted(lettuce.bc5box), residuals(lettuce.bc5box))
abline(h=0,col=2)
```
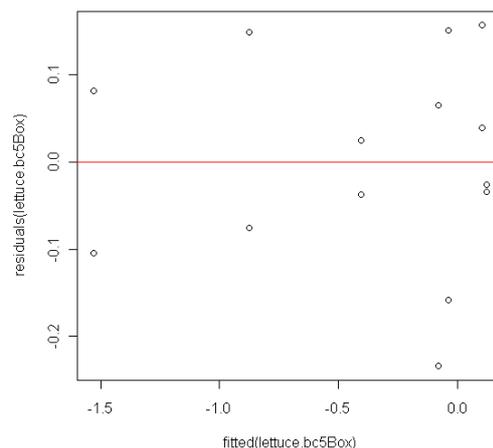

Throughout the X axis, residuals are globally distributed in a uniform way on both sides of the red line of 0 Y axis. Thus the fit is satisfactory.

```
# assessment of the quality of the lettuce.bc5box model by the use of a
'lack of fit' test #
```

```
modelfit(lettuce.bc5box)
lack-of-fit test

          modeldf      rss df f value p value
anova            7 0.14388
drc model        9 0.18230  2  0.9346  0.4368
```

The *'Lack of fit'* test is not significant (p-value >0.05), strengthening the good quality of the fit of the

`lettuce.bc5Box` model. The reduction of this kept full hormesis model is going to be considered.

```
# displaying of the estimates of the lettuce.bc5box's parameters#
summary(lettuce.bc5box)
model fitted: brain-cousens (hormesis) (5 parms)
parameter estimates:

                estimate std. error    t-value    p-value
b:(intercept)  1.190709   0.161643   7.366268 4.253e-05
c:(intercept) -0.244189   0.681644  -0.358236    0.7284
d:(intercept)  0.963425   0.094805 10.162184 3.129e-06
e:(intercept)  0.675545   0.459283   1.470871    0.1754
f:(intercept)  2.316550   2.204442   1.050856    0.3207

residual standard error:
 0.14232 (9 degrees of freedom)
non-normality/heterogeneity adjustment through optimal box-cox
transformation
estimated lambda: -0.75
confidence interval for lambda: [-1.565, 0.358]
```

Since the p-value of 'c' parameter is not significant (p-value >0.05), the lower limit of the curve seems

to be fixed to 0. In order to confirm this hypothesis the 4 parameters Brain-Cousens' model is going to

be fitted. And then its fit will be compared with the one of the 5 parameters model by an 'F' test for

nested models.

```
# fit of the 4 parameters brains-cousens's model with box-cox
transformation #
lettuce.bc4box<-drm(weight~conc,data=lettuce,fct=bc.4(),bcval=-0.75)
summary(lettuce.bc4box)
model fitted: brain-cousens (hormesis) with lower limit fixed at 0
(4 parms)
parameter estimates:
                estimate std. error    t-value    p-value
b:(intercept)  1.273794   0.026176 48.662518 3.242e-13
d:(intercept)  0.952145   0.086389 11.021615 6.473e-07
e:(intercept)  0.786673   0.510281   1.541645    0.1542
f:(intercept)  1.741208   1.386622   1.255720    0.2378
residual standard error:

 0.1373192 (10 degrees of freedom)
non-normality/heterogeneity adjustment through optimal box-cox
transformation
specified lambda: -0.75
```

```
# visualization of the fit #
plot(lettuce.bc4box,ylim=c(0,1.4),xlim=c(0,1e+06),type="all")
```
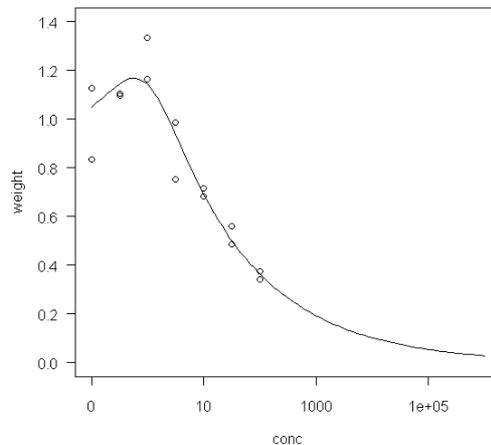

Visually, the fit is satisfactory.

