The analysis of aquatic toxicity data

S.A.L.M. Kooijman and J.J.M. Bedaux

The analysis of aquatic toxicity data

S.A.L.M. Kooijman
Professor of Theoretical Biology
Vrije Universiteit Amsterdam

J.J.M. Bedaux
Lecturer in Biostatistics
Vrije Universiteit Amsterdam

VU University Press

Published by the VU University Press
De Boelelaan 1083, 1081 HV Amsterdam

First published 1996

Printed in the Netherlands
ISBN 90-5383-477-X paperback

Contents

Preface **vii**

1 No-effect concentrations in environmental policy **1**

2 Toxic effects as process perturbations **9**
by Bas Kooijman
2.1 Introduction . . . 9
2.2 Standard descriptions . . . 13
2.3 Dynamic Energy Budgets . . . 17
2.4 Toxicokinetics . . . 20
2.5 Effects on individuals . . . 23
2.6 Effects on population dynamics . . . 31
2.7 Toxicity patterns . . . 36
2.8 Conclusions/perspectives . . . 42

3 The analysis of standardized bioassays **49**
3.1 Some statistical properties of estimates of no-effect concentrations . . . 50
by Bas Kooijman and Jacques Bedaux
3.2 Statistical Analysis of Bioassays based on hazard modelling . . . 58
by Jacques Bedaux and Bas Kooijman
3.3 Analysis of toxicity tests on fish growth . . . 72
by Bas Kooijman and Jacques Bedaux
3.4 Analysis of toxicity tests on *Daphnia* survival and reproduction . . . 93
by Bas Kooijman and Jacques Bedaux
3.5 No-effect concentrations in algal growth inhibition tests . . . 114
by Bas Kooijman, Arnbjørn Hanstveit and Niels Nyholm

4 Software package DEBtox **127**
by Matthijs Luger, Jacques Bedaux and Bas Kooijman
4.1 Introduction . . . 128
4.2 Parameter estimation . . . 129
4.3 Input . . . 131
4.4 Output . . . 135
4.5 Final remarks . . . 144

Glossary **145**

Preface

Legislation around emission of chemicals in the environment aims at minimizing ecological effects. Because of our poor understanding of ecosystem dynamics, it is usually unclear how effects on particular species translate into ecological effects. The interpretation of results from experiments with mesocosms is also far from obvious. This explains why No-Observed Effect Concentrations (NOECs) from standardized bioassays are frequently used in environmental risk assessments. This use is, however, under increased criticism due to a substantial statistical problem that is inherent to this concept: the problem of recognizing small effects in scattery data. A statistically nonsignificant effect does not imply that biologically significant effects are absent.

Small-effect concentrations have been proposed to replace NOECs in risk analysis. This approach suffers from several problems, such as: What is small?; How do small effects on species relate to ecosystem dynamics?; To what extent does the resulting value depend on those model details that do not have a mechanistic basis? How do we extrapolate acute effects to chronic effects?

This book describes how the No-Effect Concentration (NEC) can be estimated as a model parameter from data of standardized aquatic toxicity tests: acute and chronic survival, body growth (of fish), reproduction (of daphnia) and (algal) population growth. This alternative for the NOEC does not suffer from statistical problems, while its use in risk assessments still avoids the difficulty of translating observed effects into consequences for ecosystems dynamics. Being a model parameter, the point estimate of the NEC can be provided with a confidence interval. This allows positive identification of a concentration range where no effects are to be expected, which is not possible in the NOEC approach. If, on the other hand, the null hypothesis NEC=0 (implying that the toxicant has effects in all concentrations) cannot be rejected, one might consider additional research about the effects of the compound.

The method to estimate NECs is a by-product of a new process-based characterization of toxic effects. The basic idea is that the hazard rate and the parameters that quantify the energy budget of the individual are proportional to the concentration in the animal that exceeds the no-effect concentration. The inverse of the proportionality constant, which is called the tolerance concentration, quantifies the toxicity of the compound. The energy budget parameters are defined by the Dynamic Energy Budget (DEB) theory, which specifies the rules that organisms use for the energy

uptake of resources (food) and the ensuing allocations to maintenance, growth, development and propagation. The DEB theory has been tested against a wide variety of ecophysiological data. This book only summarizes some relevant points of the theory, which is fully discussed elsewhere (Kooijman 1993). At high concentrations, toxicants will affect many physiological processes simultaneously and we would need many parameters to quantify all these effects. The various physiological processes can be ordered with respect to their sensitivity to the toxicant, however, so that just one process (i.e. one parameter) is affected in the lower concentration range.

The characterization of toxic effects is built up in two steps: (i) the uptake and elimination behaviour of the compound and (ii) the translation of internal concentrations into effects. As the most simple option, one-compartment first-order toxicokinetics can be assumed. This results in an NEC, a tolerance concentration, and an elimination rate as characterizations of the various sublethal effects. The tolerance concentration is replaced by the killing rate for lethal effects. These simple characterizations are independent of exposure time and depend in a simple way on the octanol-water partition coefficient. The possibility to remove the elimination rate as an essential parameter makes the models with just two toxicity parameters the simplest possible. The results of aquatic toxicity bioassays as standardized by the OECD are suitable substrates for the models. The analysis of these results will be discussed in detail.

We consider the method as an application of the theory for energy and mass fluxes through biological systems that we are currently developing in the Department of Theoretical Biology VUA (see our homepage on WWW: http://www.bio.vu.nl/vakgroepen/thb). The biological foundation is covered by the Dynamic Energy Budget theory, which has been published separately (see Kooijman 1993, including tests against experimental data). The Department participates in the Amsterdam Centre for Environmental Science (ACES).

The book starts with an evaluation of the use of no-effect concentrations in legislation from the perspective of the Dutch Ministry for the Environment (VROM). Then follows a chapter with a discussion of the toxicological backgrounds of the new method. It is intended for ecotoxicologists, with an emphasis on scientific aspects rather than technical ones. The next chapter is more technical and is primarily aimed at statisticians and scientists who have had an introduction into applied mathematics. The sections of this chapter can be read independently and discuss the different bioassays. The last chapter describes the use of the software package DEBtox, which handles all calculations that are required to apply the method. A diskette with the Windows and Unix versions of DEBtox is delivered with this book.

Five persons contributed directly to parts of this book. Kees van Leeuwen and Jack de Bruijn wrote the introduction. They work at the Dutch Ministry for the Environment (VROM), Den Haag. Matthijs Luger, who did a great job in the coding of DEBtox, taught us a lot about the ins and outs of the programming language C and about the finer details of Windows and Unix. He did his work at the Department

of Theoretical Biology of the Vrije Universiteit Amsterdam (see final chapter). We enjoyed the collaboration with Arnbjørn Hanstveit and Niels Nyholm on algal growth very much. Arnbjørn is working at the Institute of Environmental Sciences Delft, MW-TNO (presently reorganized as TNO-nutrition Zeist); Niels is working at the Institute of Environmental Sciences & Engineering, Technical University of Denmark in Lyngby.

Many other people have contributed to this work; too many to thank them individually. We are especially grateful to those who allowed us to use their data as examples to develop our method, colleagues who discussed the technical issues on data analysis with us, and the OECD (Nicky Grandy) and the Dutch Ministry for the Environment (VROM, Jack de Bruijn and Cees van Leeuwen) for their very positive attitude and their stimulating efforts. Mike Newman, Spliid Hendrik, José Tarazona, Nico van Straalen, Wolfgang Bödeker, Reinhard Meister and Hugo van den Berg made many helpful comments on the manuscript. Theo Helder (VROM) gave valuable advice on Good Laboratory Practice and related quality standards as applied to DEBtox.

We are also very grateful to the people who tested a pre-release of DEBtox: Tom Aldenberg (RIVM), Jens Andersen (IMM, TU-Denmark), Jack de Bruijn (VROM), Cees van Gestel (VU-Amsterdam), Arnbjørn Hanstveit (TNO-Nutr.), Udo Hommen (TU-Aachen), Frans Leeuwerik (VU-Amsterdam), David Mauriello (US-EPA), Hans Toni Ratte (TU-Aachen), Theo Traas (RIVM), Manon Vaal (RIVM), Wouter Vaes (RITOX, Utrecht University), Jos van Wezel (VU-Amsterdam), Maurice Zeeman (US-EPA).

Last but not least we want to thank Mariëlle Bedaux and Truus Meijer for their support and patience during the many evenings and weekends, when office hours proved to be insufficient to meet the time schedule.

This work has been financially supported by VROM and Grant PAD 90-18 from the Alternative to Animal Experiments Platform.

Bas Kooijman
Jacques Bedaux

Dept of Theoretical Biology
Vrije Universiteit
Amsterdam

Chapter 1

No-effect concentrations in environmental policy

J.H.M. de Bruijn and C.J. van Leeuwen

Dutch Ministry of Housing, Spatial Planning and Environment
Directorate-General for Environmental Protection
Risk Assessment and Environmental Quality Division

Introduction

The use of the No-Observed Effect Concentration (NOEC) as a parameter in toxicological and ecotoxicological tests is an important point of discussion in the scientific literature, mainly because of the statistical problems associated with this concept. In the past few years several alternatives have been proposed, such as the ECx estimation (the concentration which affects x% of a test population for a specific end point after a specified exposure time (Pack 1993)), the bounded effect concentration (Hoekstra & van Ewijk 1992), the traditional L(E)C50 (Chapman et al. 1996), and a model-based no-effect concentration (NEC) (this book).

Most of these methods have been presented as alternatives based on a scientific discussion using theoretical as well as statistical arguments. Recently, Chapman et al. (1996) stated that NOECs are inappropriate for regulatory use. They illustrate this statement with an example of effluent toxicity data and propose the use of EC50 values in stead of NOECs. Their major comment is that (a) the NOEC is not a good estimate of the NEC, (b) NOECs are highly variable between tests and can lead to contradictory results, (c) relatively subtle differences in the way an analysis is carried out can also lead to quite different results, (d) EC50s or other point estimates are more consistent, more reliable and less variable than NOECs and can be compared between tests, and (e) using different taxa interchangeably in tests will increase variability.

Their comments concerning effluent control are valid, especially when they propagate the use of EC50 values for comparing effect concentrations. Another question is whether their proposal is adequate when advocating the use of ECx values. What ECx value are proposed? EC5, EC10 or EC25 values? Should the x be the same for each test or end point? What are the consequences of such a proposal for existing test guidelines, regulatory frameworks for risk assessment and current practices to derive environmental quality guidelines? What are the consequences for chemicals for which NOECs have already been reported? Is there a single conversion factor between NOEC and ECx values? Our contribution to this book is meant to stimulate the discussion on this point and to increase awareness of the consequences of some of these options.

NOECs in risk assessment of chemicals

The first phase in environmental and human health risk management involves the identification of the possible risks of chemicals resulting from production, transport, use and disposal. Traditionally this process has been directed towards the hazard identification of chemicals based on their intrinsic properties. This hazard identification may lead to classification of a chemical as 'Dangerous for the Environment' according to the EC directive on the classification and labelling of chemicals (EC 1992). In recent years, the consecutive phase of the risk management process, i.e. the risk assessment, has developed rapidly. The principles of risk assessment have been laid down in EC legislation, both for new and existing chemicals (EC 1993, 1994). According to these principles, risk characterization is the estimation of the incidence and severity of the adverse effects that are likely to occur in a human population or the environment due to actual or predicted exposure to a substance, and may include risk estimation, i.e. the quantification of that likelihood.

In order to perform a risk characterization, a dose(concentration)-response(effect) assessment and an exposure assessment have to be made. The objective of the concentration-effect assessment is to predict the concentration of the substance below which adverse effects in the environmental compartment of concern are not expected to occur. This concentration is known as the predicted no-effect concentration (PNEC). From these formulations the intention of the risk-oriented environmental policy becomes clear. It is the intention to achieve concentrations in the environment below which the risks for man and the environment are negligible. The concept "no-effect" has a dominant place in these and other legislative frameworks, often without specifying what is exactly meant by it. However, we have to realize that "no-effect" in the PNEC is quickly associated with the NOEC.

PNEC values are often derived by applying assessment factors to the lowest toxicity value in a set of toxicity data. The magnitude of these factors usually depends on the type of test (acute, chronic, or field) and on the number of data available. Typically,

assessment factors applied to NOECs are lower than the ones applied to L(E)C50 data (OECD 1992). Part of the assessment factors themselves are derived from the comparison of L(E)C50 data and NOECs (ECETOC 1993).

In addition to the use of NOECs in risk assessment, NOECs are specifically required in testing strategies that come into force when the risk assessment based on L(E)C50 data indicates a possible risk (CEC 1996). Furthermore, NOECs are used in many countries to draw up environmental quality guidelines. The US-EPA, for instance, uses NOEC values for at least eight different species in order to derive a Final Chronic Value for the aquatic ecosystem (Stephan et al. 1985). Similarly, in the Netherlands NOEC values are used in the statistical extrapolation techniques put forward by Aldenberg and Slob (1993) in order to derive maximum permissible concentrations (MPC) in water and soil. These MPC values are the basis for environmental limit and target values (Slooff 1992). The use of NOECs has been laid down in a variety of documents as well as legislation (EC 1993, 1994, OECD 1992).

It may be concluded that the term no-effect, as well as NOEC values themselves, play an important role in legislation and procedures related to risk assessment and environmental quality guidelines, both at national and international level.

What is needed in environmental effects assessment of chemicals?

As stated above, current risk assessment schemes use PNECs and compare these values with measured concentrations or with predicted environmental concentrations (PECs). The word "predicted" is important here because in many cases nothing is known about the actual concentrations and effects that may occur under environmentally realistic conditions in ecosystems. Generally the estimation of the PNEC is based on few ecotoxicological data obtained from single-species tests under standard laboratory conditions. Hence, the uncertainty in the extrapolation is great. In order to reduce this uncertainty the best estimate of the no-effect concentration for the individual species is to be preferred. If possible, this NEC should be model-based, applicable in risk assessments and independent of the fact that people use different statistical techniques. Apart from the point estimates for the NEC, there is also an increasing demand in knowledge about the possible effects on ecosystems in situations where no-effect concentrations are exceeded. These "so what" or "what if" questions require not only the NEC but also a concentration-effect curve which allows the prediction of the percentage (adverse) effect.

The NOEC versus ECx dispute

The problems associated with the use of NOECs have been clearly identified and summarized by Pack (1993). There is no need to discuss these in detail but some of his most striking observations may be repeated here. Disadvantages of the NOEC are that: (1) the NOEC must be one of the test concentrations and therefore depends on

the choice of the test concentrations; (2) no precision statements are possible with the NOEC; (3) because of the variability in an experiment the NOEC may correspond to large effects; (4) the NOEC approach gives no information on the slope of the concentration-effect curve, i.e. the range of the sensitivity of the chemical. In his review, Pack concluded that the ECx estimates would be a far better alternative since: (1) the value is interpolated and less sensitive to the choice of test concentrations; (2) its precision can be quantified with confidence intervals; (3) because of (1) and (2) the ECx values are comparable and (4) if a model is fitted to the data the whole of the toxic response of the organism may be characterized. Clearly, EC50 estimates have a number of advantages from a statistical point of view. This may also be the case for ECx values. There are, however, also a number of disadvantages with these methods, such as the choice of the model and difficulties in computation. According to Pack (1993), these problems can be solved. In our opinion the arbitrariness of the choice of the dose-response model will always be a problem since there is no theoretical basis for preferring results of one model over the other. Apart from that there are a number of other disadvantages and unresolved problems related to the use of ECx values. These will be described below.

What value of x must be chosen?

An important question is what ECx value can be regarded as having no effect at the species level. This is certainly not a trivial question. The scientific community does not seem to have a general opinion on this matter. Values ranging from 5 to 25% have been proposed, which is still a considerably large range. The choice of an effect level at the lower end of this range may have considerable consequences for the design of the test and may increase its costs. The outcome will strongly depend on the choice of the response model. Furthermore, there is little insight in the long-term ecosystem effects of higher effect concentrations. As long as this insight is lacking a serious risk is taken by accepting these relatively high effect ranges.

Consequences for test guidelines

Another question is whether for all tests the same value for x should be used. For instance, is a 5% inhibition of algal population growth similar to a 5% lethal effect level for fish populations? When establishing values for x we need to take into consideration the generation time of these organisms in deciding what adverse effect levels are acceptable or not. Do we need to address the natural variability in these discussions? This would circumvent discussions about every existing test guideline as well as new test guidelines. It is certainly not possible for all current test protocols to calculate low ECx values with a reasonable confidence limit unless changes in the design of test protocols are made.

Consequences for risk assessment

What are the implications of using different effect levels, for instance when different ECx data with different values for x are used in extrapolation techniques in order to derive PNECs? Is there a need to change assessment factors which have resulted from long discussions at international level? There is no doubt that the introduction of ECx values with varying values of x, depending on the endpoint under consideration, will probably lead to long-term discussions about test designs and extrapolation methodologies. The advantages of using ECx values instead of NOECs in terms of the overall reduction in uncertainties in effects assessment are relatively small compared to the overall uncertainties, e.g. the lack of soil/sediment data and lack of information about the actual use and exposure patterns of chemicals.

What to do with old NOEC data?

If it is decided to move away from NOEC estimations to an alternative estimation of the no-effect concentration, the question arises if the old NOEC values can still be used side by side with these alternatives in the estimation of the PNEC or if separate safety factors and extrapolation techniques have to be developed. The lack of data that currently exists makes it unacceptable to reject 'old' NOECs because they are out of date. If on the basis of a large number of chemicals it can be shown that ECx values are generally higher than the NOECs, resistance against this change-over will certainly arise. Similarly, a general shift towards lower values will not please other parties in our society. From a scientific point of view this type of analysis may be disputed as incomparable data for different chemicals and species are combined in order to predict the overall shift in the PNEC.

Costs

At this stage in the discussion it is not exactly clear what the financial consequences would be in terms of redesigning test guidelines and the transformation of NOECs to ECx values. In the event that new tests have to be generated because raw data of tests are no longer available, the financial consequences for industries and governments would certainly be unacceptable. We expect that in a transition period NOEC values and alternative parameters will have to be presented in order to gain experience and in order to correlate the results of different methods with each other.

An alternative approach

It is obvious that the NOEC suffers from a number of serious problems and that an alternative estimation of the maximum concentration without effects would be highly desirable. The ECx is often proposed as being such an alternative, but this parameter also has a number of disadvantages that seem to be difficult to overcome. Kooijman

and co-workers describe methods in this book, which can be used to estimate the No-Effect Concentration as a model parameter from data from several aquatic toxicity tests. These methods kill two birds with one stone; they provide NEC estimates as well as estimates for the toxicity of chemicals for a species. Effects of toxicants are modelled by a change in the parameters of the Dynamic Energy Budget (DEB) of an organism, as a function of the tissue-concentration of the toxicant. The DEB model specifies the rules that organisms use for energy allocation to maintenance, growth, development and reproduction. The model is applicable to organisms that do not suffer from nutritional limitations and to organisms that are limited by just one type of resource (food, nutrient).

An important aspect of the new method is that it is process-based, which allows the evaluation of population consequences of effects on individuals. It also allows the evaluation of effects when concentrations of toxicant change rather than remain constant. This makes it easier to translate the results from bioassays into expected effects of emissions and, therefore, better applicable in risk assessment. Since the NEC and the effect parameters do not depend on exposure time, contrary to the NOECs and the EC50s, it is easier to extrapolate observed effects from a particular compound to that of other compounds with a similar mode of action, but a different lipophilicity. This can reduce costs considerably, and help to choose test concentrations in bioassays effectively. The software makes the method easy to apply.

Conclusions

The question whether the NOEC approach should be replaced by a better NEC estimate is a topic that not only concerns test guideline development. The NOEC plays an important role in procedures to set environmental quality guidelines and in the estimation of PNECs. These procedures are part of legislation and are based on internationally agreed procedures for risk assessment of chemicals. Moving to ECx estimation may provoke a re-opening of the discussions on either of the above mentioned points as well as on the principles that have been agreed upon at national and international level. It goes beyond saying that amendment of texts in legislation such as EC directives would require substantial time and energy.

If the prerequisites mentioned above can be fulfilled, the next step will be to obtain national and international acceptance of the alternative parameter. In order to prevent long-lasting repetitive discussions, coordinated action at high international level should be taken. The decision to enter this process should be taken, based on a thorough analysis of the advantages and disadvantages of the turn-over. It should be realised, however, that even with international acceptance of the alternative parameter, it will probably take many years before the necessary changes in legislation and associated procedures will be effective. Keeping this in mind, as well as the fact that the uncertainty in the whole risk assessment process will usually be much

greater than the uncertainty in the estimation of NOECs, one might seriously question whether it is worth stepping into this process. What does remain in the area of effects assessment is the need for (1) good estimates of no-effect levels and (2) reliable information about the concentration-effect relationship in order to address the question what will happen if NOECs, PNECs or environmental quality guidelines are exceeded. We believe that methods are available which solve some of the statistical problems related to the derivation of NOECs, and at the same time make optimal use of test data to derive concentration-effect relationships.

We think that this book provides a major contribution to the debate regarding these alternatives. We hope that the scientific discussion will ultimately provide the necessary building blocks to make a well-considered choice of one of them.

References

Aldenberg, T. & Slob, W. 1993. Confidence limits for hazardous concentrations based on logistically distributed NOEC toxicity data. *Ecotoxicol. Environ. Saf.* **25**: 48-63.

CEC 1996. Commission of the European Communities: Risk assessment of new and existing substances. Technical Guidance Document. Directorate-General for Environment, Nuclear Safety, Brussels, Belgium, in press.

Chapman, P.M., Caldwell, R.S. & Chapman, P.F. 1996. A warning: NOECs are inappropriate for regulatory use. *Environ. Toxicol. Chem.* **15**: 77-79.

EC 1992. Council Directive 92/32/EEC of 30 april 1992 amending for the seventh time Directive 67/548/EEC on the approximation of the laws, regulations and administrative provisions relating to the classification, packaging and labelling of danger- ous substances.

EC 1993. Commission Directive 93/67/EEC of 20 July 1993, laying down the principles for the assessment of risks to man and the environment of substances notified in accordance with Council Directive 67/548/EEC.

EC 1994. Commission Regulation of 28 June 1994, laying down the principles for the assessment of man and the environment of existing substances in accordance with Council Regulation 793/93/EEC.

ECETOC 1993. Environmental hazard assessment of substances. Technical report No. 51. ECETOC, Brussels.

Hoekstra, J.A. & Ewijk, P.H. van 1992. Alternatives for the no-observed effect concentration. *Environ. Toxicol. Chem.* **12**: 187-194.

OECD 1992. Report of the OECD workshop on the extrapolation of laboratory aquatic toxicity data to the real environment. OECD Environment Monographs No. 59. Paris.

Pack, S. 1993. *A review of statistical data analyses and experimental design in OECD aquatic toxicology Test Guidelines.* Shell research Ltd. Sittingbourne, Kent, UK.

Slooff, W. 1992. RIVM Guidance Document. Ecotoxicological Effects Assessment: Deriving maximum tolerable concentrations (MTC) from single-species toxicity data. National Institute of Public Health and Environmental Protection, Report nr. 719102018. Bilthoven, The Netherlands.

Stephan, C.E., Mount, D.I., Hanson, D.J., Gentile, J.H., Chapman, G.A. & Brungs, W.A. 1985. Guidelines for deriving numeric National water Quality Criteria for the protection of aquatic organisms and their uses. PB85-227049. US-EPA, Duluth, Minnesota, USA.

Chapter 2

Toxic effects as process perturbations

2.1 Introduction

Ecotoxicology

Ecotoxicology developed from human toxicology and pharmacology; these roots are still clearly visible today. Animals originally served just as models for humans, only much later was the health of animals themselves given some interest. The extension to algae, plants and ecosystems is rather recent. This is partly due to the poor structure of ecology as a science to predict population and ecosystem dynamics. The scanty knowledge in this area is mostly based on what is called unstructured population dynamics, where individuals are treated as identical copies. Since pollutants directly affect individuals, not populations, theories on structured population dynamics had to be developed in which individuals differ in one or more respects from each other (age, size, energy reserves, toxic burden etc). These theories are also essential to give population dynamics a firm physiological rooting. Processes that define the physiology of ecosystems, such as nutrient recycling, carbon budgets, etc., can only be understood as further consequences of population dynamics. Theories about structured population dynamics are still in their second decade and they are rather complex, which explains why they still play only a minor role in ecological thinking. Furtunately, this is now changing rapidly.

Most interest in ecotoxicology derives from a costs-risk analysis where the problem is to minimize production costs of goods as well as effects of human induced chemical pollution that results from this production. The scientific problem of assessing effects of toxicants is wider, however, and also of fundamental interest. Organisms have

[0]Published as: S.A.L.M. Kooijman (1996) In: Schüürmann, G. and Markert, B. *Ecotoxicology*, Spektrum Akademischer Verlag, pp 483–519

been struggling for survival in chemically unfriendly environments from their first existence on.

The appearance of oxygen as a byproduct of photosynthesis in the atmosphere has probably been fatal for most pre-cambrian organisms. The production of cyanides, alkaloids and other secondary products by plants obviously function to deter herbivores; the tannins of acorns effectively block digestion by the European red squirrel, for instance, but the American grey squirrel found a way to deal with this defence of the oak and so managed to outcompete the red squirrel in parts of Europe (MacDonald 1995). Like plants, many species of animal use toxins to protect themselves against predation; heliconid caterpillars accumulate toxins from passion flowers; arrow frogs and the hooded pitohui (a bird) produce protective toxins themselves (Emsley 1992). More active forms of chemical defence occur in termites (Prestwich 1983), while snakes, wasps, centipeds and many other organisms use venoms to kill offensively. Biology is full of examples of chemical warfare with sometimes striking responses and defence systems (Agosta 1995). The ability of the parasitic bacterium *Wolbachia* to induce parthenogenesis in normally sexually reproducing species (doubtlessly via chemical interference) has recently attracted a lot of attention (Maran and Baumann 1994). Some bacteria quickly transform sugar into acetate for later consumption, while suppressing growth of competitors. Natural growth-suppressing compounds, such as penicillin, are intensively applied in medicine. Botulin, which is produced by the bacterium *Clostridium botulinum*, makes frequent casualties among fish and birds. The soil bacterium *Bacillus thuringiensis* produces a toxin that kills insects effectively (Holmes 1993).

These different fields of interest have not, to my knowledge, been brought together as yet. The reason is probably that the pressure to quickly 'solve' practical problems in risk assessment has always been intense and has dominated ecotoxicological methods. Science, however, would greatly benefit from a more fundamental approach to the problem of effects of toxicants (human-induced *and others*) on organisms.

The development of ecotoxicology has started for aquatic environments. This has been due to early human health problems with drinking water, but also because of the relative simplicity of this environment in being well-mixed and relatively easy to standardize. There is no such thing as a "standard soil" with a "standard humidity". Although the basic principles are the same in aquatic and soil environments, the problems of bioavailability, transport and transformation are very difficult to disentangle from toxicity in soil environments. Although standardization benefits the comparability of toxicity results, routine toxicity testing provides good examples to illustrate that standardization of experiments that lack a firm scientific basis actually hampers the development of such a basis. Applied aspects (i.e. risk assessment, cost control) rather than the scientific problem controlled experimental research and, therefore, the methods of ecotoxicology; a most undesirable situation. The slogan "no change, no progress" certainly applies to the present situation of toxicity testing.

Risk assessment

Risk analysis is based on expected effects of chemical compounds in the environment. Spatial and temporal scales are very important. There is a great difference between point and diffuse emissions and between emissions that occur only at one given moment and that continue in time. This is due to the differences in the relative importance of aspects such as transport, chemical transformation, adaptation and local effects with biological recovery due to migration from distant areas. See van Leeuwen & Hermens (1995) for a recent discussion of these topics. The most important chemical transformation to be considered in the translation of laboratory results into expected effects in the field relates to the phenomenon of bioavailability. Only a small fraction of the compound is directly available in the field due to binding to ligands (mainly dispersed organic matter), but the fraction that is not available may become available later. Such delays can be difficult to judge. The weakest topic in risk assessment, however, is the set of biological effects itself, because of the problem that we already have to predict situations without toxicants. The numerical behaviour of real-world populations is frequently erratic and difficult to understand. This makes effects of a toxic compound difficult to recognize, especially when the effects are small. This difficulty does not imply that small deviations are not important in the long run. The unpredictable behaviour is one reason why experiments with mesocosms do not always demonstrate effects that can be expected on the basis of single-species toxicity tests under well-controlled conditions (Kooijman 1988). These remarks point out that many factors contribute to expected effects in the field, but it is from the effects on biota that human-induced chemical pollution derives its interest.

Central to the problem of risk assessment is the fact that it is easy to demonstrate effects on individuals under controlled conditions, whereas the environmental problem occurs at the ecosystem level. We therefore need to know how these levels of biological organization are interrelated. Energy budgets are central in this translation because they define the processes of competition, predation and propagation, which are at the core of population dynamics and, therefore, of ecosystem dynamics. We thus have to know how energy budgets are affected and how this translates to a deviation of an ecosystem behaviour without toxicants. The latter is obviously far from straightforward, in view of the problems we already have to create models where a rich diversity of species is persistent. We are just beginning to understand how population dynamics in simple (homogeneous) environments relate to properties of energy budgets and how they change during life span.

The significance of toxicant-induced mutations and teratogenic effects for environmental risk assessment is understood extremely poorly. Some workers even deny the environmental significance of mutagenic compounds, since mutations in somatic cells hardly affect the health of the organism (except humans) and mutations in gametes are not relevant because of the abundance of unaffected gametes. Below I will

discuss the rationale behind the idea that mutagenic compounds reduce the life span of organisms via interaction with the process of aging.

If the translation from laboratory results into field predictions is feasible, it will only be so if the description of effects is based on mechanistic insight. Existing methods to describe effects of toxicants are purely descriptive, however. The most important parameters are concentrations for which the survival probability is half the value of the control (LC50), or of concentrations for which some quantity of interest such as size or number of offspring is half the value of the control (EC50). The next section will discuss the scientific problems of such a description, which also affect the estimation of concentrations that have no effect at all.

The most popular method to obtain an estimate for such a concentration is to identify the highest tested concentration that does not differ significantly from the control (the no-observed effect concentration, NOEC). See Yanagawa et al. (1994) and Hothorn (1994) for recent statistical reviews. Apart from the minor problem that the possible values are restricted to the limited set of tested concentrations, the fact that the estimate depends on the unknown power of the statistical test that is used to spot significant deviations from the control can lead to very strange results (Skalski 1981; Kooijman 1981; Hoekstra & van Ewijk 1983; Pack 1983; Stephan & Roger 1985). It is quite possible that the NOEC is larger than the EC50 for instance! Because legislation aims to prevent effects by reducing the amount of emitted chemicals, NOECs still play a major role in applied ecotoxicology. I will discuss better alternatives that eliminate the statistical problems of NOECs below.

After a brief description of standard methods to describe effects of toxicants, I will discuss the analysis of the most relevant effects of compounds: survival, mutation, reproduction. These effects are the most important in relation to the quality and quantity of life. Population dynamics depends directly on the processes of reproduction and survival. Reproduction can, however, be affected directly or indirectly via feeding (assimilation), growth, and maintenance. Why and how these physiological processes are related is the subject of the Dynamic Energy Budget (DEB) theory, which will also be discussed briefly. Although the mechanisms and analyses apply to aquatic toxicity, terrestrial toxicity as well as human toxicity, the discussion will focus on aquatic ecotoxicity.

Following Landrum & Dupuis (1990) and Landrum et al. (1992), Kooijman (1981), McCarty (1986, 1990, 1991), McCarty et al. (1989, 1993), the effect size will be related to the concentration of toxicant in the organism, and consequently the kinetics (i.e. uptake and elimination behaviour) of the compound is relevant to understanding the effects. Since the kinetics of lipophilic compounds depends on the lipid content of the organism, and, therefore, on the feeding status and the energetics, we have a second reason why the DEB theory is basic to the understanding of effect sizes. So, after the standard methods, I will first discuss elements of the DEB theory, and subsequently toxicokinetics, effects, and consequences of these effects on population dynamics.

2.2 Standard descriptions

LC50 and gradient

The results of standard tests on the lethality of toxicants usually give the number of surviving animals as a function of the concentration of toxicant, which has been constant (hopefully) during a standardized exposure. The control survival probability is typically larger than 90% and a sigmoid curve is fitted to the number of survivors as a function of the concentration of toxicant (in water). This curve is usually the log-logistic or log-probit curve, which both are characterized by a 50% point (the LC50) and a gradient parameter, which represents the maximum change in the number of survivors as a function of the logarithm of the concentration in the water.

When counts have been made at different observation times, the LC50 generally decreases as a function of exposure time. This phenomenon can be described well on the assumption that the survival probability depends on the concentration in the organism, and that the toxicant follows some simple kinetics (Kooijman 1981). The relationship between uptake and effects has meanwhile been well established (Crommentuijn et al. 1994). Death is certain as soon as the toxicant in the organism exceeds a certain individual-specific threshold value. Individuals vary in physiological condition and therefore in threshold values. The threshold value of a particular individual is assumed to be a (random) trial from a bell-shaped frequency distribution, which leads to a sigmoid concentration-response curve for the number of survivors. The effect, then, is described deterministically at the level of the individual and stochastically at the level of the cohort of tested organisms.

Problems

The reconstruction of basic assumptions in the standard model has been given above to reveal several problems that are inherent to this method of description.

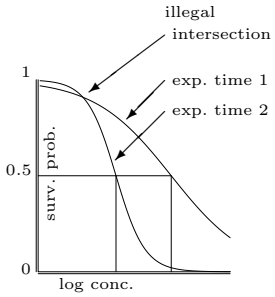
- Extreme standardization of culture conditions practically eliminates the physiological differences between individuals, but experimental practice shows that the gradient parameter cannot be increased beyond a given maximum. There is an upper limit for the maximum slope of the concentration response curve. In other words, there is a rather substantial variation of threshold values between individuals. It appears that the effect is *stochastic* at the level of the individual, not deterministic.

- The distribution that describes the variation in threshold values (log-logistic, log-probit) represents a rather arbitrary choice from the large set of possible distributions. This is of little relevance for the estimation of the LC50 value itself, as long as the selected curve fits the data. The particular choice of

response function is, however, of great importance if we wish to obtain 'small' effect concentrations, such as the LC1 or LC5, from the estimated parameters (LC50 and the gradient). The smaller the effect level, the larger the confidence interval, and more importantly, the more the result depends on the specific choice for the model.

- At high concentrations of a very toxic compound, the standard LC50/EC50 model makes the very unrealistic prediction that there are unaffected individuals. This property is due to the infinitely long upper tail of the distribution of threshold values. This is inconsistent with physical chemistry, and from long experience we simply know that no individual survives prolonged exposure to very toxic compounds.

- Since the gradient parameter reflects the variation in the (logarithms of the) threshold values, it is independent of the exposure time. In practice, however, the gradient tends to increase with the exposure time. We cannot simply parameterize this phenomenon by choosing a time-dependent function for the gradient parameter, because we then run into the problem that for certain (low or high) concentrations the survival probability will increase in time, which is obviously not possible. The only way to incorporate such phenomena is to go back to a process-oriented description for survival.



- Sublethal effects show that the reasoning behind the description of lethal effects is simply wrong. No big variations in the physiological conditions of standardized test organisms have been observed. When a toxicant affects reproduction, for instance, it does so in all individuals to about the same extent. There are no records of individuals that cease reproduction while others continue at the blank rate.

- As mentioned before, LC50/EC50 values themselves depend on exposure time. The problem is not completely solved by standardizing toxicity tests to a fixed exposure period (The standardized toxicity test with *Daphnia magna* lasts 48 h, independent from the type of compound that is tested). Surfactants react quickly; if no effect shows up after a few hours of exposure, it is unlikely that any effect will show up at that concentration. Things are totally different for toxicants such as cadmium. The LC50 for an animal as small as *Daphnia* still decreases after three weeks of exposure. The LC50-time behaviour depends on properties of the chemical as well as those of the organism (especially body size). This mixture of properties is most unfortunate in application of LC50/EC50 in risk assessment and reduces the comparability of results of standardized tests.

EC50

There is not much to say about models that are used to describe sublethal effects. The standard approach is to relate the quantity of interest, such as the cumulated number of offspring during a standardized test period, to the logarithm of the concentration of toxicant in a logistic way. Apart from the NOEC, this gives three parameters: the control value, the EC50 and a gradient parameter. This model just serves the purpose of describing a very limited data set, without bothering about the foundations. The aim seems to be to obtain an EC50 value for the quantity of interest and compare it with other EC50 values of other compounds and/or effects. A good example of a frequently applied nonsense parameter is the EC50 for biomass of algae in a 3 days test on growth inhibition. The value depends on the arbitrarily chosen test period and the (control) population growth rate (and therefore the medium composition, the temperature, light conditions, turbulence and alga species). Although these limitations are recognized (Nyholm 1985), this is apparently no reason to abandon such measures. Many people seem to think that standardization solves all problems, and leave to the poor administrator the problem of risk assessment which requires an integration of different kinds of information.

Conclusion

The standard model is based on assumptions that are not realistic. The fact that LC50 or EC50 values as well as NOECs depend on exposure time in a way that depends on both compound and organism characteristics hampers their application. The use of repeated observations to detect deviations from the control is problematic without an adequate model for the appearance of effects. In general, such observations are statistically dependent. Moreover, the standard model is inconsistent with the concept of the NOEC, because the log-logistic as well as the log-probit concentration-response curve approaches the control response for decreasing concentrations only asymptotically. The standard model can be extended to include a no-effect concentration (NEC) as a parameter (Kooijman 1981). Such a model solves the problem of statistical dependence and the unknown power of the test to spot deviations from the control response. This is because the null-hypothesis states that the NEC equals zero, while the alternative hypothesis asserts that it is positive. Twenty years of routine application indicate that point estimates for NEC are positive in about 50% of the cases and that in less than 10% of the cases the NEC differs significantly from zero. This low frequency of significantly positive NECs is due to the gradient parameter, whose unknown value reduces the information content of a concentration that shows an obvious effect to "the NEC is smaller than that concentration". Response curves with a positive NEC and with a NEC of zero are too similar. I now consider my previous attempts to improve the standard model to be a failure and advocate a radical rejection of the standard model in favour of the DEB-based models that are discussed below.

Small effect concentrations have been proposed to replace the NOEC (see Bruce & Versteeg 1992, Pack 1993). Apart from the problem of defining 'small', such parameters hardly solve the problem, due to the arbitrariness of the choice of response function. The problems become less pressing for moderate effect concentrations (e.g., LC10 of LC25), but who wants to allow such effects to occur? The larger the effect, the more important it is to have a reliable translation of the effect into consequences for the ecosystem. Such reliable translations do not exist and it is very unlikely that they will exist in the near future.

In summary, I have to conclude that the LC50 and EC50 are parameters that have desirable statistical properties (Hoekstra 1993), but nobody should attach much ecological importance to their values. They are hardly relevant for risk assessment and they are based on a model with a shaky basis. Useful descriptions should be process-oriented. The existence of extensive data bases for LC50/EC50 values should not be a reason to continue the application of the standard model.

Alternatives

Thinking about alternative methods to characterize toxic effects, it might help to have a closer look at mutagenic effects first. Traditionally, these effects are considered as a completely different category, but I will show that it is possible to construct a framework for the characterization of toxic effects into which effects on survival, growth, reproduction *and* mutation fit naturally. Several other effects, such as those on respiration and mineralization, can be understood in terms of the mentioned effects. The theoretical basis of these relationships has been worked out in Kooijman (1995).

The most frequently used bioassay for testing the mutagenic effects of compounds is the Ames test. A series of petri dishes with rich medium but a very small amount of histidine, is inoculated with a mutant of the bacterium *Salmonella typhimurium* that is not able to synthesize histidine itself. Different amounts of test compounds are added to the dishes and the number of revertant colonies is counted. The number of revertant colonies is usually a linear function of the concentration. It appears that back mutations to the wild type that is able to synthesize histidine only occur if the mutation takes place during the short period of growth, where histidine is available (van der Hoeven et al. 1990). The relevance of this bioassay for mammals (and in particular for humans) is increased by adding liver homogenate of rats to mimic metabolic transformations that might occur in mammalian cells. NOECs are usually not obtained for this test; the only interest is in the slope of the concentration-response line.

False positives can occur in the Ames test if background mutations exist (e.g. as a result of autoclavating the medium) and if the compound affects the growth rate. In that case, the compound increases the exposure period to the mutagenic compounds in the medium because the growth process lasts longer at high concentrations.

Each molecule is assumed to have an equal probability of producing a mutation, which results in a linear concentration-response relationship. Below I will show that this idea of independent effects of molecules extends to all kinds of toxic effects, *if* we focus on the right physiological target process. If we do so, we can obtain the familiar sigmoid concentration-response relationships for the number of survivors, the number of offspring and for the size of organisms, etc. The DEB theory will be used to identify the target processes and how they are interrelated. I will also show how mutagenic effects relate to the process of aging within the context of this theory.

2.3 Dynamic Energy Budgets

Energy budgets have provided a very useful vehicle for the understanding the connections between food uptake, digestion, maintenance, growth, reproduction and aging. Since these relationships change in a systematic manner during the life span, such budgets should be treated dynamically. Generally three life stages should be distinguished: embryo (which does not feed or allocate material to reproduction), juvenile (which feeds, but still does not allocate material to reproduction) and adult (male/female or both). The DEB theory gives detailed quantitative descriptions for the basic processes during the life, which are supposed to apply to all heterotrophs, from bacteria to humans to whales. Unicellular organisms are treated as juveniles because they take up resources from the environment and they do not allocate energy or material to reproduction. It is beyond the scope of this section to discuss the details of the theory; therefore, I only mention the most basic axioms. It can be shown (Kooijman 1995) that a tight connection exists between mass and energy fluxes in heterotrophs, which rests on the concept of homeostasis: the ability of organisms to keep the composition of the body constant despite variations in the chemical environment. All mass fluxes must be weighted sums of three categories of energy fluxes: assimilation, growth and dissipating energy fluxes such as maintenance, heating, etc. Autotrophs are more complex in this respect, but follow the same basic rules. Consequently, the processes of mineral recycling and other ecosystem physiological processes can all be conceived as consequences of dynamic energy budgets.

Food uptake by juveniles and adults is scaled as proportional to the surface area and depends hyperbolically on food density. The surface area is important because it is linked to encounter rates with food particles at low food density (filtering rates, searching rates) and to food processing rates at high food density (surface area of gut, which is approximately proportional to surface area of the whole animal). A hyperbolic relationship with food density results if each food particle blocks the uptake of other food particles for some time, leading to a rejection of particles when the animal is 'busy'. This handling time may relate to mechanical handling of food particles, but also to the digestion process and/or some process further down the line

of food processing. The maximum ingestion rate is realized when the animal spent all time in handling food particles and no waiting time between subsequent particles is left.

Digestion efficiency is constant, i.e. independent from food density and the size of the organism. Material and energy is extracted from food and added to the reserves. The reserves in all three life stages are used at a rate that depends on the amount of reserves and the size of the organism. A fixed fraction of this mobilized material is used for maintenance plus growth; the rest is used for development and reproduction. (Reproduction only applies to adults.) The amount of energy that is required for maintenance is proportional to the volume of structural biomass, which is synthesized as a result of resource allocation to growth. Thus, growth stops if the energy allocated to growth plus maintenance is fully consumed by the process of maintenance; reproduction is a parallel process.

The organism switches from embryo to juvenile and from juvenile to adult when it has invested a certain cumulative amount of energy in the increase of its state of maturity. The material allocated to reproduction is cumulated into a buffer and at the moment of reproduction this material is converted into one or more eggs. (Some organisms, such as most mammals, do not reproduce via eggs but via foetuses which receive nutrition from the mother during development.) Initially, the structural biomass of eggs is negligibly small, the embryo develops at the expense of its reserves.

Unicellular organisms divide when they have invested a certain amount of energy in development. Due to the partitioning rule for the energy mobilized from the reserves, this occurs at a certain structural biomass. More precisely, the cell starts to duplicate its DNA when it exceeds a certain structural biomass. This duplication process lasts a fixed period during which the cell continues to grow. Since the rate of growth depends on the amount of reserves, the cell size at division depends on the feeding conditions.

As a result of respiration (the use of oxygen to free energy from reserve materials), DNA is affected via free oxygen radicals with an efficiency that depends on the type and amount of antioxidants in the tissue. The exact link between respiration and energy fluxes on the basis of the above mentioned rules is somewhat technical, but fully specified by the set of rules for energy uptake and use. When DNA is affected, this has two consequences. The original protein (usually enzyme) is no longer produced by the affected gene and that gene produces disfunctional proteins. Affected cells do not divide. For unicellular organisms this means death or metabolic arrest. For metazoans this means that the damage, measured as the cumulated amount of disfunctional proteins, can be diluted by growth, i.e. the increase of unaffected structural biomass. It appears that a very good agreement with experimental survival patterns results if we simply take the hazard rate proportional to the cumulated damage. The hazard rate at a certain age is defined as the instantaneous death rate, given survival up to that age. The survival probability at that age equals the exponent of minus the cumulated hazard rate up to that age. Notice, however, that aging

is just one of the components that affect survival. The process of aging is described by a single parameter, called the aging acceleration. It is an acceleration because the hazard rate is proportional to *cumulated* damage; the cumulation process transforms a rate (dimension time^{-1}) into an acceleration (dimension time^{-2}).

This model for aging gives the correct predictions of the interrelationships between life span and feeding level for ectotherms, i.e. animals that do not allocate energy to heat their body to some preset temperature. Endotherms seem to decrease the efficiency of the defence system to catch free radials during life. This can be understood by realizing that organisms use free radicals to build up genetic variability in the gametes, which are treated in the same way as somatic cells with respect to mutation frequencies. There is a trade-off between the expected life span of individuals and genetic variability.

In summary, the DEB theory selects reserves, structural biomass and cumulated damage as the state variables, to describe the various processes quantitatively. A set of 11 parameters that determine all rates in the three life stages, given food density and temperature, is assumed to be fixed for a certain individual during its life span. Arrhenius relationships describe how the rate parameters depend on temperature. It is beyond the scope of this section to explain why and how these 11 parameters tend to co-vary when we compare individuals of different species. Body size scaling relationships that describe how physiological life history parameters change with adult body size can be derived on the basis of the structure of the theory without using any empirical argument. Indeed, it explains why the respiration rate is roughly proportional to biomass raised to the power 0.75; a well-known physiological enigma thus seems to be eliminated.

The DEB theory is at the heart of population dynamics. Populations are then conceived as a group of individuals that obey the rules implemented in the DEB theory and, as a first approximation, only interact via feeding on the same resource. Population dynamical theories that account for differences between organisms in terms of age, size etc. are called structured population dynamics (see Metz & Diekmann 1986 for an introduction). The laws of energy and mass conservation then lead to the rules for how population size and structure change in time (in terms of frequency distributions of body size and reserves), given a specification of the environment in which a population lives (Kooi & Kooijman 1994, 1994a, 1995). This is obviously rather complex for organisms that do not change in shape during growth, which makes their surface area proportional to their volume raised to the power 2/3. We use parallel computers to analyze the dynamics of such populations. If the organisms do change in shape during growth, such that their surface area is proportional to their volume, the rules for population dynamics simplify to what is known as unstructured population models, i.e. models in which the individuals are combined into a total population biomass. Unicellular organisms have a very interesting intermediate position, because details about their growth from baby cell to mother cell, which amounts to a factor of only two, do not greatly affect the population dynam-

ics. We use these paradigms to find simple approximations to population dynamics and the conditions under which these approximations hold. Dynamic transformation efficiencies for the conversion of food into biomass play a central role in simplified descriptions of structured population dynamics.

Populations can be tied into food chains and food webs, which can easily show very complex dynamic behaviour in simple environments. A theoretical microbial food chain of length 4 (i.e. a non-reproducing substrate, prey, predator and top-predator) can behave chaotically in a simple chemostat environment at special combinations of throughput rate and concentration of substrate in the feed. At present we are working on simplified approximate descriptions of summary statistics such as total biomass of the food web, which might contribute to ecological insight. It seems that maintenance requirements set a natural maximum to the length of a food chain, for instance, but we still cannot derive this maximum as function of the parameter sets of the species in the chain.

Although we are moving towards to an understanding of the behaviour of simplified ecosystems in terms of mass (nutrient) and energy fluxes in the compartments primary producers (plant/algae), consumers (animals) and decomposers (bacteria/fungi), progress is slow and painstaking. It is only fair to mention that we are still far away from a scientifically sound understanding of ecosystem dynamics. A major obstacle is spatial heterogeneity. It seems that this component is very important for realistic population dynamics, but very hard to incorporate and analyse in models.

2.4 Toxicokinetics

The simplest, and in many cases most realistic, model that describes the uptake and elimination process is the one-compartment model. It assumes that uptake rate is proportional to the concentration in the environment and elimination rate is proportional to the concentration in the tissue. Note that the uptake rate depends on the environment-concentration in the same way as food, provided that the 'handling' time is negligibly short with respect to the period between subsequent arrivals of molecules. This seems realistic at low concentrations of toxicant, where the 'handling' in this case refers to residence time in the exposed membranes.

At a constant concentration in the environment, this gives an exponentially satiating curve when the tissue-concentration is plotted against exposure time. The ratio of the ultimate tissue-concentration and the environment-concentration equals the ratio of the uptake and the elimination rate; it is known as the bioconcentration coefficient.

