Technische Wetenschappen

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2. Title: DEVELOPMENT OF A VERSATILE SOFTWARE PROTOCOL FOR ECOTOXICOLOGICAL RISK ASSESSMENT AT THE POPULATION LEVEL: THE MISSING LINK BETWEEN SINGLE-SPECIES TESTS AND HIGHER LEVEL TEST SYSTEMS

   Titel: ONTWIKKELING VAN EEN BREED TOEPASBAAR SOFTWARE PROTOCOL VOOR DE ECOTOXICOLOGISCHE RISICO-EVALUATIE OP POPULATIENIVEAU: DE MISSING LINK TUSSEN ENKEL-SOORTS TESTEN EN TESTSYSTEMEN OP HOGERE NIVEAUS

3. Keywords: ecotoxicology, life cycles, risk assessment, dynamic energy budgets.
   Trefwoorden: ecotoxicologie, levenscycli, risico evaluatie, dynamische energie budgetten.

4. Junior researchers / MSc students supervised by the applicants

J.E. Kammenga:	1 Post-doc, 3 PhD, 3 MSc
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5. Estimated duration of the project: 4 years

6. Abstract - Increased awareness of the potential toxic effects of chemicals on the environment has prompted the development of a range of invertebrate toxicity test systems over the last two decades. These systems comprise rapid single-species tests as well as long-term multi-species test systems (mesocosms) for population and higher level effects. In aquatic risk assessment of potentially hazardous compounds, eg. pesticides, mesocosms are required as a final tier to assess the potential impact at the ecosystem level. Yet the major drawback of this tiered approach is two-fold:

1. The extrapolation from single-species tests to mesocosm systems is difficult to perform because: a) almost all of the single-species tests involve only asexual species with relatively short life cycles, hence they do not reflect "real world" mesocosm diversity, b) sensitive single-species toxicity endpoints such as reproduction or juvenile survival may turn out not to be important from a population perspective whereas less sensitive ones may be more relevant.
2. Population responses in mesocosms are often difficult to interpret and require detailed life-cycle information.

The objective of this proposal is to develop a software protocol for the estimation of population growth rates at predicted environmental concentrations of toxicants. The protocol can be used to link single-species toxicity test outcomes to population level effects in mesocosms and will be based on animal life-cycle diversity and reproductive strategies. The work can be divided in two parts:

Part 1. Blue print design of the risk assessment protocol: Since it is not feasible to model all existing "real world" life cycles, nematodes will be used as a blue print model for life-cycle and reproductive diversity. They comprise a large variety in life cycles and reproductive strategies which mirrors biological diversity in other animal groups. The following tasks will be carried out: 1a) complete life-cycle toxicity experimentation with 2 pesticides and cadmium for 8 different nematode species including short as well as long living ones with different reproductive strategies, and 1b) estimation and comparison of population level effects using Dynamic Energy Budget (DEB) models and the toxicity data on various life-cycle traits.

Part 2. Derivation of a generalized population level risk assessment protocol: The following tasks will be carried out: 2a) from the nematode blue print design, life-cycle scenarios will be delineated which also apply to other invertebrate species, and 2b) a general software protocol will be developed for the population level risk assessment at the predicted environmental concentration for species with diverging life cycles and reproductive strategies using already existing single-species toxicity data. The protocol will be connected as a module to the recently developed Windows based DEBtox programme.

Samenvatting: Door de toenemende bezorgdheid voor de biologische effecten van milieuverontreiniging zijn gedurende de afgelopen twee decennia verschillende toxiciteitstesten met evertebraten ontwikkeld. Deze testen omvatten zowel kortdurende testen met één diersoort als langdurende zgn. mesocosmos experimenten met meerdere soorten op populatieniveau en hoger. Voor de aquatische risico-beoordeling van potentieel giftige stoffen zoals pesticiden zijn mesocosmos testen een laatste verplichte stap in de keten van testsystemen die begint met enkel-soorts testen. Aan deze ketenbenadering kleeft echter een tweetal grote nadelen:

1. de extrapolatie van enkel-soorts testen naar de mesocosmos systemen zijn moeilijk uit te voeren want: a) bijna alle enkel-soorts testen worden uitgevoerd met asexueel reproducerende kortlevende soorten terwijl in de "echte" wereld dit niet vaak voorkomt, b) gevoelige testparameters zoals reproductie en groei hoeven niet van groot belang te zijn voor de populatiegroeisnelheid. Dit hangt namelijk sterk af van het type levenscyclus van het betreffende organisme.
2. de effecten op de dierpopulaties in de mesocosmos systemen zijn vaak moeilijk te interpreteren zonder gedetailleerde informatie over de levenscyclus.

Het doel van dit onderzoek is om een software protocol te ontwikkelen voor het schatten van de effecten op populatiegroeisnelheden bij verschillende stofconcentraties. Het protocol kan gebruikt worden om resultaten van enkel-soorts testen te koppelen aan populatie effecten in mesocosmos testsystemen met als basis de levenscycli en reproductieve strategieën die veel voorkomen. Het project bestaat uit twee delen:

Deel 1: Blauwdruk ontwerp van het risico-evaluatie protocol: Omdat het ondoenlijk is de levenscycli van alle organismen te bestuderen wordt een blauwdruk ontworpen dat gebaseerd is op de diversiteit van levenscycli en reproductieve strategie binnen de nematodenfauna. Deze weerspiegelt in grote mate de diversiteit in andere diergoepen. De volgende taken zullen worden uitgevoerd: 1a) compleet levenscyclus toxiciteitsonderzoek met 8 verschillende nematoden-soorten en 2 pesticiden en cadmium, 1b) bepalen van effecten op populatieniveau op basis van toxiciteitsdata mbv. zgn. Dynamische Energie Budgetten (DEB).

Deel 2: Ontwikkeling van een algemeen protocol op populatieniveau: hier worden de volgende taken uitgevoerd: 2a) van het blauwdruk ontwerp worden levenscyclus scenarios afgeleid die ook gebruikt kunnen worden voor andere diersoorten, 2b) een algemeen toepasbaar protocol voor de risico-evaluatie op populatieniveau wordt ontwikkeld voor Windows wat als module toegevoegd kan worden aan het reeds bestaande DEBtox programma.

7. Budget

7.1 Given the considerable amount of modeling and detailed life-cycle experimentation we request support for a post-doc (modeling, 3 years) at the Vrije Universiteit and a PhD student (life-cycle research and modeling, 4 years) at Wageningen University.
7.2 The request for small equipment and consumables involves general laboratory equipment.
7.3 The large equipment needed to perform the experiments as proposed is already available.
7.4 The travel budget is limited and will be used mainly for the participation in international congresses.
7.5 At the present the Technologiestichting STW is the most logical organisation to request support.
7.6 Support

Name	Position	Employed by	hours/week
Dr. ir. J.E. Kammenga	Wp	WU	4
Prof. Dr. S.A.L.M. Kooijman	Wp	VU	4
Prof. Dr. ir. J. Bakker	Wp	WU	1
H.H.B. van Megen	Obp	WU	8
Dr.ir. A.M.T. Bongers	Wp	WU	1
Dr. J.J.M. Bedaux	Wp	VU	2
Drs. M. Luger	Wp	VU	2

7.7 The present proposal will be externally supported by the following companies: NOTOX, Aquasense, Procter & Gamble and TNO. The total sum can be estimated with a minimum of Dfl. 42,500. For more details see section 10 and accompanying support letters.

7.8 Overview of the budget (in Dutch guilders)

Year	Post-doc*	PhD-student**	Small equipment (VAT excl.)	Consumables (VAT excl.)	Travel	Chemical analysis***
2000	6 months	12 months	5,000	30,000	5,000	15,000
2001	12 months	12 months	5,000	30,000	5,000	7,000
2002	12 months	12 months	2,000	30,000	5,000	12,000
2003	6 months	12 months	2,000	30,000	5,000	7,000

The total budget can be estimated to DFl: Post-doc (210,000), PhD (191,000), equipment (14,000), consumables (120,000), travel (20,000), analyses (41,000) = 596,000.