```
# comparaison des ajustements par un test f#
anova(lettuce.bc4box,lettuce.bc5box)
1st model
 fct:       bc.4()
2nd model
 fct:       bc.5()
anova table
          modeldf      rss df f value p value
1st model       10 0.18857
2nd model        9 0.18230  1  0.3096  0.5915
```

P-value of the 'F' test is not significant (p-value >0.05). Thus, the fit of the 4 parameters Brain-Cousens' model is as satisfactory as the one of the 5 parameters model. According to the Parsimony rule, it's the 4 parameters model which is kept. Then, the 'hormesis' effect is going to be able to be assessed. For this, the 3 parameters log-logistic model (lower limit, e.g. 'c' parameter fixed to 0) is going to be fitted. Next, its fit is going to be compared with the hormesis model's one by the way of an 'F' test for nested models.

```
# fit of the 3 parameters log-logistic model#
lettuce.ll3box<-drm(weight~conc,data=lettuce,fct=ll.3(),bcval=-0.75)
summary(lettuce.ll3box)
model fitted: log-logistic (ed50 as parameter) with lower limit at 0
(3 parms)
parameter estimates:
                estimate std. error   t-value     p-value
b:(intercept)  0.608503   0.096860  6.282287    0.0001
```

```
d:(intercept)  1.118760   0.097981 11.418178 1.936e-07
e:(intercept) 28.191811  10.296944  2.737881     0.0193

residual standard error:

 0.1645451 (11 degrees of freedom)

non-normality/heterogeneity adjustment through optimal box-cox
transformation

specified lambda: -0.75


# visualization of the fit #
plot(lettuce.ll3box,ylim=c(0,1.4),xlim=c(0,1e+06), type="all")
```
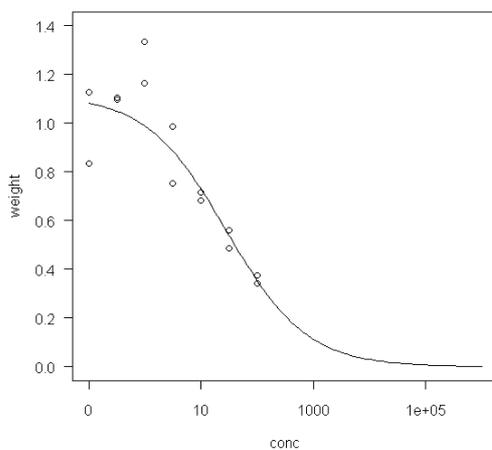


```
# assessment of the 'hormesis' effect by comparing the fits of the
'hormesis'and log-logistic models by a 'f' test#
anova(lettuce.ll3box,lettuce.bc4box)
1st model
 fct:       ll.3()
2nd model
 fct:       bc.4()
anova table

          modeldf      rss df f value  p value
1st model       11 0.29783
2nd model       10 0.18857  1  5.7943   0.0369
```

The 'F' test is significant (p-value < 0.05). Thus the fit of the 'hormesis' model is better than the one of the log-logistic model. In other words, the 'f' parameter characterizing the hormesis effect in the Brain-Cousens' model is significantly different from 0. Hence the presence of a 'hormesis' effect is validated. Now the EDp% can be estimated.