Uptake can be directly from the environment, for instance, via the respiratory system (gills or lung), the skin, or food. According to the DEB theory, food uptake is proportional to surface area so the total uptake rate of the toxicant is proportional

to surface area. This also holds for the total elimination rate, expressed as mass per time, which implies that the waiting time to reach a certain tissue-concentration is proportional to the ratio of the volume and the surface area: the volumetric length of an organism. (The volumetric length is defined as the cubic root of the volume.) Connell & Hawker (1988) arrive at similar conclusions on the basis of a diffusion model. Effects of toxicants relate to tissue-concentrations. The test on survival of 1 mm long water fleas *Daphnia*, which weighs some 0.2 mg (volume $\simeq 0.2$ mm^3), is standardized at 48 h, and of 40 mm long young *Salmo*, which weighs some 85 mg (volume $\simeq 85$ mm^3), at 96 h. If the species have the same sensitivity and surface-area specific kinetics, there must exist a factor $\sqrt[3]{85/0.2} = 7.5$ between the lengths of the tests, rather than $96/48 = 2$. Small wonder that *Daphnia* is generally considered to be a sensitive test organism.

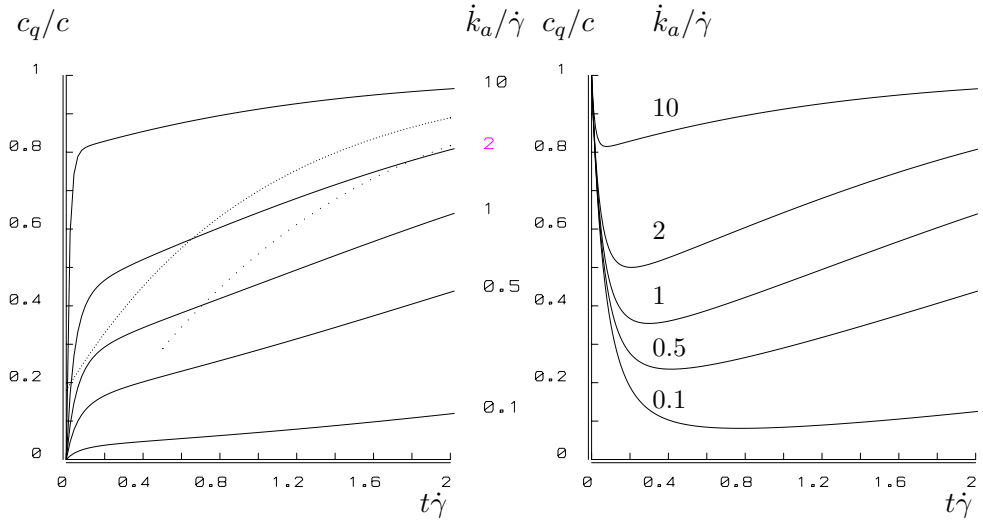
If an animal grows during exposure, the kinetics become a bit more complicated, due to the changing surface area and the dilution by growth (see Figure 2.1). A *Daphnia* reproduction test is standardized at 21 d and starts with 0.8 mm long neonates. After 21 d, the daphnids are 4 mm (in the control), which means an increase in weight by a factor $5^3 = 125$. With the DEB theory, correction for this substantial growth during exposure is straightforward.

Another cause of deviation from a one-compartment kinetics relates to changing lipid content of the animal due to changing feeding conditions, for instance. Animals such as the blue mussel *Mytilus edulis* accumulate lipids during the year; at spawning, they release most of it in gametes and drop in dry weight by more than a factor two. Lipophilic compounds accumulate rapidly in lipid-rich organisms. For this reason, eels (*Anguilla*) tend to have much higher loadings of lipophilic compounds than other fish. The DEB theory can be used to take account of these changes on the basis of the assumption that partitioning of the compound over the various body fractions is fast with respect to the uptake and elimination rates. If this partitioning is not fast, we have to turn to multicompartment models, but experience indicates that experimental data hardly justify such a move. The problem is that the number of parameters is already large enough to obtain the flexibility to fit any observed data well, irrespective of the realism of the model. Application of multicompartment models is only justified if the compartments are identified and their burden measured. If the compartments represent organs and the exchange rates between the organs have a physiological underpinning, multicompartment models are called Physiological-Based PharmacoKinetic (PBPK) models, see Baron et al. (1990) and McKim & Nichols (1992) for reviews. The use of the extremely parameter-rich multicompartment models originates from pharmacology, but such models usually have too much detail to apply in ecotoxicology.

The third class of deviations from a one-compartment model relate to metabolic transformations of the toxicant which usually occur in the liver. These transformations usually increase the hydrophilicity and thus the elimination rate. They represent a mechanism by which the organism detoxifies the compound. Both the

Figure 2.1: Uptake and elimination during growth. The scaled tissue-concentration start from $c_q(0) = 0$ (left), or $c_q(0) = c$ (right), where c stands for the environment-concentration. The different curves represent different choices for the value of the elimination rate $\dot{k}_a$, relative to the von Bertalanffy growth rate $\dot{\gamma}$. The finely dotted curve represents (scaled) body length and the coarsely dotted curve the (scaled) reproduction rate. The (scaled) lengths at the start of exposure and reproduction are realistic for the water flea *Daphnia magna* and the value $t\dot{\gamma} = 2$ corresponds with 21 d for *D. magna* at $20°C$. All curves in both graphs have an asymptote at the value 1. If the product of the von Bertalanffy growth rate and the exposure time $t\dot{\gamma} > 0.4$, the curves in the left and right panels are almost identical, i.e. independent from the initial tissue-concentration. The deviations from $c_q = c$ can therefore be attributed to 'dilution by growth'. For f denoting the scaled functional response (i.e. feeding rate), the change in scaled tissue-concentration c_q and scaled length l is given by

$$\frac{d}{dt}c_q = c\dot{k}_a f/l - c_q\left(\dot{k}_a f/l + \frac{d}{dt}\ln l^3\right) \quad \text{and} \quad \frac{d}{dt}l = \dot{\gamma}(f - l)$$



elimination and effects relate to the aqueous fraction, not to the fraction in the lipid storage of organisms, because these are metabolically inert. This is best illustrated by freshly laid birds eggs, which are almost pure reserves, the structural biomass of the embryo being negligibly small; such eggs hardly respire. During ontogeny (incubation), the respiration rate increases as the structural biomass of the embryo increases. This also means that effects are likely to show up when the reserves are used during starvation. Models for metabolic transformations are organism and compound specific.

If the transformation rate is proportional to the concentration in the aqueous fraction and partitioning is fast, we still are in the class of one-compartment models (with varying coefficients). This class of models is rather easy to implement in effect-studies. Transformation rates can, for instance, be coupled to measures for metabolic activity, such as oxygen consumption or carbon dioxide production; uptake can be linked to water uptake (for terrestrial organisms), food intake or oxygen consumption. All energy budget models that fully specify energy fluxes also specify mass fluxes, such as water uptake, oxygen consumption etc. This is explained and worked out for the DEB model in Kooijman (1995). This class of models is sometimes called 'Bioenergetic-based toxicokinetic models'. Examples of such models for static energy budgets can be found in Connolly & Tonelli (1985), Fagerström (1977), Jensen et al. (1982), Norstrom et al. (1976), Thomann (1989).

2.5 Effects on individuals

Organisms evolved in a chemically varying environment; consequently they can cope with varying concentrations of any particular compound, as long as the variations are within a certain range. The upper boundary for this range, i.e. the internal no-effect concentration, might be zero for particular compounds. Each molecule of such compounds induces effects with a certain probability, but for most compounds the upper boundary is positive. The lower boundary is zero for most compounds because they are not necessary for life. Elements such as copper are required, so that the lower boundary for copper is positive. Nitric oxide, which causes great problems in photochemical smog and acid rain at high concentrations, has many essential physiological roles at low concentrations (Young, 1993). Effects of a shortage of a compound resemble the effects of an overdose in their kinetics. The founder of ecotoxicology, Sprague (1969), studied the effects of toxicants in bioassays using oxygen shortage as an example. Although many interrelationships exist between nutrition and toxic effects, the upper boundary of the tolerance range attracted most attention in ecotoxicology, due to its application in risk assessment studies, while ecology focused on the lower boundary (see White 1993).

Discussions reveal that not every ecologist feels comfortable with the assumption of a tolerance range for chemicals. For temperature such a tolerance range is widely

accepted, however. The fact that ecological observations are usually made without a detailed specification of the chemical environment implies that most ecologists do tacitly accept the existence of a tolerance range for 'natural' compounds (whatever they might be). In fact, a complete chemical specification of any local natural environment (water, soil) is not possible. I see the assumption of a tolerance range for toxicants hardly as a problem, because we always can (and should) test the hypothesis that the upper boundary of the tolerance range (i.e. the internal no-effect concentration and therefore also the (external) No-Effect Concentration, NEC) equals zero. If we cannot reject this hypothesis, we have to face the possibility that each molecule of that compound may have an effect. If this compound still has to be released into the environment, the failure of rejecting the hypothesis that the NEC is zero might be a good reason to give priority to research on the magnitude of ecological effect of such an emission. The primary purpose of routine toxicity testing is to set priorities to further research, not to predict ecological effect sizes.

Each physiological process has its own tolerance range for any compound. The upper boundaries can be ordered, which means that at the lowest tissue-concentration range that produces effects only one physiological process is affected, while at high tissue-concentrations many processes are affected. As long as the partitioning of the compound over the various body fractions is fast with respect to the uptake/elimination kinetics for the whole animal, it is not essential to specify the tissue or organ in which the most sensitive physiological process is affected. This only becomes essential if the partitioning is slow and we have to apply multicompartment models. In that case we have to know a great deal more, which makes multicompartment models difficult to apply. Notice that one-compartment models can handle different concentrations in different organs as long as partitioning is fast. Observed deviations from one-compartment kinetics with constant coefficients frequently relate to the variations in the coefficients, not necessarily to the presence of more compartments.

Basic to the description of small effects of toxicants is the notion that each molecule that exceeds the tolerance range contributes to the effect to the same extent. Interactions between the molecules only occur at higher tissue-concentrations. This means that the effect size is, as a first approximation, a linear function of the tissue concentration. This linear relationship between the effect size and the tissue-concentration relates to the Taylor approximation. The actual relationship might be non-linear, but we use only the first term of the Taylor approximation at the upper boundary of the tolerance range. The theorem by Taylor states that we can describe any non-linear function in a given interval arbitrarily well with an appropriate polynomial function if we include enough higher order terms. So when we want to improve the description of effects, if they happen to deviate from a linear relationship with tissue-concentrations, we simply include the squared term, the cubed term, and so on. Such improvements rapidly become counter-productive because we increase the number of parameters that must be estimated and because higher tissue-concentrations affect more physiological processes simultaneously. So we are

increasing precision at the wrong points. Practice teaches that very good descriptions can be obtained by just taking effect size linear in the tissue-concentration, even at rather high effect sizes, provided that we focus at the correct physiological process.

I will now discuss a selection of frequently occurring effects of toxicants that relate to the energy budgets of organisms, all based on the above mentioned principles. Effects such as that on the immune system or resistance against parasites are not discussed here.

Effects on survival

When accidental mortality is independent of age, the hazard rate due to this cause of mortality is constant and the corresponding survival probability equals the exponent of (minus) a constant times time.

The model for aging has proved to be successful when the hazard rate is taken to be proportional to cumulative damage. The resulting survival probability then depends on age in constant environments as the exponent of (minus) a constant times age cubed for species that have a short growth period relative to their life span. If this period is not short, dilution by growth must be taken into account, which leads to a somewhat more complex survival probability in which the maintenance rate coefficient appears as a parameter. This parameter of the energy budget can also be obtained directly from measurements on growth and respiration and appears to match well in the case of the pond snail *Lymnaea stagnalis* (Kooijman 1993). This match gives strong support to the aging model.

This mortality model suggests that a toxicant may affect survival in a way similar to the cumulated damage in the case of aging. The additional idea that the individual can handle a certain concentration of toxicant without having adverse effects leads to a hazard rate that is proportional to the tissue-concentration that exceeds the internal no-effect concentration (i.e. the upper boundary of the tolerance range). The proportionality factor, called the killing rate, is a measure for the toxicity of the compound that is independent from the exposure time. In (acute) toxicity tests that are started with animals from a culture that has not been exposed to the toxicant, this cause of mortality results in a survival probability that equals the exponent of (minus) a constant times *squared* time if the exposure time is short with respect to the inverse elimination rate. (Notice that aging led to a *cubed* time in the survival probability due to the extra cumulation step.) When the animals reach steady state so that the tissue-concentration does not increase any longer, the survival probability equals the exponent of (minus) a constant times time. Since the hazard rate starts to increase from the control value at the moment that the NEC value is exceeded, we have to wait longer for effects at lower concentrations.

An important difference between the hazard and the standard model is that survival of a particular individual is *stochastic* in the hazard model and *deterministic*

in the standard model. The choice for a stochastic formulation in this case can best be motivated with two alternative models for the outcome of a tossing experiment with a dice. A very simple stochastic model just states that each of the six possible outcomes will occur with the same probability. A very complex deterministic model describes in detail how the dice is tossed, how it bounces on the table and how it will eventually come to rest. With this model it is possible to predict the outcome deterministically. The reason why this complex deterministic model fails to be practical is that we cannot control the tossing movements in sufficient detail and because of imperfections in the microscopic detail of the surface of the table and in the elasticity of the dice. Similarly, the death of an individual has too many molecular roots for a deterministic description to be practical. The different individuals are thus treated as identical (stochastic) copies rather than different (deterministic) copies.

It is of course possible to account for differences between individuals in the hazard based model, which then appear as differences in parameter values for each individual. This makes sense for animals that are collected from the field, where differences in health, age, size, feeding conditions, sex, all contribute to differences in sensitivity. The way to proceed is to describe this variation in the set of four parameters by some (multivariate) scatter distribution and obtain what is called a 'mixture' in applied probability theory. The number of parameters in this scatter distribution is obviously larger than four and the resulting survival model can easily become complex. One must be prepared to estimate these extra parameters by increasing the number of observation times and tested concentrations. When the individual sizes are measured and the mortality observations specify the individuals, it is possible to correct for these differences in size in a rather straightforward way, which does not increase the number of parameters.

The mortality is thus taken to be a function of the environment-concentration and the exposure time that is parameterized by the NEC, the killing rate, the elimination rate and the control mortality rate. Figure 2.2 illustrates the response-surface in the case where growth during exposure is of importance, such as for the chronic (3 weeks) toxicity test with *Daphnia* (see Figure 2.1). Even if the number of survivors are counted just once we have to estimate all four parameters. If the exposure time is short with respect to the inverse of the elimination rate, we can only estimate the product of the elimination and the killing rate and are unable to translate the NEC for that test to an ultimate NEC. If the exposure time is large with respect to the inverse of the elimination rate, we end up with an exponential survival model that has the (ultimate) NEC, the killing and control mortality rates as parameters. So we can sandwich the four parameter model between a pair of three parameter models. If the cultures are in good condition and the test has been done carefully, we can avoid control mortality, which further reduces the number of parameters. This low number of parameters is essential for routine applications because the data do not allow the estimation of a larger number of parameters. Although the model has better mechanistic underpinning than the standard model, the number of parameters

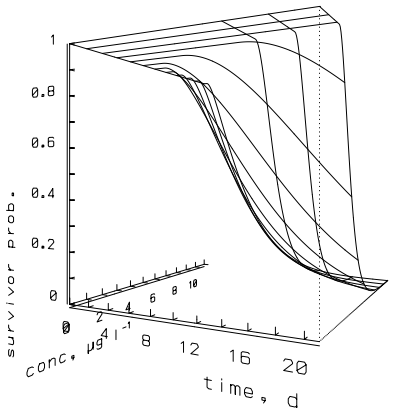


Figure 2.2: Toxic effects on survival. The elimination rate is here taken to be equal to the von Bertalanffy growth rate (0.1 d^{-1}) and the no-effect concentration is 1.5 $\mu g\ l^{-1}$

is smaller because the gradient parameter and the LC50 are replaced by the killing rate.

If the environment-concentration is not constant, correction for changes in these concentrations is rather straightforward if the concentrations are measured functions of time. This is one of the advantages of a mechanistic model. If these changes can be calculated, for instance if the compound disappears from the medium due to accumulation in the animal(s), it is in principle not necessary to measure the decline as a function of time. One measurement at the end of the experiment will do.

The elegance of hazard based modelling is that other causes of mortality can easily be built in, so that the significance of toxicant-induced mortality can be evaluated. This fits into the theory of competing risks (David and Moeschberger 1978), which is based upon the fact that an animal can die only once despite many possible causes of death. A direct application in ecotoxicology is in the toxicity of mixtures of compounds that do not interact. The contributions of different compounds to the hazard rate just have to be added, i.e. the corresponding survival probabilities have to be multiplied.

Mutagenic compounds decrease the life span of animals in a way which is very similar to the aging process. This can be illustrated with the experiments by Robertson and Salt (1981) who studied feeding, growth and life span of the rotifer *Asplanchna* using the ciliate *Paramecium* as food. These ciliates have been cultured on lettuce, which is extremely rich in nitrate. Nitrate can be transformed into nitrite, which is mutagenic. It appears that the aging acceleration is perfectly linear in the feeding level, which strongly suggests that the effect of nitrite is very similar to that of free (oxygen) radicals and therefore affects the parameter 'aging acceleration' linearly. This is consistent with the model for mutagenic effects in the Ames test for bacterial populations. I will argue in the next section that all sublethal effects of toxicants can be described by linear effects on parameter values.

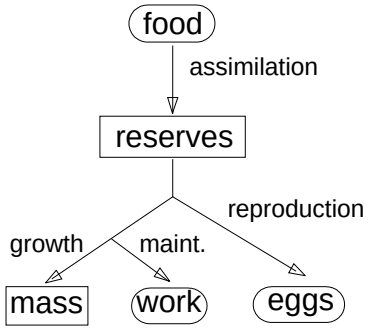


Figure 2.3: The powers as specified quantitatively by the DEB model for an ectotherm with body size and reserve density as state variables. Toxic compounds that affect reproduction can do so directly or indirectly via assimilation, growth and maintenance. The rounded boxes indicate sources or sinks.

Effects on growth and reproduction

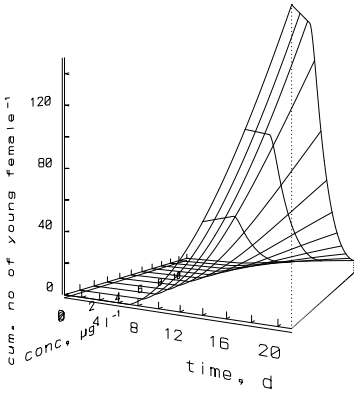
The DEB model describes how resources (i.e. products derived from food) are allocated to reproduction. Toxic effects of chemicals change the allocation via the parameter values. Since the processes of assimilation (i.e. the combination of feeding and digestion), growth, maintenance and reproductions are intimately interlinked, changes in any of these processes will result in changes in reproduction (Kooijman & Bedaux 1996b, 1996c). Two classes for the mode of action of compounds will be distinguished: direct and indirect effects on reproduction.

When reproduction is affected directly, assimilation, growth and maintenance are not affected. There exist two closely related routes within the DEB framework to affect reproduction directly. One is via survival of each ovum, and another via the energy costs of each egg.

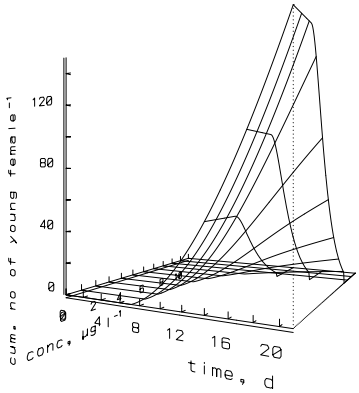
The survival probability of each ovum is affected as discussed in the previous section on effects on survival, except that the sensitive period is taken to be relatively short and fixed rather than the whole life span. (Age zero refers to the moment at which the ovum starts to develop, rather than the moment of hatching or birth.) The combination of an effect on the hazard rate of the ovum and a fixed sensitive period results in a survival probability that depends on the local environment of the ovum. This leads to another important difference with the previous section: the local environment of the ovum is the tissue of the mother rather than the environment-concentration. The relevant concentration, therefore, changes in time even if the environment-concentration is constant. The toxicity parameters that appear in the survival probability of an ovum are the NEC, as before, and the tolerance concentration, which is inverse to the product of the killing rate and the length of the sensitive period. The elimination rate defines how the effect builds up during exposure.

In terms of number of eggs per time, the reproduction rate equals the ratio of the energy allocated to reproduction and the energy costs of an egg. If the compound affects the latter, it can be modelled by making the energy costs a linear function of the tissue-concentration. The model is mathematically different from the hazard model but behaves quantitatively rather similarly. It has the same three toxicity parameters: the NEC, a tolerance concentration and the elimination rate. If we

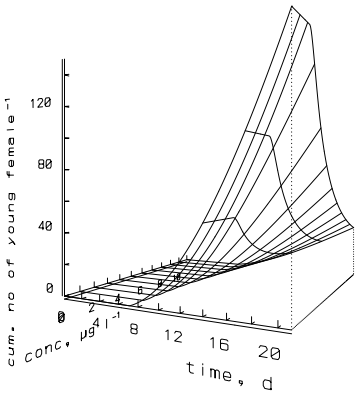
hazard, $c_H = 1.62$ μg l^{-1}



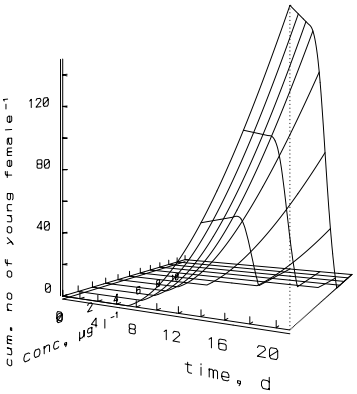
maint., $c_M = 6.02$ μg l^{-1}



costs, $c_R = 1.35$ μg l^{-1}



growth, $c_G = 0.61$ μg l^{-1}



assim., $c_A = 9.57$ μg l^{-1}

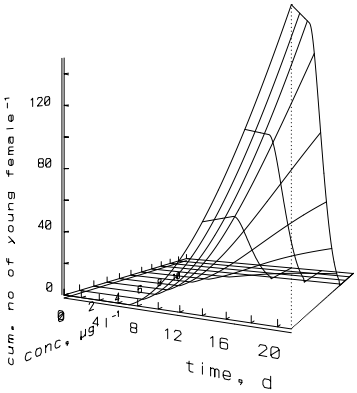
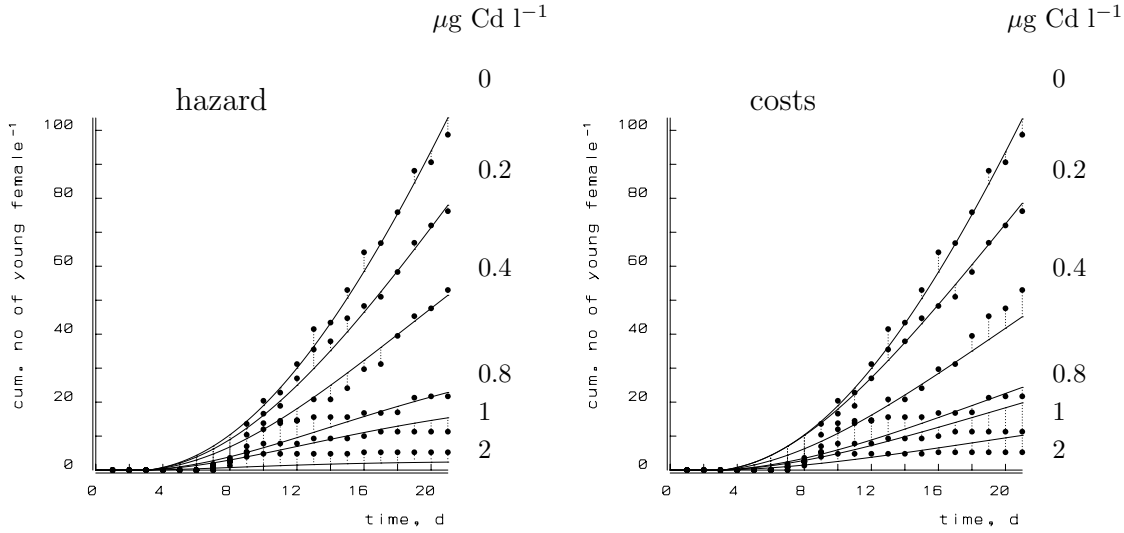


Figure 2.4: Direct (left column) and indirect (right column) effects on reproduction. The various tolerance concentrations are chosen such that the effect size is similar. The elimination rate is set equal to the von Bertalanffy growth rate, $\dot{k}_a = \dot{\gamma} = 0.1$ d^{-1} , and the no-effect concentration is $c_0 = 1.5$ μg l^{-1}.

Figure 2.5: Direct effects of cadmium on *Daphnia* reproduction. The mean cumulated number of young per female daphnid as a function of the exposure time to several concentrations of cadmium. The fitted curves represent least squares fits of the hazard (left) and the cost (right) model for effects on reproduction. Given an elimination rate of $\dot{k}_a = 0.05$ d^{-1}, the estimated values for the NEC, the tolerance concentration and the maximum reproduction rate in the control are

	c_0,μg l^{-1}	c_*,μg l^{-1}	$\dot{R}_m$,d^{-1}
hazard	0.023	0.166	13.1
cost	0.047	0.069	13.1



convert the (external) tolerance concentration into the internal one by multiplication with the bioconcentration coefficient and add the internal NEC, we arrive at the internal concentration at which the energy costs per egg doubles compared to the control.

Figure 2.4 illustrates the response-surface for the various effects on reproduction as functions of the exposure time and the environment-concentration.

Allocation to reproduction is initiated as soon as the cumulative investment in the increase of the state of maturity exceeds some threshold value. Since direct effects on reproduction only affect the translation from energy allocated to reproduction into number of offspring, these modes of action do not affect the time of the onset of reproduction. Indirect effects on reproduction via assimilation, maintenance and growth do delay the onset of reproduction. The occurrence of such delays is the best criterion to distinguish direct from indirect effects.

Indirect effects on reproduction all follow the same basic rules: the relevant parameter (surface-specific assimilation rate, volume-specific maintenance costs or

volume-specific costs of growth) is taken to be a linear function of the tissue-concentration. Since the assimilation rate represents a source of income rather than costs, it is assumed to *decrease* linearly with the tissue-concentration rather than increase. The effects on the reproduction rate as a function of environment-concentration and exposure time, all work out rather similarly and have the same three toxicity parameters: NEC, tolerance concentration and elimination rate. If growth has been measured during exposure, or if the size of the animals at the end of the exposure period is measured, it is possible to identify the mode of action. Figure 2.6 illustrates the response-surface for direct effects on growth as a function of the exposure time and the environment-concentration as well as for indirect effects on growth via assimilation and maintenance. The differences in effects on reproduction are too small to identify the mode of action on the basis of effects on reproduction alone. Figure 2.7 compares the three indirect effect models fitted to the same data. It shows that the models differ little in terms of goodness of fit.

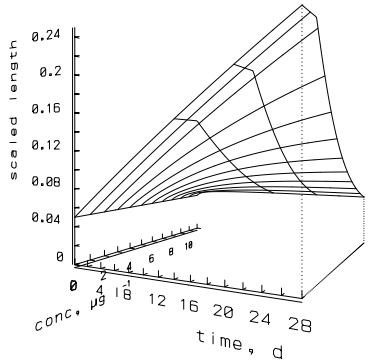
Toxicants sometimes stimulate reproduction at low concentrations rather than reduce it; a phenomenon known as 'hormesis'. The actual cause is largely unknown and therefore difficult to model. For some compounds that showed hormesis at high feeding levels, I have been able to avoid hormesis in *Daphnia* reproduction tests by reducing feeding levels. This points to an explanation in terms of suppression of a secondary stress by the toxicant at low concentrations (see under 'Toxicity patterns'). It is far from obvious to what extent this explanation is general. A wise strategy to deal with hormesis is to favour test conditions in which hormesis is avoided.

2.6 Effects on population dynamics

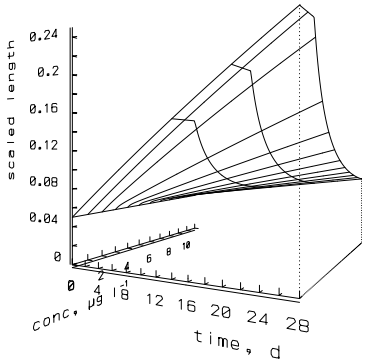
In principle, the consequences of effects on individuals on population dynamics can be analysed on the basis of the now rapidly developing theory of structured population dynamics (Webb 1985, Metz & Diekmann 1986, Hallam et al. 1988, Ebenman & Persson 1988, DeAngelis & Gross 1992). The mathematics of this theory is tedious, however, and computer simulation studies will remain necessary for cases that are of practical interest. In such simulation studies, each individual or group of individuals is followed in time, which requires substantial computation efforts. Ecotoxicological applications are beginning to appear (Hallam et al. 1988; Lassiter & Hallam 1990; Kooijman 1993) and point to the insight that effects of chemicals can work out in a rather complex way at the population level. The simulation studies will hopefully help to find useful mathematical approximations that allow a qualitative analysis of population dynamics.

Direct toxicity testing for effects on the population growth rate is only feasible for very small organisms. An example is the alga growth inhibition test, where batch cultures are followed during a short exposure period. Since alga cells are so small, it seems safe to assume that the intracellular concentration of toxicant is

maint., $c_M = 0.6$ μg l^{-1}



growth, $c_G = 0.8$ μg l^{-1}



assim., $c_A = 10$ μg l^{-1}

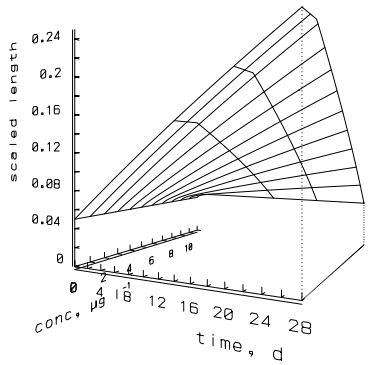
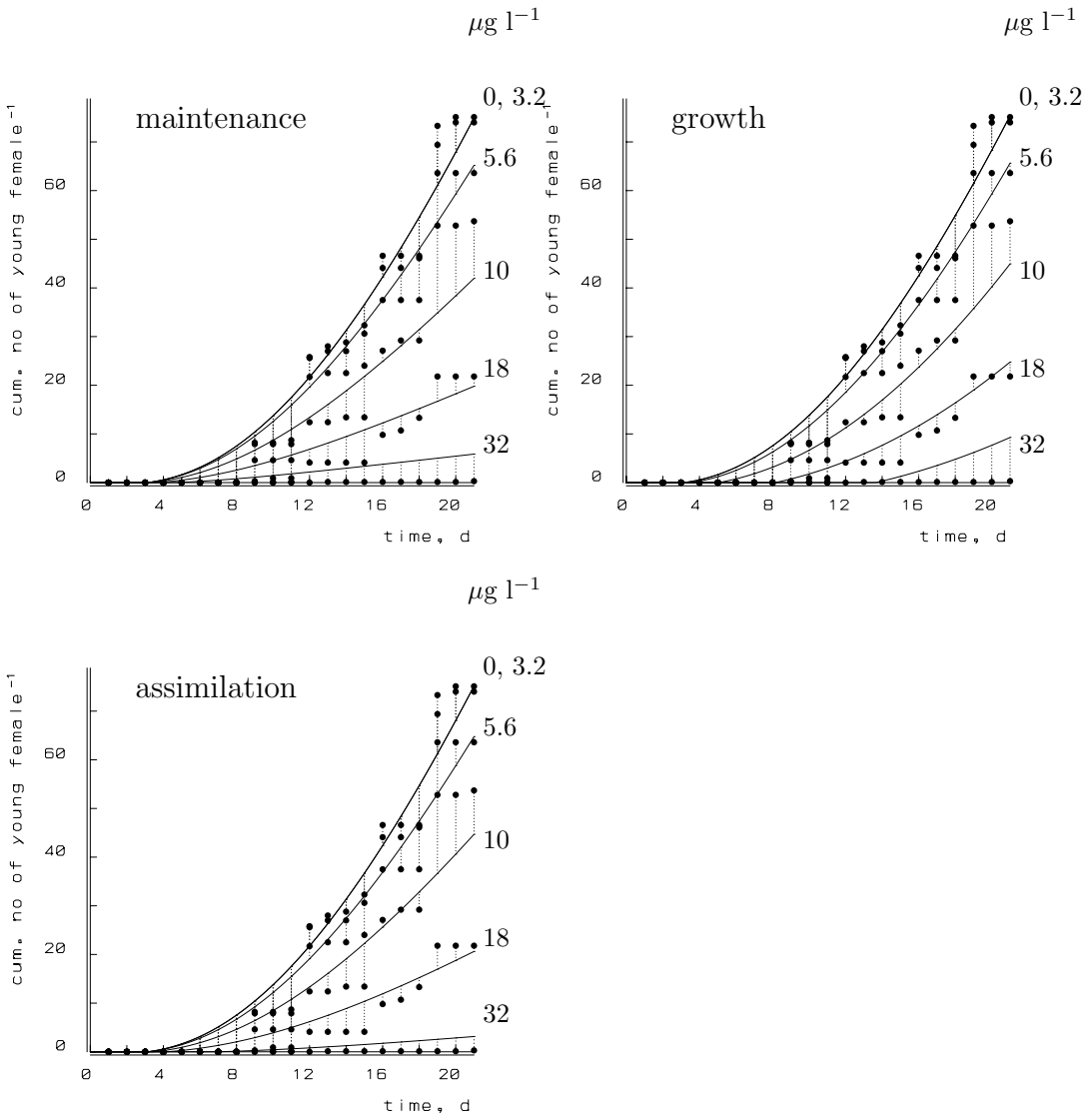


Figure 2.6: Direct and indirect effects on growth. The elimination rate is $\dot{k}_a = 0.1$ d^{-1} and the no-effect concentration is $c_0 = 1.5$ μg l^{-1}. The von Bertalanffy growth rate $\dot{\gamma} = 0.008$ d^{-1} is typical for zebrafish at 26°C.

Figure 2.7: Indirect effects of 3,4-DCAN on *Daphnia* reproduction. The mean cumulated number of young per female daphnid as a function of the exposure time to several concentrations of 3,4-dichloroaniline. The fitted curves represent least squares fits of the model for effects on reproduction via maintenance, growth and assimilation. The estimated values for the NEC, the tolerance concentration, the elimination rate and the maximum reproduction rate in the control are

	c_0,μg l^{-1}	c_*,μg l^{-1}	$\dot{k}_a$,d^{-1}	$\dot{R}_m$,d^{-1}
maint.	3.84	31.7	0.75	9.50
growth	3.51	3.85	0.90	9.56
assim.	3.37	65.8	0.87	9.53

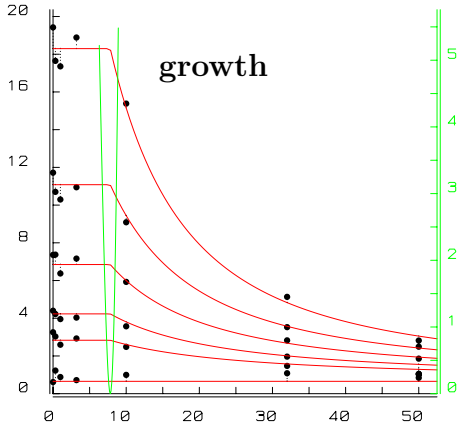


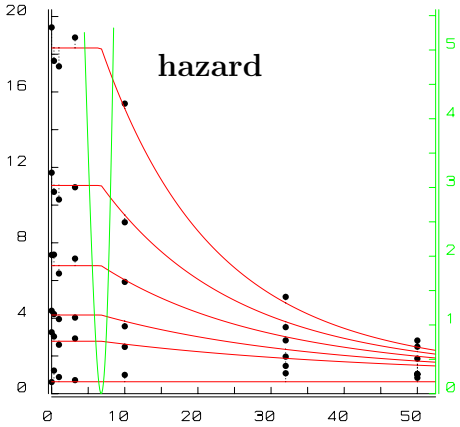
instantaneously in equilibrium with the environment for most compounds. Three modes of action can be distinguished for the toxicant. It might increase the costs of growth in terms of the required amount of energy and/or nutrient per volume of structural biomass. It might affect the hazard rate (over the generations of cells) and it might do so for a short period only. The latter refers to the process of selection. Algal cells originate from control cultures. The transition to experimental conditions that include chemical stress might be lethal for some cells; some cells might be more sensitive than others, possibly depending on where they are in the cell cycle at the moment of transition. In any case, it seems that some toxicants only delay population growth, but once a population begins growing, it does so at the control rate. All three modes of action lead to two toxicity parameters: the NEC and the tolerance concentration. The elimination rate is taken to be large with respect to the inverse of the interdivision interval of the cells. It therefore does not show up as an independent parameter.

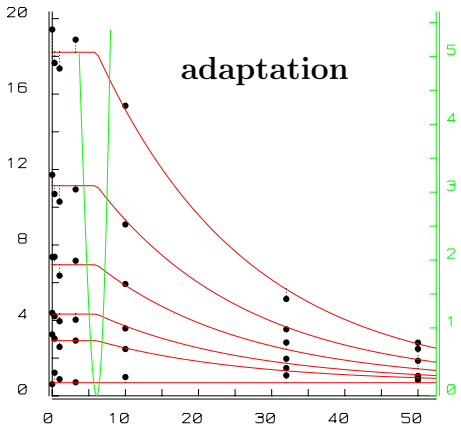
The cells in the control grow exponentially as long as the environment is constant. This results from the fact that the daughter cells repeat the physiological behaviour of the mother cell. For the same reason, all animal populations grow exponentially in constant environments. The fact that they rarely do this for a longer time in the field relates to changes in the environment, frequently due to exhaustion of the resources. How hazard and reproduction rate together determine the population growth rate is provided by a particular result in the theory of structured population dynamics: the characteristic equation. A detailed discussion is beyond the scope of this chapter. It here suffices to note that the age-specific hazard and reproduction rates not only specify the population growth rate, but also the age structure in the population. The size distribution can be obtained from the age distribution on the basis of the DEB model, which specifies the age-specific growth rates that are necessary for this translation.

This reasoning can be used to evaluate the effect of a change in parameters on the population growth rate, which reveals that the effect of toxicants on population dynamics depends on the gross production rate of the population (Kooijman & Metz 1986, Kooijman 1991, 1993). If food density is low, almost all food is used for maintenance. If a compound affects maintenance, this directly translates into an effect on the standing crop. If a compound affects reproduction, however, this hardly affects the population at low food densities because of the limitation by food. These insights can easily be verified by simple experiments with batch cultures. If we supply a population of daphnids with a fixed amount of food per time, the population will rapidly grow to a plateau value, where the incoming food just matches the maintenance of all individuals. Since the life span of daphnids is several months, death hardly plays a role, and growth and reproduction are arrested when the population reaches its plateau value. If we add a toxicant at a concentration where it affects reproduction, it might affect the time at which the plateau is reached but not the plateau itself. If we start harvesting the population at steady state, we will see that the biomass

Figure 2.8: The NEC in the algal growth inhibition test is not very sensitive to errors in the selection of the mode of action of a compound. The effects of tetrapropybenzenesulfonate on the growth of the diatom *Stephanodiscus hantzschii* are shown. The NEC is well defined; the 99% confidence interval can be read from the figure by looking at concentrations for which the profile likelihood is below 3.317. Three different models are fitted to the same data set. Data from Kooijman et al. (1983). The parameter values are the inoculum size, the population growth rate in the control, the NEC and the tolerance concentration or killing rate. The mean residual deviation, $\hat{\sigma}$, is also given to compare the goodness to fit of the three models.







par.	unit	growth	hazard	adap.
N_0	10^4 cells ml^{-1}	0.655	0.633	0.696
$\dot{\mu}_0$	d^{-1}	0.480	0.480	0.480
c_0	mg l^{-1}	7.82	6.78	6.08
c_G	mg l^{-1}	36.3		
$\dot{k}_\dagger$	l (mg d)$^{-1}$		0.0123	
c_H	mg l^{-1}			20.5
$\hat{\sigma}$	10^4 cells ml^{-1}	0.412	0.447	0.416

will settle at a value that is slightly reduced compared to the plateau value (so each individual gets more food, because food supply remains constant) and growth and reproduction are resumed such that it matches our harvesting rate. We are only now beginning to see that the toxicant affects the population biomass. If we increase the harvesting rate we will see that the maximum sustainable harvesting rate is reduced by the toxicant. These considerations show that a toxicant at a constant concentration will have a dynamic effect on a population, even apart from physical and chemical problems. This is one reason why the ecological consequences of effects of toxicants are difficult to analyse.

Toxicants reach organisms directly from the environment and food. In terrestrial environments this leads to an accumulation through the food chain (Walker 1990), which makes top predators the most vulnerable. The importance of this type of accumulation is still being debated, especially for heavy metals, because of the more intensive direct exchange with the environment in aquatic environments and the variability among species in general (Beyer 1986, Laskowski 1991). Remarkable differences exist between the various groups of animals; mammals, birds and insects obtain most toxicants via food, even in aquatic environments, because their skin/exoskeleton is not permeable to many toxicants. Even if there is no accumulation via the food chain, we should expect the tissue-concentration to increase with body size which tends to increase towards the top of the food web. This is mainly due to the amount of food that top predators have to eat.

2.7 Toxicity patterns

It is possible to obtain a wide range of experimental results from a simple toxicity experiment for almost any particular combination of organism and toxicant. This is due to a phenomenon known as secondary stress: The apparent toxicity is much higher if test conditions in the control are physiologically marginal. It is difficult to optimize test conditions because of the lack of knowledge about the detailed needs of organisms. This knowledge is only available to some extent for a very limited set of species. Even for these organisms, magic additives to medium and/or food have to be applied in chemically 'pure' environments where multiply-distilled water is used with pure salt additions and food is cultured under equally well-defined conditions. This type of problems hamper the detection of the more subtle toxicity patterns.

Temperature

All rates depend on temperature. The Arrhenius relationship describes how physiological rates depend on temperature: the logarithm of such rates depends linearly on the inverse of the absolute temperature within a certain tolerance range. The slope of this line, the Arrhenius temperature, is typically 12500 K. This corresponds approximately with an increase by a factor 3 for an increase in temperature of 10°C.

Diffusion rates are proportional to absolute temperature to the power 1.5, however. This means an almost linear increase in the range from 0 to 35 °C by a factor 1.2 only. Uptake kinetics are physiologically controlled and, therefore, increase for increasing temperature (Graney et al. 1984, Reindert et al. 1974). This also holds for elimination rates (Williamson 1975), but the data are not always conclusive (Reindert et al. 1974). Complex patterns can emerge if one process depends on temperature differently from other processes. Watkins & Simkiss (1988) found, for instance, that uptake of zinc in mussels is stimulated extra by an oscillatory temperature regime, which they explained in terms of a cellular metal-ligand homeostasis.

pH

Ionized and unionized (molecular) forms of a compound are taken up at different rates, while the pH affects their relative abundance and so the toxicokinetics (Könemann 1979). Homeostasis, a cornerstone concept in the DEB theory, ensures that the pH inside the organism is independent from that in the environment (again within a certain range of ambient pH values). The elimination rate is, therefore, independent of the pH in the environment, contrary to the uptake rate and, by consequence, the bioconcentration coefficient. The ratio of the uptake rates of the ionized and molecular forms therefore equals the ratio of the killing rates that would result if all of the compound would be present in the environment in either the ionized or the molecular form. The killing rate depends on the pH in the same way as Könemann (1979) proposed for LC50^{-1}, namely

$$\dot{k}_\dagger(\mathrm{pH}) = \frac{\dot{k}_\dagger(-\infty) + \dot{k}_\dagger(\infty) K_a 10^{\mathrm{pH}}}{1 + K_a 10^{\mathrm{pH}}}$$

where $\dot{k}_\dagger(-\infty)$ and $\dot{k}_\dagger(\infty)$ stand for the killing rate if all of the compound would be present in, respectively, the molecular and the ionized form and K_a is the dissociation coefficient $K_a \equiv [H^+][A^-][HA]^{-1}$, where $[A^-]$ and $[HA]$ stand for the concentrations of the ionized and molecular forms.

Body size and reserves

The relationship between uptake kinetics and body size has already been discussed. I mention it here just to remind the reader that the uptake and elimination rates are expected to be inversely proportional to volumetric length, so that the bioconcentration coefficient, the killing rate and tolerance concentrations for sublethal effects are all independent from body size (apart from differences in fat content). Variations in body size among individuals directly translates into variations in responses to toxicants.

Newman & Mitz (1988) found that the elimination rate of zinc in mosquitofish was about proportional to weight$^{-0.42}$ (which is consistent with the expected proportionality with length-1, in view of the scatter), but the zinc-uptake rate was

about proportional to weight-0.9. This has the unexpected consequence that the bioconcentration coefficient is proportional to weight$^{-0.48}$. The elimination rate of mercury did not seem to depend on the size of the mosquitofish, while the mercury-uptake rate tended to decrease with size, so that the bioconcentration coefficient also decreases with size (Newman & Doubet 1989). Boyden (1974) also found negative correlations between body size and concentrations of cadmium, copper, iron, lead and zinc in some species of mollusc, but no correlations for cadmium iron, nickel, lead and zinc in other species of mollusc and a positive correlation for cadmium in *Patella vulgata*.

The kinetics of these metals seems to interfere with the metabolism in a more complex way. The substantial scatter in the data hampers firm conclusions. When the experimental protocol involves a shift up and thus a transition from low to high concentrations of contaminant, negative correlations between body size and concentrations of contaminant can be expected if elimination and uptake rates decrease with body size; it takes longer for big bodies to reach equilibrium. This mechanism can at best only explain part of the observations.

The fat content tends to increase with body size when we compare different species (Kooijman 1993). This is due to the fact that reserve capacity, expressed as density, is proportional to volumetric length. Part of the fat belongs to structural body mass, and part to the reserves. The next subsection deals with fat content.

Solubility in fat

The interest in the relationship between chemical structure and physiological effect goes back to Crum-Brown and Fraser (1868), but the first application to ecotoxicological problems is very recent (Verhaar 1995). The focus is on general trends rather than precise predictions. The most obvious property of chemicals for the understanding of toxicity is the n-octanol/water partition coefficient, P_{ow}, which can be estimated from the chemical structure of the compound. Octanol serves as a model for typical lipids of animals. It has a density of 827 g dm^{-3}, a molecular weight of 130 Dalton, so that 1 dm^3 of octanol contains 6.36 mol. Most comparisons are restricted to the interval $(10^2, 10^6)$ for the P_{ow}. The size of the molecule tends to increase with P_{ow} and if the P_{ow} is larger than 10^6, the molecules are generally too big to enter cells easily (Connell & Hawker 1988). Relationships between toxicity measures and P_{ow} comprise one class of Quantitative Structure-Activity Relationships (QSARs).

The bioconcentration coefficient P_{aw} for fish relates to the octanol/water partition coefficient as $P_{aw} = 0.048\, P_{ow}$, (Mackay 1982). Hawker and Connell (1986) found $P_{aw} = 0.0484\, P_{ow}^{0.898}$ for daphnids and $P_{aw} = 0.0582\, P_{ow}^{0.844}$ for molluscs in the range $10^2 \leq P_{ow} \leq 10^6$. The scatter in the data is big enough for the relationship $P_{aw} = 0.02\, P_{ow}$ to apply for both daphnids and molluscs. The proportionality factor directly relates to the fat content. In general we can say that $P_{aw} = P_{ao} P_{ow}$, where P_{ao} stands for the mass-specific octanol equivalent of the organism, which seems to be taxon-

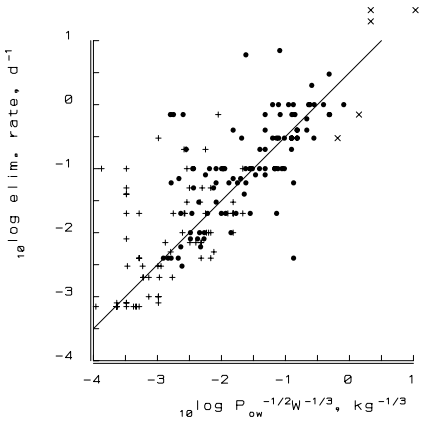


Figure 2.9: The elimination rate depends on the n-octanol-water partition coefficient P_{ow} and the weight W of an organism. It is roughly proportional to $P_{ow}^{-1/2}W^{-1/3}$ with proportionality constant $\sqrt{10}$ $\mathrm{d}^{-1}\mathrm{kg}^{-1/3}$ for 181 halogenated organic compounds in fish. Data compiled by Hendriks (1995). The marker codes are: $P_{ow} \leq 10^2$ (×), $10^2 \leq P_{ow} < 10^6$ (•), $10^6 \leq P_{ow} \leq 10^8$ (+). The range of fish weights is from 0.1 to 900 g. No corrections for differences in temperature have been made, nor for differences in fat content of the fish.

specific. High correlations between P_{aw} and P_{ao} have been found for fenitrothion in a variety of algae (Kent & Currie 1995), for instance. The DEB theory predicts that P_{ao} increases with the body size of the different species of animal because the maximum reserve capacity increases with a volumetric length as density and reserves are relatively rich in lipids. These are only general trends and many exceptions occur. The eel *Anguilla* is much fatter than other fish of similar size, for instance.