*: Post-doc (Theoretical Biology, VU): he/she will be specialized in DEB-modeling. DEB models will be developed for nematodes. The simultaneous analysis of survival, growth and reproduction data will be analysed (since this is not possible with the present version of DEBtox) and the translation of effects on survival and reproduction to effects on population growth will be modeled. Furthermore the calculation of NEC and ECx values will be conducted, including confidence intervals for data on individuals and for the population growth rate (see also 9.1.7).

**: PhD-student (OIO) (Laboratory for Nematology, WU): The Phd student will start with setting up stock populations of nematodes and will perform pilot toxicity experiments for range-finding of pesticide concentrations. He/she will perform complete life-cycle tests recording effects on important state variables for the DEB models. NEC will be derived for the compounds used for various traits.Together with the Post-doc he/she will develop DEB models for nematodes and, at a later stage, general models will be developed based on these nematode models which are applicable to other species but comparable life-cycles (see also 9.1.7).

***: chemical analysis of pesticides will be conducted by ALTERRA of the group of Dr. M. Leistra (see 9.1.7). DGL 15,000 is required for methodology development for measuring pesticides in agar. The remaining sum of DGL 21,000 will be allocated for analysis of 120 samples. Neither the laboratory of Nematology or the Department of Theoretical Biology has the experience and equipment to perform these important exposure experiments. Cadmium analysis in agar and internal nematode body burdens will be conducted by the sub-department of Soil Science and Plant Nutrition in Wageningen. Estimated costs: 500 samples, Dfl 5,000.

8. Candidates: No candidates have been selected yet, but the PhD and post-doc will be based at Wageningen and Amsterdam respectively.

9. Description of the project

9.1 Table of contents Page

9.1.1 Introduction	5
9.1.2 Problem formulation	5
9.1.3 Protocol design: reproductive strategies and life-cycle diversity	6
9.1.4 Why nematodes as a blue print model ?	8
9.1.5 The DEB approach	8
9.1.6 Why toxicity tests will be performed in this project	9
9.1.7 Methodology and time schedule	10
Part 1. Blue print design of the risk assessment protocol – selection of test compounds	10
Part 2. Derivation of a population level risk assessment protocol	11
9.1.8 Translation to other species	13
9.1.9 Application to other compounds	13
9.2 How does the project fit in the research programme of the department?	13
9.3 The scientific relevance of the project vis-à-vis related research	14
9.4 New equipment	14
9.5 Relations to other research groups in related areas in The Netherlands	14
9.6 Related research outside The Netherlands	14
10. Utilisation and protocol validation	15
10.1 Identification of users	15
10.2 Application of the protocol by end-users	16
10.3 Product outcomes and utilisation scheme	16
10.4 Product limitations	18
11. Overview articles/books	18
12. Relevant publications from the applicants	18
13. Previous developed products by the participating groups	19
14. Literature used	20

9.1.1 Introduction

The environmental impact assessment of potentially hazardous new chemicals involves a multi-tiered testing programme which comprises single-species toxicity tests as a first step. It is generally acknowledged by ecotoxicologists and environmental legislators that these single-species tests provide an adequate step toward the ecological risk assessment of toxicants in soil and water (Van Straalen 1992). To obtain safe quality criteria for pollutants in water, many tests have been developed using aquatic species (Calow 1998), some of which have been standardized and adopted by the Organisation of Economic Cooperation and Development (OECD) (Calow 1998). In addition to aquatic tests, progress is made on the development and standardization of soil toxicity tests all of them using invertebrate species (Løkke and Van Gestel 1998). In the aquatic risk assessment procedure the final step in the tiered approach often involves mesocosm testing to assess the potential impact at the ecosystem level of a new pesticide.