```
# estimation of the ed5, ed10 et ed55 and their error standard and
confidence interval#
```
```
ed(lettuce.bc4box,c(5,10,55),interval="delta",reference="control")
```
```
estimated effective doses
(delta method-based confidence interval(s))

    estimate std. error      lower      upper
5    3.67928     1.60085   0.11235    7.2462
10   4.55723     1.88486   0.35749    8.7570
55  55.94498    19.53743  12.41288   99.4771
```

8.4.5 Example of DRC application to the analysis and modelling of a hormesis dose-response relationship with quantal data

Data used in this example are those of the 'wormsHU' dataset. This is a modification of the 'eartworms' dataset included in the '*drc*' package. These data can be perceived as coming from a fictitious experiment, the purpose of which would be to assess the action of a toxic substance on the not migration of earthworms. The predictive variable would be the dose (7 levels). The total number of used earthworms would be reported in the '*total*' variable. The number of worms remaining in their contaminated container and no migrating would be reported in the '*number*' variable. The observed and modelled variable would be the percentage of non migrating worms, in others words, worms staying in their container. Five replicates per dose level would be used. Thus, at the end the dataset would contain 35 dose-response data.

The dataset is displayed in annexe 0. To be imported in R, in a first step data have to be copied and pasted in an Excel sheet with, obligatorily, a coma as a decimal separator symbol. In a second step, the Excel sheet has to be saved in 'csv' format (semicolon delimited file). By the end, importation in R is done using the `read.csv2(file.choose())`command which permits to open a window of selecting dataset (cf. annexe 8.2)

A single dataset is employed to illustrate the strategy of analysis both in absence and in the presence of an overdispersion.

```
# loading of the 'drc' dataset #
library(drc)

# import of the dataset#
wormshu<-read.csv2(file.choose())

# display of the data – not shown here#
wormshu

# visualization of the data#
with(wormshu,plot(dose,number/total,log="x"))
warning message:
in xy.coords(x, y, xlabel, ylabel, log) :
  5 x values <= 0 omitted from logarithmic plot
```
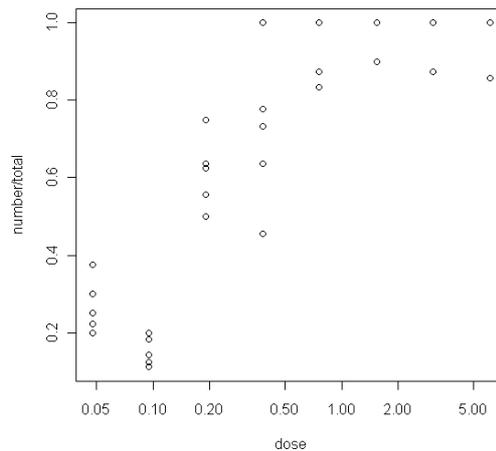
The `'Warning'` message says that the 5 control observations are not displayed on the graph because of the X axis logarithm scale.

Since the relationship has a 'U' shape only the Cedergreen-Ritz-Streibig's models can be fitted.

```
# fit of all the available full hormesis models#
wormshu.ucrs5a<-drm(number/total~dose,type="binomial",weight=total,
fct=ucrs.5a(),data=wormshu)
wormshu.ucrs5b<-drm(number/total~dose,type="binomial",weight=total,
fct=ucrs.5b(),data=wormshu)
wormshu.ucrs5c<-drm(number/total~dose,type="binomial",weight=total,
fct=ucrs.5c(),data=wormshu)

# display of the parameters' estimates #
summary(wormshu.ucrs5a)
model fitted: u-shaped cedergreen-ritz-streibig (5 parms)
parameter estimates:

              estimate std. error   t-value    p-value
b:(intercept)  2.152428   0.705520  3.050840     0.0023
c:(intercept)  0.278079   0.049853  5.577967 2.433e-08
d:(intercept)  0.972763   0.024361 39.930462     0.0000
e:(intercept)  0.312795   0.074950  4.173397 3.001e-05
f:(intercept) -1.571590   1.828073 -0.859698     0.3900


summary(wormshu.ucrs5b)
model fitted: u-shaped cedergreen-ritz-streibig (5 parms)
parameter estimates:
              estimate std. error   t-value    p-value
b:(intercept)  2.310165   0.276420  8.357446 3.205e-17
c:(intercept)  0.417944   0.085524  4.886878 1.024e-06
d:(intercept)  0.981034   0.014988 65.456285     0.0000
e:(intercept)  0.071362   0.035446  2.013220     0.0441
f:(intercept) 45.093502  42.327543  1.065347     0.2867
```