Hawker and Connell (1985, 1986) related the elimination rate $\dot{k}_a$ to P_{ow} and found $\dot{k}_a = 8.851\, P_{ow}^{-0.663}$ for fish , $\dot{k}_a = 113\, P_{ow}^{-0.507}$ for *Daphnia pulex* and $\dot{k}_a = 9.616\, P_{ow}^{-0.540}$ for molluscs. The proportionality factor is inversely proportional to the volumetric length of the animal (see above), which explains the wide range of values. The results for daphnids are most reliable, because they all have the same body size in this case, which suggests that $\dot{k}_a \propto 1/\sqrt{P_{ow}}$. In view of the finding that the bioconcentration coefficient is proportional to P_{ow}, the uptake rate should be proportional to $\sqrt{P_{ow}}$. These relationships directly follow from a symmetry argument for the rate at which a substance moves from one matrix to another: the way the uptake rate depends on P_{ow} and the elimination rate depends on P_{wo} is the same, while $P_{wo} = P_{ow}^{-1}$. (See Figure 2.9.) Notice that the elimination rate being proportional to $P_{ow}^{-0.5}$ implies that the accumulation time, i.e. the waiting time till a fixed percentage of the asymptotic concentration is exceeded, is proportional to $P_{ow}^{0.5}$.

Since the hazard model has not yet been widely applied, there is no empirical information about the relationship between the killing rate and P_{ow} at this moment. Since the equilibrium tissue-concentration is proportional to P_{ow}, we should expect to find that $\dot{k}_\dagger \propto P_{ow}$. The idea is that effects relate to the aqueous fraction of the tissue, which is proportional to P_{ow} because P_{aw} is proportional to P_{ow}. Similarly, we should expect that NEC $\propto P_{ow}^{-1}$, which also holds for the tolerance concentrations. The empirical study by de Wolf et al. (1988) confirms these expectations.

Könemann (1981) observed that the 14 days log LC50 of the guppy *Poecilia reticulata* for 50 "industrial chemicals" is LC50 $= 0.0794\ P_{ow}^{-0.87}$ mol dm^{-3}. To

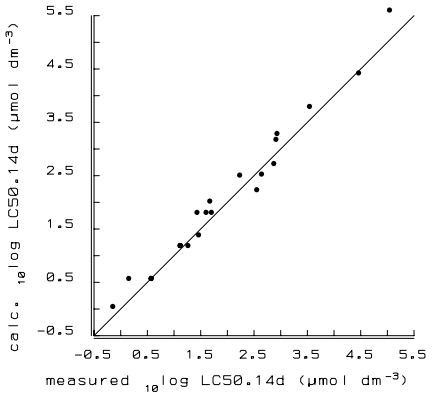


Figure 2.10: The calculated 14 days LC50 values as a function of 'measured' values for guppies (*Poecilia reticulata*) exposed to 21 chlorinated aromatic and other some chlorinated hydrocarbons whose P_{ow} ranged from $10^{-0.22}$ (pentachlorobenzene) to $10^{5.21}$ (acetone). (Data from Könemann (1979)). The calculations are based on the assumptions that the elimination rates equal $50/\sqrt{P_{ow}}$ d^{-1}, the killing rates equal $10^{-6.6}P_{ow}$ d$^{-1}\mu$mol^{-1}dm^3 and the NECs are zero (see text).

understand this relationship, we have to realize that for a large elimination rate, so a small P_{ow}, the 14 days LC50 is close to the ultimate value, but for a large P_{ow}, the ultimate LC50 is much lower than the LC50.14d. Taking these complexities into account, numerical studies confirm that $\dot{k}_\dagger \propto P_{ow}$ and $\dot{k}_a \propto P_{ow}^{-0.5}$ is indeed consistent with the finding by Könemann (see fig 2.10). The LC50 values have been obtained by Könemann by application of the standard model rather than the hazard model. The even closer match between observed and predicted LC50 values can probably obtained by application of the hazard model. Notice that the NEC, together with the killing and elimination rates of the hazard model define the survival probability as a function of time and concentration. By equating the survival probability to 0.5, we can obtain an LC50-time curve from the three parameters. Unfortunately, the data of Figure 2.10 did not allow us to check the relationship for the NEC. Although the NECs had been set to zero, adopting the function NEC $= 10P_{ow}^{-1}$ mmol dm^{-3} hardly changes the result.

The conclusion is that we can now understand the QSAR for LC50 values from first principles. Notice that the standard QSARs for LC50s require two parameters, while we need just one for killing rates; knowing the killing rate for one compound, we can guess the killing rate for another one by multiplying by the ratio of the partition coefficients of the two compounds. We can improve our guess by using more measured killing rates.

Classes of compounds

The usefulness of QSAR relationships can be greatly improved by restricting the compounds that are to be compared to compounds that have the same mode of action physiologically. Based on the work of Veith & Broderius (1987, 1990), Hermens (1989, 1990), Lipnick (1989) and Bradbury & Lipnick (1990), Verhaar et al. (1992) and Verhaar (1995), Hermens and Verhaar distinguish four main classes of organic

compounds that might contain nitrogen, sulphur and/or halogens (excluding iodine), with $1 < P_{ow} < 10^6$ and a molecular mass < 600 Daltons.

type 1 : narcotic compounds with baseline toxicity.

type 2 : less inert compounds. These include non- or weakly acidic phenols, anilines, mononitroaromatics, primary alkylamines and pyridines. Type 2 compounds might have one or two chlorine or alkyl substituents.

type 3 : compounds with unspecific reactivity.

type 4 : compounds with specific reactivity. Examples of this type of compounds are DDT and analogues, (dithio)carbamates, organotin compounds, pyrethroids, organophosphorothionate esters.

Each of these types has been further subdivided.

Sensitivity among species

Apart from compounds with specific reactivity, the only pattern in sensitivities among species that has been detected so far relates to fat content. Acute toxicity also involves body size and surface area (Hoekstra et al. 1994), but these quantities relate to kinetics. Van Straalen (1994) found a relationship between the sensitivity and the food spectrum of a species: the broader the spectrum, the better detoxification systems are developed, the less sensitive a species is. He found a correlation between the LC50 and the activity of detoxification enzyms such as cytochrome P450 and glutathion-S-transferase in terrestrial arthropods. Practical problems usually hamper the detection of such patterns. The first problem is the extremely limited feasibility of standardizing laboratory cultures of many species. Many data relate to specimens taken from the field and tested under less than optimal conditions, which amplify scatter to such an extent that sensitivity patterns disappear. Another problem is that only acute toxicity has been quantified with LC50 values. These data have not been corrected for differences in body size or other factors influencing effect (such as exposure time). It might be that useful sensitivity patterns would emerge if killing rates are used, rather than LC50s.

I have proposed a factor that can be applied to LC50 values of a very limited set of tested species to estimate the LC50 of the most sensitive species in an ecosystem (Kooijman 1987). This factor is based on the idea that the (known) LC50s of a limited set of tested species as well as the (unknown) LC50s of all species in the ecosystem represent random trials from the same frequency distribution. My purpose has been to illustrate that a very simple reasoning leads to the expectation that effects in the field can be expected when standard safety factors are applied. Mesocosm experiments usually fail to reveal this sensitivity of complex systems, first because of lack of power due to erratic behaviour of the control (a problem similar to that

connected to the NOEC), second because of lack of detail in the observations (species are not monitored separately, only categories such as 'secondary producers'). Van Straalen & Denneman (1989) and Aldenberg & Slob (1993) modified this idea of a application factor to arrive at a Hazardous Concentration for Sensitive species (HSC) and proposed an estimate for an LC50 value, the HC5, that is exceeded by a fixed percentage (namely 95%) of the species rather than all species. The value of the HCx rapidly increases with decreasing x. The practical application of this factor has been stimulated by the observation that it matches current practice. The problem that I have with the application of HC5s is in the objectives of legislation, which should not aim at the disappearence of any percentage of the species. The reason why this practice did not yet gave rise to disasters is that an increase of the variance of LC50s leads to a sharp decrease of the HC5. As has been discussed already, many factors contribute to an increase of the variance of LC50s, although this variance does not reflect a proper scatter in sensitivities among species. This is a rare example where ignorance works out to be protective.

2.8 Conclusions/perspectives

The direct application of ecotoxicology in risk assessment has given standardization and purely descriptive methods priority above developing a sound scientific basis. Reliable applications are only possible on a scientific basis, however. I have shown that mechanistic underpinnings need not lead to models that are too complex to apply on a routine basis. Scientific progress is only possible if we develop a range of *consistent* models from simple (for routine applications) to complex (for mechanistic studies). Consistency is essential for the cross fertilization between large scale routine applications and scientific progress. Routine test protocols should be developed in such a way that the simplifications make sense. This means that these protocols should be subjected to a regular update procedure. The development of a data base with raw data would facilitate the reexamination of old data in a newly developed scientific light. Existing data bases with only summary statistics, such as LC50/EC50/NOEC values, decrease in value with changing scientific insight. The rapidly decreasing costs of computer memory has removed technical obstacles for such a more extensive data base. International cooperation and organization are the main bottlenecks.

In my opinion, legislation should aim at no-effects. Spatial and time scales should be worked out in greater detail in this respect. Very local and temporary effects can, in some cases, rapidly be cancelled by immigration from the surroundings as long as the surroundings are not affected. Degradation of compounds is in such cases more important than toxicity. Immigration from unaffected surrounding areas is less likely in extensive areas with intense industrial activity that lack refuges.

Whatever the aims of legislation, ecological effects of toxicants will continue to

occur. The evaluation of the ecological significance of these effects remains necessary. The development of fundamental ecology would benefit from two endeavours in ecotoxicology:

- a widening of the problem of effects of human induced chemical disturbance to chemical disturbance in general: the understanding of the development of life in chemically changing environments
- the analysis of population dynamics in terms of the eco-physiological behaviour of individuals: the further development of structured population dynamics

Both endeavours are very complex, and progress will be slow. The reactions of organisms to changes in the chemical environment call for a deeper understanding of physiology and molecular biology. Although the field of molecular biology is developing fast as an independent discipline, little attention is given to the relationship between the molecular and organismic levels of organisation. The understanding of ecosystem dynamics in terms of population dynamics is remote and it is, in fact, still questionable whether it is feasible at all. Any list of more specific problems, such as the toxicity of poorly soluble compounds and mixtures of compounds, seems so incomplete that the effort of making such a list is useless.

References

Agosta, W. 1995. Bombardier beetles and fever trees. Addison Wesley.

Aldenberg, T. & Slob, W. 1993. Confidence limits for hazardous concentrations based on logistically distributed NOEC data. *Ecotox. Environ. Safety* **25**: 48-63.

Barron, M.G., Stehly, G.R. & Hayton, W.L. 1990. Pharmacokinetic modeling in aquatic animals I. Models and concepts. *Aquatic Toxicol.* **18**: 61-86.

Bedaux, J.J.M. & Kooijman, S.A.L.M. 1994. Statistical analysis of bioassays, based on hazard modeling. *Environ. & Ecol. Stat.* **1**: 303-314.

Beyer, W.N. 1986. A reexamination of biomagnification of metals in terrestrial food chains. *Environ. Toxicol. Chem.* **5**: 863-864.

Boyden, C.R. 1974. Trace element content and body size in molluscs. *Nature* **251**: 311-314.

Bradbury, S.P. & Lipnick, R.L. 1990. Introduction: Structural properties for determining mechanisms of toxic action. *Environ. Health Persp.* **87**: 181-182.

Bruce, R.D. & Versteeg, D.J. 1992. A statistical procedure for modelling continuous toxicity data. *Environ. Tox. & Chem.* **11**: 1485-1492.

Connell, D.W. & Hawker, D.W. 1988. Use of polynomial expressions to describe the bioconcentration of hydrophobic chemicals by fish. *Ecotoxicol. Environ. Saf.* **16**: 242-257.

Connolly, J.P. & Tonelli, R. 1985. Modeling kepone in striped bass food chain of the James River estuary. *Estuarine Coastal Shelf Sci.* **23**: 699-707.

Crommentuijn, T., Doodeman, C.J.A.M., Doornekamp, A., Pol, J.J.C.van der, Bedaux, J.J.M. & Gestel, C.A.M.van 1994. Lethal body concentrations and accumulation patterns determine time-dependent toxicity of cadmium in soil arthropods. *Environ. Toxicol. Chem.* **11**: 1781-1789.

Crum-Brown, A. & Frazer, T. 1868-9. On the connection between chemical constitution and physiological action. Part 1. On the physiological action of the ammonium bases, derived from

Strychia, Brucia, Thebaia, Codeia, Morphia, and Nicotia. *Transactions of the Royal Society of Edinburgh* **25**: 151-203.

David, H.A. & Moeschberger, M.L. 1978. *The theory of competing risks*, Griffin's Statistical Monographs & Courses **39**, C. Griffin & Co Ltd.

DeAngelis, D.L. & Gross, L.J. (eds) 1992. *Individual-based models and approaches in ecology.* Chapman & Hall.

Ebenman, B. & Persson, L. 1988. *Size-structured populations. Ecology and evolution.* Springer-Verlag.

Emsley, J. 1992. Potent painkiller from poisonous frog. *New Scientist* 30 May: 14.

Fagerström, T. 1977. Body weight, metabolic rate and trace substance turnover in animals. *Oecologia* **29**: 99-104.

Graney, R.L., Cherry, D.S. & Cairns Jr, J. 1984. The influence of substrate, pH, diet and temperature upon cadmium accumulation in the Asiatic clam (*Corbicula fluminea*) in laboratory artificial streams. *Water Res.* **18**: 833-842.

Hallam, T.G., Lassiter, R.R., Li., J. & McKinney, W. 1988. Physiologically structured population models in risk assessment. In: Riccardi, L.M. (ed.) *Biomathematics and related computational problems.*: 197-211. Kluwer Academic Press.

Haren, R.J.F.van, Schepers,H.E. & Kooijman, S.A.L.M. 1994. Dynamic Energy Budgets affect kinetics of xenobiotics in the marine mussel *Mytilus edulis*. *Chemosphere* **29**: 163-189.

Hawker, D.W. & Connell, D.W. 1985. Relationships between partition coefficient, uptake rate constant, clearance rate constant, and time to equilibrium for bioaccumulation. *Chemosphere* **14**: 1205-1219.

Hawker, D.W. & Connell, D.W. 1986. Bioconcentration of lipophilic compounds by some aquatic organisms. *Ecotox. Environ. Safety* **11**: 184-197.

Hendriks, A.J. 1995. Modelling non-equilibrium concentrations of microcontaminants in organisms: comparative kinetics as a function of species size and octanol-water partitioning.*Chemosphere* **30**: 265-292.

Hermens, J.L.M. 1989. Quantitative structure-activity relationships of environmental pollutants. In: Hutzinger, O.(ed.), *Handbook of Environmental Chemistry*, vol 2E: 111-162, Springer Verlag, Berlin.

Hermens, J.L.M. 1990. Electrophiles and acute toxicity to fish. *Environ. Health Persp.* **87**: 219-225.

Hoekstra, J.A. 1993. *Statistics in ecotoxicology.* PhD-thesis, Vrije Universiteit, Amsterdam, the Netherlands.

Hoekstra, J.A. & Ewijk, P.H.van 1992. Alternatives for the no-observed-effect level. *Environ. Toxicol. Chem.* **12**: 187-194.

Hoekstra, J.A., Vaal, M.A., Noteboom, J. & Slooff, W. 1994. Variation in the sensitivety of aquatic species to toxicants. *Bull. Environ. Cont. & Toxicol.* **53**: 98-105.

Hoeven, N.van der, Kooijman, S.A.L.M. & Raat, W.K.de 1990. *Salmonella* test: relation between mutagenicity and number of revertant colonies. *Mutation Res.* **234**: 289-302.

Holmes, B. 1993. The perils of planting pesticides. *New Scientist* 28 August: 34-37.

Hothorn, L. 1994. Multiple comparisons in long-term toxicity studies. *Environ. Health Persp. Suppl.* **102** *Suppl.* **1**: 33-38.

Jensen, A.L., Spigarelli, S.A. & Thommes, M.M. 1982. PCB uptake by species of fish in Lake Michigan, Green Bay of Lake Michigan, and Cayuga, New York, NY. *Can. J. Fish. Aquat. Sci.* **39**: 700-709.

Kent, R.A. & Currie, D. 1995. Predicting algal sensitivity to a pesticide stress. *Environ. Toxicol. Chem.* **14**: 983-991.

Könemann, W.H. 1981. Quantitative structure-activity relationships in fish toxicity studies. 1. Relationship for 50 industrial pollutants. *Toxicol.* **19**: 209-221.

Kooi, B.W. & Kooijman, S.A.L.M. 1994. Existence and stability of microbial prey–predator systems. *J. Theor. Biol.* **170**: 75-85.

Kooi, B.W. & Kooijman, S.A.L.M. 1994a. The transient behaviour of food chains in chemostats. *J. Theor. Biol.* **170**: 87-94.

Kooi, B.W. & Kooijman, S.A.L.M. 1995. Many limiting behaviours in microbial food chains. In: Arino, O., Kimmel, M. & Axelrod, D. (eds) Conf. Proc. third *Internat. Conf. Math. Pop. Dyn., Biological Systems.* Wuerz: 131-148.

Kooijman, S.A.L.M. 1981. Parametric analyses of mortality rates in bioassays. *Water Res.* **15**: 107-119.

Kooijman, S.A.L.M. 1981a. The estimation of mortality rates in toxicity tests. *Inserm* **106**: 467-474.

Kooijman, S.A.L.M. 1983. Statistical aspects of the determination of mortality rates in bioassays. *Water Res.* **17**: 749-759.

Kooijman, S.A.L.M. 1987. A safety factor for LC50 values allowing for differences in sensitivity among species. *Water Res.* **21**: 269-276.

Kooijman, S.A.L.M. 1988. Strategies in ecotoxicological research. *Environmental Aspects of Applied Biology* **17** (1): 11-17.

Kooijman, S.A.L.M. 1991. Effects of feeding conditions on toxicity for the purpose of extrapolation. *Comp. Biochem. Physiol.* **100c(1/2)**: 305-310.

Kooijman, S.A.L.M. 1993. *Dynamic Energy Budgets in Biological Systems. Theory and applications in ecotoxicology.* Cambridge University Press.

Kooijman, S.A.L.M. 1995. The stoichiometry of animal energetics. *J. Theor. Biol.* **177**: 139-149.

Kooijman, S.A.L.M. & Bedaux, J.J.M. 1996a. Statistical properties of no effects levels. *Water Res.* (to appear)

Kooijman, S.A.L.M. & Bedaux, J.J.M. 1996b. Analysis of toxicity tests on *Daphnia* survival and reproduction. *Water Res.* (to appear)

Kooijman, S.A.L.M. & Bedaux, J.J.M. 1996c. Analysis of toxicity tests on fish growth. *Water Res.* (to appear)

Kooijman, S.A.L.M., Hanstveit, A.O. & Hoeven, N.van der 1987. Research on the physiological basis of population dynamics in relation to ecotoxicology. *Wat. Sci. Tech.* **19**: 21-37.

Kooijman, S.A.L.M., Hanstveit, A.O. & Nyholm, N. 1996. No-effect concentrations in alga growth inhibition tests. *Water Res.* (to appear)

Kooijman, S.A.L.M., Hanstveit, A.O. & Oldersma, H. 1983. Parametric analyses of population growth in bioassays. *Water Res.* **17**: 727-738.

Kooijman, S.A.L.M. & Haren, R.J.F.van 1990. Animal energy budgets affect the kinetics of xenobiotics. *Chemosphere* **21**: 681-693.

Kooijman, S.A.L.M., Kooi, B.W. & Boer, M.P. 1995. Rotifers do it with delay. The behaviour of reproducers vs dividers in chemostats. *Nonlin. World* (to appear)

Kooijman, S.A.L.M. & Metz, J.A.J. 1983. On the dynamics of chemically stressed populations; The deduction of population consequences from effects on individuals. *Ecotox. Environ. Safety* **8**: 254-274.

Landrum, P.F. & Dupuis, W.S. 1990. Toxicity and toxicokinetics of pentachlorophenol and carbyl to *Pontoporeia* and *Mysis relicta*. In: Landis, W.G. & Schalie, W.H. (eds) *Aquatic Toxicology and Risk Assessment* **13**. STP 1096. Am. Soc. Testing & Materials, Philadelphia, PA: 278-289.

Landrum, P.F., Lee, H. & Lydy, M.J. 1992. Toxicokinetics in aquatic systems Model comparisons and use in hazard assessment. *Environ. Toxicol. Chem.* **11**: 1709-1725.

Laskowski, R. 1991. Are the top carnivores endangered by heavy metal biomagnification? *Oikos* **60**: 387-390.

Laskowski, R. 1995. Some good reasons to ban the use of NOEC, LOEC and related concepts in ecotoxicology. *Oikos* **73**: 140-144.

Lassiter, R.R. & Hallam, T.G. 1990. Survival of the fattest: implications for acute effects of lipophillic chemicals on aquatic populations. *Environ. Toxicol. Chem.* **9**: 585-595.

Leeuwen, C.J.van & Hermens, J.L.M. (eds) 1995. *Risk Assessment of chemicals: An introduction.* Kluwer Acad. Press, Dordrecht.

Lipnick, R.L. 1989. Base-line toxicity predicted by quantitative structure-activity relationships as a probe for molecular mechanism of toxicity. In: Magee, P.S., Henry, D.R. & Block, J.H. (Eds) *ACS Symposium Series* **413**: 366-389, American Chemical Society, Washington, DC.

MacDonald, D. 1995. *European mammals; evolution and behaviour.* HarperCollins Publishers, London.

Mackay, D. 1982. Correlation of bioconcentration factors. *Environ. Sci. Technol.* **16**: 274-278.

McCarty, L.S. 1986. The relationship between aquatic toxicology QSARs and bioconcentration for some organic chemical. *Environ. Toxicol. Chem.* **5**: 1071-1080.

McCarty, L.S. 1990. A kinetic-based analysis of quantitative structure-activity relationships in aquatic toxicology and bioconcentration bioassays with organic chemicals. Ph.D. thesis, University of Waterloo, Ontario, Canada.

McCarty, L.S. 1991. Toxicant body residues: Implications for aquatic bioassays with some organic chemicals. In: Mayes, M.A. & Barron, M.G. (eds) *Aquatic Toxicology and Risk Assessment* **14**. STP 1124. Am. Soc. Testing & Materials, Philadelphia, PA: 183-192.

McCarty, L.S., Mackay, D., Smith, A.D., Ozburn, G.W. & Dixon, D.G. 1993. Residue-based interpretation of toxicity and bioaccumulation QSARs from aquatic bioassays: Neutral narcotic organics. *Environ. Toxicol. Chem.* **11**: 917-930.

McCarty, L.S., Ozburn, G.W., Smith, A.D., Bharath, A., Orr, D. & Dixon, D.G. 1989. Hypothesis formulation and testing in aquatic bioassays: A deterministic model approach. *Hydrobiologia* **188/189**: 533-542.

McKim, J.M. & Nichols, J.W. 1992. Use of physiologically-based toxicokinetic models in a mechanistic approach to aquatic toxicology. In: Ostrander, G.K. & Malins, D.C. (eds) *Molecular biological and biochemical approaches to aquatic toxicology.* Lewis Publishers, Chelsea, MI.

Metz, J.A.J. & Diekmann, O. (eds). 1986. *The dynamics of physiologically structured populations.* Springer Lecture Notes in Biomathematics. Springer-Verlag, Berlin.

Moran, N. & Baumann, P. 1994. Phylogenetics of cytoplasmically inherited microorganisms of arthropods. *TREE*, **9**: 15-20.

Newman, M.C. & Doubet, D.K. 1989. Size dependence of mercury (II) accumulation in the mosquitofish *Gambusia affinis* (Baird and Girard). *Arch. Environ. Toxicol.* **18**: 819-825.

Newman, M.C. & Mitz, S.V. 1988. Size dependence of zinc elimination and uptake from water by mosquitofish *Gambusia affinis* (Baird and Girard). *Aquatic Toxicol.* **12**: 17-32.

Norstrom, R.J., McKinnon, A.E. & deFreitas, A.S. 1976. A bioenergetic based model for pollutant accumulation by fish. Simulation of PCB and methyl-mercury residue levels in Ottawa River. *J. Fish. Res. Board Can.* **33**: 248-267.

Nyholm, N. 1985. Response variable in algal growth inhibition tests - Biomass of growth rate? *Water Res.* **19**: 273-279.

Pack, S. 1993. A review of statistical data analysis and experimental design in OECD aquatic toxicity test guidelines. Shell Research Ltd., Sittingbourne, UK.

Prestwich, G.D. 1983. The chemical defences of termites. *Sci. Am.* **249** (2): 68-75.

Reinert, R.E., Stone, L.J. & Willford, W.A. 1974. Effect of temperature on accumulation of methylmercuric chloride and p,p'DDT by rainbow trout (*Salmo gairdneri*) *J. Fish. Res. Board Can.* **31**: 1649-1652.

Robertson, J.R. & Salt, G.W. 1981. Responses in growth, mortality, and reproduction to variable food levels by the rotifer *Asplanchna girodi*. *Ecology* **62**: 1585-1596.

Skalski, J.R. 1981. Statistical inconsistencies in the use of no-observed-effect-levels in toxicity testing. In: Branson, D.R. & Dickson, K.L. (eds) *Aquatic toxicology and hazard assessment.* Fourth Conference, ASTM STP 737. American Society for Testing and Materials: 328-338.

Sprague, J.B. 1969. Measurement of pollutant toxicity to fish I. Bioassay methods for acute toxicity. *Water Res.* **3**: 793-821.

Stephan, C.E. & Rogers, J.W. 1985. Advantages of using regression to calculate results of chronic toxicity tests. In: Bahner, R.C. & Hansen, D.J. *Aquatic toxicology and hazard assessment.*

Eight Symposium, ASTM STP 891. American Society for Testing and Materials: 328-338.
Straalen, N.M. van 1994. Biodiversity of ecotoxicological responses in animals. *Neth. J. Zool.* **44**: 112-129.
Straalen, N.M.van & Denneman, C.A.J. 1989. Ecotoxicological evaluation of soil quality criteria. *Ecotox. Environ. Saf.* **18**: 241-251.
Thomann, R.V. 1989. Bioaccumulation model of organic chemical distribution in aquatic food chains. *Environ. Sci. Technol.* **23**: 699-707.
Veith, G.D. & Broderius, S.J. 1987. Structure-toxicity relationships for industrial chemicals causing type (II) narcosis syndrome, in: Kaiser, K.L.E. (ed.) *QSAR in Environmental Toxicology II*: 385-391, D. Reidel Publ. Company.
Veith, G.D. & Broderius, S.J. 1990. Rules for distinguishing toxicants that cause type I and type II narcosis syndromes. *Environ. Health Persp.* **87**: 207-211.
Verhaar, H.J.M. 1995. *Predictive methods in aquatic toxicology.* PhD-thesis, University of Utrecht, the Netherlands.
Verhaar, H.J.M., Leeuwen, C.J.van & Hermens, J.L.M. 1992. Classifying environmental pollutants. Structure-activity relationships for prediction of aquatic toxicity. *Chemosphere* **25**: 471-491.
Watkins, B. & Simkiss, K. 1988. The effect of oscillating temperatures on the metal ion metabolism of *Mytilus edulis. J. mar. biol. Ass. U.K.* **68**: 93-100.
Williamson, P. 1975. Use of ^{6}5ZN to determine the field metabolism of the snail *Cepaea nemoralis* L. *Ecology* **56**: 1185-1192.
Walker, C.H. 1990. Kinetic model to predict bioaccumulation of pollutant. *Funct. Ecol.* **4**: 295-301.
Webb, G.F. 1985. *Theory of nonlinear age-dependent population dynamics.* Marcel Dekker, New York, pp 294.
White, T.C.R. 1993. *The inadequate environment; Nitrogen and the abundance of animals.* Berlin, Springer-Verlag, pp 425.
Wolf, W.de, Canton, J.H. Deneer, J.W., Wegman, R.C.C. & Hermens, J.L.M. 1988. Quantitative structure-activity relationships and mixture-toxicity studies of alcohols and chlorohydriocarbons: reproducibility of effects on growth and reproduction of *Daphnia magna* **12**: 39-49.
Yanagawa, T., Kikuchi, Y. & Brown, K.G. 1994. Statistical issues on the No-Observed-Adverse-Effect Level in categorical response. *Environ. Health Persp. Suppl.* **102** *Suppl.* **1**: 95-101.
Young, S. 1993. The body's vital poison. *New Scientist* 13 March: 36-40.

Chapter 3

The analysis of standardized bioassays

This chapter discusses the analysis of standardized bioassays for toxic effects on survival (sections 1 and 2), body growth (of fish, section 3), reproduction (of *Daphnia*, section 4) and population growth (of algae, section 5). The method used in the analysis of these standardized bioassays also applies to many other species of organism, since the DEB theory applies to all heterotrophs. The change from fishes to gammarids in the bioassay for body growth, for instance, only affects choices for two 'background' parameters (initial scaled length and the von Bertalanffy growth rate). Particular properties of species have to be taken into account, such as parthenogenetic vs sexual reproduction. The sections can be read independently. Here, we assume that the reader is familiar with basic concepts in statistics and mathematical modelling. This chapter describes the method that the software package DEBtox uses to analyse the results of these bioassays.

3.1 Some statistical properties of estimates of no-effect concentrations

Abstract No-effect concentrations (NECs) for toxicants are of interest from a biological and a legislative point of view. Using artificial, but typical, examples of the results of a bioassay on survival and two different models for the concentration-effect relationship, we show that the likelihood based confidence set of the NEC as parameter of the hazard model has quite acceptable statistical properties. Contrary to the hazard model, the NEC of the standard log-logistic model did not differ significantly from zero.

Introduction

The aquatic toxicity of chemical compounds with respect to the survival of animals is tested on a routine basis by exposing cohorts of individuals to a set of chosen concentrations during a standardized period. A standard measure to characterize the toxicity is the concentration at which the survival probability is half that of the control, the so-called LC50, here denoted as c_{L50}. However, the concentration that has no effect is of much greater practical interest for many purposes. The most frequent choice is the no-observed effect concentration (NOEC), i.e. the highest applied concentration that did not give statistically significant effects compared to the control. It is usually identified on the basis of (mutually dependent) tests against the control (cf. Williams 1971). An inherent problem to this procedure is that the null-hypothesis states "there is no effect at the applied concentration" (in other words: the concentration is 'safe'), sloppy experimental procedures result in high NOEC's; this is certainly an undesirable coupling. To resolve this problem, the application of 'small-effect concentrations' has recently been considered (Pack 1993), but an inherent problem to this approach is the arbitrariness of a choice for 'small'. Moreover, the estimated value for a small-effect concentration depends very sensitively on the particular concentration-effect relationship (e.g. the log-logistic one), which has no scientific justification. The basis is purely empirical and weak in the 'tails'. An attractive alternative is the no-effect concentration (NEC), treated as a model parameter (Kooijman 1981; Cox 1985). Here we study to what extent it is model-specific by comparing the extended standard log-logistic model and the hazard model for concentration-effect relationship. Both models are discussed in some detail in Kooijman (1981) and Bedaux and Kooijman (1994), respectively, and the biological backgrounds in Kooijman (1993). Here, we give a short introduction to these models and focus on the typical case where the surviving individuals are counted once only, at the end of the experiment.

[0]Published as: S.A.L.M. Kooijman and J.J.M. Bedaux (1996) Some statistical properties of estimates of no-effect concentrations. *Water Research* **30**: 1724–1728

Effect models

We choose this observation time as our time unit and consider the survival probability as a function of the concentration only. This removes the dimension time from the parameters of the survival probability. The standard procedure to model bioassays for survival is to assume that the survival probability is independent and identical for each individual in a cohort. This implies that the number of survivors follows a binomial distribution. The log-likelihood function for the experimental result to find $\{n(c_i,t)\}_{i=1}^{s}$ survivors after exposure time t to the compound, which is present in concentration c_i, is then

$$\ln l = \sum_{i=1}^{s} n(c_i,1)\ln q(c_i,1) + \sum_{i=1}^{s}(n(c_i,0)-n(c_i,1))\ln p(c_i,1) \tag{3.1}$$

where $q(c_i,t) \equiv 1-p(c_i,t)$ denotes the survival probability at time t.

The standard model for survival assumes that an individual dies as soon as the tissue-concentration exceeds a (fixed) threshold value which differs among individuals. These threshold values are conceived as random trials from a log-logistic distribution (see Ashton 1972), which just has a purely empirical basis. The standard model is extended by subtracting a no-effect-tissue-concentration from the tissue-concentration. The model has the unrealistic property that a fraction of the exposed individuals survives the toxicant, even if the exposure is very long and the concentration very high.

The hazard model assumes that the hazard rate is proportional to the tissue-concentration minus the no-effect-tissue-concentration (Kooijman 1993, Bedaux & Kooijman 1994). In contrast to the standard model, all individuals are treated as being equal in the most simple version of the hazard model, but the killing process itself is stochastic rather than deterministic. Another difference is that the compound will eventually kill all individuals that accumulate the compound above the no-effect-tissue-concentration.

For both models we will assume that the uptake and elimination behaviour of the compound follows simple first order kinetics, while the concentration in the environment remains constant and the initial concentration in the tissue is 0, which is typical for most routine toxicity tests. Extensions of this idea are easy to implement (Kooijman 1993). We also assume here that the individuals do not grow during exposure.

Extended standard model

The no-effect concentration at the time of observation relates to the (ultimate) no-effect concentration as $c_0 \equiv \frac{c_{0,\infty}}{1-\exp\{-k_a\}}$, where k_a is the elimination rate. Since the elimination rate does not affect the concentration response relationship in the standard model (at one single observation time), we have no information about k_a,

so we can only extract information about c_0 from survival data, but not about $c_{0.\infty}$. The elimination rate does affect the concentration response relationship in the hazard model, however. Obviously, information about the elimination rate can be obtained from survival data much more easily (and reliably) if several observation times had been available that reveal how effects build up in time. For the hazard model, it is thus possible to obtain both c_0 and $c_{0.\infty}$ from a single concentration response relationship.

The extended standard model for $c > c_0$ is

$$q(c) \quad = \quad q_0 \left(1 + \left(\frac{c - c_0}{c_{L50} - c_0} \right)^{1/\beta} \right)^{-1} \tag{3.2}$$

where q_0 stands for the control survival probability; β is the gradient parameter which directly relates to the (maximum) slope of the graph where the response is plotted against the concentration. The parameter c_{L50} (usually known as LC50) represents to concentration for which the survival probability is half that in the control, so $q(c_{L50}) = q_0/2$. It depends on the exposure time in a way similar to the no-effect concentration: $c_{L50} = \frac{c_{L50.\infty}}{1-\exp\{-k_a\}}$. We have the same problem here: If nothing is known about the elimination rate, we have no information about $c_{L50.\infty}$. We need more than one observation time in the standard model to obtain that information. For $c < c_0$, we have $q(c) = q_0$.

Hazard model

The hazard model for $c > c_0$ is

$$q(c) = q_0 \exp\left\{ -k_\dagger k_a^{-1} \left[c \exp\{-k_a\} + (c - c_{0.\infty}) \left(k_a - 1 + \ln\{1 - c_{0.\infty}/c\} \right) \right] \right\} \tag{3.3}$$

where $k_\dagger$ stands for the killing rate. For $c < c_0$, we have $q(c) = q_0$. This survival probability results from the hazard rate $h(c,t)$ via $q(c) = \exp\{-\int_0^1 h(c,\tau)\, d\tau\}$ (by definition), with

$$h(c,t) = h(0,t) + k_\dagger((1 - \exp\{-tk_a\})c - c_{0.\infty})_+ \tag{3.4}$$

The index + indicates that negative values between the brackets should be replaced by 0. The first term $h(0,t)$ stands for the hazard rate in the control, which is taken to be constant. So $\exp\{-\int_0^1 h(0,\tau)\, d\tau\} = q_0$. The killing rate appears here as a simple proportionality factor for the hazard rate. The term $(1 - \exp\{-tk_a\})c$ in the hazard rate is proportional to the tissue-concentration, from which the no-effect concentration is subtracted. Although the hazard model (3.3) might seem more complex than the extended standard model (3.2) at first sight, the hazard rate of the extended standard model is much more complex than (3.4); it is just a matter of presentation.

Since the information content of a concentration response relationship as regards the elimination rate is poor, the limiting cases for very large and very small elimination rates are of special interest. These limiting cases will be referred to as the exponential and the Weibull model, respectively, and amount to

$$q(c) \overset{k_a \to \infty}{=} q_0 \exp\{-k_\dagger (c - c_0)\} \tag{3.5}$$

$$q(c) \overset{k_a \to 0}{=} q_0 \exp\left\{-\frac{1}{2} kc (1 - c_0/c)^2\right\} \tag{3.6}$$

The exponential model (3.5) can be derived from the hazard model (3.3) in a straightforward way, but the relationship between the Weibull model (3.6) and the hazard model is more complex. This is because effects relate to tissue-concentrations, while the model is formulated in terms of environment-concentrations. Tissue-concentrations build up linearly in time for $k_a \to 0$, so that $c_{0.\infty} \to 0$ and the waiting time till the tissue-concentration exceeds the no-effect-tissue-concentration is inversely proportional to the environment-concentration. The interpretation of c_0 in (3.6) is the limit of $c_{0.\infty}/k_a$. It here has the dimension of a concentration because we have chosen the exposure time as unit of time, which is somewhat misleading. We also have that $k_\dagger \to \infty$ such that the killing acceleration $k \equiv k_\dagger k_a$ remains fixed, which leads to the hazard rate $h(c,t) = h(0,t) + kc(t - c_0/c)_+$ and so to (3.6).

Examples

Practice teaches that it is hard to obtain partial effects in more than one or two concentrations, for most compounds. This gives problems in obtaining point estimates for c_0. A practical solution to this problem is to choose a variety of values for c_0 and obtain the maximum likelihood estimates for the other parameters via a Newton Raphson procedure, for instance. We then inspect the profile log-likelihoods (see e.g. McCullagh & Nelder 1991, p 254, or Carroll et al. 1995), i.e. the log-likelihood function that is maximized with respect to all parameters except c_0, as a function of c_0. The difference of the profile log-likelihoods and the maximum log-likelihood is plotted in Figure 3.1. Analyses are given for four artificial but typical examples of experimental results, where $n(c_i, 0) = 10$ has been chosen for all c_i in all examples (see Table 3.1). The concentrations have a fixed difference in one pair of examples and a fixed factor in the other pair, while the number of survivors have been chosen identically. This is done to investigate the effect of the morphology of the observed concentration response relationship on the estimate for c_0. A second comparison is based on the interchange between the number of survivors in the control and the lowest concentration. This is done to investigate how uncertainty about the cause of death (i.e. control vs toxicant-induced mortality) affects the estimate for c_0.

Figure 3.1: The five dots in each graph represent numbers of 'observed' survivors out of 10. The expected number of survivors is plotted for the hazard model (solid curve) and the extended standard model (dotted curve), based on the maximum likelihood estimates of the parameters, which are given below. The difference between the maximum log-likelihood and the profile log-likelihood is also plotted as a function of the NEC, for both models (dotted and bold curve) as well as for the Weibull (left thin curve) and the exponential model (right thin curve).

example	hazard model					extended standard model			
	q_0	c_0	k	$k_\dagger$	k_a	q_0	c_0	c_{L50}	β
1	0.950	1.303	5.143			0.950	0.763	2.045	0.225
2	1.000	0.749	2.510			1.000	0.000	1.858	0.228
3	0.945	1.000		1.407	2.507	0.945	0.981	2.066	4.259
4	1.000	0.654	1.635			1.000	0.000	1.986	0.300

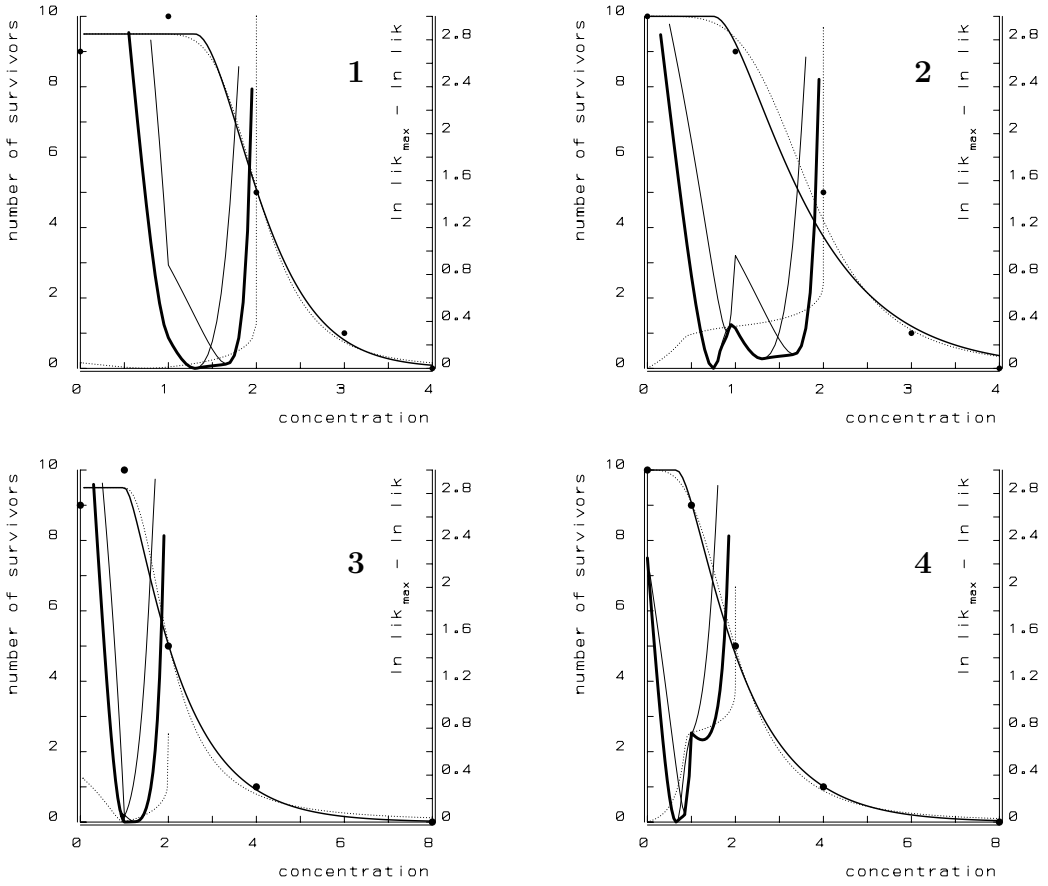


Table 3.1: The chosen concentrations and surviving individuals out of ten.

example	1 & 2	0	1	2	3	4	conc.
example	3 & 4	0	1	2	4	8	conc.
example	1 & 3	9	10	5	1	0	surv.
example	2 & 4	10	9	5	1	0	surv.

Conclusions

Both the extended standard and the hazard model appear to fit the data well. Since the shape of the concentration response relationship in examples 1 and 2 differs from that of examples 3 and 4, the goodness of fit is not very sensitive to details of the shape. The best fitting hazard model proved to be the Weibull model (3.6) in three examples (see Table 3.1). The examples being typical, we expect that the tiny differences in goodness of fit cannot be used to choose between the models in practice. This means that scientific arguments must be used for the choice rather than statistical ones. The hazard model (with the exponential and Weibull model as special cases) has a statistical advantage above the standard one because it has fewer parameters, which generally leads to smaller confidence intervals. This is even more obvious if several observation times are considered simultaneously, because then the elimination rate also appears in the extended standard model while no new parameters show up in the hazard model.

The profile log-likelihood functions are plotted in the same Figure 3.1 to compare these functions with the concentration response relationships. The profiles can be used to obtain confidence sets for the no-effect concentration c_0. When the large sample theory for likelihood ratios applies (see Silvey 1975), the likelihood based α-level confidence set for c_0 is given by $\{c_0 | 2\,(\ln l(\hat{c}_0) - \ln l(c_0)) \leq \chi^2_1(\alpha)\}$, where $\ln l(c_0)$ denotes the profile log-likelihood in c_0 and $\chi^2_1(\alpha)$ is a number such that for a random variable $\underline{z}$ that is χ^2-distributed with 1 degree of freedom we have $\text{Prob}\{\underline{z} \leq \chi^2_1(\alpha)\} = \alpha$. Likelihood-based confidence sets seem to be more robust against deviations from 'large samples' than the interval $\{c_0 | (c_0 - \hat{c}_0)^2 \hat{\text{var}}(\hat{c}_0) \leq \chi^2_1(\alpha)\}$ in models like these (Kooijman 1983, Carroll et al. 1995). Application of this idea in Figure 3.1 means that the 90% or 95% confidence set contains the c_0-values for which the log-likelihood is 1.35 or 1.92 lower than the maximum log-likelihood.

Deviations from the large-sample theory can be translated into corrections on the χ^2-values. Figure 3.1 shows that such corrections would hardly affect the confidence limits of $\hat{c}_0$ since the profile log-likelihood functions are very steep. Practice has little interest in a high accuracy at this point. We can conclude that the NEC for the extended standard model is not significantly different from 0, but for the hazard model it is for all examples. This reflects an important structural difference between

both models. If the hazard model is acceptable on scientific grounds, it should be preferred above the extended standard model, not because of tiny differences in goodness of fit but because of its structural properties.

The bold curves in Figure 3.1 represent the profile log-likelihood functions of the hazard model. They coincide with the profile log-likelihood functions of the Weibull model for the lower concentrations and that of the exponential model for the higher concentrations. The relatively small shift between the profile log-likelihood functions of the Weibull and the exponential model corresponds with the change of the elimination rate k_a from 0 to ∞. We conclude that a single observation time for surviving individuals results in poor knowledge about the elimination rate, but this has little effect on the estimate for the no-effect concentration.

Examples 2 and 4 have no deaths in the control and one in the lowest concentration. The profile log-likelihood functions for these examples have two local maximums, which correspond with two possible interpretations for the cause of death: is it control mortality (right local maximum), or toxicant-induced (left local maximum)? The profiles show that the interpretation in terms of toxicant-induced mortality is more likely in these two examples. Examples 1 and 3 have one death in the control and none in the lowest concentration. Here, we have just one possible interpretation for the cause of death (namely control mortality) and just one local maximum for the profile log-likelihood function. The profile log-likelihood functions thus quantify the probabilities for the alternative causes of death.

Acknowledgements

This work was supported by Grant PAD 90-18 from the Alternative to Animal Experiments Platform.

References

Ashton, W.D. 1972. *The logit transformation with special reference to its uses in bioassay.* Griffin's statistical monographs & courses 32. Griffin, pp. 88.

Bedaux, J.J.M. & Kooijman, S.A.L.M. 1994. Statistical analysis of bioassays, based on hazard modeling. *Journal of Environmental Statistics* **1**: 303-314.

Carroll, R.J., Ruppert, D. & Stefanski, L.A. 1995. *Measurement error in nonlinear models.* Monographs on Statistics and Applied Probability 63, London: Chapman & Hall, pp 305.

Cox, C. 1987. Threshold dose-response models in toxicology. *Biometrics* **43**: 511-523.

Kooijman, S.A.L.M. 1981. Parametric analyses of mortality rates in bioassays. *Water Research* **15**: 107-119.

Kooijman, S.A.L.M. 1983. Statistical aspects of the determination of mortality rates in bioassays. *Water Research* **17**: 749-759.

Kooijman, S.A.L.M. 1993. *Dynamic Energy Budgets in Biological Systems. Theory and applications in ecotoxicology.* Cambridge University Press, pp. 350.

McCullagh, P. & Nelder, J.A. 1991. *Generalized linear models.* Monographs on Statistics and Applied Probability 37. Chapman & Hall, pp. 511.

Pack, S. 1993. A review of statistical data analysis and experimental design in OECD aquatic toxicity test guidelines. Shell Research Ltd., Sittingbourne, UK.

Silvey, S.D. 1975. *Statistical inference.* Monographs on Statistics and Applied Probability 7. Chapman and Hall, pp. 192.

Williams, D.A. 1971. A test for differences between treatment means when several dose levels are compared with a zero dose control. *Biometrics* **27**: 103-117.

3.2 Statistical Analysis of Bioassays based on hazard modelling

Abstract A stochastic model is proposed to describe time–dependent lethal effects of toxic compounds. It is based on simple mechanistic assumptions and provides a measure of the toxicity of a chemical compound, the so-called killing rate. The killing rate seems a promising alternative for the LC50. The model also provides the no–effect level and the LC50, both as a function of exposure time. The model is applied to real data and to simulated data.

Introduction

The analysis of survival data is important in toxicological studies. In many laboratories, bioassays are carried out routinely to investigate toxicological properties of new chemical compounds. Determination of LC50-values and no–effect concentrations (NEC) is the main objective. The LC50-value of a compound is the concentration expected to cause death of 50% of the population within a fixed time. The no–effect concentration is the maximum concentration which has no lethal effect within the duration of the experiment. Both LC50 and NEC depend on the species chosen, the exposure time, the temperature at which the experiment is performed, the age of the experimental animals, etc. In routine experiments, animals are exposed to a compound in a range of concentrations. After a fixed time chosen on the basis of experience and intuition and depending on the species used, the numbers of survivors are counted for every concentration. The resulting LC50- and NEC-estimates only tell something about exposure during that fixed time and as such only have limited meaning. In more elaborate experiments survivorship is measured after several exposure times. This enables the study of the time-dependence of LC50 and NEC.

For simple experiments, a wide variety of statistical methods is used to estimate LC50 and NEC. Nonparametric methods, such as moving average or (trimmed) Spearman-Kärber, as well as parametric methods, such as probit or logit analysis, are used to estimate LC50; for a review see Hoekstra (1991). Morgan (1988) reviews several extensions of the classical logit and probit models. Estimation methods of NECs can be found in Cox (1987). Kooijman (1981) proposed models to estimate NEC and (time-dependent) LC50 in various experimental designs.

In most parametric procedures, distribution functions are chosen *ad hoc* to describe the stochastic behaviour of the data. Biological knowledge is rarely incorporated in the stochastic model. In this paper we develop a stochastic model based on simple assumptions that are still realistic from a biological point of view. The key assumption is that the hazard rate is proportional to the concentration of the compound in the animal. The idea of relating the hazard rate to the dose is not new.

[0]Published as: J.J.M. Bedaux and S.A.L.M. Kooijman (1994) Statistical Analysis of Bioassays based on hazard modelling. *Environmental and Ecological statistics*, **1**: 303-314.

Puri and Senturia (1972; Laurence and Morgan, 1989; Morgan, 1992, Ch. 5) proposed a stochastic model in which the hazard rate is a function of the concentration of the compound.

A second assumption concerns the kinetics of the compound. We assume a simple linear one–compartment model. The incorporation of the kinetics of the compound in the dose-response model is not new either. Puri and Senturia (1972) constructed a stochastic process underlying the concentration in the animal. Van Ryzin and Rai (1987) used Michaelis–Menten nonlinear kinetics to describe the internal concentration as a function of the external concentration. They only considered steady–state conditions, so their approach cannot be used in the study of time–dependent toxicity.

The usual experimental data sets consist of counts of surviving organisms that have been exposed to a chemical compound at a range of concentrations during a fixed time of exposure. Most statistical methods only provide estimates in simple designs involving a single exposure time. In order to analyze experiments involving several exposure times, the time-dependence of the parameters must be modelled. Kooijman (1981) proposed an extension of the log-logistic tolerance distribution. His model will be compared with the present model. Carter and Hubert (1984) proposed a growth–curve model approach.

Maximum likelihood methods are used to estimate the parameters of the model. To study the statistical properties of the estimators, we applied the method to data obtained by Monte-Carlo simulation. In addition, we applied the method to experimental data.

Modelling survivorship

The key assumption in this paper is that the hazard rate is proportional to the concentration of the chemical compound in the animal, as far as it exceeds a so-called no-effect level. To be more precise, we assume the hazard $\dot{h}(t)$ to be proportional to the (positive) difference between the concentration $[Q](t)$ and the no-effect level $[Q]_0$. Generally, we do not know the actual concentrations in the animal. We only know the concentration in the environment $c(t)$. (Note that throughout this section $[Q]$ is used to denote the concentration in the animal and c for the concentration in the environment. Symbols used are shown in Table 3.2.) We therefore have to make assumptions about the uptake dynamics of the compound in the animal. A simple model, which is still realistic from a biological point of view, is the so-called one–compartment model (Jacquez, 1985). That is,

$$\frac{d[Q](t)}{dt} = \dot{k}_u c(t) - \dot{k}_a [Q](t) \,, \tag{3.7}$$

where $\dot{k}_a$ is the elimination rate and $\dot{k}_u$ the uptake rate. The solution of (3.7) is easily found to be

$$[Q](t) = [Q](0)\mathrm{e}^{-\dot{k}_a t} + \dot{k}_u \int_0^t \mathrm{e}^{-\dot{k}_a (t-\tau)} c(\tau)\, d\tau \,. \tag{3.8}$$

Table 3.2: List of symbols. T, M and L denote the dimensions time, mass and length.

Symbol	dimension	interpretation
$\dot{h}$	T^{-1}	hazard rate
t	T	time
$[Q]$	ML^{-3}	concentration of the compound in the animal
$[Q]_0$	ML^{-3}	no–effect level in the animal
c	ML^{-3}	concentration of the compound in the environment
c_0	ML^{-3}	no–effect level in the environment
$\dot{k}_u$	T^{-1}	uptake rate
$\dot{k}_a$	T^{-1}	elimination rate
c_0	ML^{-3}	no–effect level in the environment
t_0	T	time at which $[Q]$ exceeds the no-effect level
$\dot{k}_\dagger$	$\mathrm{L}^3\mathrm{M}^{-1}\mathrm{T}^{-1}$	killing rate
$\dot{\lambda}$	T^{-1}	control mortality rate
$\ddot{k}_\dagger$	$\mathrm{L}^3\mathrm{M}^{-1}\mathrm{T}^{-2}$	killing acceleration
c_{L50}	ML^{-3}	ultimate LC50 value
β	-	slope parameter of the logistic distribution
$q(t;c)$	-	survivor probability at time t and concentration c
x_{ij}	-	number of surviving animals at time t_i and conc. c_j
p_{ij}	-	probability of an animal to die between t_{i-1} and t_i, at c_j
n_{ij}	-	number of animals died between t_{i-1} and t_i, at c_j

Here we consider experimental situations in which $c(t)$ is constant, say $c(t) = c$. We also assume that $[Q](0)$ is negligibly small. Then (3.8) reduces to

$$[Q](t) = \frac{\dot{k}_u}{\dot{k}_a} c(1 - \mathrm{e}^{-\dot{k}_a t}) \,. \tag{3.9}$$

The ultimate concentration is given by $\lim_{t\to\infty}[Q](t) = c\dot{k}_u/\dot{k}_a$, the ratio $\dot{k}_u/\dot{k}_a$ being known as the bioconcentration factor. If this value is smaller than the no-effect level $[Q]_0$, *i.e.* if $c < [Q]_0\dot{k}_a/\dot{k}_u$, there will be no effect at all, even after long exposure times. This defines the environmental (ultimate) no-effect level $c_0 = [Q]_0\dot{k}_a/\dot{k}_u$.

If $c > c_0$ there is a point in time t_0 at which $[Q](t)$ exceeds $[Q]_0$ (see Figure 3.2). From (3.9) t_0 can be calculated to be

$$t_0 = -\frac{1}{\dot{k}_a} \ln(1 - \frac{c_0}{c}) \,. \tag{3.10}$$

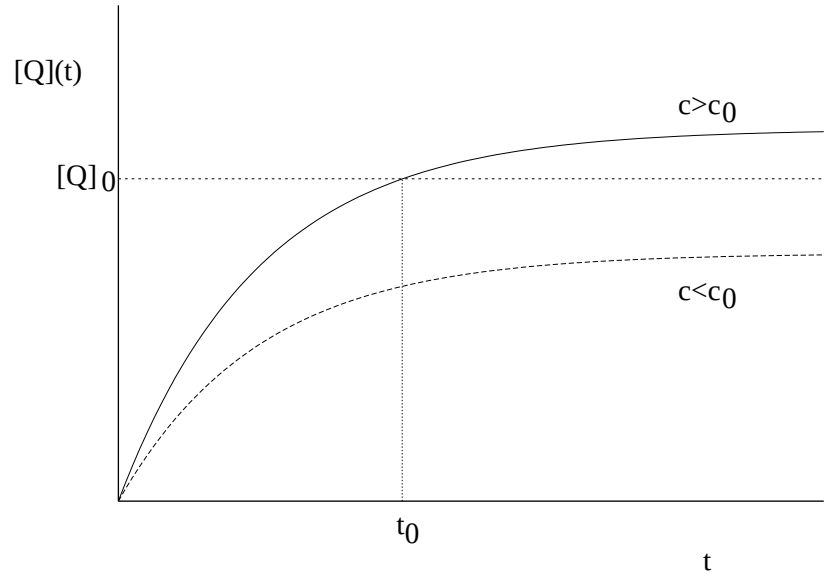


Figure 3.2: Accumulation curves for two values of the environmental concentration c, one below and one above c_0.

The hazard rate is now given by

$$\dot{h}(t;c) \propto [Q](t) - [Q]_0 = \frac{\dot{k}_u}{\dot{k}_a}(c(1 - \mathrm{e}^{-\dot{k}_a t}) - c_0)_+$$

or

$$\dot{h}(t;c) \propto (c(1 - \mathrm{e}^{-\dot{k}_a t}) - c_0)_+ \,, \tag{3.11}$$

where $(x)_+$ means the maximum of x and 0. This notation will be used frequently in the following. The proportionality constant in (3.11), written as $\dot{k}_\dagger$, will be called the killing rate, as proposed in Kooijman (1993, p. 277). It has dimension (concentration·time)$^{-1}$ and can be viewed as a measure of the toxicity of the compound with respect to survival. The hazard rate can now be written as

$$\dot{h}(t;c) = \dot{k}_\dagger(c(1 - \mathrm{e}^{-\dot{k}_a t}) - c_0)_+ \,.$$

The survivor function $q(t;c)$ of the time of dying caused by the chemical compound at concentration c, is then given by

$$q(t;c) = \begin{cases} \exp\left(\frac{\dot{k}_\dagger}{\dot{k}_a} c(\mathrm{e}^{-\dot{k}_a t_0} - \mathrm{e}^{-\dot{k}_a t}) - \dot{k}_\dagger(c - c_0)(t - t_0)\right) & \text{if } c > c_0 \text{ and } t > t_0 \\ 1 & \text{otherwise} \end{cases} . \tag{3.12}$$

Control mortality is readily included in this model formulation. Assuming independence of death caused by the chemical compound and death caused by natural circumstances, we can simply add the corresponding hazard rates. The hazard rate

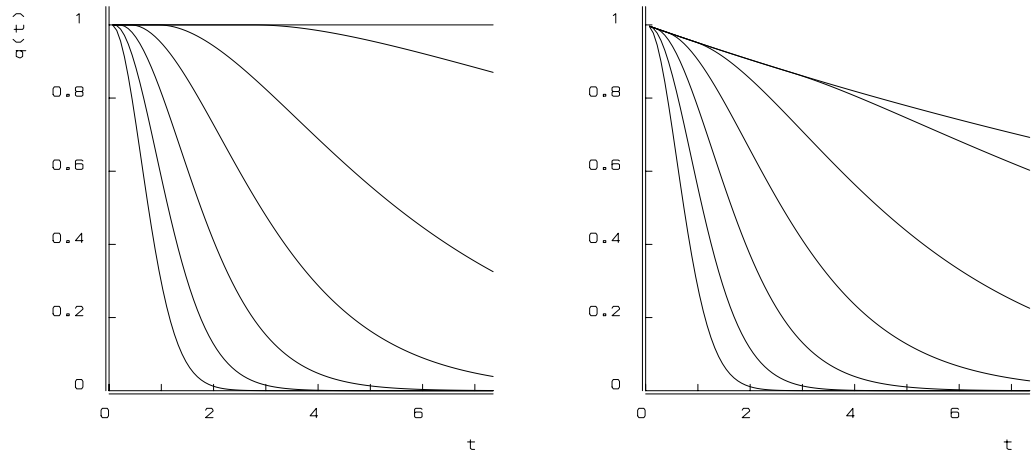


Figure 3.3: Survivor functions for various values of c (from top downwards 1, 2, 4, 8, 16, 32 and 64). Parameter values are $c_0 = 1.5$, $\dot{k}_\dagger = 0.1$ and $\dot{k}_a = 0.5$. Left $\dot{\lambda} = 0$, right $\dot{\lambda} = 0.05$.

due to control mortality, $\dot{\lambda}$, will be taken constant. This is reasonable because the duration of the experiments is mostly short compared with the mean lifetime of the organisms used. The resulting survivor function is the one obtained in (3.12) multiplied by $\exp(-\dot{\lambda}t)$. In Figure 3.3 some survival curves are plotted with and without control mortality, for one choice of parameter values.

An interesting special case concerns extremely small elimination rates, so $\dot{k}_a \to 0$. This occurs for instance with cadmium in some soil arthropods (Janssen et al. 1991). The accumulation process reduces to $\frac{d}{dt}[Q] = \dot{k}_u c$, so that $[Q](t) = \dot{k}_u c t$ if again the initial concentration in the tissue is negligibly small. The no–effect level (in the environment) now equals 0 because a very small concentration in the environment will ultimately result in a very high concentration in the tissue. A no–effect level in the tissue, *i.e.* the upper boundary of the tolerance range, still exists, of course, and is exceeded at $t_0 = [Q]_0(\dot{k}_u c)^{-1}$. The hazard rate amounts to $\dot{h}_c = \ddot{k}_\dagger c(t - t_0)_+$. The relationship between the killing acceleration $\ddot{k}_\dagger$ and the killing rate $\dot{k}_\dagger$ is $\ddot{k}_\dagger = \lim_{\dot{k}_a \to 0} \dot{k}_\dagger \dot{k}_a$. The survival probability is

$$q(t;c) = \exp\{-\frac{1}{2}\ddot{k}_\dagger c((t - t_0)_+)^2\} \,. \tag{3.13}$$

This represents a Weibull distribution with shape parameter 2.

For very large values of $\dot{k}_a$, on the other hand, the survivor function becomes an exponential function

$$q(t;c) = \mathrm{e}^{-\dot{k}_\dagger t(c - c_0)_+} \,,$$

which can also be seen as a Weibull function, with shape parameter 1. In Figure 3.4 some possible shapes of $q(t;c)$ for varying elimination rates are shown.

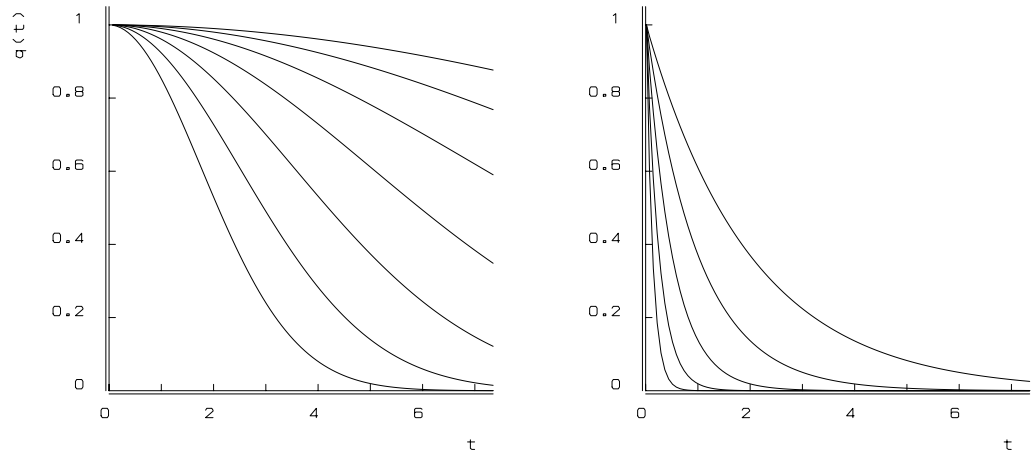


Figure 3.4: Possible shapes of survivor curves with changing elimination rate. Parameter values ($c_0 = 0$, $\dot{k}_\dagger = 0.5$, $\dot{\lambda} = 0$, $\dot{k}_a = 0.01$ (left) or 100 (right).

As an alternative we will discuss the model proposed by Kooijman (1981), which is an extension of the standard log-logistic model. The extension involves a no–effect level and a LC50–time relation consistent with the first–order kinetics (3.7). It can be summarized as follows. The probability of surviving an exposure time t at concentration c is given by

$$q(t;c) = \left(1 + \left(\frac{\left(c(1 - \mathrm{e}^{-\dot{k}_a t}) - c_0\right)_+}{c_{L50} - c_0}\right)^{\beta}\right)^{-1}, \tag{3.14}$$

where c_{L50} is the ultimate LC50, i.e. the LC50-value after a very long exposure time. Kooijman (1993, p. 279) compares the models (3.12) and (3.14). An important difference is the behaviour after long exposure times, if $c > c_0$: in model (3.12) we have $\lim_{t\to\infty} q(t;c) = 0$ and in (3.14) $\lim_{t\to\infty} q(t;c) > 0$.

Estimation of parameters

In experiments which are set up to evaluate the lethal effect of toxic compounds, the resulting data sets consist of counts x_{ij} of surviving organisms on fixed times t_i, $i = 0, \ldots, r$, exposed to a chemical compound at concentration c_j, $j = 1, \ldots, k$. An example of such a data set is given in Table 3.3. To fit the model (3.12) to such a data set we use the maximum likelihood (ML) method. For this experimental set-up the likelihood function is not the product of the density function of $\underline{t}_\dagger$ in the data points (t_i, c_j), since we do not know the exact times of death of the organisms. We only know the time intervals at which death has occurred. Before deriving the likelihood function we have to introduce some new symbols.

Table 3.3: Number of surviving guppies *Poecilia reticulata* in natural sea water after exposure to the pesticide dieldrin. Data were kindly provided by Ms T. Adema (IMW-TNO Laboratories, Delft).

	concentration *dieldrin* (μg l^{-1})							
time (d)	0	3.2	5.6	10	18	32	56	100
0	20	20	20	20	20	20	20	20
1	20	20	20	20	18	18	17	5
2	20	20	19	17	15	9	6	0
3	20	20	19	15	9	2	1	0
4	20	20	19	14	4	1	0	0
5	20	20	18	12	4	0	0	0
6	20	19	18	9	3	0	0	0
7	20	18	18	8	2	0	0	0

The probability p_{ij} of an organism, exposed to concentration c_j, to die between t_{i-1} and t_i is given by $p_{ij} = q(t_{i-1}, c_j) - q(t_i, c_j)$. The number of organisms n_{ij} which died in that period is given by $n_{ij} = x_{i-1,j} - x_{ij}$. The number of organisms surviving at t_r will be denoted by $n_{r+1,j}$. The probability of surviving at t_r is denoted by $p_{r+1,j}$ and equals $q(t_r, c_j)$.

The probability of obtaining the counts x_{ij} can now be written as a product of multinomial probabilities:

$$\text{Prob}(\underline{x}_{ij} = x_{ij}) = \text{Prob}(\underline{n}_{ij} = n_{ij}) = \prod_{j=1}^{k} x_{0j}! \prod_{i=1}^{r+1} \frac{p_{ij}^{n_{ij}}}{n_{ij}!} \,. \tag{3.15}$$

The log-likelihood function is then given by

$$\ell(\theta; (x_{ij})) = \sum_{i=1}^{r+1} \sum_{j=1}^{k} n_{ij} \ln(p_{ij}) \qquad \text{with } \theta = (c_0, \dot{k}_\dagger, \dot{k}_a, \dot{\lambda})' \,, \tag{3.16}$$

where the constant term has been ignored. Maximum likelihood estimates can be found by solving the vector equations

$$G(\theta) = \frac{\partial \ell}{\partial \theta} = \sum_{i=1}^{r+1} \sum_{j=1}^{k} \frac{n_{ij}}{p_{ij}} \frac{\partial p_{ij}}{\partial \theta} = 0 \,. \tag{3.17}$$

The information matrix $I(\theta)$, defined as minus the expectation of the matrix of second derivatives, can be shown to be

$$I(\theta) = -E\left(\frac{\partial^2 \ell}{\partial \theta^2}\right) = \sum_{j=1}^{k} x_{0j} \sum_{i=1}^{r+1} \frac{1}{p_{ij}} \left(\frac{\partial p_{ij}}{\partial \theta}\right) \left(\frac{\partial p_{ij}}{\partial \theta}\right)' \,. \tag{3.18}$$

This matrix can be used to estimate the asymptotic variance-covariance matrix. It can also be used in the so-called method of scoring, an iteration scheme to find the ML estimates:

$$\theta_{i+1} = \theta_i + I^{-1}(\theta_i)G(\theta_i)\,. \tag{3.19}$$

As a rough measure of goodness-of-fit we use the deviance of the model (McCullagh and Nelder, 1989). The deviance is defined as twice the difference between the maximum achievable log likelihood and that attained under the fitted model. The maximum achievable log likelihood $\ell_{sup}((x_{ij}))$ is obtained by estimating each p_{ij} without any constraint, *i.e.*, $\hat{p}_{ij} = n_{ij}/x_{0j}$. Substituting this in (3.16) we get

$$\ell_{sup}((x_{ij})) = \sum_{i,j} n_{ij} \ln(\frac{n_{ij}}{x_{0j}})\,,$$

where the summand should read 0 if $n_{ij} = 0$. The difference in deviances between nested models can be used as a test statistic. It is equivalent to the usual likelihood-ratio test. The approximate distribution of the test statistic is $\chi^2_{[d]}$, where d is the difference in the number of parameters between the two nested models. The deviance should not be used to test the absolute goodness-of-fit. Usually the asymptotic theory does not apply, because some of the expected numbers $\mathrm{E}(\underline{n}_{ij})$ are too small.

Equations (3.17), (3.18) and (3.19) were implemented in a computer program written in APL. The numerical procedure appeared to be sensitive to starting values of the parameters, which cannot be easily found. If $\dot{\lambda} = 0$, relation (3.10) can be used to guess starting values for c_0 and $\dot{k}_a$.

Monte-Carlo Simulation

We studied the performance of ML estimation by Monte Carlo simulation. Data sets resembling the data of Table 3.3 were generated: 8 concentrations (an exponential series, $10^{j/4}$ for $j = 0, \ldots, 7$), 8 time points (0, 1, ..., 7) and fixed parameters ($c_0 = 2$, $\dot{k}_\dagger = 0.1$ and $\dot{k}_a = 0.5$) were chosen. Four different values of x_{0j} (5, 10, 20 and 50) were chosen to study the influence of sample size. For every value of x_{0j}, 1000 data sets were generated by simulating multinomial distributions according to (3.15) and (3.12). In every data set we estimated the parameters by solving (3.17) numerically, where the 'true' parameters were chosen as starting values. The resulting 1000 vectors of parameter estimates $(\hat{c}_0, \hat{\dot{k}}_\dagger, \hat{\dot{k}}_e)'$ were analyzed by calculating means, standard deviations and correlation matrices. The latter were compared with the theoretical asymptotic values.

The results are shown in Table 3.4. The parameter estimates behave quite differently with respect to sample size. Estimation of c_0 is accurate, even for 5 animals per concentration, while reliable estimation of $\dot{k}_\dagger$ apparently needs large sample sizes. The estimates of the standard deviations, which can be compared with the theoretical value after multiplication with $\sqrt{x_{0j}}$, and the estimates of the correlation coefficients

Table 3.4: Results of ML estimation on simulated data. In the first horizontal block theoretical values are shown: asymptotic expectations, standard deviations according to (3.18), multiplied by $\sqrt{x_{0j}}$, and correlation coefficients. In the following blocks simulation results are shown: means, standard deviations ($\times\sqrt{x_{0j}}$) and correlation coefficients of parameter estimates of 1000 simulated data sets.

		mean	sd	$\text{sd}\sqrt{x_{0j}}$	correlation coefficients		
theoretical	c_0	2	-	1.425	1		
values	$\dot{k}_\dagger$	0.1	-	0.0875	–0.393	1	
	$\dot{k}_a$	0.5	-	0.5827	0.755	–0.790	1
$x_{0j} = 5$	c_0	2.095	0.6442	1.441	1		
	$\dot{k}_\dagger$	0.1453	0.1052	0.2353	–0.377	1	
	$\dot{k}_a$	0.5262	0.3721	0.8321	0.431	–0.454	1
$x_{0j} = 10$	c_0	2.033	0.4013	1.269	1		
	$\dot{k}_\dagger$	0.1179	0.0431	0.1364	–0.335	1	
	$\dot{k}_a$	0.5053	0.1916	0.6057	0.635	–0.703	1
$x_{0j} = 20$	c_0	2.018	0.2970	1.328	1		
	$\dot{k}_\dagger$	0.1100	0.0251	0.1121	–0.308	1	
	$\dot{k}_a$	0.4919	0.1286	0.5749	0.655	–0.748	1
$x_{0j} = 50$	c_0	1.995	0.1863	1.318	1		
	$\dot{k}_\dagger$	0.1042	0.0137	0.0968	–0.354	1	
	$\dot{k}_a$	0.4927	0.0815	0.5761	0.695	–0.799	1

indicate that asymptotic theory should only be applied at large values of x_{0j}, say $x_{0j} \geq 20$.

Application to experimental data

We have fitted model (3.12)—with and without control mortality—to the data set of Table 3.3. Solutions were checked by Monte Carlo searches. We also fitted model (3.14) to the same data. This led to some numerical problems. All parameters but β grew very small and c_0 even became zero. In the limit situation for small $\dot{k}_a$ we can then rewrite model (3.14) to

$$q(t;c) = \left(1 + \left(\frac{ct}{A}\right)^{\frac{1}{\beta}}\right)^{-1} \qquad \text{where } A = \lim_{\dot{k}_a \to 0} \frac{c_{L50}}{\dot{k}_a}\,. \tag{3.20}$$

The results of parameter estimation are given in Table 3.5. Inclusion of control mortality in model (3.20) did not noticeably affect the results.

The data and estimated survivor curves are plotted in Figure 3.5. The improvement in fit from inclusion of control mortality in (3.12) is apparent for $c = 5.6$ and

Table 3.5: Results of ML estimation on empirical data given in Table 3.3. For each parameter, point estimates, standard deviations, and correlation coefficients are given.

model	par.	units	estimate	s.d.	correlation coefficients			
	$\dot{\lambda}$	d^{-1}	0	-	-			
(3.12)	c_0	$\mu\mathrm{g\ l}^{-1}$	2.77	0.303	-	1		
	$\dot{k}_\dagger$	$\mathrm{l\ \mu g}^{-1}\mathrm{d}^{-1}$	0.0309	0.00549	-	–0.233	1	
	$\dot{k}_a$	d^{-1}	0.727	0.201	-	0.511	–0.790	1
	$\dot{\lambda}$	d^{-1}	0.00835	0.00490	1			
(3.12)	c_0	$\mu\mathrm{g\ l}^{-1}$	5.20	0.465	0.309	1		
	$\dot{k}_\dagger$	$\mathrm{l\ \mu g}^{-1}\mathrm{d}^{-1}$	0.0376	0.00777	0.046	–0.024	1	
	$\dot{k}_a$	d^{-1}	0.791	0.281	–0.049	0.281	–0.811	1
	$\dot{\lambda}$	d^{-1}	0	-	-			
(3.20)	c_0	$\mu\mathrm{g\ l}^{-1}$	0	-	-	-		
	A	$\mu\mathrm{g\ l}^{-1}\mathrm{d}$	62.8	4.05	-	-	1	
	β		2.72	0.263	-	-	–0.052	1

10 $\mu\mathrm{g\ l}^{-1}$. For $c = 0$ the fit is worse. The deviances are 40.21 and 36.43, respectively. The difference is 3.78, which means that inclusion of control mortality does not lead to a significant improvement ($\alpha = 0.05$). The deviance of model (3.20) equals 43.57, which is more than the previous values. However, because the models (3.12) and (3.20) are not nested, we cannot draw strong conclusions from the deviances.

The second data set which we analyzed is given in Table 3.6. As mortality occurs at $c = 0$, we are forced to include control mortality in the model.

Fitting model (3.12) we found several (local) maximums of the likelihood. The deviance at the global maximum equals 35.55. The estimation procedure for model (3.14) ran into the same numerical problems as encountered with the first data set. Again c_0 became zero. The resulting deviance is 38.38. Results of parameter estimation are given in Table 3.7 and Figure 3.6. The estimates of control mortality are more or less the same in both models, with relatively large standard deviations. The other parameters in model (3.12) have small standard deviations. In model (3.14) only β has a small standard deviation. The extremely high correlation between c_{L50} and $\dot{k}_a$ and the small value of the latter indicate that the resulting model approximates model (3.20).

Discussion

In the present paper we introduce a new model for the analysis of survival data. There are several advantages to our approach. First, our approach provides an alternative measure of the toxicity of a compound with respect to survival, the killing rate $\dot{k}_\dagger$. The killing rate can be interpreted as the probability of dying, per unit of time and

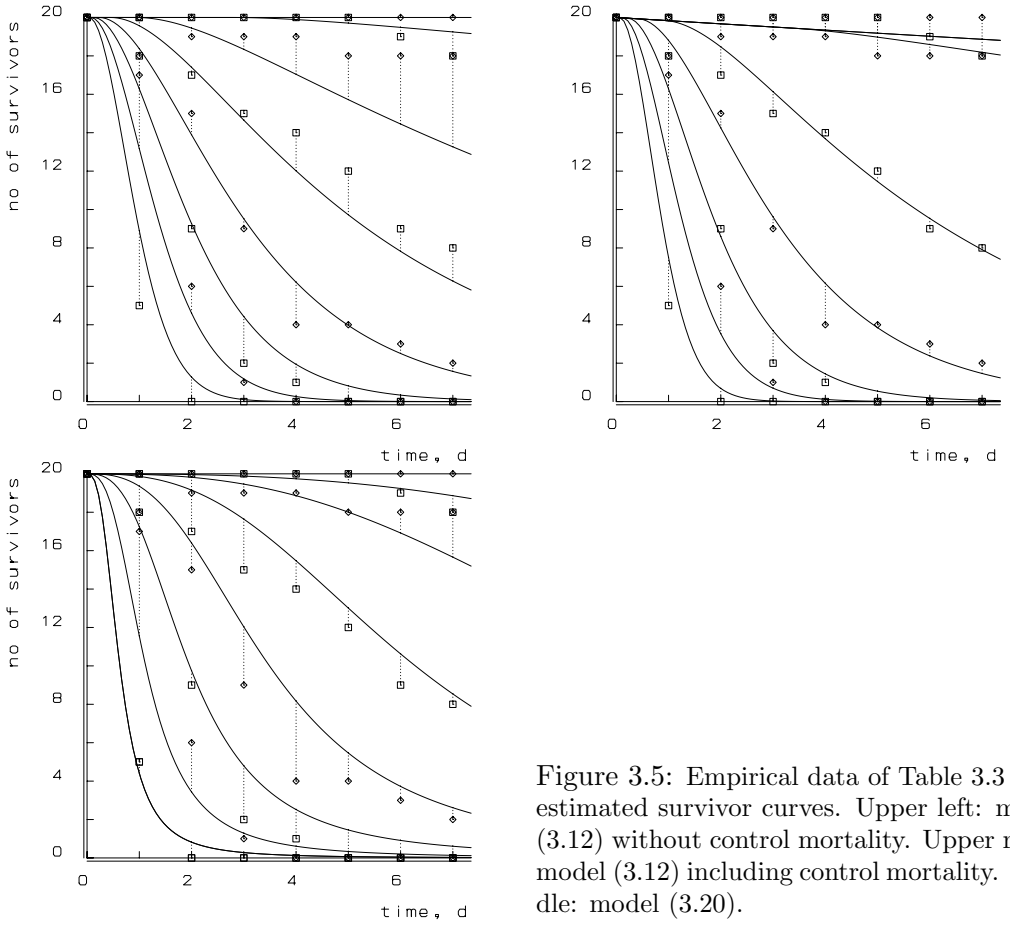


Figure 3.5: Empirical data of Table 3.3 with estimated survivor curves. Upper left: model (3.12) without control mortality. Upper right: model (3.12) including control mortality. Middle: model (3.20).

Table 3.6: Number of surviving daphnids *Daphnia magna* in potassium dichromate. Data were kindly provided by Ms T. Adema (IMW-TNO Laboratories, Delft).

	concentration $K_2Cr_2O_7$ (mg l^{-1})					
time (d)	0	0.1	0.18	0.32	0.56	1
0	50	50	50	50	50	50
2	50	50	50	50	50	48
5	50	50	50	50	48	36
7	50	50	50	50	48	35
9	49	50	50	50	48	31
12	49	50	50	50	40	15
14	49	50	50	48	32	9
16	49	50	50	47	30	3
19	49	50	50	47	23	0
21	49	50	50	45	16	0

Table 3.7: Results of ML estimation on empirical data are given in Table 3.6. For every parameter point estimates, standard deviations and correlation coefficients are given.

model	par.	units	estimate	s.d.	correlation coefficients			
	$\dot{\lambda}$	d^{-1}	$3.08\ 10^{-4}$	$2.89\ 10^{-4}$	1			
(3.12)	c_0	mg l^{-1}	0.272	0.0179	0.058	1		
	$\dot{k}_\dagger$	l $mg^{-1}d^{-1}$	0.278	0.0414	0.024	0.016	1	
	$\dot{k}_a$	d^{-1}	0.214	0.0397	0.029	0.657	–0.501	1
	$\dot{\lambda}$	d^{-1}	$3.94\ 10^{-4}$	$3.97\ 10^{-4}$	1			
(3.14)	c_{L50}	mg l^{-1}	0.161	0.106	0.136	1		
$c_0 = 0$	$\dot{k}_a$	d^{-1}	0.0186	0.0143	0.130	0.998	1	
	β		3.848	0.412	0.251	0.526	0.539	1

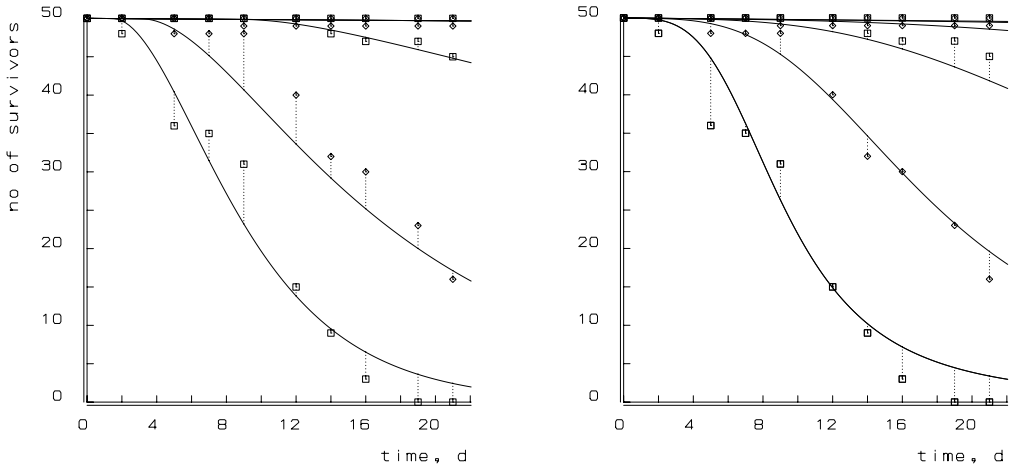


Figure 3.6: Empirical data of Table 3.6 with estimated survivor curves. Left: model (3.12) including control mortality. Right: model (3.14) including control mortality.

per unit of (environmental) concentration exceeding the no–effect concentration. It does not depend on exposure time, as does LC50. In addition, the model also provides NEC as well as LC50 as functions of time, in a single estimation procedure. The NEC-time relation can be found by calculating c from $[Q](t) > [Q]_0$ in (3.9) at a fixed time t, leading to $\text{NEC}(t) = c_0(1 - \exp(-\dot{k}_a t))^{-1}$. The LC50–time relation can be found by (numerically) solving c from $q(t; c) = 0.5$. Second, we model a survival function based on simple mechanistic assumptions which may, at least in theory, be tested in independent experiments. Most other parametric approaches assume some distribution function without any biological or mechanistic justification.

A key assumption in the model is that the hazard rate is proportional to the concentration of the toxicant. This implies that the lethal effects of a toxicant should disappear as soon as the concentration decreases below the no–effect level, that is, the animals should instantaneously recover completely. A different approach could be to assume that the toxicant causes irreparable damage to the animal, again proportional to the concentration. Diggle and Gratton (1984; Morgan, 1992) put forward a comparable idea in their extension to the model of Puri and Senturia (1972). If, in our model, the hazard is taken to be proportional to the total damage, this results in a hazard proportional to the accumulated concentration. In an analogous way Kooijman models aging processes (Kooijman, 1993, pp. 105–112). For small values of $\dot{k}_a$, the survivor function approximates a Weibull function with shape parameter 3 instead of 2 as in (3.13).

The model can, of course, be extended by changing the assumptions. For instance, the assumption about the accumulation process in the animal can be changed in a two-compartment model if a one-compartment model does not make sense. However, this will probably cause estimation problems unless the data set is very detailed. Another extension of our model could be the introduction of stochasticity between animals. As a matter of fact animals are supposed to be identical in the present model: they all have the same kinetic parameters. The only stochastic component is in the process of dying. In the log–logistic model (3.14) stochasticity is located entirely between animals. There the process of dying is completely deterministic. As a result, both approaches lack reality. To meet this objection we might consider the elimination parameter $\dot{k}_a$ as a random variable. Stochastic parameters in accumulation models are discussed in Bedaux and Kooijman (1994).

Acknowledgements

This work was supported by Grant PAD 90-18 from the Alternative to Animal Experiments Platform. We like to thank our colleagues C. Zonneveld and H.A. van den Berg and three anonymous referees for their comments on the manuscript, and Miss S.L. Yeung for her assistance in the computations.

References

Bedaux, J.J.M. and Kooijman, S.A.L.M. (1994). Stochasticity in deterministic models. In *Handbook of Statistics*, volume 12: *Environmental Statistics*, C.R. Rao, G.P. Patil, and N.P. Ross, eds., North Holland: 561-581.

Carter, E.M. and Hubert, J.J. (1984). A growth–curve model approach to multivariate quantal bioassay. *Biometrics* **40**: 699-706.

Cox, C. (1987). Threshold dose-response models in toxicology. *Biometrics* **43**: 511-523.

Diggle, P.J., and Gratton, R.J. (1984). Monte Carlo methods of inference for implicit statistical models. *Journal of the Royal Statistical Society*, B **46**: 193-227.

Hoekstra J.A. (1991). Estimation of the LC50, A Review. *Environmetrics* **2**: 139-152.

Jacquez, J.A. (1985). *Compartmental Analysis in Biology and Medicine*. Elsevier Publishing Company, Amsterdam.

Janssen, M.P.M., Bruins, A., De Vries, T.H. and Van Straalen, N.M. (1991). Comparison of cadmium kinetics in four soil arthropods species *Archives of Environmental Contamination and Toxicology* **20**: 305-312.

Kooijman, S.A.L.M. (1981). Parametric Analyses of mortality rates in bioassays. *Water Research* **15**: 107-119.

Kooijman, S.A.L.M. (1993). *Dynamic Energy Budgets in Biological Systems. Theory and Applications in Ecotoxicology*. Cambridge University Press.

McCullagh, P. and Nelder, J.A. (1989). *Generalized Linear Models*. Chapman and Hall, London.

Laurence, A.F. and Morgan, B.J.T. (1989). Observations on a stochastic model for quantal assay data. *Biometrics* **45**: 733-744.

Morgan, B.J.T. (1988). Extended models for quantal response data. *Statistica Neerlandica* **42**: 253-272.

Morgan, B.J.T. (1992). *Analysis of Quantal Response Data*. Chapman and Hall, London.

Puri, P.S. and Senturia, J. (1972). On a mathematical theory of quantal response data. *Proceedings of the 6th Berkeley Symposium on Mathematical Statistics* **4**: 231-247.

Van Ryzin, J. and Rai, K. (1987). A dose–response model incorporating nonlinear kinetics. *Biometrics* **43**: 95-105.

3.3 Analysis of toxicity tests on fish growth

Abstract We present a statistical analysis of bioassays for fish growth, such as the routine toxicity test that is described in the OECD guideline 210. The analysis is based on the Dynamic Energy Budget theory and a one-compartment kinetics for the toxic compound. It is fully process oriented. We compare a formulation in terms of direct effects on growth with indirect effects via assimilation and maintenance. All formulations characterize the effects by a no-effect concentration, a tolerance concentration and the elimination rate. Simplified formulations are obtained for very small and very large elimination rates. The accuracy of estimates for the no-effect concentration is assessed by the profile likelihood function. The method is illustrated with applications to several data-sets for body size versus concentration of toxicant.

Introduction

This paper is one in a series that aims to analyze the full set of routine aquatic toxicity experiments (Bedaux & Kooijman 1994; Kooijman & Bedaux 1996a, 1996b; Kooijman et al. 1996; Kooijman 1996). The main feature of these analyses is to provide a method to estimate the no-effect concentration (NEC) on the basis of mechanistic models for the effects of chemicals on the various endpoints (survival, growth, reproduction). It offers an alternative to the frequently used no-observed-effect concentration (NOEC). The use of the latter is under increased pressure due to the statistical problems with this characteristic (Kooijman 1981, 1995a; Pack 1993; Laskowski 1995). The second aim is to provide process-based characterizations of the various effects of toxic chemicals that are independent of exposure time.

Growth can be affected directly by toxic chemicals, or indirectly via effects on feeding or maintenance, because these processes are intimately linked. The Dynamic Energy Budget (DEB) theory provides a mechanistic basis for this link that has been tested against experimental data for many animal species (Kooijman 1993). Reproduction, as is routinely tested with *Daphnia*, can be indirectly affected via growth, feeding or maintenance (Kooijman & Bedaux 1996b). Satisfactory analyses of toxicity data for growth must be consistent with those of the indirect effects on reproduction. This is why the analyses of effects on growth and reproduction are linked.

In this paper, we present and apply a statistical analysis of routine toxicity tests on fish growth based on insights from the DEB theory. The choice for fish conforms to the OECD guideline 210 (OECD 1992), but the analysis applies equally to the growth of other animals because the DEB theory applies to all heterotrophs. The application of the DEB theory is simple only if the growth conditions are constant (food density, temperature etc.). We first summarize the relevant details of the test guideline, then we work out a new analysis of the toxicity test on growth and compare

[0]Published as: S.A.L.M. Kooijman and J.J.M. Bedaux (1996) Analysis of toxicity tests on fish growth. *Water Research* **30**: (to appear)

Table 3.8: List of symbols. The symbols in the dimension-column stand for t time, m mass, l length (of environment), L length (of organism).

symbol	dimension	interpretation
c	ml^{-3}	concentration in environment
c_q	ml^{-3}	ratio of conc. in tissue and P_{vd}
c_0	ml^{-3}	no-effect conc. for growth
c_*	ml^{-3}	tolerance conc. for $* \equiv M$, G or A
$[Q]$	mL^{-3}	concentration in tissue
$\mathcal{C}_q$	$ml^{-3}t$	product of conc. in tissue and uptake rate
P_{vd}	l^3L^{-3}	bioconcentration coefficient
L	L	body length
W	m	body weight
t	t	exposure time
$\dot{k}_a$	t^{-1}	elimination rate
$\dot{\gamma}$	t^{-1}	von Bertalanffy growth rate
$\dot{m}$	t^{-1}	maintenance rate coefficient
g	-	energy investment ratio
s, S	-	stress function

it with the standard analysis. Since we link effects to concentrations in the fish, we present our analysis in a brief discussion of growth, uptake kinetics, and effects.

Routine toxicity tests on growth

The routine toxicity test on fish growth according to Guideline 210 of the OECD (1992) requires that small (young) fish are exposed to a range of concentrations of test compound during a period of 28 d. The zebrafish *Brachydanio rerio* and the rainbow trout *Oncorhynchus mykiss* (formerly *Salmo gairdneri*) are frequently selected for this type of experiment. The zebrafish is a popular small fish for research purposes (Laale 1977), the rainbow trout is an example of a large fast growing fish (Weatherley & Gill 1984) which is of commercial interest. Although the test protocol does not mention the feeding conditions prior to the start of the test, enhanced growth can be expected after starvation (Quinton & Blake 1990) that is hard to analyse. We therefore assume that the fish were well-fed prior to the experiment. Rainbow trout can be triploid, but this does not seem to affect the energetics (Oliva-Teles & Kaushik 1990).

Five concentrations are suggested. Although the guidelines recommend a housing of sixteen fish per tank, social interactions in the feeding can easily increase the variation in growth rates in a way that is hard to analyse. Social interactions in feeding are well described, both for the zebrafish (Craig & Fletcher 1984; Steele et al. 1991; Lucas & Priede 1992) and the trout (Brown 1946; Phillips 1989), and affect uptake kinetics and effects of the toxicant (Arthur & Dixon 1994). We will assume that housing is such that social effects can be excluded.

The length or the weight is measured at the start of the experiment and at 14 and 28 days. (It will help the analysis if size observations are made during the growth process as well.) The temperature and concentration of compound in the media are as constant as possible.

Typical maximum sizes for the zebrafish are 45 mm fork-length (Laale 1977), 760-990 mg wet weight and 215-330 mg dry weight. The males are typically more slender than the females. The sexes start to deviate after one month of age.

A variant of the growth test starts from eggs to include effects on survival. The most sensitive period is usually at the initiation of the feeding process. The incubation period of zebrafish typically lasts 96 h from fertilization at 26°C. We will assume that differences in hatching times are small.

Growth according to the DEB theory

Although a comprehensive discussion of the DEB theory is outside the scope of this paper, the discussion of some of its basic assumptions will help to clarify the analysis of effects of compounds.

The feeding rate depends on food density and is proportional to the surface area of the organism. This holds for the mean feeding rate over a longer time period (Staple & Nomura 1976). (The amount of food eaten after a period of starvation is proportional to body weight (Grove et al. 1978), because stomach volume is proportional to body weight.) Although small changes in the relative sizes of various organs in immature rainbow trout have been observed (Denton & Yousef 1976), they are remarkably conservative (Weatherley & Gill 1983). The shape of the organism during growth is taken to be constant in this paper, although the theory for changing shapes has been worked out. In this case, surface area is proportional to (structural) biovolume to the power 2/3. The digestion efficiency is taken to be independent of the size of the organism and the food density (Staples & Nomura 1976).

Material derived from food is added to the reserves, which are rich in fat (Denton & Yousef 1976; Atherton 1975). The reserve density (i.e. the reserves per structural biovolume) is utilized at a rate proportional to reserve density and inversely proportional to a length measure. (The latter is because of homeostasis for the reserves during juvenile growth.) A fixed fraction of energy that is utilized from the reserves is spent on growth plus maintenance, the rest is spent on development plus reproduction. The maintenance costs are proportional to the structural biovolume. The costs

for growth are proportional to the increase in structural biovolume. Endotherms, such as mammals and birds, also spend energy on thermoregulation. Since the primary interest in this paper is in fish, heating costs are excluded here. The detailed motivation and derivation of the various assumptions are given in Kooijman (1993).

Body weight combines contributions from structural biovolume and reserves. If food is abundant, however, body weight is just proportional to biovolume due to the assumption of homeostasis. A length measure (such as the snout-fork length) is proportional to the cubic root of the biovolume, and so of body weight, due to the assumption that the shape does not change. If food density is not constant, the relationship between length and weight measures is more complex.

At abundant food, these assumptions specify that growth is given by von Bertalanffy growth equation

$$\frac{d}{dt}V = 3\dot{\gamma}(V^{2/3}V_m^{1/3} - V) \tag{3.21}$$

where V is structural biovolume, V_m is the maximum structural biovolume and $\dot{\gamma}$ is the von Bertalanffy growth rate (dimension per time). The maximum volumetric length $V_m^{1/3}$ is proportional to the ratio of the surface area-specific assimilation rate and the volume-specific maintenance costs. The (maximum) von Bertalanffy growth rate is given by

$$\dot{\gamma} = \frac{1}{3}\frac{\dot{m}g}{1+g} \tag{3.22}$$

where the maintenance rate coefficient $\dot{m}$ stands for the ratio of the volume-specific maintenance and growth costs. The investment ratio g stands for the ratio of the volume-specific growth costs and the fraction of the maximum reserve density that is spent on growth plus maintenance. Note that $\dot{\gamma}V_m^{1/3}$ is independent of the maintenance costs. Formulation of growth under food limitations shows that the von Bertalanffy growth rate correlates negatively with the ultimate size (Kooijman 1993), as has frequently been observed empirically (Galliucci & Quinn 1979; Xiao 1994).

Since weight is proportional to structural volume at abundant food (Kooijman 1993), (3.21) also applies if we substitute weight for V and maximum weight for V_m. Alternatively we can substitute cubed length for V and cubed maximum length for V_m, so

$$\frac{d}{dt}W = 3\dot{\gamma}(W^{2/3}W_m^{1/3} - W) \tag{3.23}$$

$$\frac{d}{dt}L = \dot{\gamma}(L_m - L) \tag{3.24}$$

The von Bertalanffy growth equation has been fitted frequently to fish data and usually fits quite well (Chen et al. 1992; Hearn & Leigh 1994). Figure 3.7 gives a test for model (3.24) against experimental data for zebrafish and rainbow trout. To simplify the discussion that follows, we only use length measures as in (3.24), but cubic roots of weights can always be substituted for lengths. Length measurements

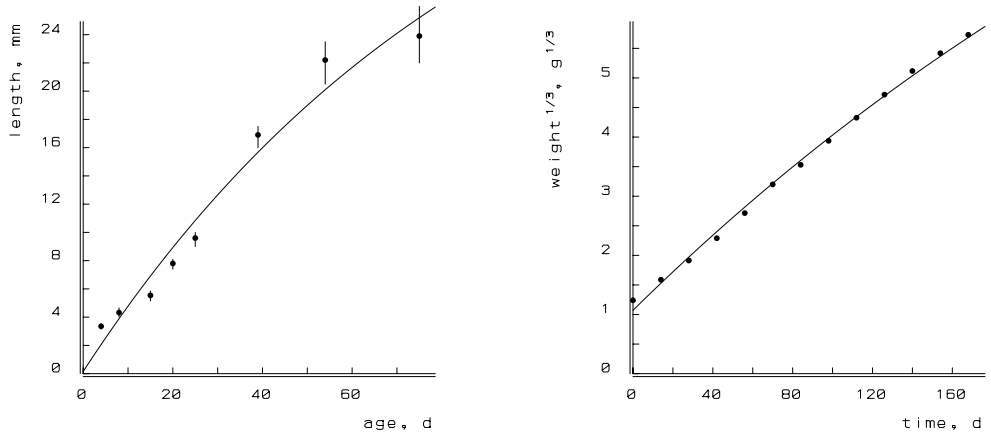


Figure 3.7: Length-at-age for the zebrafish *Brachydanio rerio* at 25.5°C (left) and weight-at-age for the rainbow trout *Oncorhynchus mykiss* at 12°C (right). Data from Eaton & Farley (1974) and Weatherley & Gill (1983). The fitted curves are the von Bertalanffy growth curve (3.24). The parameter estimates (with standard deviation) for the zebrafish are the initial length 0.15 (1.33) mm, ultimate length 45.1 (17.6) mm and von Bertalanffy growth rate 0.0109 (0.0063) d^{-1}. For the rainbow trout with initial weight 1.23 g, the estimated von Bertalanffy growth rate was $\dot{\gamma} = 2.36\ 10^{-3}$ $(3.55\ 10^{-5})$ $d^{-1} = 0.86(0.013)$ a^{-1}. The ultimate weight has been set at 3.5 kg (Ruting 1958). The length-at-age is almost proportional to time and the weight-at-age is almost proportional to cubed time as long as the actual size is much smaller than the ultimate size.

have the advantage over weight measurements that the fish need less handling, which allows repeated length measurements during growth of particular individuals. On the other hand, weight measurements are usually more accurate.