```
summary(wormshu.ucrs5c)
```
```
model fitted: u-shaped cedergreen-ritz-streibig (5 parms)
parameter estimates:
                estimate std. error    t-value     p-value
b:(intercept)   2.149295   0.419078   5.128627  2.919e-07
c:(intercept)   0.416251   0.089831   4.633722  3.592e-06
d:(intercept)   0.980798   0.014875  65.935022      0.000
e:(intercept)   0.189352   0.040773   4.644025  3.417e-06
f:(intercept)   1.998914   1.037598   1.926482      0.054
```
```
# visualization of the fits #
par(mfrow=c(2,2))# permet de diviser la fenetre graphique en 4#
plot(wormshu.ucrs5a, type="all",lwd=2)
plot(wormshu.ucrs5b, type="all",lwd=2)
plot(wormshu.ucrs5c, type="all",lwd=2)
```
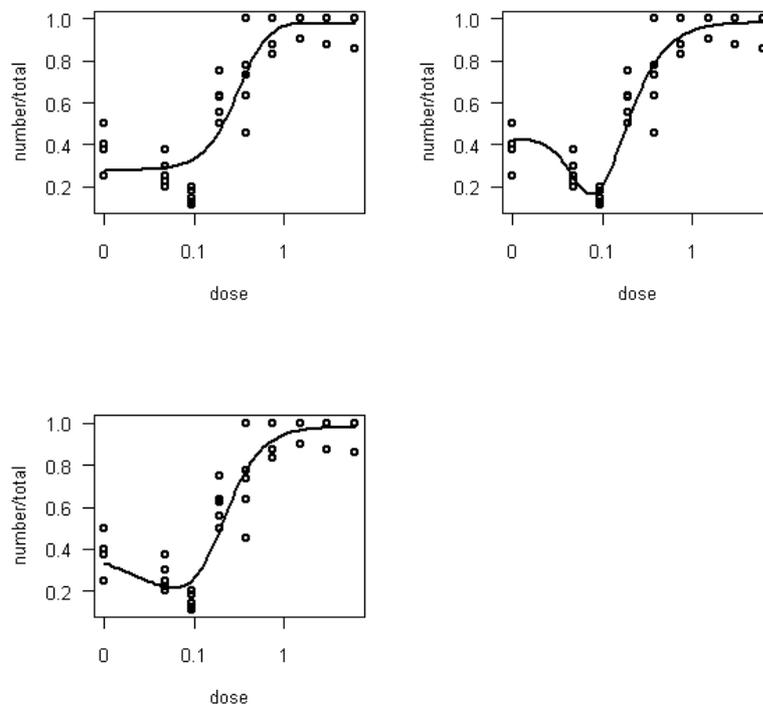

Visually, the best fit is the one of the `WormsHU.ucrs5b` model (high right corner). This model is those which describe the data best.

```
# estimation of the log-likelihood of the different models#
loglik(wormshu.ucrs5a)
'log lik.' -51.37844 (df=5)
loglik(wormshu.ucrs5b)
'log lik.' -47.99751 (df=5)
loglik(wormshu.ucrs5c)
'log lik.' -49.23641 (df=5)
```

(D-N°:5.1) – Experimental plan
Dissemination level: PU
Date of issue of this report: **31/10/2011**

The `WormsHU.ucrs5b` model is also the one who has the highest 'Log-likelihood' value.