A practical problem in the application of these ideas in the analysis of routine toxicity data is that only very limited data on size change are available. To reduce the number of parameters that are to be estimated, we treat the investment ratio g and the von Bertalanffy growth rate $\dot{\gamma}$ as known parameters, while the maximum size, i.e. L_m or W_m is to be estimated from the concentration-response relationship. This leaves one free parameter for the response in the control. Translated into the elementary components of the energy budget, we assume that maximum surface area-specific assimilation rate can differ from one experiment to another, but the specific costs for maintenance and growth are fixed. The assimilation rate depends, among other things, on food quality. At first glance, it might seem an odd choice to set the von Bertalanffy growth rate in the analysis of a growth experiment. At second glance, however, we must realize that the von Bertalanffy growth rate is not a growth rate in the strict sense of the word. It has dimension per time, not length or weight per time. If the actual length is small with respect to the maximum length, the growth rate in length per time is about equal to the product $\dot{\gamma} L_m$.

Uptake/elimination kinetics

Exposure is assumed to start from previously unexposed individuals at a constant environment-concentration c of toxic compound. We choose $t = 0$ as the start of the exposure period.

Suppose that absorption of the compound to the food particles is instantaneous and that the concentration of food particles is constant. Uptake can occur directly from the water and indirectly via food (Karlsson-Norrgren & Runn 1985), but both uptake rates are proportional to the surface area of the animal, which is proportional with $V^{2/3}$ of L^2 for isomorphs. Most fish grow roughly isomorphically, from an energetics perspective. The direct elimination is again assumed to be proportional to the surface area and to the concentration in the (aquatic fraction of the) tissue $[Q]$. The partitioning of the compound over the different body fractions (including the lipid fraction) is again assumed to be instantaneous. The uptake/elimination kinetics reduces to

$$\frac{d}{dt}[Q] = cP_{vd}\dot{k}_a L_m/L - [Q]\left(\dot{k}_a L_m/L + \frac{d}{dt}\ln(L/L_m)^3\right) \tag{3.25}$$

where c is the concentration in the environment (dissolved plus absorbed to food particles), P_{vd} is the bioconcentration coefficient and $\dot{k}_a$ the elimination rate. The term $\frac{d}{dt}\ln(L/L_m)^3 = 3\frac{L_m}{L}\frac{d}{dt}\frac{L}{L_m}$ in (3.25) accounts for the dilution by growth. This correction has been found to be essential empirically as well as numerically (Borgmann & Whittle 1992; Hammar et al. 1993).

The tissue-concentration is usually not measured in routine toxicity tests, so that it plays the role of a hidden variable. It proves to be convenient to introduce the scaling $c_q \equiv [Q]/P_{vd}$, which has the dimensions of an environment-concentration, but is just proportional to the tissue-concentration. The kinetics of the scaled tissue-concentration reduces to

$$\begin{aligned}\frac{d}{dt}c_q &= c\dot{k}_a L_m/L - c_q\left(\dot{k}_a L_m/L + \frac{d}{dt}\ln(L/L_m)^3\right) &(3.26)\\ &= \dot{k}_a\left(c - c_q - \frac{3c_q}{\dot{k}_a L_m}\frac{d}{dt}L\right)\frac{L_m}{L} &(3.27)\end{aligned}$$

Although this simple first order differential equation with variable coefficients can be solved, this hardly helps because the solution still has integrals that must be obtained numerically. The behaviour only depends on the scaled elimination rate $\dot{k}_a$ relative to the von Bertalanffy growth rate $\dot{\gamma}$.

Without effects on growth, the concentration c_q exceeds level c_0 at t for

$$c_0(t) = \frac{c_0}{\dot{k}_a}\frac{\exp\{-3\dot{\gamma}t + (3\dot{\gamma}+\dot{k}_a)\int_0^t \Gamma(t_1)\,dt_1\}}{\int_0^t \exp\{-3\dot{\gamma}t_1 + (3\dot{\gamma}+\dot{k}_a)\dot{k}_a\int_0^{t_1}\Gamma(t_2)\,dt_2\}\Gamma(t_1)\,dt_1} \overset{t\to\infty}{\to} c_0 \tag{3.28}$$

where $\Gamma(t) \equiv L_m/L(t)$. The practical significance of this result is that the no-effect concentration c_0 that will appear in the description of effects has the interpretation

of the ultimate no-effect concentration, while the apparent no-effect concentration $c_0(t)$ for exposure time t is higher, so $c_0(t) > c_0$, because the tissue-concentration builds up gradually.

Effects

We distinguish three types of effects on growth: direct effects and indirect effects via maintenance and assimilation. However, we assume that only one of these effects occurs at the same time, in the lower effect range of the compound. This assumption relates to the concept of the most sensitive physiological process that is affected.

Direct effects on growth

Direct effects on growth will be described by a change in the parameter for the costs of growth, which occurs in the numerator of energy investment ratio g and the denominator of the maintenance rate coefficient $\dot{m}$. We assume that the energy investment ratio at concentration c_g relates to that in the control g_0 as $g_c = g_0(1 + s(c_q))$ with stress function $s(c_q) = c_G^{-1}(c_q - c_0)_+$, where c_G is the tolerance concentration for growth and c_0 the no-effect concentration. The index $+$ is defined as $(x)_+ \equiv \max\{0, x\}$. So we have $s(c_q) = 0$ and $g_c = g_0$ for $c_q \leq c_0$. Similarly we have that $\dot{m}_c = \dot{m}_0(1 + s(c_q))^{-1}$. The product $\dot{m}g$ is thus unaffected by compounds with a direct effect on growth. The effect size is thus proportional to the tissue-concentration that exceeds the internal no-effect concentration. Each molecule that exceeds the handling capacity acts independently. Interactions between the molecules are likely to occur at higher concentrations. At high concentrations, not only growth will be affected, but probably several other physiological processes as well. We refrain from modelling such simultaneous effects because this is not practical in view of the simplicity of the experimental data. Due to these practical constraints, we simply accept the possibility that this description is not accurate at high concentrations.

Substitution of the effect on growth into the growth rate (3.24) leads to

$$\frac{d}{dt}L = \dot{\gamma}(L_m - L)\frac{1 + g}{1 + g(1 + s(c_q))} \tag{3.29}$$

Indirect effects on growth

Effects on maintenance and assimilation indirectly affect growth by the principle of conservation of energy: maintenance competes with growth investment for the allocation of energy that is utilized from the reserves and a decrease of assimilation translates into a decrease of the amount of energy that is utilized from the reserves.

Maintenance

Many toxic compounds are likely to affect maintenance requirements, which translates into an increase in the maintenance costs. Because maintenance has priority

over growth in the DEB theory, such an increase leads to a reduction of the growth rate. Since the feeding rate depends on body size, the feeding rate is affected as well. In analogy with the direct effect on growth, we now assume that the maintenance rate coefficient is $\dot{m}_c = \dot{m}_0(1 + s(c_q))$ with stress function $s(c_q) = c_M^{-1}(c_q - c_0)_+$, where c_M is the tolerance concentration for maintenance and c_0 the no-effect concentration; $\dot{m}_0$ stands for the maintenance rate coefficient in the control. Substitution of the effect on maintenance into the growth rate (3.24) leads to

$$\frac{d}{dt}L = \dot{\gamma}(L_m - L(1 + s(c_q))) \tag{3.30}$$

where $\dot{\gamma}$ is the von Bertalanffy growth rate in the control and L_m is the maximum length in the control.

Assimilation

If assimilation is affected, i.e. the incoming energy is reduced, growth is affected as well. The maximum assimilation rate does not occur in the von Bertalanffy growth rate, only in the maximum length. In analogy with the direct effect on growth, we now assume that the maximum length is $L_{m,c} = L_m(1 - s(c_q))$ with stress function $s(c_q) = c_A^{-1}(c_q - c_0)_+$, where c_A is the tolerance concentration for assimilation and c_0 the no-effect concentration. Note that the stress function appears with a negative rather than a positive sign, to model adverse effects on assimilation. The consequence is that $c_g < c_A + c_0$ must hold to avoid death, so also $c < c_A + c_0$, for all chosen test concentrations c. The constraint on the value for c_A is in fact somewhat stronger than this, because the assimilation rate must exceed the maintenance requirements. The DEB theory states that the individual dies by starvation if it is unable to mobilize enough energy from its reserves for maintenance purposes.

Substitution of the effect on assimilation into the growth rate (3.24) leads to

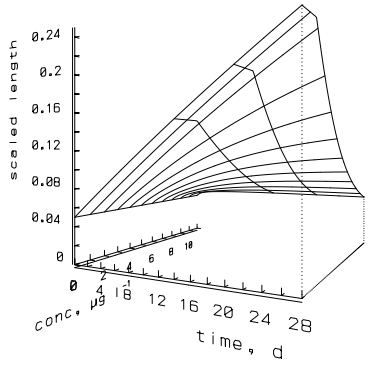
$$\frac{d}{dt}L = \dot{\gamma}(L_m(1 - s(c_q)) - L) \tag{3.31}$$

The model for direct effects on growth and the two indirect ones are illustrated as response surfaces above the exposure time-concentration plane in Figure 3.8. For $L \ll L_m$, length is increasing almost linearly in time at rate $\dot{\gamma}L_m$. This growth rate is decreasing hyperbolically as a function of the concentration for a direct effect on growth and linearly for an effect on assimilation. Increase in length is nonlinear for effects via maintenance, where both the ultimate size and the von Bertalanffy growth rate are affected.

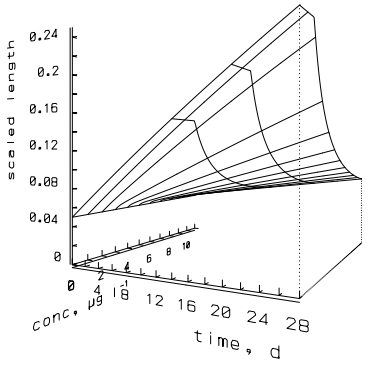
Reduced models

Many organic compounds have an elimination rate that is high with respect to the von Bertalanffy growth rate (Hawker & Connell 1986), so $\dot{k}_a \gg \dot{\gamma}$, (3.27) reduces to

maint., $c_M = 0.6$ μg l^{-1}



growth, $c_G = 0.8$ μg l^{-1}



assim., $c_A = 10$ μg l^{-1}

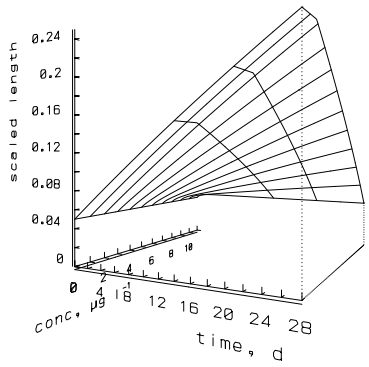


Figure 3.8: Direct and indirect effects on growth. The von Bertalanffy growth rate is chosen $\dot{\gamma} = 0.008$ d^{-1}, which is typical for zebrafish at 26°C. The elimination rate is $\dot{k}_a = 0.1$ d^{-1} and the no-effect concentration is $c_0 = 1.5$ μg l^{-1}. The tolerance concentrations for maintenance, growth and assimilation are chosen 0.6, 0.8 and 10 μg l^{-1}, respectively, to produce similar response levels.

$c_q = c$ and (3.24) leads to the explicit growth curve

$$L(t) = L_{m,c} - (L_{m,c} - L_0)\exp\{-\dot{\gamma}_c t\} \tag{3.32}$$

with L_0 standing for the initial length, $L_{m,c}$ for the ultimate length at concentration c and $\dot{\gamma}_c$ the von Bertalanffy growth rate at concentration c. The latter two parameters are given by

model	no	$L_{m,c}$	$\dot{\gamma}_c$
growth	(3.29)	$L_{m,0}$	$\dot{\gamma}_0 \frac{1+g}{1+g(1+s(c))}$
maintenance	(3.30)	$L_{m,0}(1+s(c))^{-1}$	$\dot{\gamma}_0(1+s(c))$
assimilation	(3.31)	$L_{m,0}(1-s(c))$	$\dot{\gamma}_0$

(3.33)

where the stress function is $s(c) = c_*^{-1}(c - c_0)_+$ for $* \in \{G, M, A\}$ as before. The three modes of action of the compound lead to effects on either the ultimate length, the von Bertalanffy growth rate, or both.

If the elimination rate is small with respect to the von Bertalanffy growth rate, so $\dot{k}_a \ll \dot{\gamma}$, we have to reconsider the scaling from the tissue-concentration $[Q]$ to the environment-concentration c_q via the bioconcentration coefficient P_{vd}, because the latter is the ratio between the uptake rate and the elimination rate $\dot{k}_a$. The limit of interest is $\dot{k}_a \to 0$ and $P_{vd} \to \infty$ such that $\dot{k}_a P_{vd}$ is constant. The stress function is in fact a function of the tissue-concentration, so we define a new stress function $S(\mathcal{C}_q) = \mathcal{C}_*^{-1}(\mathcal{C}_q - \mathcal{C}_0)_+$ for $* \in \{G, M, A\}$, where $\mathcal{C}_q \equiv c_q/\dot{k}_a$, $\mathcal{C}_0 \equiv c_0/\dot{k}_a$ and $\mathcal{C}_* \equiv c_*/\dot{k}_a$. The latter equality, for instance, should be read as the limit for $c_* \to 0$ and $\dot{k}_a \to 0$ such that $c_*/\dot{k}_a$ is constant at value $\mathcal{C}_*$. The dimension of the $\mathcal{C}$'s is concentration times time. We will refer to $\mathcal{C}_0$ as the no-effect concentration-time. Notice that $S(\mathcal{C}_q) = s(c_q)$. The equations (3.27) and (3.29), (3.30), (3.31) now become

$$\frac{d}{dt}\mathcal{C}_q = cL_m/L - \mathcal{C}_q \frac{d}{dt}\ln(L/L_m)^3 \tag{3.34}$$

$$\text{growth } \frac{d}{dt}L = \dot{\gamma}(L_m - L)\frac{1+g}{1+g(1+S(\mathcal{C}_q))} \quad \text{for} \quad S(\mathcal{C}_q) = \mathcal{C}_G^{-1}(\mathcal{C}_q - \mathcal{C}_0)_+ \tag{3.35}$$

$$\text{maint. } \frac{d}{dt}L = \dot{\gamma}(L_m - L(1+S(\mathcal{C}_q))) \quad \text{for} \quad S(\mathcal{C}_q) = \mathcal{C}_M^{-1}(\mathcal{C}_q - \mathcal{C}_0)_+ \tag{3.36}$$

$$\text{assim. } \frac{d}{dt}L = \dot{\gamma}(L_m(1 - S(\mathcal{C}_q)) - L) \quad \text{for} \quad S(\mathcal{C}_q) = \mathcal{C}_A^{-1}(\mathcal{C}_q - \mathcal{C}_0)_+ \tag{3.37}$$

In practice, it may be difficult to obtain the three toxicant parameters $\dot{k}_a$, c_0 and c_* for $* \in \{G, M, A\}$ from a single length-concentration curve. However, we can sandwich this full model between two marginal models for very small and very large values for the elimination rate $\dot{k}_a$. These marginal models have two toxicant parameters only. Moreover, we may use other information to obtain an estimate for the elimination

rate, such as a known elimination rate of a related compound, corrected for differences in the octanol-water partition coefficient, the size of the animal that has been used and the temperature.

Statistics

Given observation times $\{t_1, t_2, \cdots, t_r\}$ and test concentrations $\{c_1, c_2, \cdots, c_k\}$, the mean lengths of individuals in a cohort, L_i, are assumed to follow a normal distribution with a mean value that is described by the model for growth. It can be shown that simple stochastic models for the fine structure of the feeding process end up in a variance of a length measure that is proportional to the squared mean (Kooijman 1993, page 121). The variance of the mean length is inversely proportional to the number of individuals in that cohort. This might be important if mortality occurs. The easiest way to obtain parameter estimates is by non-linear regression where the weight coefficients are chosen inversely proportional to the product of squared mean observed values and the number of values that is used to calculate the mean. If the model fits well, this method will produce results similar to the maximum likelihood method (Carroll et al. 1995) but is much easier to implement.

The parameters that have to be estimated are L_m, c_0, c_* and $\dot{k}_a$, where the initial lengths, the investment ratio g and the von Bertalanffy growth rate are treated as given. We assume that the length measurements are individual-specific, i.e. the initial length and the length during growth (or at least at the end of the experiment) is measured for each individual. In that case, it is no problem that the initial lengths of the individuals differ. If the initial lengths do not differ too much, the mean value might be estimated as a parameter or again treated as a known value.

In the next section we will use profile ln likelihood functions (see e.g. McCullagh & Nelder 1989 or Carroll et al. 1995) for the no-effect concentration to obtain information about its confidence interval. These functions are defined as the difference between maximum ln likelihood and the ln likelihood given the value for a particular parameter, as a function of this parameter. We here take this difference as positive for graphical purposes. The profile ln likehood function $p(c_0)$ for the no-effect concentration c_0 is in this case of a normally distributed 'error' given by

$$p(c_0) = n \ln \sigma(c_0)/\sigma_{\min} \tag{3.38}$$

where n is the number of observations of data points; $\sigma(c_0)$ is the mean residual deviation $\sqrt{n^{-1} \sum_i (L_i - \mu_i(c_0))^2}$, where $\mu_i(c_0)$ is the model expectation for length L_i given the value c_0; $\sigma_{\min}$ is the minimum of $\sigma(c_0)$, for all $c_0 \geq 0$ (thus when c_0 equals the maximum likelihood estimate). We assume that the mean residual from a weighted regression is sufficiently close to the maximum likelihood value that it can be treated as such. The model expectation $\mu_i(c_0)$ is obtained by minimizing the sum of (weighted) squared deviations for all parameters, except c_0, which is kept fixed at the chosen value. A practical problem arises when the chosen value for c_0 is so

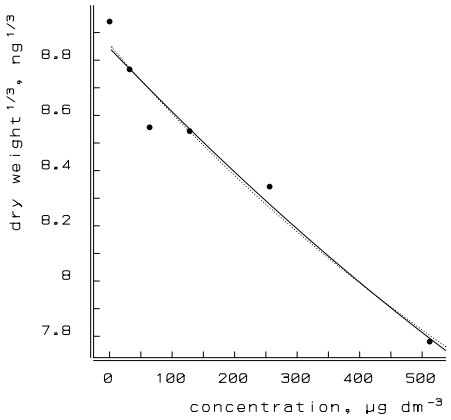


Figure 3.9: Effects of seven days of exposure of the fathead minnow *Pimephales promelas* to sodium pentachlorophenol on growth. Data from Weber et al. (1989) as given by Bruce & Versteeg (1992). The curves represent model expectations for direct effects on growth while the elimination rate is zero (drawn curve) or infinitely large (dotted curve). The cubic root of the initial dry weight has been set at 4 $\mu g^{1/3}$, the von Bertalanffy growth rate at 0.008 d^{-1}. The estimated parameter values are given in Table 3.9.

far from the maximum likelihood value that the corresponding model expectations deviate strongly from the observed values. In that case, the minimum of the sum of squared deviations will be hard to identify as a function of the free parameters. We can avoid this problem by starting from the maximum likelihood estimate for c_0 and then gradually increase or decrease the value for c_0 till the profile ln likelihood is too low to be of further interest.

If the large sample theory of the likelihood ratio statistic would apply, the 95 % confidence set for c_0 is approximated by the set of values for which the profile ln likelihood is less than 1.92 (see e.g. Carroll et al. 1995). Deviations from the large sample theory can be translated into deviations from this threshold-value. The examples will illustrate, however, that the profile ln likelihood functions are so steep that such deviations hardly affect the confidence set for c_0.

In an egg-larval test, where eggs rather than young fish are exposed, part of the variation in incubation period translates into variations in growth at a certain age. Growth during the embryonal period is at the expense of reserves and still continues a short period after hatching. Late hatching frequently correlates with big size at hatching. For the present purpose, the onset of feeding is more important than the moment of hatching. In view of the simplicity of the data, we do not take these complexities into account and assume that such variations are minor.

Tests against experimental data

Figure 3.9 illustrates the application of the model for effect on growth in a growth test with the fathead minnow *Pimephales*. This is an example where the models for effects on growth, maintenance and assimilation prove to be very similar, see Table 3.9. The maximum likelihood estimate for the NEC is zero in all cases, so that there is no need to test the hypothesis that differs significantly from zero.

The second example concerns a growth test with zebrafish that have been exposed

Table 3.9: Parameter estimates (and standard deviations) and mean residual deviations for the models for the effects on growth, maintenance and assimilation, applied to the minnow data given in Figure 3.9.

	growth		maintenance		assimilation	
parameter	$\dot{k}_a = 0$	$\dot{k}_a = \infty$	$\dot{k}_a = 0$	$\dot{k}_a = \infty$	$\dot{k}_a = 0$	$\dot{k}_a = \infty$
$\mathcal{C}_0$,c_0 (d mg/l,mg/l)	0(1.12)	0(0.035)	0(1.56)	0(0.041)	0(1.46)	0(0.044)
$\mathcal{C}_*$,c_* (d mg/l,mg/l)	29.9(2.9)	0.900(0.092)	5.94(0.53)	0.159(0.015)	85.4(8.06)	2.46(0.24)
$W_\infty^{1/3}$ (μg$^{1/3}$)	92.9(1.35)	93.1(1.34)	92.5(1.42)	92.8(1.36)	92.3(1.45)	92.6(1.39)
σ (μg$^{1/3}$)	0.0737	0.0732	0.0772	0.0742	0.0793	0.0755

to benzo(k)-fluoranthene for 37 days as larvae. The data points represent means of up to ten fish. (The last data point involved a single fish only). The model for effects on assimilation gave the best fit (see Figure 3.10), but the differences in goodness of fit are small. The limit for small elimination rates fit best for the growth and the assimilation model. The profile ln likelihood for the growth model changes sharply when the estimate for the elimination rate becomes infinitely large. This shows that these data hardly contain any information about the elimination rate. The parameter estimates are given in Table 3.10. The profile likelihood functions can be used to test the null-hypothesis $c_0 = 0$. The profile ln likelihood function at $c_0 = 0$ for the maintenance model is 0.4, which corresponds with a tail probability for the likelihood ratio statistic under the null hypothesis of 0.37. The profile ln likelihood function at $\mathcal{C}_0 = 0$ for the assimilation model is 0.6, which corresponds with a tail probability of 0.27. The no-effect concentration-time $\mathcal{C}_0$ differs significantly from 0 for the growth model. The applicability of the large sample theory to these examples has not been tested and can be questioned in view of the small number of data points. Notice that the curves for the best fitting model parameters show a no-effect-concentration for 37 days of exposure, while c_0 relates to the NEC for infinitely long exposures.

The third example gives the results for the effects of phenanthrene on the growth of zebrafish (see Figure 3.11). The parameter estimates are given in Table 3.11. The high control value is not included in the estimation. The point estimate for the no-effect concentration is zero. The model for the effect on assimilation again fits best, but the differences in goodness of fit are small.

The last example gives the results for the effects of dilutions of a mixture of polycyclic hydrocarbons on the growth of zebrafish (see Figure 3.12). A litre of the undiluted mixture contained 3.2 μg phenanthrene, 10 μg fluoranthene, 0.18 μg benzo(k)fluoranthene, 1.8 μg chrysene, 10 μg benzo(a)pyrene and 0.32 μg benzo(ghi)-perylene. The parameter estimates are given in Table 3.12. The model for the direct

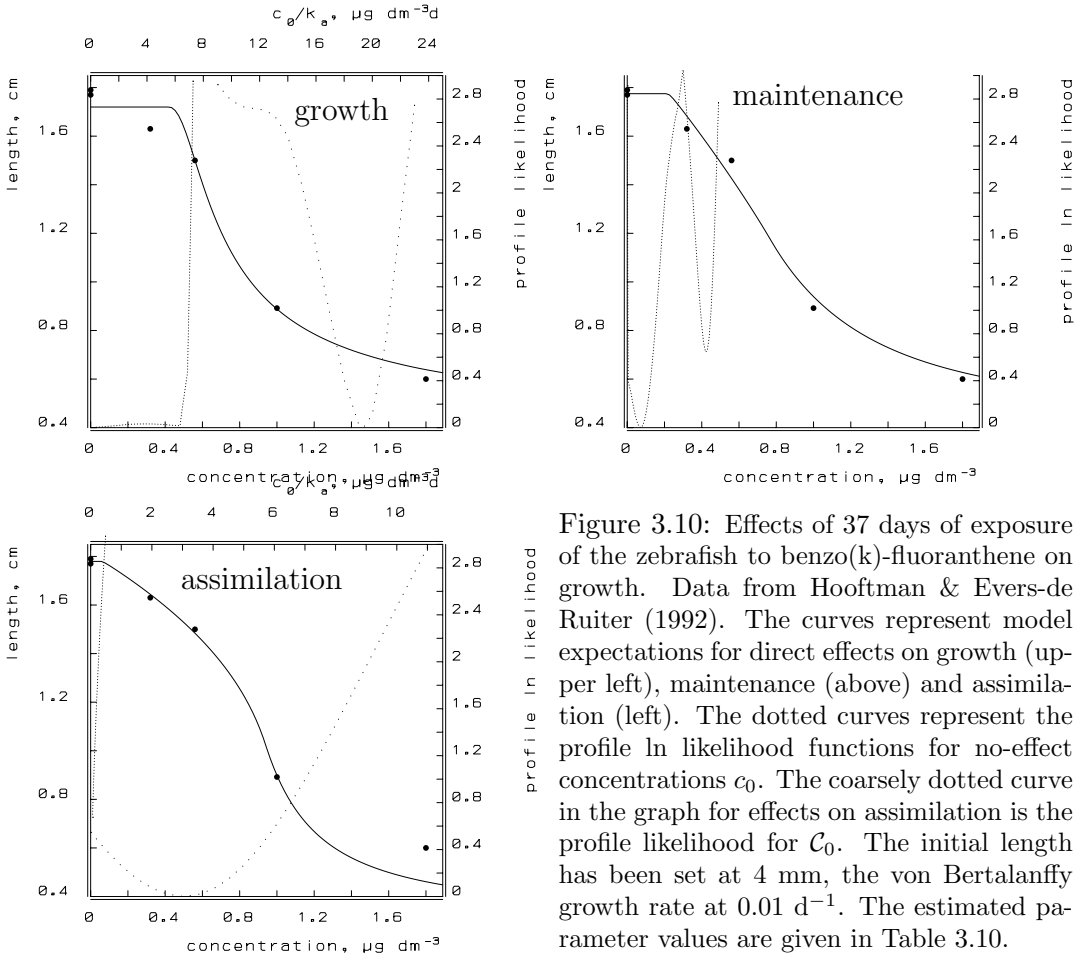


Figure 3.10: Effects of 37 days of exposure of the zebrafish to benzo(k)-fluoranthene on growth. Data from Hooftman & Evers-de Ruiter (1992). The curves represent model expectations for direct effects on growth (upper left), maintenance (above) and assimilation (left). The dotted curves represent the profile ln likelihood functions for no-effect concentrations c_0. The coarsely dotted curve in the graph for effects on assimilation is the profile likelihood for $\mathcal{C}_0$. The initial length has been set at 4 mm, the von Bertalanffy growth rate at 0.01 d^{-1}. The estimated parameter values are given in Table 3.10.

effect on growth fits best, but the differences in goodness of fit are small again. The profile ln likelihood functions for $c_0 = 0$ for the growth, maintenance and assimilation model are 0.498, 1.042 and 0.080 respectively, so that the corresponding upper tail probabilities are 0.32, 0.15 and 0.69; little reason to reject the null-hypothesis that $c_0 = 0$.

Discussion and conclusions

The examples indicate that the set of models fit the data well. The number of data points in the standardized bioassays on fish growth is small, while from a scientific and application point of view, three parameters are minimally required to describe the data: the response in the control, a no-effect concentration and a toxicity parameter. Therefore, present models cannot be simplified meaningfully in terms of numbers of parameters. This implies that standard deviations that are calculated on the basis of large sample theory are not reliable (McCullagh & Nelder 1989, p 255); at best

Table 3.10: Parameter estimates (and standard deviations) and mean residual deviations for the models for the effects on growth, maintenance and assimilation, applied to the zebrafish data given in figure 3.10.

parameter	growth	maintenance	assimilation
$\mathcal{C}_0$,c_0,$\mathcal{C}_0$ (d μg/l,μg/l,d μg/l)	19(1.33)	0.07(0.17)	2.49(2.49)
$\mathcal{C}_G$,c_M,$\mathcal{C}_A$ (d μg/l,μg/l,d μg/l)	8.33(1.63)	0.121(0.264)	116.6(5.96)
$\dot{k}_a$ (d^{-1})		0.0105(0.0357)	
L_∞ (cm)	4.67(0.098)	4.85(0.118)	4.86(0.071)
σ (cm)	0.0547	0.0509	0.0307

Table 3.11: Parameter estimates (and standard deviations) and the mean residual deviation for the models for the effects on growth, maintenance and assimilation, applied to the zebrafish data given in figure 3.11.

parameter	growth	maintenance	assimilation
c_0,(mg/l)	0(0.0038)	0(0.014)	0.00(0.009)
c_G,c_M,c_A (mg/l)	0.0385(0.478)	1.58(0.20) 10^{-5}	0.407(1.34)
$\dot{k}_a$ (d^{-1})	1.77(65) 10^{-5}	1.84(25) 10^{-3}	0.00595(0.0245)
L_∞ (cm)	2.819(0.023)	2.88(0.047)	2.88(0.045)
σ (cm)	0.0159	0.0148	0.0139

they give some indication. The application of profile likelihood functions to obtain confidence sets is less sensitive to deviations from large sample theory (Carroll et al 1995), but small sample theory based on computer simulation studies is required for firm conclusions from small samples. Modifications of the experimental protocol, such as the inclusion of more observation points in time, will both help the estimation of parameter values and the identification of the mode of action of the compound. The standardized bioassay can be used to determine a no-effect concentration. The present examples, however, did not reveal significant deviations from zero.

The statistical analysis of growth data is less standardized than that of survival data. Kamakura & Takizawa (1994) discussed multiple comparison methods in logistic growth models. Bruce & Versteeg (1992) applied a log-probit model, where the weights at the end of the exposure experiment are taken to be proportional to

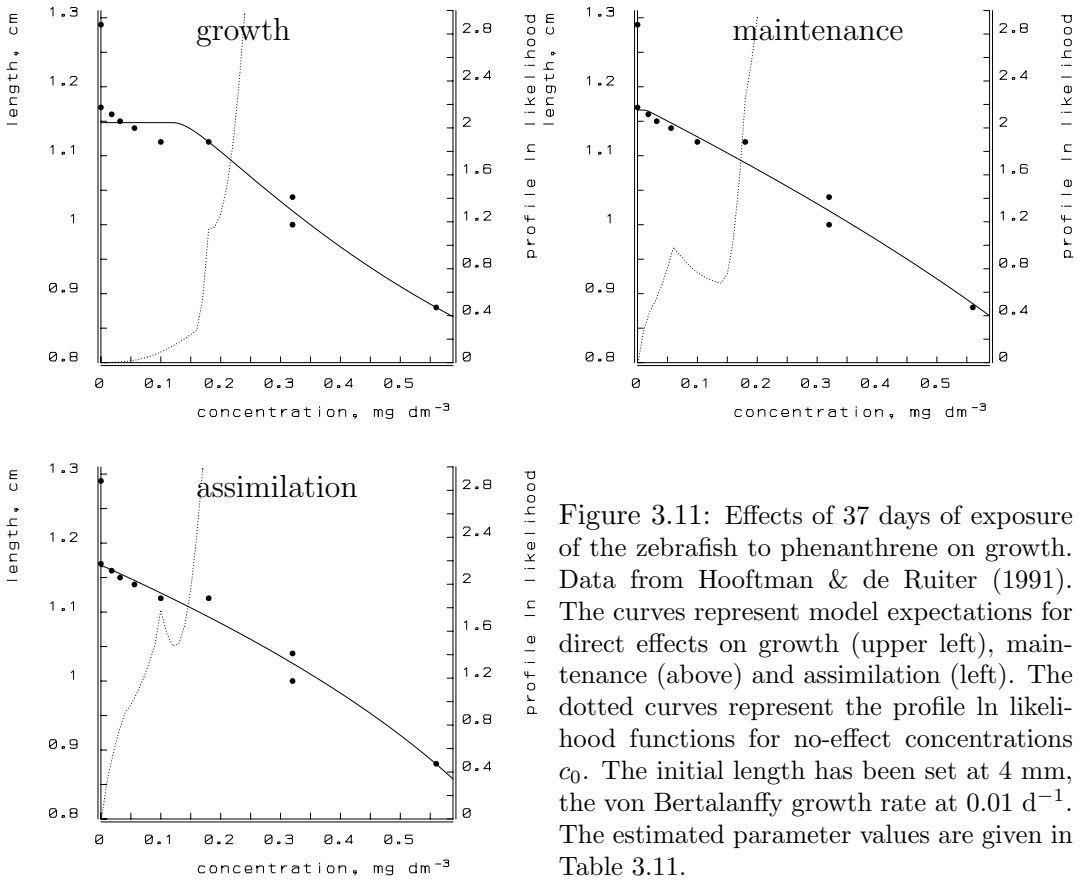


Figure 3.11: Effects of 37 days of exposure of the zebrafish to phenanthrene on growth. Data from Hooftman & de Ruiter (1991). The curves represent model expectations for direct effects on growth (upper left), maintenance (above) and assimilation (left). The dotted curves represent the profile ln likelihood functions for no-effect concentrations c_0. The initial length has been set at 4 mm, the von Bertalanffy growth rate at 0.01 d^{-1}. The estimated parameter values are given in Table 3.11.

the survivor function of the normal distribution when plotted against the logarithm of the concentration. A log-logistic model is also used, which is very similar (Finney 1971). These purely descriptive models are also applied to other toxicity data, such as for invertebrate (*Daphnia*) reproduction, (algal) population growth and survival. To circumvent the problems that are inherent to the determination of the No-Observed Effect Concentration, the EC20 is proposed as a 'small'-effect concentration. See Kooijman (1995) for a discussion of the problems with this approach.

Although few effect models exist, several models for toxicokinetics in growing fish have been proposed in the literature (e.g. Madenjian et al. 1993; Borgmann & Whittle 1992). Bioenergetic models for fish growth are frequently based on the assumption that the energy allocation to growth equals ingestion minus egestion minus respiration and excretion (and specific dynamic action). These mass fluxes are converted to energy fluxes using fixed conversion coefficients. The DEB theory shows that respiration and excretion themselves relate to assimilation, growth and dissipating energy fluxes, such as maintenance (Kooijman 1995). This means that we

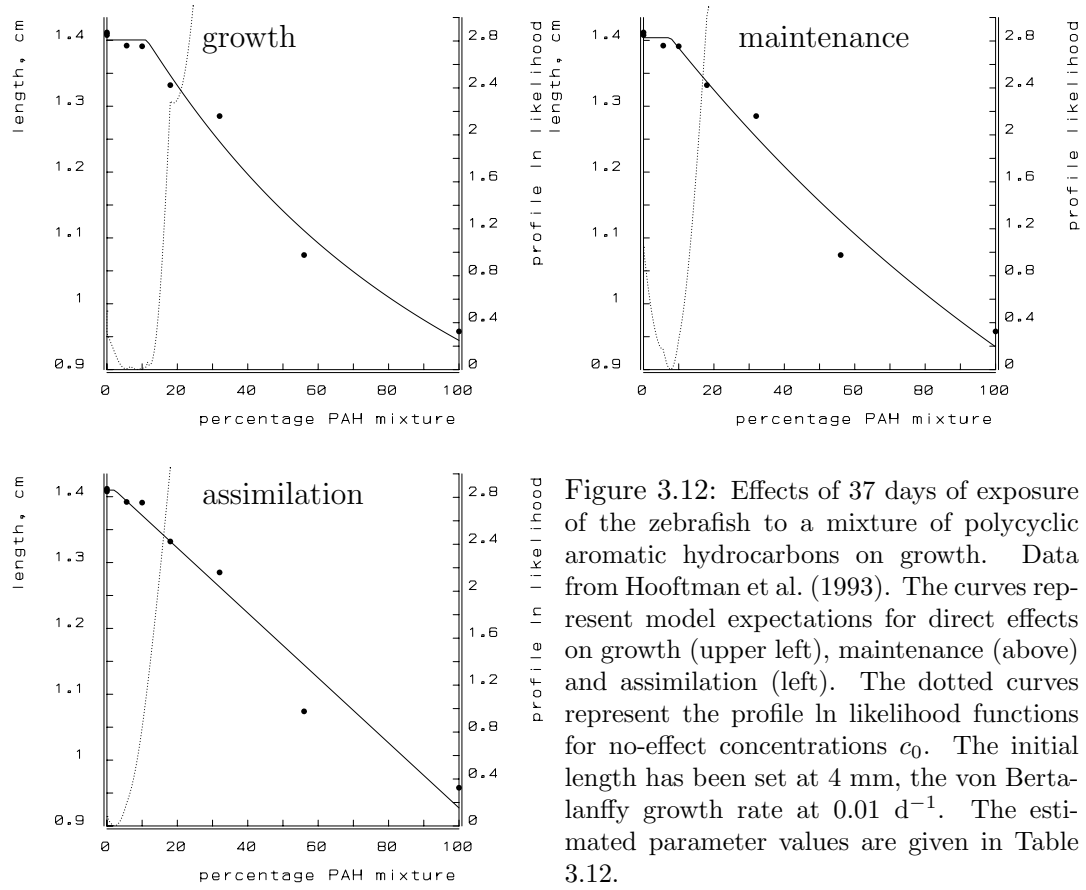


Figure 3.12: Effects of 37 days of exposure of the zebrafish to a mixture of polycyclic aromatic hydrocarbons on growth. Data from Hooftman et al. (1993). The curves represent model expectations for direct effects on growth (upper left), maintenance (above) and assimilation (left). The dotted curves represent the profile ln likelihood functions for no-effect concentrations c_0. The initial length has been set at 4 mm, the von Bertalanffy growth rate at 0.01 d^{-1}. The estimated parameter values are given in Table 3.12.

Table 3.12: Parameter estimates (and standard deviations) and the mean residual deviation for the models for the effects on growth, maintenance and assimilation, applied to the zebrafish data given in figure 3.12.

parameter	growth	maintenance	assimilation
c_0 (%)	8.7(13.1)	7.66(3.30)	2.22(5.45)
c_G,c_M,c_A (%)	36.3(31.8)	44.6(3.2)	230(17.4)
$\dot{k}_a$ (d^{-1})	0.0756(0.366)	∞	∞
L_∞ (cm)	3.635(0.034)	3.647(0.044)	3.666(0.067)
σ (cm)	0.0213	0.0236	0.0291

cannot obtain the flux to growth via simple subtraction. Moreover, most models use allometric functions to describe how basic fluxes, such as ingestion, respiration and toxicokinetics depend on body size. This technique has serious drawbacks (Kooijman 1993). The DEB model avoids these complexities and has relatively few parameters. The main advantage is that other processes, such as reproduction and aging, fit in naturally (Kooijman 1993), which allows the evaluation of population consequences. Kooijman & Bedaux (1996c) and Kooijman (1996) discussed the properties of the present approach relative to the standard empirical EC50-based approach.

We assumed that the food density is constant, so that the reserve density is also constant. This, of course, only holds if the animal is 'in equilibrium' with this food availability. If food density does change, or if there is no food at all, we have to account for the change in lipid content of the animal because the uptake/elimination behaviour can be rather sensitive to such changes. The details of effects of changes in lipid content have been worked out (Kooijman & van Haren 1990; Kooijman 1993). Because the present description of effects of compounds is based on the tissue-concentrations, variations in time can be taken into account. Such variations include metabolic transformation of the compound.

Acknowledgements

This study has been partially supported by Grant PAD 90-18 from the Alternative to Animal Experiments Platform. We thank Ria Hooftman, TNO-MW, Delft, for making her data available to us, and Mike Newman and an unkown referee for helpful comments.

References

Atherton, W.D. 1975. The effect of different levels of dietary fat on the growth of rainbow trout (*Salmo gairdneri* Richardson). *J. Fish Biol.* **7**: 565-571.

Arthur, A.D. & Dixon, D.G. 1994. Effects of rearing density on the growth response of juvenile fathead minnow (*Pimephales promelas*) under toxicant-induced stress. *Can. J. Fish. Aquat. Sci.* **51**: 365-371.

Bedaux, J.J.M. & Kooijman, S.A.L.M. 1994. Statistical analysis of bioassays, based on hazard modeling. *Envir. & Ecol. Stat.* **1**: 303-314.

Borgmann, U. & Whittle, D.M. 1992. Bioenergetics and PCB, DDE, and mercury dynamics in Lake Ontario Trout (*Salvelinus namaycush*): A model based on surveillance data. *Can. J. Fish. Aquat. Sci.* **49**: 1086-1096.

Brown, M.E. 1946. The growth of brown trout (*Salmo trutta* Linn.) I. Factors influencing the growth of trout fry; II. The growth of two-year-old trout at a constant temperature of 11.5°C. *J. Exp. Biol.* **22**: 118-144.

Bruce, R.D. & Versteeg, D.J. 1992. A statistical procedure for modelling continuous toxicity data. *Environ. Toxicol. Chem.* **11**: 1485-1494.

Burden, R.L. & Faires, J.D. 1983. *Numerical analysis.* Boston: Prindle, Weber & Schmidt, 676 pp.

Carroll, R.J., Ruppert, D. & Stefanski, L.A. 1995 *Measurement error in nonlinear models.* Monographs on Statistics and Applied Probability 63, London: Chapman & Hall, pp 305.

Craig, J.F. & Fletcher, J.M. 1984. Growth and mortality of zebra fish, *Brachydanio rerio* (Hamilton Buchanan), maintained at two temperatures and on two diets. *J. fish Biol.* **25**: 43-55.

Chen, Y., Jackson, D.A. & Harvey, H.H. 1992. A comparison of von Bertalanffy and polynomial functions in modelling fish growth data. *Can. J. Fish. Aquat. Sci.* **49**: 1228-1235.

Denton, J.E. & Yousef, M.K. 1976. Body composition and organ weights of rainbow trout, *Salmo gairdneri. J. Fish Biol.* **8**: 489-499.

Eaton, R.C. & Farley, R.D. 1974. Growth and the reduction of depensation of zebrafish, *Brachydanio rerio*, reared in the laboratory. *Copeia*, **1974** (1): 204-209.

Finney, D.J. 1971. *Probit analysis.* Cambridge University Press, Cambridge.

Galliucci, V.F. & Quinn, T.J. 1979. Reparameterizing, fitting, and testing a simple growth model. *Trans. Am. Fish. Soc.* **108**: 14-25.

Grove, D.J., Loizides, L.G. & Nott, J. 1978. Satiation amount, frequency of feeding and gastric emptying rate in *Salmo gairdneri. J. Fish Biol.* **12**: 507-516.

Hammar, J., Larsson, P. & Klavins, M. 1993. Accumulation of persistent pollutants in normal and dwarfed arctic char (*Salvelinus alpinus* sp. complex). *Can. J. Fish. Aquat. Sci.* **50**: 2574-2580.

Hearn, W.S. & Leigh, G.M. 1994. Comparing polynomial and von Bertalanffy growth functions for fitting tag-recapture data. *Can. J. Fish. Aquat. Sci.* **51**: 1689-1691.

Hooftman, R.N. & Evers-de Ruiter, A. 1992. The toxicity and uptake of benzo(k)-fluoranthene using *Brachydanio rerio* in an early life stage test. *TNO-Report* IMW-R 92/218.

Hooftman, R.N. & de Ruiter, A. 1991. The influence of phenanthrene on the early life stages of *Brachydanio rerio* (semi static test). *TNO-Report* IMW-R 91/059.

Hooftman, R.N., Henzen, L. & Roza, P. 1993. The toxicity of a Polycyclic Aromatic Hydrocarbon mixture in an early life stage toxicity test carried out in an intermittent flow-through system. *TNO-Report* IMW-R 93/253.

Kamakura, T. & Takizawa, T. 1994. Multiple comparison among groups of growth curves. *Environ. Health Persp. Suppl.* **102** *Suppl.* **1**: 39-42.

Karlsson-Norrgren, L. & Runn, P. 1985. Cadmium dynamics in fish: pulse studies with ^{109}Cd in female zebrafish, *Brachydanio rerio. J. Fish Biol.* **27**: 571-581.

Kooijman, S.A.L.M. 1981. Parametric analyses of mortality rates in bioassays. *Water Res.* **15**: 107-119.

Kooijman, S.A.L.M. 1983. Statistical aspects of the determination of mortality rates in bioassays. *Water Res.* **17**: 749-759.

Kooijman, S.A.L.M. 1993. *Dynamic Energy Budgets in Biological Systems. Theory and applications in ecotoxicology.* Cambridge University Press, pp 350.

Kooijman, S.A.L.M. 1996. Process-oriented descriptions of toxic effects. In: Schüürmann, G. and Markert, B. (eds) *Ecotoxicology*, Spectrum Akademischer Verlag. (to appear)

Kooijman, S.A.L.M. 1995a. An alternative for NOEC exists, but the standard model has to be replaced first. *Oikos* (to appear)

Kooijman, S.A.L.M. & Bedaux, J.J.M. 1996a. Some statistical properties of estimates of no-effect levels. *Water Res.* (to appear)

Kooijman, S.A.L.M. & Bedaux, J.J.M. 1996b. Analysis of toxicity tests on *Daphnia* survival and reproduction. *Water Res.* (to appear)

Kooijman, S.A.L.M., Hanstveit, A.O. & Nyholm, N. 1996. No-effect concentrations in alga growth inhibition tests. *Water Res.* (to appear)

Kooijman, S.A.L.M. & Haren, R.J.F.van 1990. Animal energy budgets affect the kinetics of xenobiotics. *Chemosphere* **21**: 681-693.

Laale, H.W. 1977. The biology and use of zebrafish, *Brachydanio rerio* in fisheries research. A literature review. *J. Fish Biol. 10*: 121-173.

Laskowski, R. 1995. Some good reasons to ban the use of NOEC, LOEC and related concepts in ecotoxicology. *Oikos* **73**: 140-144.

Lucas, M.C. & Priede, I.G. 1992. Utilization of metabolic scope in relation to feeding and activity by individual and grouped zebrafish, *Brachydanio rerio* (Hamilton-Buchanan). *J. Fish Biol.* **41**: 175-190.

Madenjian, C.P., Carpenter, S.R., Eck, G.W. & Miller, M.A. 1993. Accumulation of PCBs by lake trout (*Salvelinus namaycush*): an individual-based model approach. *Can. J. Fish. Aquat. Sci.* **50**: 97-109.

McCullagh,P. & Nelder, J.A.1989 *Generalized linear models.* Monographs on Statistics and Applied Probability 37, London: Chapman & Hall, pp 511.

OECD 1992. Draft OECD test guideline 210 Fish, Early-life Stage Toxicity Test (adopted 17th July 1992); Fish, Toxicity Test on Egg and Sac-fry Stages (draft March 1992); Fish, Juvenile Growth Test-28 days (draft March 1992)

Oliva-Teles, A. & Kaushik, S.J. 1990. Growth and nutrient utilization by 0+ and 1+ triploid rainbow trout, *Oncorhynchus mykiss. J. Fish Biol.* **37**: 125-133.

Pack, S. 1993. A review of statistical data analysis and experimental design in OECD aquatic toxicity test guidelines. Shell Research Ltd., Sittingbourne, UK.

Phillips, M.J. 1989. The feeding sounds of rainbow trout, *Salmo gairdneri* Richardson. *J. Fish Biol.* **35**: 589-592.

Quinton, J.C. & Blake, R.W. 1990. The effect of feed cycling and ration level on the compensatory growth response in rainbow trout, *Oncorhynchus mykiss. J. Fish Biol.* **37**: 33-41.

Silvey, S.D. 1975. *Statistical inference.* Monographs on applied probability and statistics. Chapman and Hall, pp 192.

Staples, D.J. & Nomura, M. 1976. Influence of body size and food ration on the energy budget of rainbow trout *Salmo gairdneri* Richardson. *J. Fish Biol.* **9**: 29-43.

Ruting, J. 1958. *Welke vis is dat?* W.J. Thieme & Cie, Zutphen.

Steele, C.W., Scarfe, A.D. & Owens, D.W. 1991. Effects of group size on the responsiveness of zebrafish, *Brachydanio rerio* (Hamilton Buchanan), to alanine, a chemical attractant. *J. Fish biol.* **38**: 553-564.

Tytler, P., Tataner, M. & Findlay, C. 1990. The ontogeny of drinking in the rainbow trout, *Oncorhynchus mykiss* (Walbaum). *J. Fish Biol.* **36**: 867-875.

Weatherley, A.H. & Gill, H.S. 1983. Relative growth of tissues at different somatic growth rates in rainbow trout *Salmo gairdneri* Richardson. *J. Fish Biol.* **22**: 43-60.

Weatherley, A.H. & Gill, H.S. 1984. Growth dynamics of white myotomal muscle fibers in the bluntnose minnow, *Pimephales notatus* Rafinesque, and comparison with rainbow trout, *Salmo gairdneri* Richardson. *J. Fish Biol.* **25**: 13-24.

Weber, C.I., Peltier, W.H., Norberg-King, T.J., Horning, W.B., Kessler, F.A., Menkedick, J.R., Neiheisel, T.W., Lewis, P.A., Klemm, D.J., Pickering, Q.H., Robinson, E.L., Lazorchak, J.M.. Wymer, L.J. & Freyberg, R.W. 1989. Short-term methods for estimating the chronic toxicity of effluents and surface waters to freshwater organisms. EPA 600/4-89-001, Cincinnati, OH.

Xiao, Y. 1994. Von Bertalanffy growth models with variability in, and correlation between, K and L_∞. *J. Fish Biol.* **51**: 1585-1590.

3.4 Analysis of toxicity tests on *Daphnia* survival and reproduction

Abstract We present a statistical analysis of bioassays for *Daphnia* survival and reproduction, such as the routine toxicity test that is described in the OECD guideline 202. The analysis is based on the Dynamic Energy Budget theory and a one-compartment kinetics for the toxic compound. It is fully process-oriented. We compare a formulation in terms of effects on survival during oogenesis to various direct and indirect effects on the energetics of reproduction. All formulations characterize the effects by a no-effect concentration, a tolerance concentration and the elimination rate. We conclude that all options lead to similar no-effect levels. We compare the analysis to the standard NOEC/EC50 analysis and conclude that our analysis is both simpler and more effective.

Introduction

Toxicity tests are of interest for scientific purposes as well as for legislation and risk assessment purposes. The no-observed-effect concentration (NOEC) still plays an important role in the latter purposes, but its use is under increased scrutiny due to associated statistical problems. Earlier attempts to solve these problems by the incorporation of the no-effect concentration (NEC) as a model parameter suffers from difficulties that will be discussed later. Small-effect concentrations (EC5, EC10) recently gained interest, but these characteristics are also far from ideal, as will be explained later. In this paper, we propose a solution that seems free of statistical and practical problems but that requires a radical change in the way that we look at toxic effects: a fully process-based approach.

Reproduction can be affected directly by toxic chemicals, or indirectly via effects on feeding, growth or maintenance. This is because these processes are intimately linked. The Dynamic Energy Budget (DEB) theory provides a mechanistic basis that has been tested against many experimental data (Kooijman 1993). Figure 3.13 gives a summary of the energy flows in an organism that the DEB theory specifies quantitatively.

In this paper we present and apply a statistical analysis of routine toxicity tests on *Daphnia* reproduction based on insights from the DEB theory. Since this test is the best standardized among all aquatic toxicity tests and applied on a routine basis all over the world, it is useful to work out an analysis for this particular test. However, many features apply to other tests as well. We first summarize the relevant details of the test guideline, then we work out a new analysis of these tests and compare it with the standard analysis.

[0]Published as: S.A.L.M. Kooijman and J.J.M. Bedaux (1996) Analysis of toxicity tests on *Daphnia* survival and reproduction. *Water Research* **30**: 1711–1723

Table 3.13: List of symbols. The symbols in the dimension-column stand for t time, m mass , l length (of environment), L length (of organism), # number.

symbol	dimension	interpretation
c	ml^{-3}	concentration in environment
c_q	ml^{-3}	ratio of conc. in tissue and P_{vd}
$[Q]$	mL^{-3}	concentration in tissue
P_{vd}	l^3L^{-3}	bioconcentration coefficient
NOEC	ml^{-3}	no-observed effect concentration
c_0	ml^{-3}	no-effect conc. for survival, reprod.
c_{L50}, $c_{L50.\infty}$	ml^{-3}	50 % effect conc. for surv., ultimate c_{L50}
c_{E50}	ml^{-3}	50 % effect concentration for reprod.
β, β_R	-	gradient parameter for surv., reprod.
c_*	ml^{-3}	tolerance conc. for $* \equiv H$, R, M, G or A
$\dot{k}_\dagger$	$m^{-1}l^3t^{-1}$	killing rate
$\dot{k}_a$	t^{-1}	elimination rate
q	-	tolerance function for survival
s	-	stress function for reproduction
t	t	exposure time
$\dot{h}_0$, $\dot{h}_c$	t^{-1}	hazard rate in control, compound induced
f	-	ingestion as fraction of its max. given l
l	-	body length as fraction of its max.
l_b, l_p	-	l at birth, puberty
$\dot{\gamma}$	t^{-1}	von Bertalanffy growth rate
$\dot{m}$	t^{-1}	maintenance rate coefficient
$\dot{v}$	Lt^{-1}	energy conductance
g	-	energy investment ratio
$\mathcal{F}$	-	survival probability
$\dot{R}$, $\dot{R}_m$	$\#t^{-1}$	reprod. rate, max. reprod. rate
N	#	number of offspring
$\dot{\mu}$	t^{-1}	population growth rate

Routine toxicity tests

The routine toxicity test on *Daphnia magna* reproduction according to guideline 202 of the OECD (1994) requires that young females (< 1 d) are individually exposed to a range of concentrations of test compound during a period of 21 d. Survival and number of offspring are observed on a daily basis when food (green algae such as *Chlorella* or *Scenedesmus*) is supplied. The media are renewed three times a week. The temperature and concentration of the compound in the media are as constant a possible (in the range of 18-22°C). At least 10 replicates are required for each chosen test concentration, one of them being the control. The coefficient of variation of the number of living offspring in the control should be less than 0.3.

The female daphnids typically grow from 0.8 mm to 4 mm at day 21 during exposure, which means an increase by a factor 5 in length and a factor 125 in weight.

Models

A practical problem for the analysis of reproduction data resulting from a routine toxicity test is that variables such as feeding, growth and respiration rates are usually not measured. This makes it hard to select the pathway for the effect on reproduction that applies to the compound tested. We will see, however, that several parameters that characterize the toxicity of the compound do not depend sensitively on the selection of the correct pathway. We first discuss toxicokinetics, then reproduction and finally effects because we link effects to tissue-concentrations, not environment-concentrations. The equations directly follow from the DEB theory for growth at constant food density.

Growth

Although body size is usually not measured in routine toxicity tests, growth has to be considered because toxicokinetics depends on body size and effects on survival and reproduction depend on the amount of compound in the body. Not being measured, body length has the role of a hidden variable. It proves to be convenient to work with

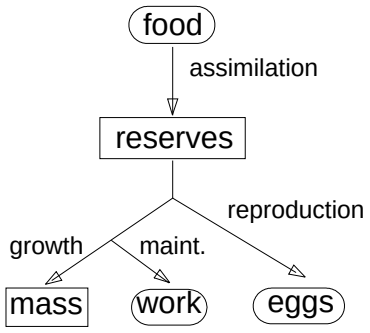


Figure 3.13: The powers as specified quantitatively by the DEB model for an ectotherm with body size and reserve density as state variables. Toxic compounds that affect reproduction can do so directly, or indirectly via assimilation, growth and maintenance. The rounded boxes indicate sources or sinks.

scaled length rather than actual length $l \equiv (V/V_m)^{1/3}$, where $V_m^{1/3}$ is the maximum volumetric length an adult can reach.

If food density is kept constant, the DEB theory states that scaled length behaves as

$$\frac{d}{dt}l = \dot{\gamma}(f - l) \tag{3.39}$$

where f is the ingestion rate as a fraction of the maximum ingestion rate for an individual of that body size and $\dot{\gamma}$ is the von Bertalanffy growth rate. This rate parameter depends on energy parameters as $\dot{\gamma} = \frac{\dot{m}g}{3(f+g)}$, where the maintenance rate coefficient $\dot{m}$ stands for the ratio of the volume-specific maintenance costs (per unit of time) and the volume-specific costs for growth, i.e. the amount of energy required to synthesize a unit volume of biomass. The investment ratio g is the ratio of the volume-specific costs for growth and the fraction of the reserve density that is allocated to growth plus maintenance. The maximum volumetric length depends on energy parameters as $V_m^{1/3} = \frac{\dot{v}}{\dot{m}g}$, where the energy conductance $\dot{v}$ represents the ratio of the surface area-specific assimilation rate and the volume-specific costs for growth. We need the energy interpretations of the von Bertalanffy growth rate and the maximum volumetric length in the discussion of indirect effects on reproduction.

If food density is kept constant and if the compound does not affect feeding, growth or maintenance, scaled length as a function of time amounts to the von Bertalanffy growth curve

$$l(t) = f - (f - l_b)\exp\{-\dot{\gamma}t\} \tag{3.40}$$

where l_b is the scaled length at birth, i.e. $l(0) = l_b$. Notice that for abundant food, we have that $f = 1$ and that it is not important that food density is constant, as long as food is abundant. For *D. magna* at 20°C and abundant food we typically find $l_b = 0.18$ and $\dot{\gamma} = 0.1$ d^{-1}. Figure 3.15 gives the typical growth curve for *D. magna* during the 21 days of exposure.

Reproduction

Energy allocation to reproduction is a continuous process, while reproduction itself is event-driven. This means that energy allocated to reproduction first accumulates in a buffer and that the content of this buffer is instantaneously converted into (a clutch of) eggs. *Daphnia* moults at a frequency that depends on temperature. When a clutch of hatchlings is delivered, the female moults, converts the buffer of energy allocated to reproduction into eggs and deposits freshly laid eggs into the broodpouch. So the intermoult period equals the incubation time. These details are specific to *Daphnia*. We first discuss toxic effects on reproduction, treated as a continuous process, because this applies to a wide variety of animals and then discuss details that relate to the clutches of *Daphnia*.

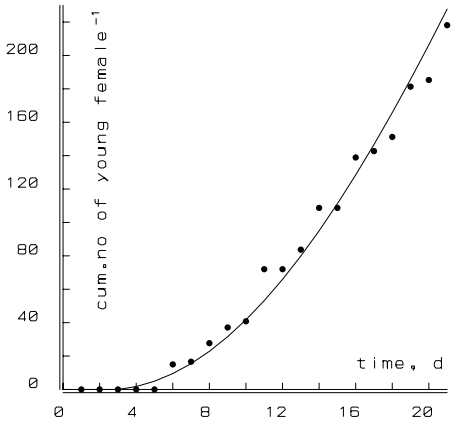


Figure 3.14: The cumulative number of young per female *D. magna*, starting from individuals of age ≤ 1 d with a scaled length of 0.18. The data represent a control from a test that has been executed under OECD guideline 202. The curve is the least squares fit, assuming that the reproduction rate is linear in the squared and cubed length, while length grows with a von Bertalanffy growth rate of 0.1 d^{-1}. The scaled length at puberty is 0.4. The estimated maximum reproduction rate is 28.9 d^{-1}.

Reproduction depends on body size and energy reserves, so indirectly on food availability. This means that in a control situation, where an organism such as *D. magna* is followed individually from an age less than 1 d up to 21 d at a constant or high food density, the reproductive effort can be described by a satiating function of age. According to the DEB theory, the reproduction rate at constant food density as a function of scaled length is

$$\dot{R}(l) = \frac{\dot{R}_m}{1 - l_p^3}\left(\frac{g + l}{g + f} f l^2 - l_p^3\right) \tag{3.41}$$

for $l_p < l < f$, where l_p is the scaled length at puberty, which amounts to $l_p = 0.4$ for *D. magna*. The value for the energy investment ratio for *D. magna* is less certain, but $g \simeq 1$ is realistic. It might be somewhat smaller, but numerical studies indicate that this hardly affects toxicity parameters. Substitution of the values for l_p and g into (3.41) gives $\dot{R}(l) \simeq 0.53\dot{R}_m(l^2 + l^3 - 0.128)$. The parameter $\dot{R}_m$ can be interpreted as the maximum reproduction rate, i.e. the reproduction rate of an individual of maximum size at abundant food. It is proportional to maintenance costs^{-2}, assimilation rate^3 and independent of the growth costs. Figure 3.14 illustrates that (3.41), together with (3.40), fits the data well.

Uptake/elimination kinetics

As is relevant for the OECD toxicity test with *D. magna* (OECD 1994), exposure is assumed to start at birth at a constant environment-concentration c of toxic compound. We choose $t = 0$ as the start of the exposure period and the time at which $l = l_p$, say t_p, as the start of the reproductive effort, so $l(t_p) = l_p$.

Suppose that absorption of the compound to the food particles is instantaneous and that the concentration of food particles is constant. Uptake can occur directly from the water and indirectly via food, but both uptake rates are proportional to

the surface area of the animal, which is proportional to $V^{2/3}$ for an isomorph such as *Daphnia*. The direct elimination is assumed to be proportional to the surface area again and to the concentration in the aqueous fraction of the tissue $[Q]$. The partitioning of the compound over the different body fractions, including the lipid fraction, is assumed to be instantaneous again. The uptake/elimination kinetics reduces to

$$\frac{d}{dt}[Q] = cP_{vd}\dot{k}_a f/l - [Q]\left(\dot{k}_a f/l + \frac{d}{dt}\ln l^3\right) \tag{3.42}$$

where c is the concentration in the environment (dissolved plus absorbed to food particles), P_{vd} is the bioconcentration coefficient and $\dot{k}_a$ the elimination rate. The term $\frac{d}{dt}\ln l^3 = 3l^{-1}\frac{d}{dt}l$ in (3.42) accounts for the dilution by growth.

The tissue-concentration is usually not measured in routine toxicity tests, so that it plays the role of a hidden variable, just like body length. It proves to be convenient to introduce the scaling $c_q \equiv [Q]/P_{vd}$, which has the dimensions of an environment-concentration but is just proportional to the tissue-concentration. The kinetics of the scaled tissue-concentration reduces to

$$\frac{d}{dt}c_q = c\dot{k}_a f/l - c_q\left(\dot{k}_a f/l + \frac{d}{dt}\ln l^3\right) \tag{3.43}$$

Although this simple first order differential equation with variable coefficients can be solved, this hardly helps because the solution still has integrals that must be obtained numerically. At abundant food, where $f = 1$, the behaviour only depends on the scaled elimination rate $\dot{k}_a$ relative to the von Bertalanffy growth rate $\dot{\gamma}$. Figure 3.15 illustrates how the shape of the uptake/elimination curve depends on the elimination rate. It also shows that the process of dilution by growth is important for the kinetics.

Effects on survival

The hazard rate depends linearly on the tissue-concentration, thus also on the scaled tissue-concentration. The idea behind this linear response function is that each molecule that exceeds the physiological capacity to handle the compound has an effect that is independent of that of other molecules. We can relax this strict interpretation by stating that the actual way in which the hazard rate is affected is complex and non-linear, but that we approximate the description with two terms of the Taylor expansion around the no-effect concentration. This approximation is only valid for small effects, of course, but small effects on the hazard rate (and on energy parameters that will be discussed later) end up having considerable effects on survival (and reproduction). The hazard rate and the survival probability are given by

$$\dot{h}_c = \dot{k}_\dagger(c_q - c_0)_+ \tag{3.44}$$

$$\mathcal{F}(c,t) = q(c,t)\mathcal{F}(0,t) = \exp\{-\int_0^t \dot{h}_c(t_1)\,dt_1\}\mathcal{F}(0,t) \tag{3.45}$$

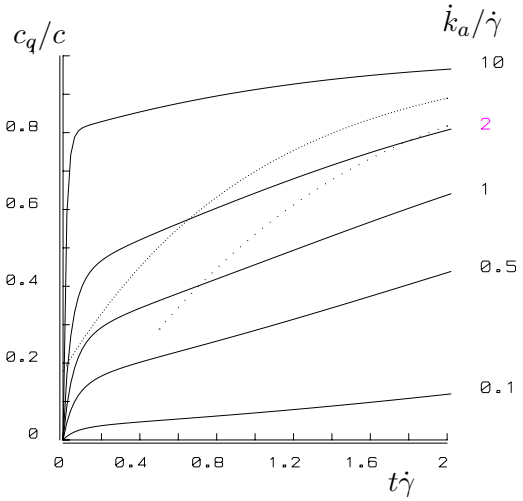


Figure 3.15: The scaled tissue-concentration for an organism that grows at constant food density with von Bertalanffy growth rate $\dot{\gamma}$. The uptake and elimination rate from the water is proportional to the surface area of the organism. Uptake and elimination can be directly from the water as well as via food. The hatchling originates from a culture in this example. The finely dotted curve represents the scaled length, the coarsely dotted curve is the scaled reproduction rate, the solid curves the tissue-concentrations for different choices of the elimination rate $\dot{k}_a$. The scaling is such that all curves have asymptote 1. The parameter values $l_b = 0.18$, $l_p = 0.5$, $g = 1$ are typical for *D. magna* at 20°C and $t\dot{\gamma} = 2$ corresponds with 21 d.

where c_0 is the no-effect concentration (for survival), $\dot{k}_\dagger$ the killing rate and $\mathcal{F}(0,t) = \exp\{-\dot{h}_0 t\}$ is the control survival probability, with $\dot{h}_0$ being the hazard rate in the control, which is considered to be constant. The function $q(c,t)$ will be called the 'tolerance' function (for survival). Notice that $q(0,t) = 1$ is the maximum value for the tolerance function. The index + means: $(x)_+ \equiv \max\{0,x\}$.

Statistics

Given observation times $\{t_1, t_2, \cdots, t_k\}$ and test concentrations $\{c_1, c_2, \cdots, c_r\}$, the number of dead (or immobilized) individuals in the time-interval (t_{i-1}, t_i) follows a multinomial distribution with probability parameter $q(c_j, t_{i-1}) - q(c_j, t_i)$. Since the parameters $\dot{h}_0$, $\dot{k}_\dagger$, $\dot{k}_a$ and c_0 occur in the probability parameters of all observation times and test-concentrations, all observations should be considered simultaneously to extract these four parameters using the maximum likelihood method (see Silvey 1975, McCullagh & Nelder 1989, Carroll et al. 1995).

Direct effects on reproduction

We will discuss two slightly different views on the direct effect on reproduction rate, one representing an effect on survival during oogenesis and the other an effect on energetics.

Hazard model

The first view interprets effects on reproduction as a mortality during oogenesis.

The sensitive period t_H is assumed to be short and fixed. The tolerance function for reproduction $q_R(c,t)$ can then be written as

$$q_R(c,t) = \exp\{-\int_t^{t+t_H} \dot{h}_{cH}(t_1)\, dt_1\} \simeq \exp\{-t_H \dot{h}_{cH}(t)\} \tag{3.46}$$

where $\dot{h}_{cH}$ is the hazard rate for the ovum, which is taken to be a linear function of the concentration of toxic compound in the female. It proves to be convenient to introduce the 'stress function' $s(c_q) = t_H \dot{h}_{cH} = t_H \dot{k}_{\dagger H}(c_q - c_0)_+ = c_H^{-1}(c_q - c_0)_+$, where $\dot{k}_{\dagger H}$ is the killing rate during oogenesis (Kooijman 1993, Bedaux & Kooijman 1994, Kooijman & Bedaux 1996). Note that the killing rate relates to the tolerance concentration as $t_H \dot{k}_{\dagger H} = c_H^{-1}$.

This effect on survival is consistent with the DEB theory for aging, which states that the hazard rate is proportional to the concentration of accumulated disfunctional proteins in the body. These proteins are produced by DNA that is affected by free radicals inherent to the respiration process. The respiration rate is roughly proportional to the use of energy from the reserves (Kooijman 1995). See Kooijman (1993) for a derivation of the relationship between aging and energetics, the relationship with effects of mutagenic compounds and tests against experimental data. The hazard model for the description of toxic effects on reproduction takes the postulated short sensitive period during oogenesis as the only difference with effects on survival of the reproducing female.

Substitution of the hazard rate into the reproduction rate leads to

$$\dot{R}(c,t) = \dot{R}(0,t) \exp\{-s(c_q)\} = \dot{R}(0,t) \exp\{-c_H^{-1}(c_q - c_0)_+\} \tag{3.47}$$

where c_q is given in (3.43) and the parameter c_H will be called the tolerance concentration (for reproduction).

Costs model

The second view on direct effects on the reproductive output is an increase in the energy costs per egg; the latter is again taken to be a linear function of the concentration of toxic compound in the animal. Each molecule that exceeds the handling capacity of the individual has the same effect. This strict view can be relaxed by stating that the actual way in which the compound affects the energy cost per egg is complex and non-linear, but that we approximate the description with two terms of the Taylor expansion around the no-effect concentration. This leads to the stress function $s(c_q) = c_R^{-1}(c_q - c_0)_+$, where the parameter c_R will be called the tolerance concentration (for effects on the costs per egg). The resulting reproduction rate is

$$\dot{R}(c,t) = \dot{R}(0,t)\,(1 + s(c_q))^{-1} = \dot{R}(0,t)\left(1 + c_R^{-1}(c_q - c_0)_+\right)^{-1} \tag{3.48}$$

where c_q is given in (3.43).

Both views on the direct effects on reproduction involve the same three parameters: the no-effect concentration c_0, the tolerance concentration c_H, c_R and the elimination rate $\dot{k}_a$. The last parameter also occurs in the description of effects on survival of the reproducing female. Numerically, the two reproduction functions behave very similarly.

Indirect effects on reproduction

Effects on maintenance, growth and assimilation indirectly affect reproduction by the conservation of energy principle. This section evaluates these indirect effects on reproduction, which will not only result in a decrease of the reproduction rate but also in a delay of the onset of reproduction. The occurrence of such a delay is the best indication for indirect effects.

Maintenance

Many toxic compounds are likely to directly affect maintenance requirements rather than reproduction, which translates into an increase in the maintenance costs. Because maintenance has priority over growth in the DEB theory, such an increase leads to a reduction of the growth rate. If the animal would compensate this reduction of growth by increasing the allocation to maintenance plus growth and thus decreasing the allocation to reproduction, this would be indistinguishable from the costs model of the direct effect on reproduction which has just been discussed. Therefore, we now assume that the animal does not compensate the reduction of growth. Since the feeding rate depends on body size, the feeding rate is affected as well. Reproduction also depends on body size as well as on feeding rate (via the effect on reserve dynamics), so reproduction is also affected, not only via a reduced allocation to reproduction but also via a delay in the start of reproduction.

In analogy with the direct effects, we now assume that the maintenance rate coefficient is $\dot{m}_c = \dot{m}_0(1 + s(c_q))$ with stress function $s(c_q) = c_M^{-1}(c_q - c_0)_+$, where c_M is the tolerance concentration for maintenance and c_0 the no-effect concentration; $\dot{m}_0$ stands for the maintenance rate coefficient in the control. The formulation of the reproduction rate does not account for effects on the energy costs of eggs, only for the reduced energy allocation to reproduction.

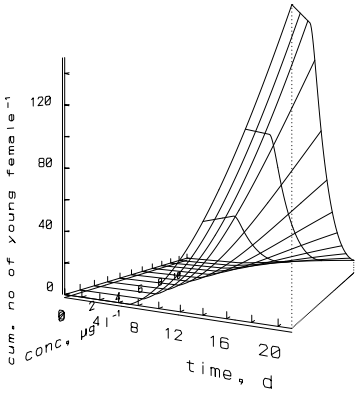
Substitution of the effect on maintenance into the growth rate (3.39) and the reproduction rate (3.41) leads to

$$\frac{d}{dt}l = \dot{\gamma}(f - l(1 + s(c_q))) \tag{3.49}$$

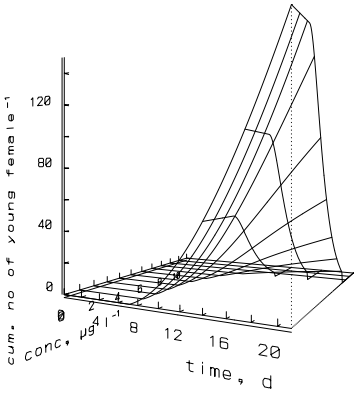
$$\dot{R} = (1 + s(c_q))^{-2}\frac{\dot{R}_m}{1 - l_p^3}\left(\frac{g + l}{g + f}fl^2 - l_p^3\right) \tag{3.50}$$

where $\dot{\gamma}$ is the von Bertalanffy growth rate in the control and $l \equiv (V/V_m)^{1/3}$ is the scaled length. The maximum volume V_m in the scaling refers to the maximum

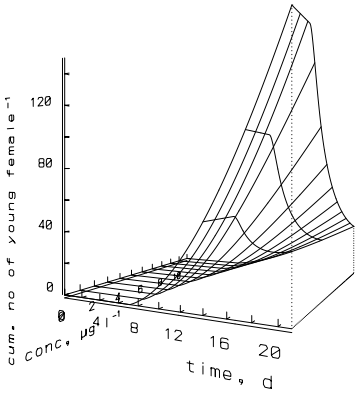
hazard, $c_H = 1.62$ μg l^{-1}



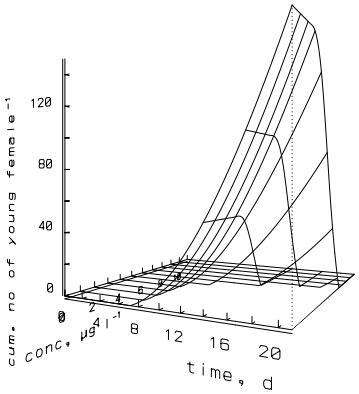
maint., $c_M = 6.02$ μg l^{-1}



costs, $c_R = 1.35$ μg l^{-1}



growth, $c_G = 0.61$ μg l^{-1}



assim., $c_A = 9.57$ μg l^{-1}

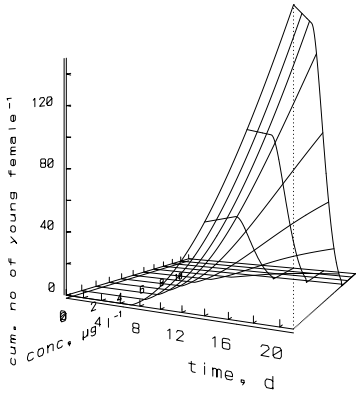


Figure 3.16: Direct (left column) and indirect (right column) effects on reproduction. The various tolerance concentrations are chosen such that the effect size is similar. The elimination rate is set equal to the von Bertalanffy growth rate, $\dot{k}_a = \dot{\gamma} = 0.1$ d^{-1} , and the no-effect concentration is $c_0 = 1.5$ μg l^{-1}.

volume in the control. The coupled equations (3.49) and (3.43) together describe growth and the kinetics of the compound.

Growth

If growth is affected, reproduction will be affected as well because feeding depends on body size. Since the growth rate does not affect the ultimate size, the effect is a delay in reproduction. In analogy with the direct effects, we now assume that the energy investment ratio is $g_c = g_0(1+s(c_q))$ with stress function $s(c_q) = c_G^{-1}(c_q - c_0)_+$, where c_G is the tolerance concentration for growth and c_0 the no-effect concentration.

Substitution of the effect on growth into the growth rate (3.39) and the reproduction rate (3.41) leads to

$$\frac{d}{dt}l = \dot{\gamma}(f-l)\frac{f+g}{f+g(1+s(c_q))} \tag{3.51}$$

$$\dot{R} = \frac{\dot{R}_m}{1-l_p^3}\left(\frac{(1+s(c_q))g+l}{(1+s(c_q))g+f}fl^2 - l_p^3\right) \tag{3.52}$$

Assimilation

If assimilation is affected, i.e. the incoming energy is reduced, reproduction and growth are affected as well. In analogy with the direct effects, we now assume that the energy investment ratio is $\dot{v}_c = v_0(1-s(c_q))$ with stress function $s(c_q) = c_A^{-1}(c_q - c_0)_+$, where c_A is the tolerance concentration for assimilation and c_0 the no-effect concentration. Note that the stress function appears with a negative, rather than a positive sign, to model adverse effects on assimilation. The consequence is that $c_g < c_A + c_0$ must hold, so also $c < c_A + c_0$ for all chosen test concentrations c. The constraint on the value for c_A is in fact a bit stronger than this, because the assimilation rate must exceed the maintenance requirements. The DEB theory states that the individual dies from starvation if it is unable to mobilize enough energy from its reserves for maintenance purposes.

Substitution of the effect on assimilation into the growth rate (3.39) and the reproduction rate (3.41) leads to

$$\frac{d}{dt}l = \dot{\gamma}(f(1-s(c_q)) - l) \tag{3.53}$$

$$\dot{R} = (1-s(c_q))^3\frac{\dot{R}_m}{1-l_p^3}\left(\frac{g+l}{g+f}fl^2 - l_p^3\right) \tag{3.54}$$

The two direct models for effects on reproduction and the three indirect ones are illustrated as response surfaces above the exposure time-concentration plane in Figure 3.16.

Statistics

The translation of the reproductive effort into the number of offspring involves the moulting cycle and details of the experimental protocol. The test media are usually renewed three times a week and the number of surviving parents and offspring are counted on a daily basis. The intermoult period is somewhat flexible and the animal tends to synchronize its moulting cycle with the renewal cycle; soon after the change of test medium it delivers and moults. When counting daily at 20°C, moults, and thus young, will be observed only three times a week. We now focus on those observations at time points t_i^*, where a moult has been present. (If a moult is present, it is likely to be present for almost a day because moulting occurs just after renewal of the medium and counting; but we will neglect this detail.) The expected number of offspring of a particular female at exposure time t_i^* in concentration c_j equals

$$N(t_i^*, c_j) = q_R(c_j, t_{i-1}^*) \int_{t_{i-2}^*}^{t_{i-1}^*} \dot{R}(0,t)\, dt \simeq q_R(c_j, t_{i-1}^*)(t_{i-1}^* - t_{i-2}^*)\dot{R}(0, t_{i-1}) \quad (3.55)$$

This formulation does justice to the biological fact that the allocation to reproduction takes place during the intermoult period prior to the one that delivers the hatchlings.

The moults are usually not observed, so that this analysis is perhaps too detailed for practical purposes. We might try to neglect the moulting cycle and just fit the number of offspring as if the reproduction process is continuous. The frequent zeros correct the broods for the right mean, but the variance is large, of course. This gives problems in the parameter estimation. Alternatively, we might fit the cumulative reproduction. This reduces the estimation problem, but the counts are now statistically dependent. Numerical studies indicate that this hardly affects point estimates.

The observed number of offspring is empirically assumed to follow the normal distribution approximately, with a fixed coefficient of variation. This coefficient should be less than 0.25 for the toxicity test to be acceptable according to the OECD guideline. The estimation of the parameter values using the maximum likelihood method can be simplified as a first approximation by a least squares method with weight coefficients inversely proportional to 1 plus the squared number of observed offspring (McCullagh & Nelder 1989, Carroll et al. 1995). Note that the estimation of the no-effect concentration c_0 and the elimination rate $\dot{k}_a$ depend on the type of effect on reproduction. The scaled lengths, scaled tissue-concentrations and the cumulative reproductive output has to be obtained numerically, using e.g. Adams predictor-corrector method (Burden & Faires 1985).

If effects on reproduction and survival occur at the same time, the observations should be analyzed simultaneously because both descriptions have the same elimination rate.

Reduced models

Many organic compounds have an elimination rate that is large with respect to the von Bertalanffy growth rate (Hawker & Connell 1986), so that for $\dot{k}_a \gg \dot{\gamma}$ (3.43) reduces to $c_q = c$. The survival probability and reproduction rate reduce to

$$\mathcal{F}(c,t) = \mathcal{F}(0,t)\exp\{-t\dot{k}_\dagger(c-c_0)_+\} \tag{3.56}$$

$$\dot{R}(c,t) = \dot{R}(0,t)\exp\{-c_H^{-1}(c-c_0)_+\} \tag{3.57}$$

$$= \dot{R}(0,t)\left(1+c_R^{-1}(c-c_0)_+\right)^{-1} \tag{3.58}$$

The stress functions for the indirect effects on reproduction are constants, but the reproduction rate must still be obtained numerically.

For the chronic *Daphnia* test we can hardly neglect the change in body size during exposure, but for a number of other toxicity tests we can. For $l = f$, the scaled tissue-concentration (3.43) reduces to $c_q(t) = c(1-\exp\{-t\dot{k}_a\})$, and the survival probability and reproduction rate become for $c > c_0$ and $t > t_0 \equiv -\dot{k}_a^{-1}\ln\{1-c_0/c\}$

$$\mathcal{F}(c,t) = \mathcal{F}(0,t)\exp\left\{\dot{k}_\dagger\dot{k}_a^{-1}c(\exp\{-t_0\dot{k}_a\}-\exp\{-t\dot{k}_a\}) - \dot{k}_\dagger(c-c_0)(t-t_0)\right\}$$

$$\overset{\dot{k}_a \text{ small}}{=} \mathcal{F}(0,t)\exp\left\{-(t-t_0)^2\ddot{k}_\dagger c\right\} \tag{3.59}$$

$$\dot{R}(c,t) = \dot{R}(0,t)\exp\{-c_H^{-1}(c(1-\exp\{-t\dot{k}_a\})-c_0)_+\} \tag{3.60}$$

$$\overset{\dot{k}_a \text{ small}}{=} \dot{R}(0,t)\exp\{-c_H^{-1}(ct\dot{k}_a - c_0)_+\} \tag{3.61}$$

$$\dot{R}(c,t) = \dot{R}(0,t)\left(1+c_R^{-1}(c(1-\exp\{-t\dot{k}_a\})-c_0)_+\right)^{-1} \tag{3.62}$$

$$\overset{\dot{k}_a \text{ small}}{=} \dot{R}(0,t)\left(1+c_R^{-1}(ct\dot{k}_a - c_0)_+\right)^{-1} \tag{3.63}$$

The derivation of (3.59) is less straightforward, because our strategy to deal with environment-concentrations rather than tissue-concentrations works out a bit more complex here. We have to consider the limit $\dot{k}_a \to 0$, while the bioconcentration coefficient $P_{vd} \to \infty$, such that the uptake rate $[\dot{k}_{da}] \equiv P_{vd}\dot{k}_a$ remains fixed, and the killing rate $\dot{k}_\dagger \to \infty$, such that the 'killing acceleration' $\ddot{k}_\dagger \equiv \dot{k}_\dagger\dot{k}_a$ remains fixed as well. The tissue-concentration amounts to $[Q](t) = [\dot{k}_{da}]ct$ and the hazard rate to $\dot{h}_c = \ddot{k}_\dagger c(t-t_0)$. The time parameter t_0 in (3.59) has the interpretation $t_= [Q]_0([\dot{k}_{da}]c)^{-1}$, where $[Q]_0$ stands for the no-effect-tissue-concentration. This time parameter is thus inversely proportional to c.

Notice that the description of the effects have only two parameters if the elimination rate is small, because only $\dot{k}_a/c_H$, $\dot{k}_a/c_R$ and c_0/c_R, c_0/c_R can be estimated in (3.61) and (3.63). It will be hard to identify indirect effects on reproduction if the exposure time is short relative to the inverse of the von Bertalanffy growth rate.

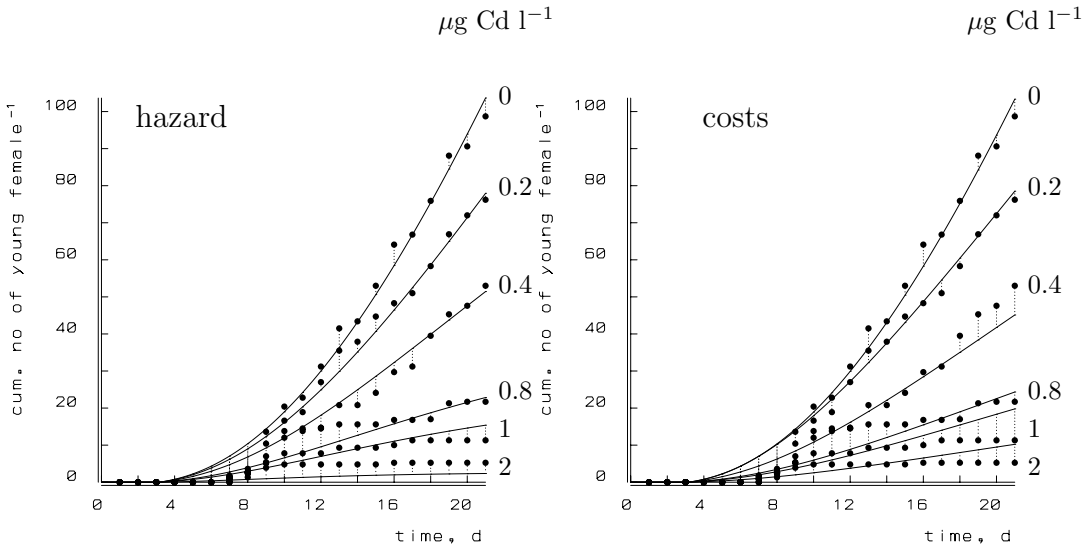


Figure 3.17: The mean cumulated number of young per female daphnid as a function of the exposure time to several concentrations of cadmium. The fitted curves represent least squares fits of the hazard (left) and the cost (right) model for effects on reproduction. Given an elimination rate of $\dot{k}_a = 0.05$ d^{-1}, the estimated parameters (and standard deviations) are $c_0 = 0.023(0.004)$ μg l^{-1}, $c_H = 0.166(0.006)$ μg l^{-1}, $\dot{R}_m = 13.1(0.2)$ d^{-1} and $c_0 = 0.047(0.002)$ μg l^{-1}, $c_R = 0.069(0.003)$ μg l^{-1}, $\dot{R}_m = 13.1(0.2)$ d^{-1} respectively.

Tests against experimental data

We give one example of a direct effect and two of indirect effects, all taken from a ring test that was organized by the OECD. We used the cumulative number of young per female, to facilitate the estimation of parameters and to improve the presentation of the results. The standard deviations are meant to be indicative. An analysis based on moulting cycles was not possible due to lack of observations of the moults.

Figure 3.17 shows the effects of cadmium on the reproduction of *D. magna*, with the least squares fit of the hazard and the cost model. Both models fit well, the hazard model perhaps slightly better. The no-effect concentrations of both models differ by a factor two, but in view of the fact that they represent just 11 and 23 % of the lowest tested concentration, the accuracy seems quite acceptable. The elimination rate could not be estimated from these data. Since no cadmium-induced delay of the onset of reproduction has been observed, we have no indications for indirect effects of cadmium on reproduction.

The second example (Figure 3.18) shows the effects of phenol on the reproduction of *D. magna*, with the least squares fit of the maintenance, growth and assimilation model. Direct effects can be excluded on the basis of a phenol-induced delay of the onset of reproduction. The three models fit well; the mean deviation between the observed number of young and model expectations are 5.7, 5.4 and 5.3 for effects

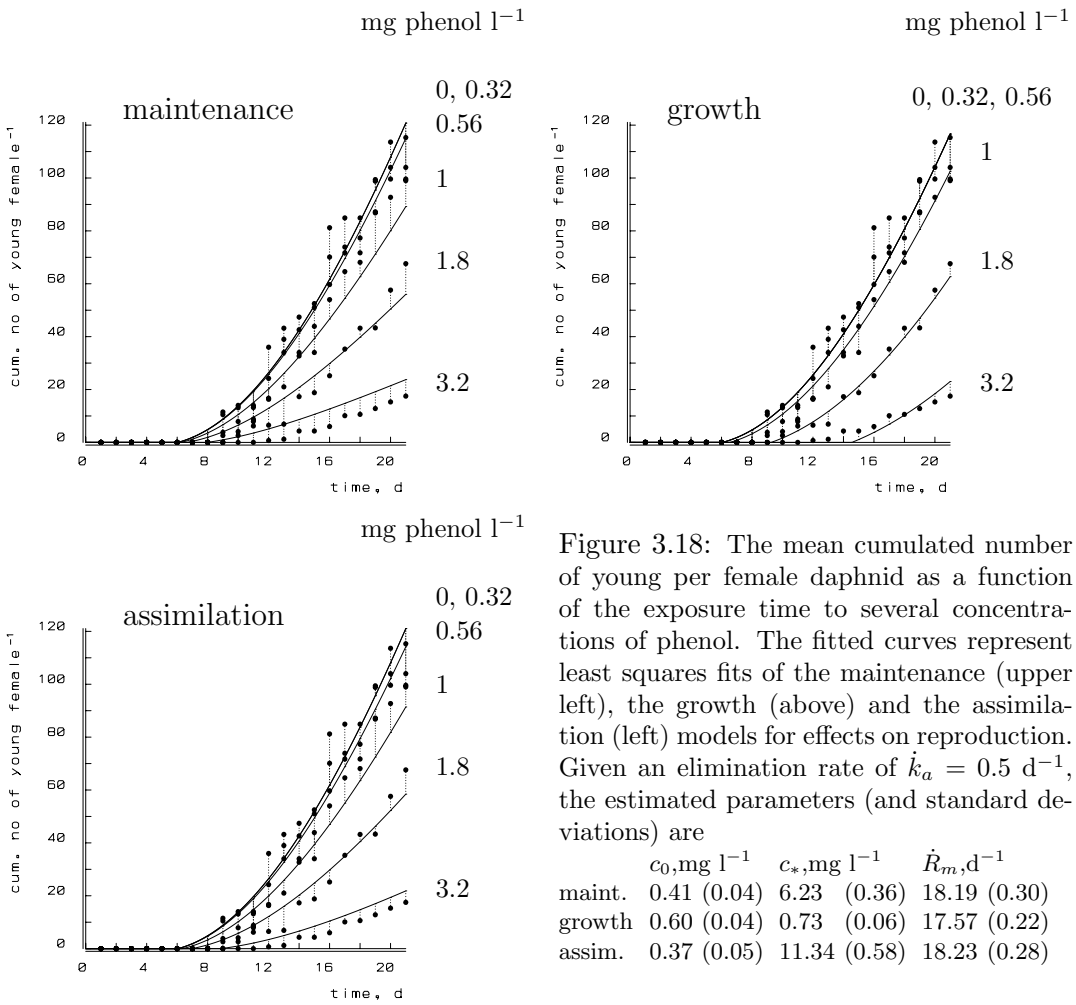


Figure 3.18: The mean cumulated number of young per female daphnid as a function of the exposure time to several concentrations of phenol. The fitted curves represent least squares fits of the maintenance (upper left), the growth (above) and the assimilation (left) models for effects on reproduction. Given an elimination rate of $\dot{k}_a = 0.5$ d^{-1}, the estimated parameters (and standard deviations) are

	c_0,mg l^{-1}	c_*,mg l^{-1}	$\dot{R}_m$,d^{-1}
maint.	0.41 (0.04)	6.23 (0.36)	18.19 (0.30)
growth	0.60 (0.04)	0.73 (0.06)	17.57 (0.22)
assim.	0.37 (0.05)	11.34 (0.58)	18.23 (0.28)

on maintenance, growth and assimilation, respectively. The no-effect concentration hardly depends on the type of effects. The elimination rate could not be estimated from these data. The onset of reproduction in the control is slightly later than usual, which has been taken into account by choosing $l_p = 0.55$.

The third example (Figure 3.19) shows the same for 3,4-dichloroaniline. The delay of the onset of reproduction points to an indirect effect. The three models fit well; the mean deviation between the observed number of young and model expectations are 5.0, 4.6 and 4.7 for effects on maintenance, growth and assimilation, respectively.

Standard statistical analyses

The statistical analysis of survival data is better standardized than that of reproduction data. Frequently applied survival and reproduction models are based on the

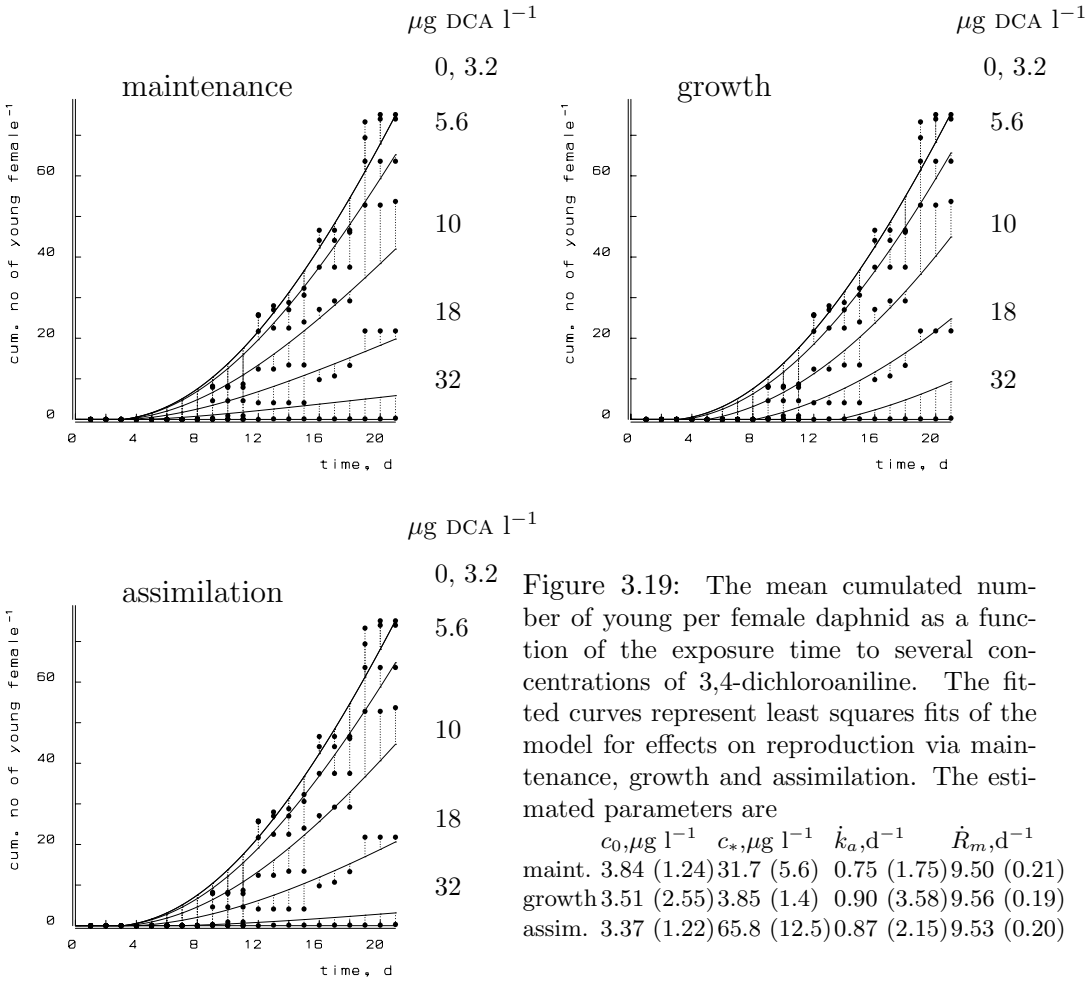


Figure 3.19: The mean cumulated number of young per female daphnid as a function of the exposure time to several concentrations of 3,4-dichloroaniline. The fitted curves represent least squares fits of the model for effects on reproduction via maintenance, growth and assimilation. The estimated parameters are

	c_0,μg l^{-1}	c_*,μg l^{-1}	$\dot{k}_a$,d^{-1}	$\dot{R}_m$,d^{-1}
maint.	3.84 (1.24)	31.7 (5.6)	0.75 (1.75)	9.50 (0.21)
growth	3.51 (2.55)	3.85 (1.4)	0.90 (3.58)	9.56 (0.19)
assim.	3.37 (1.22)	65.8 (12.5)	0.87 (2.15)	9.53 (0.20)

log-logistic function

$$q(c,t) = \left(1+\left(\frac{c}{c_{L50}(t)}\right)^{1/\beta}\right)^{-1} \tag{3.64}$$

$$N(c) = N(0)\left(1+\left(\frac{c}{c_{E50}}\right)^{1/\beta_R}\right)^{-1} \tag{3.65}$$

where c_{L50} denotes the LC50, i.e. the concentration at which the survival probability is half that in the control, β is the gradient of the concentration-response function, $N(c) \equiv \int_0^{21} \dot{R}(c,t)\,dt$ is the expected cumulative number of offspring at 21 d, c_{E50} is the 50% effect concentration (usually known as EC50) and β_R is the gradient parameter for reproduction. Kooijman (1981) tied the $c_{L50}(t)$ parameter to the

tissue-concentration, which leads for a one-compartment uptake/elimination kinetics to $c_{L50}(t) = c_{L50.\infty}(1 - \exp\{-t\dot{k}_a\})^{-1}$. The survival probability in the control is described by $\mathcal{F}(0,t) = \exp\{-t\dot{h}_0\}$. The estimation of LC50 values has been discussed by Hoekstra (1993).