```
# assessment of the model's fit by a 'residuals vs. fitted'#
plot(fitted(wormshu.ucrs5b ), residuals(wormshu.ucrs5b ))
abline (h=0, col=2)
```
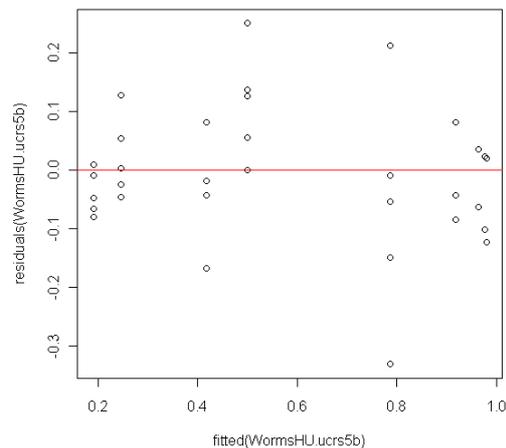

Globally, during the X axis, residuals are distributed on both sides of the 0 X-axis red line. The fit of the model WormsHU.ucrs5b is thus satisfactory.

```
# assessment of the model's fit by a 'goodness of fit' test #
modelfit(wormshu.ucrs5b)
goodness-of-fit test
          df chisq value p value
drc model   40      31.941  0.8143
```

Since the '*Goodness of fit*' test is not significant (p-value ≥ 0.05), then the fit of the wormsHU.ucrs5b model is satisfactory.

```
# assessment of a possible overdispersion #
31.941/40
[1] 0.798525
```

The ratio of the statistic of the '*Goodness of fit*' test out of its degrees of freedom being less than 1, then no overdispersion is highlighted. Thus, the result of the *'Goodness of fit'* test is valid. The reduction of the full hormesis model is going to be done. For this, in a first step, a model specifying the upper limit ('d' parameter) is fixed to 0 is fitted. Taking in account that the 'd' parameter's estimate of the full hormesis model equals 0.981, this hypothesis is very likely.

```
#reduction of the best full 'hormesis' model#
```

```
# fit of the model specifying that the upper limit = 1, and the alpha
parameter = 0.25 as it in the case in the wormshu.ucrs4b model #
wormshu.ucrs4b<-drm(number/total~dose,type="binomial",weight=total,
fct=ucedergreen(fixed=c(na,na,1,na,na),names = c("b", "c", "d", "e",
"f"),alpha=0.5),data=wormshu)
```

Since no 'hormesis' model directly specifying that the upper limit ('d' parameter)=1 is available in '*drc*', it is needed to use the `ucedergreen` general function. The `fixed=c(NA,NA,1,NA,NA)` command permits to fix the 'd' parameter to 1. The first element in the parenthesis corresponds to the 'b' parameter, the second to the 'c' parameter, the third to the 'd' parameter, the forth to the 'e' parameter, and the fifth to the 'f' parameter. The `NA` symbols permit to not fix the value of the corresponding parameter.

```
# displaying of the estimations#
summary(wormshu.ucrs4b)
model fitted: u-shaped cedergreen-ritz-streibig (4 parms)
parameter estimates:
                  estimate std. error    t-value    p-value
b:(intercept)  2.063757    0.154216 13.382245 3.840e-41
c:(intercept)  0.402674    0.087278  4.613722 3.955e-06
e:(intercept)  0.050064    0.032346  1.547778    0.1217
f:(intercept) 80.449217   94.662702  0.849851    0.3954
```

```
# visualization of the fit#
plot(wormshu.ucrs4b, type="all", col=3, lwd=2)
```
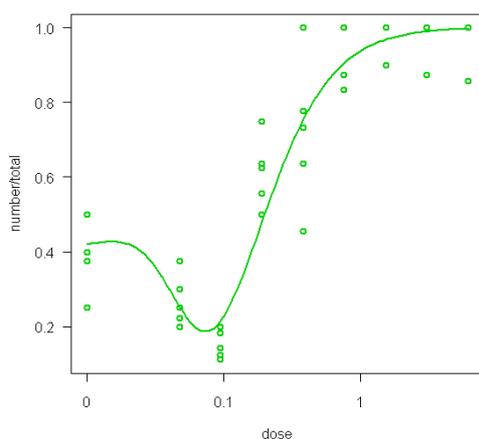


```
# superimposition of fits of the 4 and 5 parameters models #
plot(wormshu.ucrs5b, type="all",lwd=2)
```

```
plot(wormshu.ucrs4b, type="all",lwd=2,col=3,add=t)
```
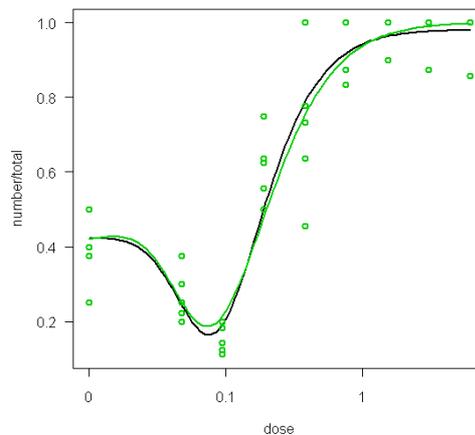

In black colour there is the fit of the full 5 parameters model, in green the one of the 4 parameters model. Looking at this plot, the 4 parameters model seems to describe a little bit less fine the hormesis effect, but is still satisfactory.

```
# comparison of the 5 and 4 parameters models using a 'likelihood ratio'
test #
anova(wormshu.ucrs4b,wormshu.ucrs5b)
1st model
 fct:       ucedergreen(fixed = c(na, na, 1, na, na), names = c("b",
"c",
2nd model
 fct:       ucrs.5b()
anova-like table
          modeldf  loglik df lr value p value
1st model       4 -49.806
2nd model       5 -47.998  1    3.617  0.0572
```

P-value of the *'likelihood ratio'* test is slightly higher of the significant level (0.05). Nevertheless, since it is exceeded, the fit of the 4 parameters model is considered as satisfactory that the one of the 5 parameters model. By the end, the restricted model is kept.

If an overdispersion would have been detected, the 4 parameters model would also has been kept because of the 'd' parameter's estimate is very close to 1.

```
# assessment of the 'hormesis' effect #
```

Since no overdispersion has been highlighted, in a first step, the log-logistic model corresponding to the restricted 'hormesis' model is fitted. It is the 3 parameters model having the higher limit ('d' parameter) fixed to 1.

```
# fit of the log-logistic model#
wormshu.ll3u<-drm(number/total~dose,type="binomial",weight=total,
fct=ll.3u(),data=wormshu)

# display of the estimatation #
summary(wormshu.ll3u)
model fitted: log-logistic (ed50 as parameter) with upper limit at 1
(3 parms)
parameter estimates:
                estimate std. error    t-value    p-value
b:(intercept) -1.830545   0.274881 -6.659413 2.749e-11
c:(intercept)  0.262651   0.049109  5.348298 8.879e-08
e:(intercept)  0.288021   0.045116  6.383980 1.725e-10

# visualization of the fit #
plot(wormshu.ll3u, type="all",lwd=2,col=4)
```
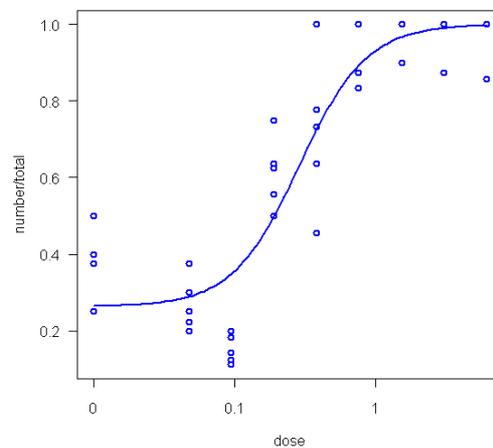


```
# comparison of the fits of the hormesis and log-logistic models #
anova(wormshu.ll3u,wormshu.ucrs4b)
1st model
 fct:       ll.3u()
2nd model
 fct:       ucedergreen(fixed = c(na, na, 1, na, na), names = c("b",
"c",

anova-like table

          modeldf  loglik df lr value p value
1st model       3 -53.974
2nd model       4 -49.806  1    8.337  0.0039
```

The *'likelihood ratio'* test is significant (p-value < 0.05), and then the 'f' parameter of the 'hormesis'

model is significantly different from 0. In other words, the hypothesis of a hormesis effect is accepted.

If an overdispersion would have been detected, then hormesis effect would has been assessed from the p-value pf the 't' test corresponding to the 'c' parameter, which is given by the `summary` function used with `od=T` argument.