Independent of and inconsistent with these models, the no-observed-effect concentration (NOEC) for survival and reproduction is estimated, which is the highest tested concentration that does not differ significantly from the control. Extensions of the standard log-logistic model to include a no-effect concentration as a parameter have also been proposed (Kooijman 1981). The motivation was to solve the problem of making an error of the second kind by not rejecting the null-hypothesis that the compound in the tested concentration has no effect, while in fact it does have an effect. The consequence of such an error is that sloppy test procedures lead to high no-effect concentrations, a most unfortunate coupling of properties.

The parameters to be estimated are $\dot{h}_0$, $\dot{k}_a$, $c_{L50.\infty}$, β and c_0 for survival and $N(0)$, c_{E50}, β_R and c_0 for reproduction.

These standard models are primarily descriptive rather than mechanistic. Kooijman (1993) gives arguments why interpretations of the log-logistic response models in terms of scatter distributions of threshold values are weak for effects on survival. The arguments do not seem to apply to effects on reproduction, because we can easily observe that the reproduction rate is affected for each individual. Effects on reproduction of a cohort of individuals cannot be understood in terms of affected and unaffected individuals, which is inherent to the concept of threshold value.

The gradient parameters do not have a useful function. In fact, they complicate the estimation of the no-effect concentrations considerably, because a free gradient parameter reduces the information content of an observed effect at a particular concentration to "the no-effect concentration must be lower than this particular concentration". If the description of effects does not include a free gradient parameter, such as in the present study, observed effects do contain information about the value of the no-effect concentration. The use of the gradient parameter to calculate small-effect concentrations is of limited value, because such values depend sensitively on the detailed shape of the tail of the sigmoid response function. This is problematic because a sigmoid response function only has an empirical basis, with hardly any support for the detailed shape of the tails. It is almost impossible to justify a particular choice empirically. Practice teaches that it is possible to estimate a positive no-effect concentration as a parameter built into the log-logistic model in about half of the results from typical toxicity tests, and in about one out of ten cases it differs statistically from zero. Consequently, it is of limited practical value.

Discussion and conclusions

We assumed that the food density is constant, so that the reserve density is also constant. This, of course, only holds if the animal is 'in equilibrium' with this food

availability. If food density changes, or if there is no food at all, we have to account for the change in lipid content of the animal because the uptake/elimination behaviour can be rather sensitive to such changes. We also need reserves to model elimination via reproduction, since freshly produced eggs consist almost exclusively of reserves. The details of effects of changes in lipid content have been worked out (Kooijman & van Haren 1990, Kooijman 1993). Because the present description of effects of compounds is based on the tissue-concentrations, variations in time can be taken into account. Such variations include metabolic transformation of the compound.

Sometimes a stimulation of reproduction is observed at low concentration of compounds that reduce reproduction at higher concentrations. This poorly understood phenomenon is called hormesis. Sometimes it is present when food is supplied in large amounts, but absent when food is supplied in moderate amounts. This indicates that secondary stress is involved, at least in some cases. One source might be polysaccharides that some green alga (*Chlorella*) produce when they occur in high densities and/or bacteria that decompose cells in poor conditions (algae are usually kept in stock in a concentrated form for some time, which does not do their condition any good, and test media are less suitable for algal growth). Because of these uncertainties, hormesis is not incorporated in the model analysis and we believe that it can be avoided by optimizing test conditions, at least in some cases.

The advantages of the proposed analysis over the standard method are

- The basis is much more mechanistic, which facilitates
    - ◦ the link with physico-chemical properties of the compounds
    - ◦ the comparison with data from other toxicity tests (such as the survival or growth tests of fish larvae), effects of differences in exposure times, body size, etc.
    - ◦ the construction of a series of consistent models, ranging from simple ones for routine applications to advanced ones for research purposes. It is, for instance, straightforward to deal with compounds that disappear from the medium, due to degradation. We first have to model the disappearance process, by a first order process for instance, and insert the result in (3.43). This is all we have to do to arrive the correct effect-characteristics.
    - ◦ the incorporation of additional information, such as the body length at 21 d. Such extra information helps to identify the mode of action of the compound (i.e. to choose between the indirect effect models) and to reduce the confidence intervals for the parameters that have to be estimated.
- The models do not have gradient parameters. This solves the estimation problems of the no-effect concentrations (see section 'standard statistical analysis'). These no-effect concentrations are now integrated in the model as a parameter that can assume values for concentrations that have not been tested (as

opposed to NOEC's). The reduction of the number of parameters that have to be estimated simplifies the estimation procedure and reduces the confidence intervals.

- The parameters (no-effect concentration, killing rate and tolerance concentration) are independent of the exposure time. Notice that the standard parameters (NOEC, LC50, EC50, gradients) do depend on the exposure time, which reduces the usefulness in risk assessment and the comparison with other toxicity data.

- The present characterization of the effects on survival and reproduction is fully process-oriented, which means that

  ◦ effects are analyzed as a response surface above the exposure time-concentration plane. This allows the calculation of other characteristics, such as the EC50 and gradient values.

  ◦ effects can be translated into effects on the population growth rate. This is not possible for the standard method because reproduction is not treated as a process, so that a delay in reproduction cannot be distinguished from a reduction of the reproduction rate.

Although the various pathways to affect reproduction might be hard to distinguish from reproduction data, the consequences for the population level can differ considerably (Kooijman & Metz 1983). The reason is that effects on maintenance are strongly felt when the population is at its carrying capacity and almost all of the energy budget is spent on maintenance, but when the population is in a productive phase, these effects are hardly felt because maintenance takes only a small part of the energy budget.

The population growth rate $\dot{\mu}$ can be obtained from the survival probability $\mathcal{F}(a)$ at age a and the reproduction rate $\dot{R}(a)$ in constant environments via the characteristic equation

$$1 = \int_0^\infty \exp\{-\dot{\mu}t\}\mathcal{F}(t)\dot{R}(t)\,dt$$

The aging process can become important for the survival probability at high ages (see the section on the hazard model).

If the environment is not constant, we have to fall back to the theory of structured population dynamics to evaluate the population consequences of the effects of compounds on survival and reproduction. See Metz & Diekmann (1986) for an introduction.

The comparison of the toxicity of compounds with different effects on reproduction is rather complex. One possibility is to compare the population growth rate as a function of the concentration, as obtained from $1 = \int_0^\infty \exp\{-\dot{\mu}t\}\dot{R}(t)\,dt$. This has

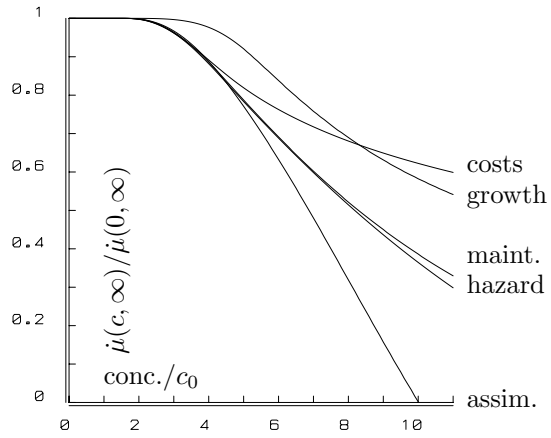


Figure 3.20: The population growth rate as a function of the environment-concentration for compounds that have direct and indirect effects on reproduction. The parameter values are the same as in Figure 3.16.

the advantage that delays in the onset of reproduction are taken into account and that it better links up with the translation of observed effects on individual reproduction into the laboratory to population consequences outdoors. Figure 3.20 illustrates how the population growth rate depends on the environment-concentration for the various modes of action of the compound.

The present description requires the application of rather advanced numerical techniques. This practical handicap can be eliminated by application of software package DEBtox. The rapid increase of the performance of low-cost personal computers minimizes this problem of application.

The existence of large data bases of LC50/EC50 values will doubtlessly delay the implementation of any improvement on the standard description of effects. For compatibility reasons with these data bases, it might be interesting to calculate LC50 and EC50 time curves from the present description. This can be done by numerically solving the equations $q(c_{L50},t) = \frac{1}{2}$ and $\dot{R}(c_{E50},t) = \dot{R}(0,t)/2$. Although the present description has fewer parameters than the standard one, it is possible to obtain the standard parameters from the present one, but it is not possible to obtain the new parameters from the standard ones. The original survival/reproduction data are required to obtain them.

We conclude that the present models for the analysis of data from reproduction toxicity tests have substantial advantages over the standard ones and merit implementation on a routine basis. The choice of the proper model depends on the mode of action of the compound; a decision that is up to the user. The observation that the no-effect concentration seems to be insensitive to this choice is encouraging.

Acknowledgements

This study has been partially supported by Grant PAD 90-18 from the Alternative to Animal Experiments Platform and by the OECD. The OECD made the toxicity

data available to us. We like to thank Cor Zonneveld, Wout Slob, Mike Newman, an unknown referee and the participants of the OECD meeting of march 27 in Sheffield for helpful comments and discussions and Annemarie Pielaat for assistance with the computations.

References

Bedaux, J.J.M. & Kooijman, S.A.L.M. 1994. Statistical analysis of bioassays, based on hazard modeling. *J. Envir. Stat.* **1**: 303-314.

Burden, R.L. & Faires, J.D. 1983. *Numerical analysis.* Boston: Prindle, Weber & Schmidt, pp 676.

Carroll, R.J., Ruppert, D. & Stefanski, L.A. 1995 *Measurement error in nonlinear models.* Monographs on Statistics and Applied Probability 63, London: Chapman & Hall, pp 305.

Hawker, D.W. & Connell, D.W. 1986. Bioconcentration of lipophilic compounds by some aquatic organisms. *Ecotox. Envir. Safety* **11**: 184-197.

Hoekstra, J.A. 1993. *Statistics in ecotoxicology.* PhD-thesis Vrije Universiteit, Amsterdam.

Kooijman, S.A.L.M. 1981. Parametric analyses of mortality rates in bioassays. *Water Res.* **15**: 107-119.

Kooijman, S.A.L.M. 1983. Statistical aspects of the determination of mortality rates in bioassays. *Water Res.* **17**: 749-759.

Kooijman, S.A.L.M. 1993. *Dynamic Energy Budgets in Biological Systems. Theory and applications in ecotoxicology.* Cambridge University Press, pp 350.

Kooijman, S.A.L.M. 1995. The stoichiometry of animal energetics. *J. Thoer. Biol* **177**: 139-149.

Kooijman, S.A.L.M. & Bedaux, J.J.M. 1996. Some statistical properties of estimates of no effect levels. *Water Research* (to appear)

Kooijman, S.A.L.M. & Haren, R.J.F.van 1990. Animal energy budgets affect the kinetics of xenobiotics. *Chemosphere* **21**: 681-693.

Kooijman, S.A.L.M. & Metz, J.A.J. 1983. On the dynamics of chemically stressed populations; The deduction of population consequences from effects on individuals. *Ecotox. Envir. Safety* **8**: 254-274.

McCullagh,P. & Nelder, J.A.1989 *Generalized linear models.* Monographs on Statistics and Applied Probability 37, London: Chapman & Hall, pp 511.

Metz, J.A.J. & Diekmann, O. (eds). 1986. *The dynamics of physiologically structured populations.* Springer Lecture Notes in Biomathematics. Springer-Verlag, Berlin.

OECD 1994. Draft OECD test guideline 202 part II *Daphnia magna* reproduction test to be used in the final ring test.

Silvey, S.D. 1975. *Statistical inference.* Monographs on applied probability and statistics. Chapman and Hall, pp 192.

3.5 No-effect concentrations in algal growth inhibition tests

Abstract We propose three simple models for effects of chemical compounds on the growth of batch cultures of algae that allow the estimation of the no-effect concentration. The growth model assumes that the costs for growth is proportional to the concentration that exceeds the no-effect level. The hazard model assumes that the hazard rate is proportional to the concentration that exceeds the no-effect level. The adaptation model is similar to the hazard model, but the effects only occur at the start. The no-effect concentrations of the three models turn out to be very similar.

Introduction

The effects of chemical compounds on aquatic biological systems are tested routinely with a set of simple toxicity tests, in which groups of individuals of a single species, usually originating from a laboratory culture, are exposed to a set of concentrations of a chemical during some standardized period. The No-Observed Effect Concentration (NOEC) is defined as the highest tested concentration that gives no significant deviation from a control without the chemical (Bartlett et al. 1974, Bringmann & Kühn 1980). The usefulness of this frequently used statistic suffers from lack of knowledge about the power of the statistical test that is used. This power also depends, of course, on the probability of an error of the first kind (usually taken to be 5%), which is rather arbitrary. In addition, the NOEC is highly dependent on the test design since it can only assume values of tested concentrations. A compromise exists between the number of different concentrations that are used in the test and the number of replicates per concentration. Precision increases with the number of different concentrations and the power increases with the number of replicas. In the environmental risk assessment of chemicals, the prediction of environmental no-effect concentrations relies heavily on laboratory test derived NOEC values. However, most standard ecotoxicity tests were originally designed for determining EC50 values.

In an attempt to address the problems inherent to the NOEC, it is current practice to derive PNECs (Predicted Environmental No-Effect Concentrations) from EC50 data using a scheme of fixed application factors. The draft March 1995 version of the EU technical guidance document for environmental risk assessment of new and existing substances suggests factors of 10, 50, 100 or 1000, depending on the amount and quality of the data available. Because dose-response slopes can be very compound-specific, some workers proposed EC5, EC10 or other "small"-effect values. Such an approach is difficult to apply to risk assessment due to lack of consensus about the precise definition of "small". The smaller the effect size in descriptive models, the larger the confidence interval and the more the estimate becomes dependent on the

[0]Published as: S.A.L.M. Kooijman, A.O. Hanstveit, N. Nyholm (1996) No-effect concentrations in algal growth inhibition tests. *Water Research* **30**: 1625–1632

specific model that has been used to describe the results. Since the empirical log-logistic model does not have a scientific basis, this is quite an obstacle. This problem becomes less important for the larger effect sizes, such as the EC15 or EC20. The problem then becomes how "small" effects in the laboratory translate into effects outdoors and how the effects of emissions of various compounds in a certain area combine when each is allowed to have a "small" effect.

In this paper we discuss the applicability of a No-Effect Concentration (NEC) in algal growth inhibition tests. There are at least four internationally accepted standard test descriptions, i.e. the nearly identical OECD guideline 201 (OECD 1984), the International Standard ISO 8692 (ISO 1989) and the EU Guideline C3 (EU 1992) covering the freshwater environment, and the International Standard ISO 10253 (ISO 1994) covering the marine environment. The assessment of the NOEC value in these methods is, for lack of better methods, only superficially defined, and it is mostly left to the judgement of the particular scientist whether a standard statistical test should be applied or not. The weakness of this approach has been recognized by the working groups developing these methods. The NOEC value will depend on the variation among replicate test vessels and the deviations of threated algal populations from the control. Relatively high NOEC values may therefore result from a badly performed test. The NEC does not suffer from the statistical problems of the NOEC because the null hypothesis is that the NEC equals zero. A poor power results in an inability to reject the null hypothesis and leads to the conclusion that the tested compound requires further research.

The present paper is one of a series (Bedaux & Kooijman 1994, Kooijman & Bedaux 1996, 1996a) that deals with similar NECs in the other OECD toxicity test methods. All models behind these analyses assume that the effect size on the various physiological target processes is proportional to the concentration of compound that exceeds a no-effect concentration in the organisms. The Dynamic Energy Budgets (DEB) theory is used to identify the target processes. This theory is described in Kooijman (1993). The choice of a linear relationship between effect size and the tissue-concentration relates to the idea of a Taylor-approximation to the 'real' effect size. So the actual effect size might be a complex function of the tissue-concentration, but we use only the first term of its Taylor approximation. For highly non-linear relationships, this only works for small effect sizes. The inclusion of more terms of the Taylor-approximation hardly makes sense in the light of the idea that physiological processes can be ordered with respect to sensitivity for a particular compound. At low concentrations only the most sensitive process is affected, but at high concentrations many processes are affected. It will be very difficult to make reliable models for large effects. Since risk assessment requires knowledge of small effects, not of large effects, the situation that large effects are possibly not well captured by model predictions is hardly relevant.

To simplify the reasoning, we assume that the elimination rate is small with respect to the inverse of the interdivision interval, so the intra-cellular concentration

is almost instantaneously in equilibrium with the environment-concentration, which makes sense for minute algal cells. As long as the aqueous bioavailable concentration of the test compound remains approximately constant and is not reduced by sorption or other elimination mechanisms, the toxic dose can be regarded as constant throughout the test. So the focus is on population growth at constant environment-concentrations of test compound. Irrespective of the physiological complexity of the cell cycle, the fact the daughter cells repeat the physiological behaviour of the mother cell implies that the population will grow exponentially in cell numbers as long as the environment is constant.

Models

A summary of the algal growth inhibition test according to the standard test methods is as follows: Batch cultures of one of the recommended algal species (i.e. the fresh water green alga *Selenastrum capricornutum* or *Scenedesmus subspicatus*, or the marine diatom *Skeletonema costatum* or *Phaeodactylum tricornutum*) are started with a fixed cell density of 10^4 cells ml^{-1} in the prescribed media with different additions of the test compound, usually below the solubility in water. Although the guideline prescribes concentrations in terms of effect sizes, these choices are less important for the analyses that we propose here. Temperature and light intensity are constant during exposure, but precise temperature and light intensity are not prescribed, only an allowable range. The test has been designed so that ideally exponential growth in the control cultures can be sustained for the entire duration of the test, which has been fixed at 72 hours. It is specified as a validity criterion that the growth rate must be high enough to allow the biomass in the control cultures to increase by at least a factor 16, which corresponds to a minimum growth rate of 0.92 d^{-1}. The growth rates normally obtained for the standard test species *S. capricornutum* and *S. subspicatus* may vary from about 1.2 to 2.0 d^{-1}, increasing with light intensity and temperature (Hanstveit 1982, 1991). The mean growth rates for *S. costatum* and *P. tricornutum* are 2.40 d^{-1} and 1.73 d^{-1}, respectively, determined in an international ring test (Hanstveit 1991). The corresponding biomass increase during 3 days amounts to a factor 37 and 400, respectively. In practice a 72 h exponential growth is only achieved with *S. capricornutum* and *P. tricornutum*. The other species grow approximately logistically, due to the large cell volume of *S. subspicatus* (resulting in a large inoculated biomass) or to the high growth rate of *S. costatum*. The guidelines, however, allow for the use of the exponential growth phase for the evaluation of effects. The biomass is usually measured by electronic particle counting, by spectrophotometry (optical density) or by fluorometry (in vivo chlorophyll fluorescence), which implies that both the living and dead cells contribute (dead cells somewhat less than living cells with fluorometry). Formally, the biomass (i.e. dry weight or total cell volume) is the proper measure for the algal growth. The cell density (i.e. cell numbers per volume) may be used as long as it relates to the biomass (which is

not the case when cells form chains).

We assume here that the cell number in the control grows exponentially, that is

$$\begin{aligned} \frac{d}{dt}N &= \dot{\mu}_0 N \\ N(t) &= N(0)\exp\{\dot{\mu}_0 t\} \end{aligned} \tag{3.66}$$

where $\dot{\mu}_0$ is the control population growth rate (dimension time^{-1}). We consider three different effects.

Growth model

The costs of growth in terms of nutrients or energy is inversely proportional to the population growth rate. The linear effect model as mentioned in the introduction amounts to the assumption that the costs for growth are linear in the intracellular concentration of test compound. For tiny organisms such as unicellular algae, these costs are thus linear in the environment-concentration. This leads to

$$\begin{aligned} \frac{d}{dt}N &= \dot{\mu}_c N \\ N(t,c) &= N(0,c)\exp\{\dot{\mu}_c t\} \quad \text{with} \quad \dot{\mu}_c = \dot{\mu}_0\left(1 + c_G^{-1}(c-c_0)_+\right)^{-1} \end{aligned} \tag{3.67}$$

where c is the concentration of test compound in the environment, c_0 is the NEC and c_G is the 'tolerance concentration' which just serves as a proportionality constant. It is called this because the less toxic the compound, the higher its value. Note that an interpretation of this parameter is $c_G = \text{EC50} - \text{NEC}$, where the EC50 is the concentration that causes a reduction of the population growth rate by a factor of two. The concept EC50 is very familiar in the analysis of toxicity tests. Despite the simple relationship with the tolerance concentration, we will not use the parameter EC50. The first reason is that the combination EC50 and NEC behaves worse than the combination c_G and c_0 in a statistical sense, because their estimates have a higher (negative) correlation coefficient. The second reason is that the relationship between EC50 and the tolerance concentration is less simple in other models (see the adaptation model). The notation $(c-c_0)_+$ indicates that we replace negative values of $c - c_0$ by zero. We assume that no death occurs if the compound affects the energetics of the cells.

Hazard model

The second mechanism of toxic effect is via the hazard rate that is assumed to be proportional to the intra-cellular concentration that exceeds the no-effect concentration. The surviving cells grow at the same rate as those in the control. The change

in the numbers of living and dead cells becomes

$$\begin{aligned}\frac{d}{dt}N_1 &= \dot{\mu}_c N_1 = (\dot{\mu}_0 - \dot{k}_\dagger(c-c_0)_+)N_1 \\ \frac{d}{dt}N_0 &= \dot{k}_\dagger(c-c_0)_+ N_1\end{aligned}$$

where we have no dead cells at the start of the experiment, so $N_0(0) = 0$. The parameter $\dot{k}_\dagger$ just serves as a proportionality constant and is called the 'killing rate'. The total (living plus dead) number of cells amounts to

$$N(t,c) = N(0,c)\left(\frac{\dot{\mu}_0}{\dot{\mu}_c}\exp\{\dot{\mu}_c t\} + 1 - \frac{\dot{\mu}_0}{\dot{\mu}_c}\right) \tag{3.68}$$

Ignoring cell lysis during the 3 days of the test, the counted number of cells corresponds with the total number of cells. Note that the total number of cells does not grow exponentially if effects on the hazard rate occur.

Adaptation model

The third mechanism of toxic effects is via the change from the control situation of the stock culture to the experimental test condition. The effect is the same as in the hazard model, but it occurs only during a short (fixed) period of exposure. If the cells survive this transition, they are not affected by the compound, so the resistant cells are selected. The survival probability then amounts to $\mathcal{F} = \exp\{-c_H^{-1}(c-c_0)_+\}$ and the total (living plus dead) number of cells to

$$N(t,c) = N(0,c)\left(\mathcal{F}\exp\{\dot{\mu}_0 t\} + 1 - \mathcal{F}\right) \tag{3.69}$$

where 'tolerance concentration' c_H just serves as proportionality constant. It is inverse to the product of the killing rate and the length of the sensitive period. The survival probability $\mathcal{F}$ can be interpreted as the fraction of resistant cells in the control culture.

Statistics

The number of cells in any experimental unit is assumed to be normally distributed with a mean that behaves as explained in the model section and a variance that is (approximately) proportional to the squared mean. So the coefficient of variation is assumed to be constant. This depends, however, on the accuracy and the method of measurement of the biomass. A constant variance, independent of the mean, is an attractive alternative. The most straightforward estimation criterion is the maximum likelihood method. To find the parameter estimates, we have to evaluate the matrix of second derivatives of the cell numbers to the three parameters: $\dot{\mu}_0$, c_0 and c_G, c_H or $\dot{k}_\dagger$. Since the formulae become lengthy, a less elegant but useful alternative

is to apply weighted non-linear regression, where the weight coefficients are taken to be inversely proportional to the squared observed cell numbers. This gives the additional advantage that we can give less weights to cultures that show a large effect. The applicability of the likelihood method should be tested, however, if the weight coefficients affect the results substantially. If the (control) cultures grow too fast (depending of the algal species), the last data point will show a deviation from exponential growth because the cultures become nutrient limited. This will happen if the light intensity and temperature are both approaching the upper limits of the prescribed ranges. The weight of such a deviating data point can also be reduced for biological reasons. (One should always be extremely careful not to exclude data points because the model does not fit.)

The profile ln likelihood (cf McCullagh & Nelder 1989) for c_0 is

$$l(c_0) = \sum_{ij} w_{ij} \ln \hat{\sigma}_0 / \hat{\sigma}_1$$

where $\hat{\sigma}_0$ stands for the estimated standard deviation, given the value for c_0, and $\hat{\sigma}_1$ stands for the estimated standard deviation given the maximum likelihood estimation of c_0. The estimated variance, i.e. the squared standard deviation, is $\hat{\sigma}^2 = \sum_{ij}^{-1} w_{ij} \sum_{ij} w_{ij}(N_{ij} - N(t_i, c_j))^2$. The factor $\sum_{ij} w_{ij}$ stands for the sum of all weight coefficients, where the summation is over all time points and concentrations, including the control. The confidence set for c_0 can be obtained from the profile ln likelihood, where we use the property that two times the profile ln likelihood at any given value for c_0, under the null hypothesis that this is the correct value, is asymptotically χ^2 distributed with one degree of freedom. The α-level confidence set for c_0 is then given by $\{c_0 | l(c_0) \leq \chi_1^2(\alpha)/2\}$, where $\chi_1^2(\alpha)$ is a number such that $\int_0^{\chi_1^2(\alpha)/4} (\pi x)^{-1/2} \exp\{-x\}\, dx = \alpha$ (cf Silvey 1975, Kooijman 1983).

Examples

Figure 3.21 shows the experimental results of 14 algal growth inhibition tests for a variety of compounds and algal species. The parameters of the three models are given in Table 3.14.

The 99% confidence interval for the no-effect concentration c_0 is approximately the point estimate plus and minus 2.56 times the standard deviation. So for the growth model of the test with TPBS we obtain a confidence interval for c_0 of $\{6.86, 8.77\}$ mg l^{-1} (see Table 3.14). A χ^2-distributed variable with one degree of freedom exceeds the value 6.635 with a probability of 1%. The 99% confidence interval can be read from Figure 3.21 by looking at the values of c_0 for which the profile ln likelihood is below 6.635/2=3.317. This gives a very similar confidence interval, i.e. $\{6.75, 8.7\}$ mg l^{-1}. This illustrates the applicability of the large sample theory for the likelihood ratio test: the shape of the ln likelihood function is in most cases perfectly parabolic. In two other cases, we see that the profile ln likelihood

function has two local minimums and, in the test with ethoxylated alcohol, we see odd behaviour of this function due to the stimulatory effect of the compound at low concentrations, which is usually referred to as hormesis (Stebbing 1982). This little understood phenomenon must be left unexplained here.

In many cases the three models all fit well to the same data; they would be hard to tell apart graphically. The no-effect concentration proves to be very insensitive to the choice of model. (This is in contrast with EC-small values, see Introduction.) In a few cases the mean deviation $\hat{\sigma}$ differs by a factor two between the models; here we can choose between the different modes of action of the compound on the basis of goodness of fit. The maximum deviation occurs in the test for potassium dichromate with *Cyclotella*. The fact that the c_0 differs here by a factor of three is no problem because we should select the value of the best fitting model. The NEC differs significantly from zero in 10 out of 14 cases. The cases where the NEC does not differ from zero indicate that the experiment should be repeated with an adjusted concentration series.

Discussion

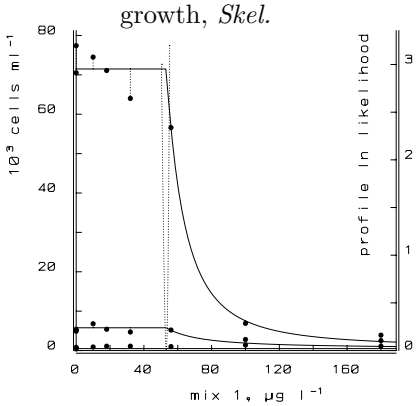
Our method shows that simple mechanistic models can be used successfully to describe the results of algae inhibition growth tests. It has fewer parameters than the standard analysis, which relates the population growth rate to the concentration of compound according to the log-logistic model (cf Kooijman et al. 1983). Independent of and consistent with this model, a no-observed effect concentration (NOEC) is usually identified for risk assessment purposes. This implies that four rather than three parameters are usually estimated: the control population growth rate, the EC50, the gradient parameter, and the NOEC. The proposed method replaces the EC50 plus gradient parameter by either the tolerance concentration or the killing rate, and the NOEC by the NEC. The latter is now a real parameter with a confidence interval, not just one of the tested concentrations.

Our method avoids the complexities inherent to NOEC and EC-"small" values.

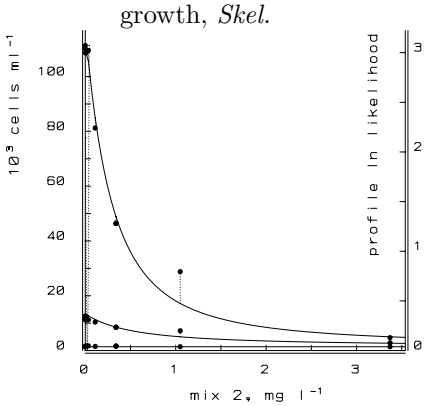
Table 3.14: Parameter estimates and standard deviations of the examples given in Figure 3.21. The three rows for each compound/species combination correspond with the growth, hazard and adaptation model, respectively. The units of the parameters are: N_0, $\hat{\sigma}$: units of cells density (given for each example); $\dot{\mu}$: d^{-1}; c_0, c_G, c_H: units of compound concentration (given for each example); $\dot{k}_\dagger$: (units of compound conc. $\times$ d)$^{-1}$ Compounds: 3,5-DCP = 3,5-dichlorophenol, TPBS = Tetrapropylenebenzene sulphonate, PEA = polyethylene amine, SDE = substituted diphenoxyethane, EA = ethoxylated alcohol, Mix 1,2 = mixture of organic N,S-compounds, Mix 3 = mixture of nonionic surfactants. Species: *Cycl.* = *Cyclotella operculata*, *Phae.* = *Phaeodactylum tricornutum*, *Skel.* = *Skeletonema costatum*, *Nitz.* = *Nitzschia palea*, *Step.* = *Stephanodiscus hantzschii*, *Scen.* = *Scenedesmus subspicatus*, *Sele.* = *Selenastrum capricornutum*.

comp/spec.	N_0	s.d.	$\dot{\mu}_0$	s.d.	c_0	s.d.	$c_G/\dot{k}_\dagger/c_H$	s.d.	$\hat{\sigma}$
PEA, mg l^{-1}	1.04	0.273	1.93	0.095	0.0191	0.021	1.93	0.222	6.95
Skel., 10^3 cells ml^{-1}	1.10	0.212	1.91	0.070	0	0.020	0.943	0.062	5.32
	1.42	0.449	1.78	0.115	0.146	0.055	0.390	0.070	8.92
3,5-DCP, mg l^{-1}	0.766	0.352	2.88	0.267	0	0.091	3.75	0.880	4.49
Nitz., 10^3cells ml^{-1}	0.768	0.393	2.86	0.298	0	0.102	0.731	0.137	5.17
	0.910	0.496	2.76	0.316	0	0.106	1.05	0.186	5.40
3,5-DCP, mg l^{-1}	0.646	0.312	2.89	0.261	0.167	0.050	5.12	0.798	4.66
Cycl., 10^3cells ml^{-1}	0.824	0.239	2.76	0.157	0.113	0.043	0.547	0.036	3.29
	1.11	0.304	2.60	0.149	0.101	0.043	1.27	0.077	3.23
$K_2Cr_2O_7$, mg l^{-1}	1.10	0.238	1.44	0.123	0	0.355	11.7	2.85	1.50
Phae., 10^2cells ml^{-1}	1.09	0.218	1.44	0.106	0	0.421	0.138	0.026	1.43
	1.29	0.232	1.35	0.094	0	0.423	5.45	0.914	1.37
$K_2Cr_2O_7$, mg l^{-1}	4.355	0.337	1.26	0.027	3.48	0.237	26.1	1.85	3.68
Phae., cell ml^{-1}	4.30	0.375	1.26	0.030	2.44	0.413	0.0418	$2.8\ 10^{-3}$	4.25
	5.24	0.548	1.19	0.036	1.80	0.597	12.5	0.930	5.29
$K_2Cr_2O_7$, mg l^{-2}	5.66	0.685	2.10	0.063	0.777	0.031	3.46	0.307	7.21
Skel., 10^3 cells ml^{-1}	5.67	0.573	2.10	0.053	0.710	0.036	0.553	0.0311	6.17
	6.59	0.511	2.022	0.040	0.683	0.031	1.33	0.0547	4.90
$K_2Cr_2O_7$, μg l^{-1}	0.800	0.372	2.72	0.254	24.8	4.83	59.9	40.2	5.16
Cycl., 10^3cells ml^{-1}	0.739	0.151	2.78	0.112	10.5	1.17	0.0200	$1.6\ 10^{-3}$	2.23
	0.851	0.123	2.71	0.079	7.96	0.57	38.1	1.51	1.61
SDE, mg l^{-1}	0.980	0.195	1.43	0.069	0.410	0.055	2.13	0.435	3.21
Sele., 10^4 cells ml^{-1}	0.962	0.184	1.43	0.067	0.364	0.066	0.637	0.106	3.11
	1.057	0.199	1.40	0.065	0.295	0.040	0.823	0.092	3.09
Bioban, μg l^{-1}	0.336	0.087	2.72	0.140	16.1	1.43	203	25.0	1.46
Skel., 10^3 cells ml^{-1}	0.341	0.093	2.71	0.147	14.8	1.88	0.0132	$1.4\ 10^{-3}$	1.55
	0.369	0.105	2.67	0.154	14.4	2.03	52.9	5.31	1.61
TPBS, mg l^{-1}	0.655	0.036	0.481	$8.6\ 10^{-3}$	7.82	0.365	36.2	3.07	0.412
Step., 10^4cells ml^{-1}	0.633	0.037	0.487	$9.3\ 10^{-3}$	6.78	0.559	0.0123	$8.3\ 10^{-4}$	0.447
	0.696	0.038	0.472	$8.5\ 10^{-3}$	6.08	0.610	20.5	1.11	0.416
EA, mg l^{-1}	2.68	0.581	2.01	0.104	0.923	0.032	3.45	0.413	5.18
Skel., 10^3 cells ml^{-1}	2.88	0.403	1.97	0.067	0.895	0.029	0.566	0.0334	3.58
	3.46	0.427	1.88	0.059	0.886	0.029	1.16	0.0575	3.29
Mix 1, μg l^{-1}	0.494	0.147	2.62	0.157	53.2	0.87	56.7	15.2	2.17
Skel., 10^3 cells ml^{-1}	0.495	0.148	2.62	0.158	51.5	1.16	0.0346	$6.1\ 10^{-3}$	2.21
	0.514	0.160	2.60	0.165	51.1	1.25	20.7	3.34	2.28
Mix 2, mg l^{-1}	1.59	0.131	2.07	0.042	0	$1.3\ 10^{-3}$	0.363	0.018	1.35
Skel., 10^3 cells ml^{-1}	1.61	0.229	2.06	0.073	0	$2.8\ 10^{-3}$	6.09	0.435	2.39
	1.78	0.308	2.01	0.088	0	$3.5\ 10^{-3}$	0.115	9.41	2.88
Mix 3, mg l^{-1}	1.39	0.201	2.10	0.070	0.0228	$8.0\ 10^{-3}$	1.39	0.101	2.51
Skel., 10^3 cells ml^{-1}	1.36	0.278	2.11	0.099	0.0075	0.012	1.508	0.109	3.58
	1.50	0.351	2.06	0.112	0	0.015	0.435	0.032	4.02

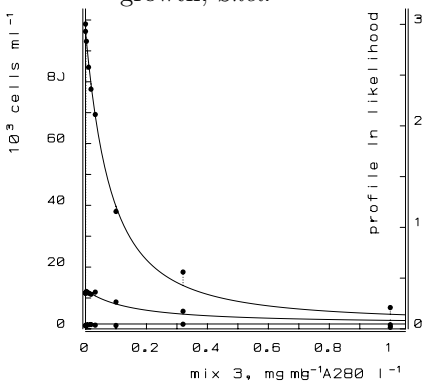
growth, *Skel.*



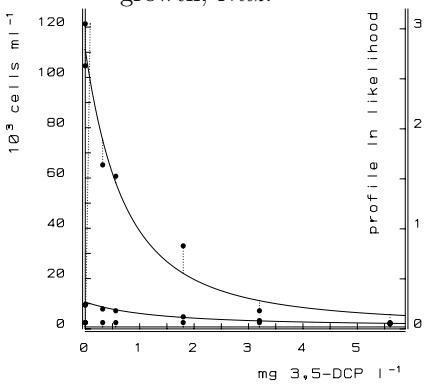
growth, *Skel.*



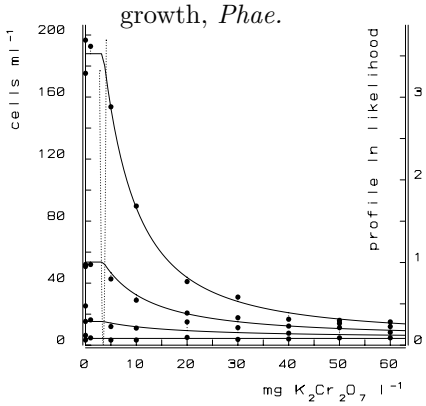
growth, *Skel.*



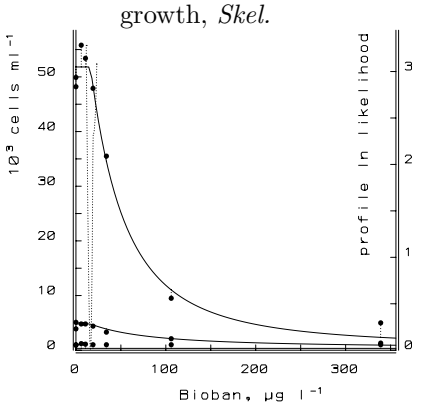
growth, *Nitz.*



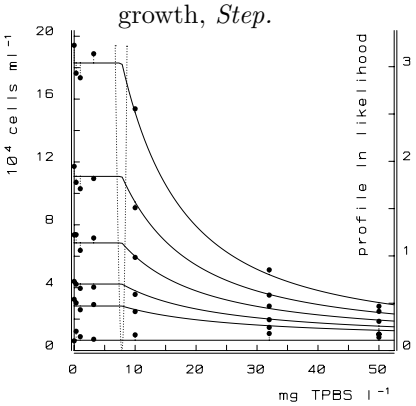
growth, *Phae.*



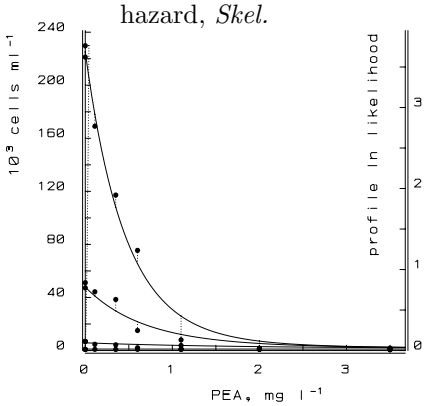
growth, *Skel.*



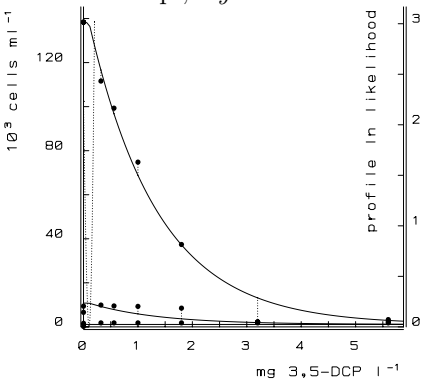
growth, *Step.*



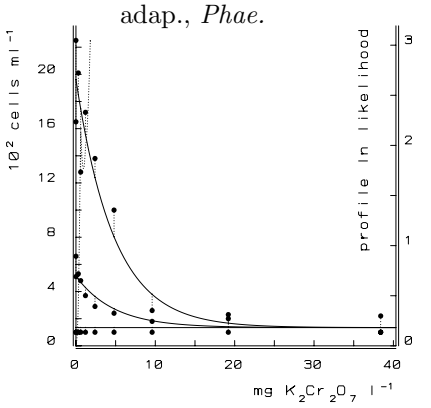
hazard, *Skel.*



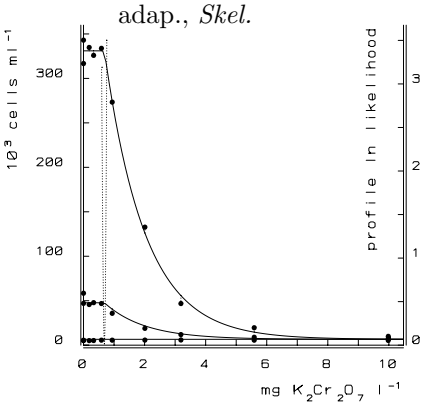
adap., *Cycl.*



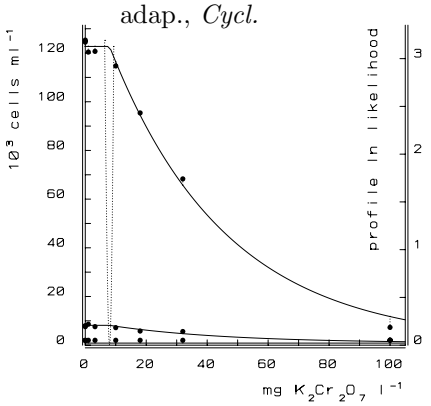
adap., *Phae.*



adap., *Skel.*



adap., *Cycl.*



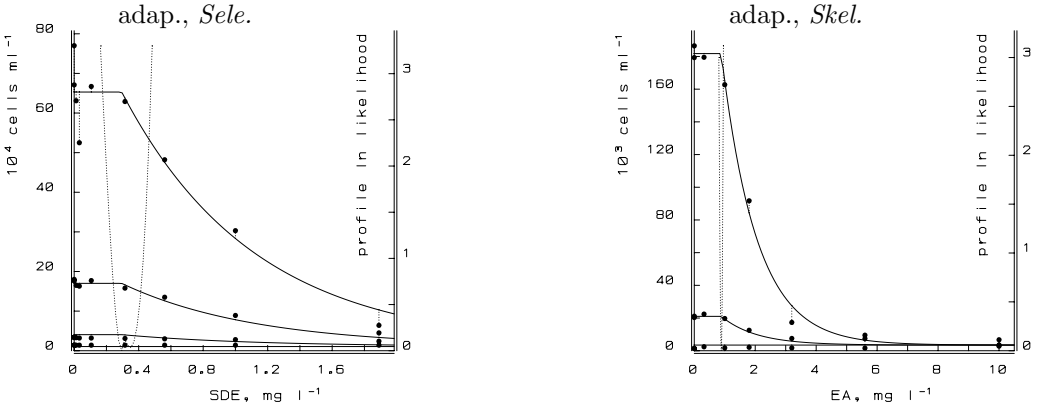


Figure 3.21: The best fitting of the three models (the growth, adaptation or hazard model) is shown in these examples, together with the profile ln likelihood function for the no-effect concentration c_0. The various curves in each figure correspond with an observation time. Observations at times at which the control deviates from exponential growth have not been included. The abbreviations for the compounds and algal species are given in the legends to Table 1.

The parameter c_0 does not suffer from the statistical problems of the NOEC. It seems not to be very sensitive to error in the identification of the specific mode of action of the compound. This is of importance because routine toxicity tests are not very suitable for this purpose. It would help, for instance, to distinguish the living from the dead cells, but this requires extra effort. Our method also avoids the complexities that are inherent to small-effect concentrations. Similar conclusions apply to other standard routine toxicity tests, such as the chronic reproduction *Daphnia* test. The combined evidence supports a rejection of the conventional NOEC/EC50-based analysis in favour of the NEC-based analysis with specific effects to the various biological endpoints. The examples presented in this paper have been analyzed earlier with the method described in Kooijman et al. (1983), supplemented with NOEC 'estimates'. Application of the new method shows that the estimated NECs correspond well with the old NOECs. The control population growth rates tend to be slightly lower than estimated using a logistic growth model. This is partly due to the problem of detecting deviations from exponential growth, but also to the estimation of the carrying capacity of the logistic growth model.

An additional advantage of our mechanistic approach is that assumptions about the kinetics of the compounds that prove to be too simple can easily be replaced by more complex (and hopefully more appropriate) ones for scientific purposes. This obviously requires a more elaborate experimental set-up. Being process-oriented, the analysis can be extended to include the effects of degradation and metabolic transformation. Such an effort is essential to evaluate the consequences of emissions

in the environment. This consistency between models for risk assessment and for scientific purposes is essential if we take risk assessment seriously.

The observation that the three different models frequently fit well to the same data set invites an attempt to convert the three toxicity measures (c_G, c_H, $\dot{k}_\dagger$) into each other. We can do so by equating the EC50 for biomass at the moment at which the control population exceeds n times the inoculated value. The test requires that $n \geq 15$. Simple mathematics reveals the following relationships

$$\dot{k}_\dagger c_G = (1-x)\dot{\mu}_0 \left(\frac{\ln n}{\ln 2} - 1\right) \quad \text{and} \quad \frac{c_G}{c_H} = \left(\frac{\ln n}{\ln 2} - 1\right) \ln \frac{n-1}{n/2-1}$$

where x is the solution of $x \ln n = \ln\{1 + x(n/2-1)\}$. For $n = 15$ this simplifies to $\dot{k}_\dagger c_G = 1.233\dot{\mu}_0$ and $c_G = 2.86 c_H$. This exercise also shows that the EC50 for biomass itself is totally useless to characterize the effects of compounds because this measure depends on the length of the test (choice for n) and on growth conditions (value for $\dot{\mu}_0$, which depends on media, light and temperature). For further discussion of these points, see Nyholm (1985). Nonetheless, it is frequently used and current standard protocols prescribe that this figure be reported along with an EC50 for the population growth rate.

Acknowledgements

We would like to thank Harry Oldersma (TNO-MW) for his careful experimental work and Jacques Bedaux and Cor Zonneveld for helpful discussions and Mike Newman and an unknown referee for comments.

References

Bedaux, J.J.M. & Kooijman, S.A.L.M. 1994. Statistical analysis of bioassays, based on hazard modeling. *Environmental & Ecological Statistics* **1**: 303-314.

Bartlett, L., Rabe, F.W. & Funk, W.H. 1974. Effects of copper, zinc and cadmium on *Selenastrum capricornutum*. *Water Res.* **8**: 179-185.

Bringmann, G. & Kühn, R. 1980. Comparison of the toxicity thresholds of water pollutants to bacteria, algae and protozoa in the cell multiplication inhibition test. *Water Res.* **14**: 231-241.

Cox, C. 1987. Threshold dose-response models in toxicology. *Biometrics* **43**: 511-523.

EU 1992. EU Guideline No C.3. *Growth inhibition test with algae.* Off. J. Eur. Comm. L 383A, 179-186.

Hanstveit, A.O. 1982. *Evaluation of the results of the third ISO-interlaboratory study with an algal toxicity test.* ISO Document ISO/TC 147/SC 5/WG 5/N64; TNO-Report CL 82/128, TNO-Environmental Sciences, Delft.

Hanstveit, A.O. 1991. *The results of an international ring test of the marine algal growth inhibition test according to ISO/DP 10253.* TNO-Report R 91/236, TNO-Environmental Sciences, Delft.

ISO 1989. International Standard, ISO 8692: 1989 (E). Water Quality - Fresh water algal growth inhibition test with *Scenedesmus subspicatus* and *Selenastrum capricornutum*, Genève.

ISO 1994. Draft International Standard, ISO/DIS 10253: Water quality - Marine algae growth inhibition test with *Skeletonema costatum* and *Phaeodactylum tricornutum*, Genève.

Kooijman, S.A.L.M. 1983. Statistical aspects of the determination of mortality rates in bioassays. *Water Res.* **17**: 749-759.

Kooijman, S.A.L.M. 1993. *Dynamic Energy Budgets in Biological Systems. Theory and applications in ecotoxicology.* Cambridge University Press, pp. 350.

Kooijman, S.A.L.M. & Bedaux, J.J.M. 1996. Some statistical properties of estimates of no-effects levels. *Water Research* (to appear)

Kooijman, S.A.L.M. & Bedaux, J.J.M. 1996a. Analysis of toxicity tests on *Daphnia* survival and reproduction. *Water Research* (to appear)

Kooijman, S.A.L.M., Hanstveit, A.O. & Oldersma, H. 1983. Parametric analyses of population growth in bioassays. *Water Res.* **17**: 727-738.

McCullagh, P. & Nelder, J.A. 1989. *Generalized linear models.* Monographs on Statistics and Applied Probability 37. Chapman & Hall, pp. 511.

Nyholm, N. 1985. Response variable in algal growth inhibition tests - Biomass or growth rate? *Water Res.* **19**: 273-279.

OECD 1984. OECD Guideline for Testing of Chemicals 201: *Alga, growth inhibition test.*, Paris.

Silvey, S.D. 1975. *Statistical inference.* Monographs on Statistics and Applied Probability 7. Chapman and Hall, pp. 192.

Stebbing, A.R.D. 1982. Hormesis - the stimulation of growth by low levels of inhibitors. *Sci. Total Environ.* **22**: 213-234.