```
summary(wormshu.ucrs4b,od=t)
model fitted: u-shaped cedergreen-ritz-streibig (4 parms)
parameter estimates:

                estimate std. error    t-value   p-value
b:(intercept)   2.063757   0.220386   9.364290 3.828e-21
c:(intercept)   0.402674   0.124726   3.228474    0.0012
e:(intercept)   0.050064   0.046224   1.083065    0.2788
f:(intercept)  80.449217 135.279818   0.594688    0.5521
```

Here the p-value of the 't' test corresponding to the 'f' parameter being higher than 0.05, the 'f' parameter would has been considered as no different from 0. The hypothesis of a hormesis effect would has been rejected. Thus in case of overdispersion, the conclusion would be contrary to that one obtained in absence of this phenomenon. This opposition is explained, by part, by the increase of the standard error of the 'f' parameter during the overdispersion correction, which leads a 't' test more difficulty significant. Another reason which can be evoked is the less robustness of the 't' test with regard to the *'likelihood ratio'* test.

WormsHU dataset

dose	number	total
0	2	5
0	2	5
0	1	4
0	3	8
0	4	8
0.0475	3	10
0.0475	2	8
0.0475	1	5
0.0475	2	9
0.0475	3	8
0.095	1	9
0.095	1	7
0.095	1	5
0.095	2	11
0.095	1	8
0.19	7	11
0.19	5	9
0.19	6	8
0.19	5	8
0.19	2	4
0.38	7	9
0.38	4	4
0.38	5	11
0.38	7	11
0.38	11	15
0.76	11	11
0.76	5	6
0.76	7	8
0.76	9	9
0.76	8	8
1.53	6	6
1.53	9	10
1.53	10	10
1.53	10	10
1.53	8	8
3.05	5	5
3.05	9	9
3.05	7	7
3.05	8	8
3.05	7	8
6.11	3	3
6.11	7	7
6.11	11	11
6.11	10	10
6.11	6	7

8.5 Specific Glossary

Definitions are all adapted from (OECD, 2003). Terms are listed by alphabetic order.

■**Confidence interval**

A *x* % confidence interval for a parameter is an interval of values that theoretically covers the true value of the estimated parameter with x % of confidence. Note that the confidence level reflects the proportion of cases that the confidence interval would contain the true parameter value in a long series of repeated random samples under identical conditions.

■**Continuous data**

Data are continuous when they can theoretically take any value in an open interval

■**ECp , EDp, EDRp**

In ecotoxicology, the term *ECp* is defined as the concentration associated with an effect *p* where *p* is defined as the percent change in the (average) level of the endpoint considered $p\% = 100\left(\dfrac{y(EC_{p\%})}{y(0)} - 1\right)\%$. The same definition can apply for the Dose (*ED$_{p\%}$*) or the dose rate (*EDRp*). These parameters are estimated by modelling (concentration-effects, dose-effects or dose rate-effect modelling).

■**Effect**

An effect is the change in an endpoint under consideration when it is compared to a control.

■**Endpoint**

In toxicity testing and evaluation it is the biological response that is measured. Endpoints vary with the level of biological organization being examined and include response at the subcellular level to the community level such as biomarkers (subcellular level), survival, growth, reproduction (individual level), primary production, and changes in structure (and abundance) and function in a community (population or community level). Endpoints are used in toxicity tests as criteria for effects.

■**Experimental Unit/replicate**

The experimental unit is the smallest unit of experimental material to which a treatment can be allocated independently of all other units. By definition, experimental units (*e.g.* aquariums, beakers, or plant pots) must be able to receive different treatments. Each experimental unit may contain multiple sampling units (*e.g.* fish, daphnia or plants) on which measurements are taken. Within each experimental unit, sampling units may not be independent. However, in some special case situations, individual organisms (housed in common units) can be treated as the experimental units: these special cases require some proof or strong argument of independence of organisms

■**Exposure concentration, dose or dose rate**