Chapter 4

Software package DEBtox

Abstract DEBtox is a user-friendly software package designed to analyze the results of the standard set of aquatic toxicity tests: tests on survival, the (37 d) fish growth test, the (21 d) reproduction test with *Daphnia* and the (4 d) alga growth inhibition test. It basically serves six purposes:
- Estimation of parameters, their asymptotic standard deviations and their correlation coefficients. The different modes of action of the compound are described by different models: one for the survival experiment, three for body growth, five for reproduction and three for population growth.
- Measurement of the goodness of fit of the selected model and a graphical presentation of data together with the model expectations for the time and the concentration profiles.
- Calculation of the profile ln likelihood function for the no-effect concentration. The results are presented graphically. DEBtox reads this graph and can produce confidence intervals if the user has selected the confidence level.
- Calculation of EC$e.t$ values, ET$e.c$ values and ER$c.t$ values and their standard deviations if the user has selected the concentration c, the exposure time t and/or the effect level e. DEBtox can graphically present the effect as a function of concentration and exposure time, using isoclines.
- Statistical tests on any parameter value or combination of parameter values. The tests are based on the likelihood ratio test.
- Analysis of the residuals. The residuals, i.e. the differences between observed values and model expectations, can be plotted as functions of the concentration, the exposure time or the response.
This chapter describes some background, which is meant to complement the extensive help files of DEBtox.

4.1 Introduction

DEBtox is a user-friendly software package designed to analyze the results of the standard set of aquatic toxicity tests: acute and chronic tests on survival, the (37 d) fish growth test, the (21 d) reproduction test with *Daphnia* and the (4 d) alga growth inhibition test. DEBtox refers to these toxicity tests as 'experiments' (i.e. bioassays) to avoid confusion with statistical tests on parameter values, or goodness of fit. The measured biological variable is called 'response'. The principle aim is to characterize the effect of the chemical compound on the response.

The occurrence of effects is considered as a function of the exposure time and the concentration of the toxic compound. So observations in all four experiments typically comprise a data-matrix, with test concentrations in the first row, exposure times in the first column and response values in the body of the data-matrix. The data at exposure time zero specify the initial conditions for each cohort, i.e. the set of individuals that receive identical treatment. These data are required for the survival experiment only. The data at concentration zero describe the behaviour of the control. Although we advice to include one or more controls, DEBtox can do without. The effect is analyzed as a process, so the data are conceived as a set of simultaneous time series; not as a series of concentration-response curves for the different exposure times.

The times in the first column of the data-matrix are interpreted as times since the start of the experiment; the concentrations in the first row are interpreted as environment-concentrations, which are supposed to be constant during exposure. The responses are the number of surviving individuals, the mean body length, the mean cumulative number of young per female or the population size. The means refer to the various individuals in a cohort, so individuals that receive the same exposure. The numbers of individuals in the cohort in the body growth and the reproduction experiment might change (to some extent); these changes can be compensated by a proper choice of weight coefficients: the larger the number of individuals in a cohort, the smaller the deviation from an expected response.

When you try DEBtox for the first time, you might wonder why your computer is busy so long, especially when you select the body growth or the reproduction experiment. The amount of time that you have to wait obviously depends on the performance of your computer; we do not recommend application on machines slower that an 486DX2; it would stretch your patience beyond reasonable limits. A mathematical coprocessor is required. A table of run times for sample files on different PCs is included in the help files of DEBtox. This gives some idea what run time you should expect on your PC. The reason why it takes some time is that the program has a lot to calculate, as will soon become obvious.

4.2 Parameter estimation

The main task of DEBtox is the estimation of the parameter values. This is done by maximizing the (ln) likelihood function, which is described by (3.16) for the survival experiment and by minus a weighted sum of squared deviations between observed and expected responses for the other three experiments. The latter method is known as weighted least squares and can be interpreted as a maximum likelihood method under certain restrictions and assumptions. This interpretation is used in statistical tests on the parameter values and in the estimation of variances, covariances and confidence sets.

The likelihood function that is maximized as a function of the model parameters is non-linear. The numerical procedures that will (hopefully) find the maximum require an 'educated' initial guess for the best fitting parameter values. DEBtox removes this sometimes difficult task from the user by trying many different initial guesses. DEBtox chooses the initial guesses strategically in a model-specific way. For each initial guess, DEBtox will find a maximum of the likelihood function and will select the maximum of these maximums. Many thousands of these function evaluations are typically required for one estimation of the parameters.

In the case of the body growth and the reproduction experiment, the likelihood function is not known explicitly, only as a set of differential equations, together with initial conditions. This set of differential equations has to be integrated numerically before the function value can be given for each value of the set of parameters. If the elimination rate (which is one of the parameters) is large, the set of equations becomes stiff and requires a very small time increment for the numerical integration and/or other special treatment. Even if the resulting estimate for the elimination rate is not large, large values might occur during the estimation procedure. DEBtox solves this problem by making use of the explicit solution of the equation for the toxicokinetics, when integrating body length (and reproduction) numerically. This results in a robust, but time consuming procedure. A fourth order Runge Kutta method is used for these numerical integrations.

DEBtox uses the 'amoeba' algorithm to find the maximum of the likelihood function (see Press et al. 1994). Actually it finds the minimum of minus the log likelihood. This downhill simplex method by Nelder and Mead (1965) is very inefficient, but it is robust. Less robust algorithms require a high degree of education for the initial guess, and even then they frequently fail to find the proper maximum. We need robustness because likelihood functions sometimes have a very irregular surface, depending on the responses. If DEBtox has found the maximum of the likelihood function, it directly continues to calculate the best fitting parameters in the case that the elimination rate is very small with respect to the inverse of the exposure time (slow kinetics), or very large (fast kinetics). It does so for any selection of model and presents only the best fitting of the three results. The models of the population growth experiment, however, assume that the kinetics is always fast.

The elimination rate determines how fast the effect builds up during exposure. If the observation is only at the end of the experiment, hardly anything is known about this rate. The problem is that a trace of information about the value of the elimination rate is still left in the shape of the concentration-response relationship at any single observation time, so we cannot simply refrain from estimating the elimination rate. This lack of knowledge becomes visible in a huge standard error for the elimination rate and gives considerable numerical problems in finding the proper maximum of the likelihood function. We *strongly* recommend using several observation times. This extra information is usually already available and removes these statistical and numerical problems. A two-day survival experiment usually requires inspection after one day. Our suggestion is to count the number of survivors at this inspection and include this in the input for DEBtox. This simple extra information is already very helpful.

This essay makes clear why DEBtox is busy for some time when you hit the start-button, especially for the body growth and reproduction experiments. You can interrupt the calculations at any time, or use your computer for other purposes, while DEBtox is running in the background. The waiting time is shorter for 'beautiful' data where the model fits well than for 'less beautiful' data. If there are hardly any effects, DEBtox will be unable to find parameter estimates. Practice will have to reveal the extent of gray area between 'beautiful' and 'less beautiful'. We *strongly* recommend that the goodness of fit of any model be *always* inspected graphically from the concentration and the time profiles for the responses that DEBtox produces. Nothing is as effective as the human eye in this respect, and no procedure or method should replace common sense!

Validation

The estimation of the parameters of all models of DEBtox has first been coded independently in the programming language APL (A Programming Language). The APL functions use a Newton Raphson procedure to find the maximum likelihood estimates rather than an amoeba algorithm. We tested many data sets, including all the sample files that are included in DEBtox. We arrived at results with the APL functions that were very similar to the results with DEBtox, i.e. the results were identical in the first four decimal places. We are convinced that the small differences are due to the rules for stopping the Newton Raphson and amoeba procedure; the longer we continued the procedures, the smaller the differences became. The asymptotic standard deviations of all parameter estimates and the profile ln likelihood functions for the NECs were likewise very similar for the APL functions and DEBtox.

DEBtox is coded in C++ and it runs under DOS using Windows 3.1 or Windows95 on a PC. We did not try Windows 3.0. We also included a command line version that works under Unix SunOS 4.1.3. Current information about DEBtox will be available on WWW (page http://www.bio.vu.nl/vakgroepen/thb).

```
Code            Example 2
Experiment      Survival
Model           hazard
Compound        Dieldrin
Species         Poecilia reticulata
Time            day
Concentration   microgram/liter
Response        number of survivors
Date            18 January 1996
Note            From IMW-TNO
Data
0 1 2 3 4 5 6 7
0 3.2 5.6 10 18 32 56 100
20  20  20  20  20  20  20  20
20  20  20  20  18  18  17  5
20  20  19  17  15  9   6   0
20  20  19  15  9   2   1   0
20  20  19  14  4   1   0   0
20  20  18  12  4   0   0   0
20  19  18  9   3   0   0   0
20  18  18  8   2   0   0   0
```

Figure 4.1: An example of a DEBtox input file.

Below we will describe the input and output for DEBtox and discuss some of its rationale. Button-by-button information is included in the extensive help files of the Windows version, which is meant to be adequate. The Unix version is not interactive. The choices have to be set by flags, which are described in the manual file (type 'debtox -h' or debtox.doc under Unix).

4.3 Input

The data input has two segments: documentation and numerical data. The documentation consists of a number of fields, some are required, some are optional. The fields are identified by their names: 'Code', 'Time', 'Concentration', 'Response', 'Species', 'Compound', 'Date', 'Experiment', 'Model', 'Date', 'Experimentalist', 'Notes', 'Data' and 'Weights'. See Figure 4.1 for an example. The order is not important, as long as the 'Weights' is not before the 'Data' field; each field should start on a new line with its name followed by a at least one space- (or tab-) character. DOS uses two characters to indicate a new line, Unix uses just one character. DEBtox can handle both methods in the Windows version as well as in the Unix version. If you do not have data for the optional fields, there is no need to mention empty fields.

The required fields comprise:

- **Code**, which is used to identify the data set; it has no effect on the results.

- Names for the chemical **Compound** and the **Species** of test organism; it has no effect on the results.
- Units for **Time**, **Concentration** and **Response**.
- The measured responses go into the **Data** field. The numerical data comprise three series: Observation times, concentrations and responses.

  Each of the three series of numbers should start on a new line. This is how DEBtox recognizes which numbers relate to times, which to concentrations or which to responses.

  The concentrations should be ordered from low to high, starting from zero in column two. Multiple concentrations are allowed. The exposure times should also be ordered from low to high; the data for the survival experiment require that exposure time zero is present in row two. No multiple exposure times are allowed. The number of responses should be equal to the product of the number of times and concentrations. If a response has value -1, it will be ignored during the estimation procedure.

The optional fields comprise:

- **Experiment** and **Model**; if these fields are provided, DEBtox automatically selects the correct settings.
- **Weights**; 0 (zero; the default) for all weight coefficients equal; 1 (one) for weight coefficients inversely proportional to the responses; 2 (two) for weight coefficients inversely proportional to the squared responses (see under weight coefficients below); Apart from a choice of one of these three numbers, weight numbers can be specified for the body growth and reproduction experiments (see below). Each series of weight numbers that belong to a particular exposure time should start on a new line. The number of weight numbers should be equal to the number of responses and the weight numbers should always be integers.
- **Date** of the experiment; it has no effect on the results.
- Name of the **Experimentalist**; it has no effect on the results.
- **Notes**; a possibility to add any information; it has no effect on the results.

The weight numbers, together with the selection in the 'Weights' field, are used to calculate the weight coefficients. The way in which this is done depends on the selection of the type of experiment.

Weight coefficients for the body growth experiment are calculated from the numbers of individuals in the cohorts n_{ij}, i.e. the weight numbers. The default is that the numbers of individuals in all cohorts are equal. (The default is selected if the weight numbers are not included into the 'Weights' field.) The weight coefficients

also depend on the specification that the variance of the response is independent of the response (the default), proportional to the response (the Poisson-case) or proportional to the squared response (constant variation coefficient). The choice is made in the field 'Weights' by the values 0, 1 or 2, respectively. DEBtox calculates the weight coefficient w_{ij} that belongs to ith time and the jth concentration with response x_{ij} and n_{ij} individuals in that cohort as

$$w_{ij} = \frac{n_{ij} x_{ij}^{-s}}{\sum_{k,l} n_{kl} x_{kl}^{-s}} \sum_{k,l} (n_{kl} \neq 0)$$

where $s = 0$ (default), 1, or 2, i.e. the contents of the field 'Weights'. The summation is over all exposure times (including zero) and concentrations (including the control(s)). The expression $(n_{kl} \neq 0)$ is either zero (if false) or one (if true). Multiplication of the numbers of individuals in a cohort (or the responses) by an arbitrary positive factor does not affect the weight coefficients. The sum of all weight coefficients is equal to the number of responses from cohorts with a positive number of individuals. The default is $n_{ij} = 1$ and $s = 0$, so that $w_{ij} = 1$ for all i and j.

If the number of individuals in a cohort is zero, the response of that cohort no longer contributes to the results. If some response has become lost, you simply insert the response -1 in that cell of the response-matrix and specify a zero for the corresponding weight number, i.e. the number of individuals in that cohort.

The weight coefficients in the reproduction experiment are calculated in the same way as for the body growth experiment, except that x_{ij} is not the measured response, i.e. the cumulative number of young per female, but one plus the reproduction rate, i.e. the ratio of the differences between subsequent cumulative numbers of young and observation times. We added the number one to avoid the problem that weight coefficients can become infinite. This addition is always made to calculate the weight coefficients for the reproduction experiment, but if $s = 0$, this has no effect on the results.

Since DEBtox applies a non-linear regression technique, we assume that the responses are independently distributed. This assumption is violated if the intervals between subsequent observation times are too small. This hardly affects the point estimates for the parameters, but the asymptotic standard deviations are rather sensitive to this problem. The root of the problem lies in the interpretation of the stochasticity in terms of measurement error. This point of view is questionable for the body growth and reproduction experiments, but more realistic approaches are very complex (Bedaux & Kooijman 1994). These remarks do not apply to the survival experiment, where DEBtox uses a maximum likelihood method.

Weight numbers are not used in the population growth and survival experiments, but if the response has value -1, it is ignored.

A practical application of the exclusion of responses in the population growth experiment is that the population density in the control and in the lower concentrations became too high and started to deviate from exponential growth. This problem

will probably not occur in the higher concentrations, where the toxicant reduces the growth rate. Exclusion of the responses in the lower concentrations allows you to make use of the valuable measurements at the higher concentrations. The selection in the 'Weights' field applies to the population growth experiment in the same way as to the body growth and reproduction experiments.

The exclusion of responses in the survival experiment makes it possible to handle data sets with missing observations. The reason for these missing observations might be accidental or on purpose. Suppose, for instance, that a cohort of n_j individuals is exposed to concentration c_j and after observation time t_i a number n_k of *living* individuals is sacrificed for analyzing the tissue-concentration. We should then insert concentration c_j two times in the data matrix. The numbers of surviving individuals, excluding the sacrificed ones, go into one of these columns, so starting with $n_j - n_k$ individuals; the number n_k is inserted into all cells of the second column till observation time t_i, and -1 is inserted for all responses after. In this way we still make use of the knowledge that the sacrificed individuals were still alive at exposure time t_i. We would introduce a bias if we subtracted n_k from all cells prior to t_i in that concentration. We insert concentration c_j three times if we removed living individuals for the experiment at two different observation times. If all of the cohort became lost after time t_i, we enter concentration c_j just once and insert value -1 for all responses of that cohort after time t_i. The number of surviving individuals in these cells are then ignored.

Although DEBtox allows you to choose any prefix before the units of time, concentration and response, you have to choose these prefixes such that most numbers appear with one digit before the point, if possible. You must be prepared to end up having numerical problems if you ignore this advice.

You can prepare your input file in Excel, for instance, or any text editor that can save plain ASCII. A simple spreadsheet for data input from the keyboard is built into DEBtox. You can also use this spreadsheet to change your data. DEBtox will save the data before starting to calculate anything. DEBtox checks the consistency of the data and refuses to calculate anything if the data prove not to be consistent. Consistency is obviously no guarantee that the data make sense, it is only a minimum requirement.

Fixed parameters

In addition to data input, and selections for type of experiment and model, DEBtox requires the setting of some fixed parameters for the description of the response in the control in the case of the body growth and the reproduction experiment. These parameters are fixed because the results of routine toxicity experiments do not have sufficient information to estimate the values from the responses.

The default settings are realistic for experiments with zebra fish (*Brachydania rerio*) at 25°C and daphnids (*Daphnia magna*) at 20°C, respectively. The parameter

values depend on the species (and even strain) and the culture conditions, however, and need to be checked.

The fixed parameters for the body growth experiment comprise the von Bertalanffy growth rate, the initial body length and the energy investment ratio. If you have measured body lengths at time zero, you should choose the initial body length close to the mean of these lengths. (The cubic root of the body weight can play a role that is similar to body length.) There is no strict need for measured body lengths at time zero, however; if you insert the value of the fixed parameter in the data-matrix at time zero for each concentration, the point estimates for parameters are unaffected, but the variances of the parameters are reduced, because DEBtox counts more measured responses. The data matrix should therefore contain only real measurements.

The initial body length is replaced by the scaled lengths at birth and puberty in the reproduction experiment, i.e. the ratio of the lengths at birth and puberty and the maximum (adult) body length. *D. magna* typically hatches at 0.8 mm, starts allocating resources to reproduction at 2.5 mm and grows to a maximum of 6 mm at abundant food. The scaled lengths at birth and puberty are therefore $0.8/6 = 0.13$ and $2.5/6 = 0.42$, respectively. Graphical inspection of the time profiles for the responses will reveal any need to adjust the value of the scaled length at puberty.

The energy investment ratio has the interpretation of the ratio of the volume-specific costs for growth and the product of the maximum reserve energy density and the fraction of catabolic energy that is allocated to growth plus maintenance, as opposed to development plus reproduction. (The maximum reserve energy density is the reserve energy per volume of structural body mass of an adult that lives at abundant food for a long time; the catabolic energy is the flux of energy that is released from the reserves.) This elusive parameter is dimensionless. Our experience is that the toxicity parameters do not depend sensitively on its value. The default value is 1, realistic values are in the range 0.1 till 100, depending on the species; the energy investment ratio is roughly inversely proportional to the volumetric adult body length of a species.

The von Bertalanffy growth rate can be interpreted as the change in length, as a fraction of the difference of the ultimate and the actual length in the control. The DEB theory explains why this growth rate is constant at constant food availability. The von Bertalanffy growth rate is roughly inversely proportional to the volumetric adult body length of a species.

4.4 Output

Besides output on the screen, DEBtox can write to printers or to postscript or ASCII files. Most word processors, such as Word, WordPerfect or LaTeX, have facilities to paste postscript files into the text. (This book has been written in LaTeX, and the

Code	Example 2
Experiment	Survival, Hazard
Compound	Dieldrin
Species	Poecilia reticulata
Date	18 January 1996
Experimentalist	
Note	From IMW-TNO

	Time: day, Conc,: microgram/liter							
	0.0	3.2	5.6	10.0	18.0	32.0	56.0	100.0
0	20	20	20	20	20	20	20	20
1	20	20	20	20	18	18	17	5
2	20	20	19	17	15	9	6	0
3	20	20	19	15	9	2	1	0
4	20	20	19	14	4	1	0	0
5	20	20	18	12	4	0	0	0
6	20	19	18	9	3	0	0	0
7	20	18	18	8	2	0	0	0

Figure 4.2: Example of a DEBtox report for the notes of the data for the sample file 'survival.dtd'. These files are stored under the names 'surviva1.eps' and 'surviva2.eps' in the directory DEBTOX after the proper selection under 'Save PostScript' (DOS limits file names to 8 characters).

figures in this section are output from DEBtox.) The ASCII files are editable. The selection of the various files can be done in the File Menu. Each output file has an identification of the data and the model that is used. The files show tabular and/or graphical information. Public domain gnuplot is used for the graphics on screen in the Unix environment. This program can also produce postscript files.

Tabular information

Data plus notes

This table just presents your input. Some quality control procedures require that you present this file with the results in one print. DEBtox can do this, but you can also include this file separately in your report. See Figure 4.2 for an example.

Parameters

The parameters are given with (asymptotic) standard deviations and correlation coefficients, including a deviance or a residual standard deviation. The latter quantifies the goodness of fit and can be used for comparative purposes. If the sample size is large enough, the parameter estimates are approximately normally distributed. Figure 4.3 gives an example of a parameter report as postscript file; the report can also be exported as ASCII file.

Most reports on model fits do not include correlation coefficients. However, this is valuable information. You will observe large (asymptotic) standard deviations

Survival, Hazard, Normal kinetics			ASD	Correlation coefficients		
Blank mortality rate	0.008358	d^{-1}	0.0049			
No-effect concentration	5.206	$\mu g\ l^{-1}$	0.4651	0.3093		
Killing rate	0.03763	$l\ \mu g^{-1}\ d^{-1}$	0.0078	0.0457	-0.0244	
Elimination rate	0.7902	d^{-1}	0.2177	-0.0488	0.2806	-0.8115
Deviance	36.4281					

Figure 4.3: Example of a DEBtox report for the parameters that results from the survival.dtd file. The file is stored under the name 'surviva3.eps' in the directory DEBTOX after the proper selection under 'Save PostScript'.

if correlation coefficients approach the values -1 or $+1$. If two parameters have a correlation coefficient near -1, each of their values are poorly determined by the data, but their difference or ratio might be well determined; if they have a correlation coefficient near $+1$, the same holds for their product or sum. You can reduce the problem of extreme correlations by using more observation times in the data, thus changing the experimental protocol.

If the exposure time is short with respect to the inverse of the elimination rate, DEBtox will probably select the 'slow kinetics' case as fitting best. In this case, the estimate of the elimination rate and no-effect concentration is zero by consequence, and the ratio of the no-effect concentration and the elimination rate, the no-effect concentration-time, appears as a free parameter that can be estimated. The same holds for the tolerance concentration; the ratio of the tolerance concentration and the elimination rate is called the tolerance concentration-time. The killing rate combines with the elimination rate as a product, which then becomes the killing acceleration. It is important to realize that the exposure time should be extended to estimate the primary toxicity parameters, if the slow kinetics variant fits best.

Parameter table

This table gives parameter estimates and the deviance in each row to allow comparisons between different choices for model and/or parameter values. You can specify one or more parameter values and estimate the remaining parameters given this specification.

By comparing the deviances (mean residual deviations), you can test the values of parameters statistically on the basis of the likelihood ratio test. Suppose, for instance, that we know the killing rate of a reference compound and want to test whether the estimated killing rate from a particular experiment deviates significantly. (Laboratories sometimes follow this procedure to check the condition of their cultures of test-animals.) We first estimate all parameters (including the killing rate) and obtain the deviance. We then set the killing rate at the known value, estimate the other parameters again and obtain a new deviance. The likelihood ratio theory

says that, if the known killing rate is the real value, two times the difference between the deviances that we calculated represents a random trial from the Chi-square distribution with one degree of freedom. If the calculated difference is too large, we reject the hypothesis that the killing rate has the known value.

More complex tests on the parameter values are also possible. If we want to test the values for the NEC and the killing rate simultaneously, for example, we first estimate all parameters and obtain the deviance. We then fix the NEC and the killing rate at the given values, estimate the other parameters again and obtain a new deviance. The likelihood ratio theory tells that, if the known NEC and killing rate are the real values, two times the difference between the deviances that we calculated represents a random trial from the Chi-square distribution with two degrees of freedom. If the calculated difference is too large, we reject the hypothesis that the two parameters have the specified values.

The parameter table facility allows you to produce profile ln likelihood functions of any parameter (while testing your patience). The Unix version is not interactive and you can use DEBtox as a subroutine in your own program, where you scan the parameter of interest and plot the deviance as a function of the parameter value.

When choosing different models to match the same data, it is tempting to use differences in the deviance to identify the mode of action of the compound. Unfortunately, no statistical test can help you to decide that one model fits the data *significantly* better than the other. (The reason is rather technical but boils down to the fact that statistics is about parameter values, not about model structure.) Small differences between deviances frequently prove not to be reproducible and should be ignored. You will notice, however, that the NECs of different models that fit the data equally well turn out to differ little.

Confidence set for NEC

The confidence set for the no-effect concentration consists of one or more confidence intervals. These intervals are obtained from the profile likelihood function, which has to be calculated before. The facility is included under 'Statistics NEC' and interpolates linearly in the table that is produced when the function is calculated. This is why the confidence sets are calculated so rapidly after a new selection of the confidence level. The results can be exported as ASCII file.

Effect surface

Values for the effect e, concentration c and/or exposure time t can be obtained from the effect surface by specifying two of these three variables. The three different selection options are labelled ER, EC and ET for the variables effect, concentration and time, respectively. The parameters determine the whole effect surface, so that these

calculations do not involve the measurements directly. Here, the effect is defined as

$$\text{effect}(c,t) \equiv 1 - \text{response}(c,t)/\text{response}(0,t)$$

This facility makes the new method to quantify toxicity compatible with the standard methods. The EC100$e.t$ and ET100$e.c$ values are found by solving the equations effect(EC100$e.t, t$) $= e$ and effect(c,ET100$e.c$) $= e$ numerically. DEBtox uses a hybrid between a Newton Raphson and a bisection method. This hybrid is necessary to solve the problem that the effect is absent for certain combinations of c and t, which would lead to an infinite step size in the Newton Raphson procedure. The numerical method assumes that the EC is less than 100 times the maximum concentration and that ET is less than 10 times the maximum exposure time in the data matrix.

The most frequently used standard toxicity measure is the EC50 at the end of the exposure time. It is usually called LC50 in the case of the survival experiment. You obtain this value by specifying effect 0.5 and the proper exposure time in the row labelled EC. The default choices anticipate these specifications. An interesting variant is the EC0 at some observation time. It relates to the no-effect concentration as NEC=EC0.∞. Since all models for the population experiment assume that the elimination rate is large with respect to the inverse exposure time, we have the EC0.t =NEC for all t. All other experiments have EC0.t >NEC if the kinetics is not fast.

We did not implement ET for the body growth and the reproduction experiment because the effect does not always increase monotonically with the exposure time in these experiments. Not one but several points in time can exist, where an effect of given size occurs at a given concentration.

The presented estimates for the standard deviations are

$$\hat{\text{sd}}\, g(\theta) = \sqrt{(\frac{d}{d\theta} g(\hat{\theta})^T \hat{\mathbf{\Sigma}} \frac{d}{d\theta} g(\hat{\theta})} \tag{4.1}$$

where θ denotes the vector of parameters of the selected model, g the function of parameters that gives EC, ET or ER, and $\mathbf{\Sigma}$ the variance-covariance matrix of the parameters. The hats indicate that the estimated values are used. The estimates for the standard deviations for EC, ET or ER should be used as approximations to the real values, which can be crude.

The effect surface directly relates to the response surface, which is the population size (density of cells) in the case of the population growth experiment. The standard analyses of the population growth experiment frequently include the EC50 for the population growth rate rather than the biomass. The EC100x for the population growth rate relates to the NEC, c_0, and the tolerance concentration, c_G, (or killing rate, $\dot{k}_\dagger$, and population growth rate in the control, $\dot{\mu}_0$) as

growth model

$$\begin{aligned} \hat{\text{EC}}x &= \hat{c}_0 + \hat{c}_G \frac{x}{1-x} \\ \hat{\text{var}}\,\text{EC}x &= \begin{pmatrix} 1 \\ \frac{x}{1-x} \end{pmatrix}^T \begin{pmatrix} \hat{\text{var}}\, c_0 & \hat{\text{cov}}\, c_0, c_G \\ \hat{\text{cov}}\, c_G, c_0 & \hat{\text{var}}\, c_G \end{pmatrix} \begin{pmatrix} 1 \\ \frac{x}{1-x} \end{pmatrix} \end{aligned}$$

hazard model

$$\begin{aligned} \hat{\text{EC}}x &= \hat{c}_0 + x\hat{\dot{\mu}}_0/\hat{\dot{k}}_\dagger \\ \hat{\text{var}}\,\text{EC}x &= \begin{pmatrix} x\hat{\dot{k}}_\dagger^{-1} \\ 1 \\ x\hat{\dot{\mu}}_0\hat{\dot{k}}_\dagger^{-2} \end{pmatrix}^T \begin{pmatrix} \hat{\text{var}}\, \dot{\mu}_0 & \hat{\text{cov}}\, \dot{\mu}_0, c_0 & \hat{\text{cov}}\, \dot{\mu}_0, \dot{k}_\dagger \\ \hat{\text{cov}}\, c_0, \dot{\mu}_0 & \hat{\text{var}}\, c_0 & \hat{\text{cov}}\, c_0, \dot{k}_\dagger \\ \hat{\text{cov}}\, \dot{k}_\dagger, \dot{\mu}_0 & \hat{\text{cov}}\, \dot{k}_\dagger, c_0 & \hat{\text{var}}\, \dot{k}_\dagger \end{pmatrix} \begin{pmatrix} x\hat{\dot{k}}_\dagger^{-1} \\ 1 \\ x\hat{\dot{\mu}}_0\hat{\dot{k}}_\dagger^{-2} \end{pmatrix} \end{aligned}$$

The expressions for the variances directly follow from (4.1). Note that we have $\text{EC}50 = c_0 + c_G$ for the growth model, which means that the tolerance concentration c_G has the interpretation of the difference between the EC50 for the population growth rate and the NEC.

The adaptation model for effects on population growth only delays rather then reduces growth, which implies that the EC100x for population growth does not apply to this model.

The results can be exported as ASCII file. We think that the model parameters specify effects much better than any set of values for EC, ET and/or ER. The model parameters have a direct relationship with the biological process that is affected and they are independent of the exposure time.

Graphical information

Concentration and time profiles

These graphs show the model fits: they present the measured responses and the model expectations as functions of exposure time and concentration. See Figure 4.4 for an example. These graphs should always be inspected to judge the goodness of fit of the model. If a model happens to fit badly, the estimates for the model parameters and all other results from DEBtox should be ignored. You can try other model choices, of course. Alternatively, you can repeat the bioassay and judge the consistency of your measurements.

Note that the models for the results of bioassays for population growth assume that population growth is exponential, at least in the control. Batch cultures will usually start to grow exponentially but then deviate for a variety of reasons. This makes it necessary to delete responses for "long" exposure times. We recommend checking the validity of the assumption of exponential growth in the control by graphical inspection.

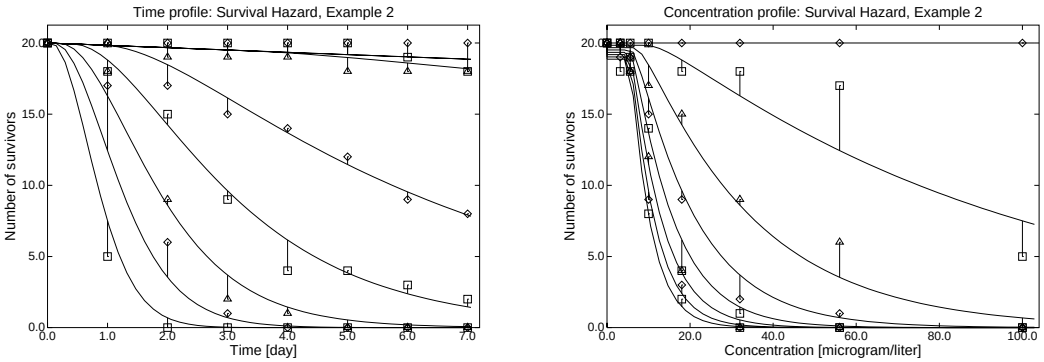


Figure 4.4: Example of a DEBtox report for the time and concentration profiles that results from the survival.dtd file. The files are stored under the name 'surviva4.eps' and 'surviva5.eps' in the directory DEBTOX after the proper selection under 'Save PostScript'.

Profile likelihood function

The profile likelihood function for the no-effect concentration directly relates to its confidence set. See Figure 4.5 for an example and under 'confidence set' for numerical values for the NEC. Given that the kinetics is slow, the point estimate for the NEC itself is always zero, but this result is rather sensitive to the choice of the (maximum) exposure time. See also the comments in the section on 'parameter estimation'. The confidence interval for the NEC still gives some idea what to expect for the NEC if the design of the experiment is adjusted to prevent that slow kinetics fits best.

The function is calculated starting from the maximum likelihood value of the

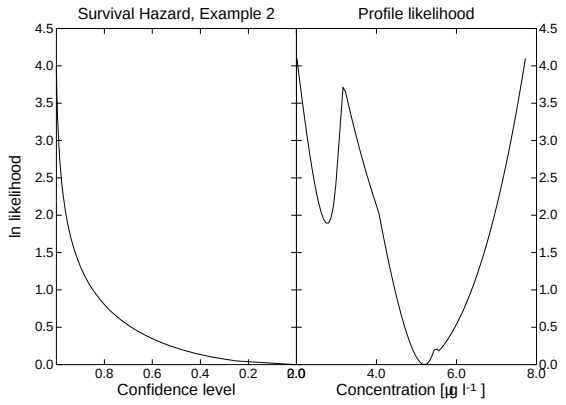


Figure 4.5: Example of a DEBtox report for the profile likelihood function that results from the survival.dtd file. The file is stored under the name 'surviva6.eps' in the directory DEBTOX after the proper selection under 'Save PostScript'.

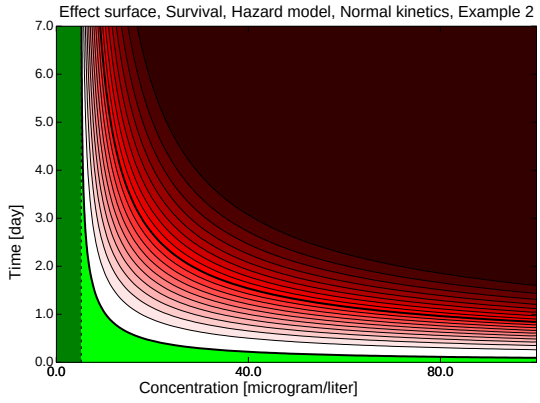


Figure 4.6: Example of a DEBtox report for the effect surface that results from the survival.dtd file. The file is stored under the name 'surviva7.eps' in the directory DEBTOX after the proper selection under 'Save PostScript'.

NEC. At each incremental increase of the NEC, all other parameters are estimated, and the maximum of the ln likelihood function is obtained, given the value of the NEC. If the difference of this maximum and the maximum for a free NEC exceeds 4.1, the process of incremental *in*crease is terminated and the procedure is repeated for an incremental *de*crease, starting again for the maximum likelihood value. It is unlikely that the difference of the deviances becomes less than 4.1 again outside the evaluated interval, but it is not impossible for data sets that fit badly. In that case, *no* results should be trusted.

Effect surface

The effect surface is graphically presented by isoclines: curves of equal effect. The effect levels are $e = 0,\ 0.05,\ 0.10,\ 0.15, \cdots$ The postscript files shade the effect levels with colours, which black-and-white laser printers convert into grey levels. See figure 4.6 for an example. The EC0 and EC50 curves are plotted with a large linewidth, the EC5, EC15, EC25, $\cdots$ with a small linewidth. The area with no effects is coloured light green, the area with no ultimate effects is coloured dark green. The maximum concentration and exposure time for the effect surface can be costumized, the default values correspond with those in the input data matrix.

The isoclines are calculated, starting from the maximum exposure time and using the EC routine to find the concentrations for the various effects levels e. The isocline is then followed ($i = 0, 1, \cdots$) according to the scheme

$$c^*_{i+1} = c^*_i + g^*_c d + g^*_t f \quad \text{and} \quad t^*_{i+1} = t^*_i + g^*_t d - g^*_c f$$

where $c^* = c/c_{\max}$, $t^* = t/t_{\max}$, $g^*_c = g_c/g$, $g_c = \frac{d}{dc^*} e(c_i, t_i)$, $g^*_t = g_t/g$, $g_t = \frac{d}{dt^*} e(c_i, t_i)$, $g = g_c^2 + g_t^2$, $d = e - e(c_i, t_i)$ and $f = \sqrt{h^2 g - d^2}$, for h being a small step size. This scheme has the nice property that $(c^*_{i+1} - c^*_i)^2 + (t^*_{i+1} - t^*_i)^2 = h^2$. The values for g_c

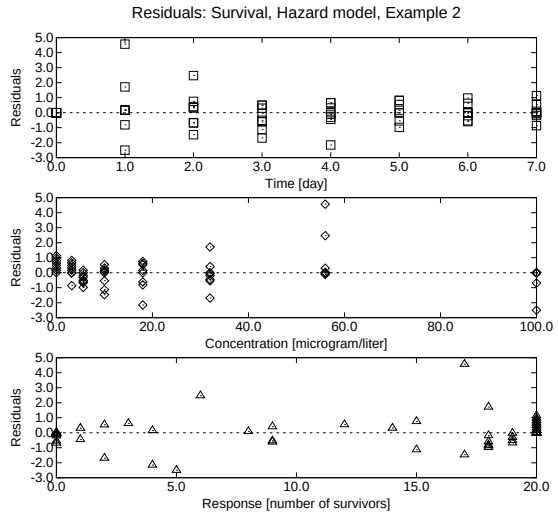


Figure 4.7: Example of a DEBtox report for the residuals that results from the survival.dtd file. The file is stored under the name 'surviva8.eps' in the directory DEBTOX after the proper selection under 'Save PostScript'.

and g_t are obtained numerically. Given that $e(c_0, t_0) = e$, the scheme is designed to keep the error d small for a sufficiently small value for stepsize h.

Residuals

The residuals are given as functions of time, concentration or response, to detect systematically deviating model expectations. Note that the theory assumes that the effect on the control parameter is small, so that deviations can be expected for large effects. Practice indicates that small changes in the control parameter translate into substantial changes in response, so that adequate fits are frequent.

The residuals for the survival experiment are expected to be small for small and large response values, and larger in between. More specifically, the mean residual is approximately $\sqrt{nq(1-q)}$, were n denotes the number of individuals of a cohort at the start of the experiment, and q the survival probability; the expected response is nq. See Figure 4.7 for an example. The graph of the residuals is difficult to interpret if the cohorts differ in size at the start.

The residuals as a function of the response are expected to follow the pattern as specified in the Weights field for the reproduction, body and population growth experiments. So, if the Weights field has the selection zero (the default), no trends in residuals should be visible; if it has the selection 1, the residuals should be approximately proportional to the square root of the response; for selection 2, the residuals are expected to be proportional to the response. The residuals are more difficult to interpret for the reproduction experiment, because the weight coefficients relate to the reproduction rate, rather than to the cumulative number of young.

The role of weight coefficients should not be overestimated. If the model fits

well, i.e. if the residuals are small, the weight coefficients hardly effect the parameter estimates. If the model does not fit well, the choice of weight coefficients is important but the scientific significance of the result is doubtful.

4.5 Final remarks

The primary purpose of DEBtox is to analyze the results of existing standardized toxicity tests. We hope that frequent application of this package will lead to adjustments of the experimental design to optimize the efficiency of the bioassays. In our opinion, the details of the design of any bioassay should be based on the analysis of the results, rather than vice versa. The optimization of the design might aim at a minimization of the confidence interval for the no-effect concentration and/or the toxicity measure, given constraints on the total financial costs of the bioassay.

A consequence of our primary purpose is that we did not (yet) include the analysis of simultaneous sets of measurements. For example, the lengths of the daphnids at the end of a reproduction experiment would be of great help to identify the mode of action of the compound. These simple additional measurements hardly increase the total costs of the experiment and the inclusion of this extra information into the analysis would considerably reduce the uncertainty in some parameters.

Because the analysis is process-oriented, including deviations from a constant environment-concentration in the bioassay is rather straightforward. The concentrations must then be measured during exposure. Substantial reductions of the measurement schemes could be realized if the mechanisms are known that cause such deviations. For instance, if the mechanism is that the compound is accumulating in the fish, one measurement of the environment-concentration at the end of the bioassay in principle defines the whole time-profile. The same holds when the compound disappears by degradation, absorption or evaporation, according to a first-order or other known kinetics.

Please read the 'readme' file for the latest changes and installation directions.

References

Bedaux, J.J.M. & Kooijman, S.A.L.M. 1994. Stochasticity in deterministic models. In: Rao, C.R., Patil, G.P. & Ross, N.P. (eds) Handbook of Statistics 12: Environmental Statistics. North Holland: 561-581.

Nelder, J.A. & Mead, R. 1965. A simplex method for function minimisation. *Computer Journal* **7**: 308-313.

Press, W.H., Teukolsky, S.A., Vetterling, W.T. & Flannery, B.P. 1994. *Numerical recipes in C.* Cambridge University Press.

Glossary

coefficient of variation The dimensionless ratio of the (sample) standard deviation and the mean. It is a useful measure for the scatter of realizations of a random variable that has a natural origin. The measure is useless for temperatures measured in degrees Celsius, for example.

compound parameter A function of original parameters. It is usually a simple product and/or ratio.

concentration-time The ratio of a concentration and the elimination rate. The dimensions are mass per volume per time. This concept results if the observed exposure time is short which respect to the inverse elimination rate. In that case, the tolerance or no-effect concentrations cannot be obtained from measured responses, nor can the elimination rate. However, the responses still allow the estimation of the concentration-times.

DEB Initials of the Dynamic Energy Budget model or theory. The term 'dynamic' refers to the contrast with the frequently used static energy budget models, where the specifications of the individual do not change explicitly in time.

ectotherm An organism that is not an endotherm.

endotherm An animal that usually keeps its body temperature within a narrow range by producing heat. Birds and mammals do this for most of the time that they are active. Some other species (insects, tuna fish) have endothermic tendencies.

energy investment ratio The ratio of the energy costs of growth per unit of volume of structural biomass and the product of the (maximum) capacity of energy reserves per unit of volume and the fraction of catabolic energy that is allocated to growth plus maintenance, as opposed to development plus reproduction.

estimation The use of measurements to assign values to one or more parameters of a model. This is usually done in some formalized manner that allows evaluation of the uncertainty of the result.

expectation The theoretical mean of a function of a random variable. For a function g of a random variable $\underline{x}$ with probability density $\phi_{\underline{x}}$, its formal definition is $\mathcal{E}g(\underline{x}) \equiv \int_x g(x)\phi_{\underline{x}}(x)\,dx$. For $g(\underline{x}) = \underline{x}$, the expectation of $\underline{x}$ is the theoretical mean.

exponential distribution The random variable $\underline{t}$ is exponentially distributed with parameter $\dot{r}$ if the probability density is $\phi_{\underline{t}}(t) = \dot{r}\exp\{-\dot{r}t\}$. The mean of $\underline{t}$ equals $\dot{r}^{-1}$.

first order process A process that can be described by a differential equation where the change of a quantity is linear in the quantity itself. If the quantity represents the contents of a compartment, the compartment is said to follow a one-compartment kinetics.

functional response The ingestion rate of an organism as a function of food density.

growth Increase in structural body mass, measured as an increase in volume in most organisms. Anabolic processes that are part of maintenance are not included into growth.

hazard rate The probability per time increment that death strikes at a certain age, given survival up to that age.

heterotroph An organism that uses organic compounds as a source of energy.

homeostasis The ability of most organisms to keep the chemical composition of their body constant despite changes in the chemical composition of the environment.

isomorph An organism that does not change its shape during growth.

likelihood function A function of the parameters of a model that quantifies the probability of finding the measurements as observed, if the measurements really obey that model with the specified parameter values.

maintenance A rather vague term denoting the collection of energy demanding processes that life seems to require to keep going, excluding all production processes. Heat production in endotherms is excluded from maintenance for practical purposes.

mass action law The law that states that the meeting frequency of two types of particles is proportional to the product of their densities, i.e. number of particles per unit of volume.

maximum likelihood estimate A value for a parameter that maximizes the likelihood function.

parameter A quantity in a model that describes the behaviour of state variables. It is usually assumed to be a constant.

partition coefficient The ratio of the equilibrium concentrations of a compound dissolved in two immiscible solvents, which is taken to be independent of the actual concentrations. The concentrations are here expressed per unit of weight of solvent (not per unit of volume or per mole of solvent).

polynomial A polynomial of degree n of argument x is a function of the type $\sum_{i=0}^{n} c_i x^i$, where $c_0, c_0, ., c_n$ are fixed coefficients.

probability density function A non-negative function, here called ϕ, belonging to a continuous random variable, $\underline{x}$ for instance, with the property that

$$\int_{x_1}^{x_2} \phi_{\underline{x}}(x)\, dx = \text{Prob}\{x_1 < \underline{x} < x_2\}$$

survivor function A rather misleading term which stands for the probability that a given random variable exceeds a specified value. All random variables have a survivor function, even those without any connection to life span. It equals one minus the distribution function. The term is sometimes synonymous with 'upper tail probability'.

tolerance concentration The EC50 minus the NEC for a particular parameter (such as the specific costs for growth or maintenance). The parameter value of interest depends on the ratio of the EC50 minus the NEC and the tolerance concentration. All three concentrations originally refer to internal concentrations, but they can be translated into external concentrations by multiplication with the bioconcentration coefficient.

Taylor expansion The approximation of a function by a polynomial of a certain degree that is thought to be accurate for argument values around a specified value. The coefficients of the polynomial are obtained by equating the function value and its first n derivatives at the specified value to that of the n degree polynomial.

volumetric length The cubic root of the volume of an object. It has dimension length.

weighted sum The sum of terms that are multiplied with weight coefficients before addition. If the terms do not have the same dimension, the dimensions of the different weight coefficients convert the dimensions of weighted terms to the same dimension.

zero-th order process A process that can be described by a differential equation where the change of a quantity is constant.

Index

adaptation, 118
aging, 18, 27, 70, 100
Ames test, 16
approximation
  Taylor, 24, 98, 115
assimilation, 79, 84, 103

biomass, 116, 125

coefficient
  bioconcentration, 20, 38, 60, 77, 98, 105
  correlation, 136
  maintenance rate, *see* maintenance
  partition, 38
  variation, 118
  weight, 82, 119, 132
concentration
  -time, 81, 137
  hazardous, 42
  intracellular, 117
  lethal, *see* LC50
  no-effect, *see* NEC,NOEC,PNEC
  threshold, 13
  tissue, 25, 51, 52, 77, 98
  tolerance, 30, 100, 101, 103, 117, 118
confidence set, 55, 119

DEB theory, 17, 74, 115
density
  cell, 116
deviance, 65, 67
distribution
  binomial, 51
  chi-square, 119
  log-logistic, 13, 50, 51, 59
  log-normal, 13
  multinomial, 64, 99
  normal, 104, 118
  Weibull, 62

EC50, 15, 114, 117, 120, 139
energy
  budget, 17
  conductance, 96
  flow, 93
  investment ratio, 75, 96, 97, 103
error
  first kind, 114
  second kind, 42

feeding, 17

gradient, 13–15, 52, 108, 120
growth
  costs, 75, 78, 96, 117
  exponential, 34, 116, 117
  logistic, 87, 116, 124
  von Bertalanffy, 75, 96
guideline, 73, 95, 115

homeostasis, 17, 37
hormesis, 31, 110, 120
hypothesis
  null, 15, 85, 119

isocline, 142

killing
  acceleration, 62, 105

rate, *see* rate
kinetics
first order, 51, 63, 98
one-compartment, 59, 70
toxico, 20, 77, 97
two-compartment, 70

LC50, 13, 39, 50, 52, 58, 139
likelihood, 51, 63, 118, 129
estimate, 53, 64
profile, 53, 55, 82, 119, 141
ratio, 55, 65, 83, 119, 137

maintenance
costs, 75, 78, 101
rate coefficient, 75, 96, 101
matrix
covariance, 65
information, 64
mixture
of compounds, 27
of densities, 26
mutation, 16, 27

NEC, 24, 50, 58, 59, 115, 117, 120, 138
NOEC, 12, 50, 72, 109, 114, 120

oogenesis, 99

pH, 37
PNEC, 114
population
dynamics, 19, 31
growth, *see* rate
prefix, 134
profile, 140

rate
assimilation, 97
control mortality, 25, 62
elimination, 39, 51, 59, 77, 105, 115
hazard, 51, 52, 59, 70, 98, 100, 117
killing, 25, 52, 61, 99, 118
pop. growth, 111, 117
von Bert. growth, 75, 96
references, 7, 43, 56, 71, 90, 113, 125
reproduction, 95, 96
costs, 100
residual, 143
risk assessment, 9, 11, 114
run time, 128

standard deviation, 139
symbols, 60, 73, 94

temperature, 36
tolerance
concentration, *see* concentration
function, 99, 100

weight, 132
coefficient, *see* coefficient
number, 132

Standardized bioassays are frequently used to assess the toxicity of chemical compounds. This book presents a new analysis of the results of such bioassays for effects on survival, body growth (fish), reproduction (daphnia) and population growth (algae). The analysis provides a no-effect concentration and one process-based toxicity measure that is independent of the exposure time. One parameter, the elimination rate, describes how fast the effect builds up during exposure. The different modes of action of the compound are evaluated with respect to the energy budget of the organism. The biological, toxicological and statistical backgrounds are analyzed in depth. The book discusses the problems that are inherent to the standard NOEC/EC50 analysis of this type of bioassays and shows how the new analysis solves these problems.

The application of the analysis is very simple using the software package DEBtox, which is provided with the book. It runs under Windows on a PC (486 or higher) and under Unix and is GLP-validated. The data input is easy from packages such as Excel, or directly from keyboard. The resulting graphs and tables are presented on screen but they can also be saved in postscript files, which are easy to include in user reports. The results comprise parameter estimates with standard deviations for the various selections of mode of action of the compound, a goodness of fit measure for the model, a residual analysis, confidence intervals for the no-effect concentration for any choice of confidence level, statistical tests on parameter values as well as a full analysis of the effect as a function of concentration and exposure time. The latter includes the standard toxicity measures EC50 and ET50 with standard deviations, but other choices of effect levels are possible as well. Extensive help files will answer all likely questions by the user.

After a general introduction, the book presents the scientific background to the analysis, followed by a series of more technical chapters that explain the mathematical models and the statistical aspects. The final chapter describes the use of DEBtox in non-technical terms. This book will be of interest to all people involved in the assessment of the toxicity of chemical compounds.

Cover Illustration:
The hermetic vase containing crow, peacock, dragon and roses symbolizes alchemists' pursuit of the ideal state (gold) by a skilful process of decomposition and reconstruction.

VU University Press