The exposure concentration, dose or dose rate is the "amount" that an organism is exposed to. For a chemical element radioactive or stable, it can be expressed as a concentration (quantity of the substance per volume or mass of the exposure source, *in mol or g or Bq per L or per g)*. For a radionuclide, the dose is the total quantity of ionising radiation absorbed by the organism (*in Gy)*; the absorbed dose rate refers to the quantity of ionising radiation released over a specified unit of time (*e.g. μGy/h*).

■**Hormetic /non-hormetic**

Hormetic- or dose(rate)-effect relationship exhibits a biphasic shape with a stimulatory effect at the low dose levels, and then an inhibitory effect at the high dose levels; Hormetic etic dose-response curves can have two shapes :"inverted U" and "U" shapes.

▪LOEC (or LOED or LOEDR) and NOEC (or NOED or NOEDR)

The Lowest Observed Effect-Concentration is the lowest Concentration out of the tested Concentration at which a statistically significant difference from the control group is observed.

The No Observed Effect -Concentration is the tested concentration just below the LOEC. They are obtained by hypothesis testing.

The same definitions apply for Dose and Dose Rate (in place of Concentration).

▪Monotonous /non-monotonous

A monotonic concentration- or dose(rate)-effect relationship exhibits an increase or a decrease over the range of concentrations or dose(rate)s in the study. In a non-monotonic relationship, the variation in effects are not consistent across the concentrations or dose(rate)s.

▪Power

Power is the probability of rejecting the null hypothesis in favour of the alternative hypothesis, given that the alternative hypothesis is the true. Power of a test varies with sample size, variance of the measured response, the size of an effect that it is of interest to detect, and the choice of statistical test. Power to detect differences can be increased by increasing the sample size and/or reducing variation in the measured responses.

▪Quantal data

These data can exhibit two states: *eg* an individual shows an effect or not. Typically, these data are presented as the number of individuals showing the property out of a total number in the experimental unit.

▪Response

A response corresponds to an observed value of any endpoint. This term has been avoided as far as possible to avoid confusion.

▪Statistical significance

In hypothesis testing, a result is statistically significant at the chosen level α if the test statistic falls in the rejection region. The finding of statistical significance implies that the observed deviation from what was expected under the null hypothesis is unlikely to be attributable to chance variation. In general, the α-level will be 0.05 unless otherwise stated.

▪Type I and Type II errors

Type I errors (false positives) occur when the null hypothesis is the true but the hypothesis test results in a rejection of the null hypothesis in favour of the alternative hypothesis. The probability of making a Type I error is often referred to as α and is usually specified by the data analyst – often at 0.05, or 5 %. Type II errors (false negatives) occur when the alternative hypothesis is true but the test fails to reject the null hypothesis (*i.e.* there is insufficient evidence to support the alternative hypothesis). The probability of making a Type II error is often referred to as β (1 – power).

▪OMICS
Refers to the study of biological systems, and includes genomics (DNA), transcriptomics (mRNA transcripts), proteomics (proteins) and metabolomics (products of biological reactions).

▪DEB and DEBTox
The Dynamic Energy Budget (DEB) theory describes how individuals acquire and use energy based on simple rules for metabolism. Organisms are represented as dynamic systems with mass and energy

balance. The DEBTox version, based on the DEB theory, is a biology-based model describing how toxicants accumulate over time in exposed organisms and alter energy acquisition and allocation to growth and reproduction. The approach analyses jointly effects induced on several endpoints (survival, growth and reproduction) by one or several toxicants and delivers estimates of No Observed Effect Concentration and insights on the physiological/metabolic mode of action.