The objective of this proposal is to rationalize the risk assessment of toxic compounds by developing a software protocol at the population level based on animal life-cycle diversity and reproductive strategies. It is believed that this protocol may be a versatile and cost-effective tool which links single-species tests to mesoscosm test systems.

9.1.2 Problem formulation

Current ecotoxicological risk assessment methods of a chemical compound adopted by the European Union involves (Ahlers and Diderich 1998):

- *exposure assessment*: leading to predicted environmental concentrations (PEC) of a chemical from release due to its production, processing, use and disposal.
- *effect assessment*: data obtained from acute or long-term toxicity tests are used to extrapolate concentrations with expectedly no adverse effects on organisms (no effect concentration, NEC).
- *risk characterization*: the PEC is compared with the NEC. In case PEC > NEC then an attempt should be made to revise data of exposure and/or effect in an iterative process to conduct a refined risk characterization. In case the PEC remains larger than the NEC, risk reduction measures have to be considered.

The aquatic effect assessment is often based on a tiered approach starting with single-species toxicity tests (EC_{50} or LC_{50}) from which data are then extrapolated to the ecosystem level by application of safety factors or calculation rules to obtain NEC-values (Ahlers and Diderich 1998). In the case of manufacturing new potentially hazardous compounds (eg. pesticides) mesocosm data are required as a final tier to negate the presumptions of risk assessment (Urban 1994; EU 1996). A mesocosm can be defined as an intermediate-sized system, such as a pond or enclosure, that can be replicated and manipulated to test population performance and functions (Touart 1994). Mesocosms aim to bridge the gap between laboratory single-species experiments and field observations. The advantages are that complex ecosystem functions such as nutrient behaviour or interactions between species can be observed and monitored even as any indirect effects. Moreover replicates can be used in contrast to large scale field studies (Jak 1997).

An important aspect of mesocosms is that population responses can be adequately assessed and recovery can be monitored following toxic insults (Graney et al. 1994).

At present a number of risk assessment tools have been developed for population and higher organisation levels such as communities and food weds. To bridge the gap between theoretical analysis and empirical fitting, (Hendriks and Enserink, 1996) designed an analytical model linking traditional ecological variables to common toxicological endpoints. To allow generalization of the model for different species, ecological variables were correlated to species size using reported allometric equations. They reported that populations that exhibit low life-time fecundity relative to survival effects produce the greatest reductions in population growth rate. The model was applied to laboratory populations of waterfleas and to a field population of cormorants. Model calibration indicated that the observed population trend could be explained largely by exposure to polychlorinated biphenyls although other factors will have contributed too. Traas et al. (1998) constructed an integrated fate and effects food web model to simulate effects of chlorpyrifos in experimental indoor ecosystems (microcosms). Direct effects were simulated by integrating a sigmoidal concentration-effect relationship with the population growth model for functional groups and not for groups with specific life-history characteristics. Klepper et al. (1998) calculated the potentially affected fraction of species, defined as the fraction of species exposed to a concentration above their NOEC. The approach was used to show the utility in ranking substances and areas. Further approaches were focused on the accumulation of toxic compounds along the food chain or a food web (Jongbloed et al., 1996; Hendriks et al., 1996).

Yet these studies do not provide a step toward generalizing the results for species with divergent life-histories. The proposed software protocol for instance can be used to interpret mesocosm population responses of Daphnids using life-cycle information and food availability such as reported by Jak et al. (1996; 1998).

The major drawback of current risk assessment approaches is two-fold and therefore requires rationalization:

1) the extrapolation from single-species tests to mesocosm test systems is difficult to perform. There is no connection between the single-species tests and the mesocosms because almost all single-species tests involve asexual species with relatively short life cycles. Moreover they do not account for the diversity in life cycles and reproductive strategies which exist in the "real" world (see section 9.1.3). Another aspect which has largely been ignored is that sensitive single-species toxicity endpoints such as reproduction or juvenile survival may turn out not to be important from a population perspective whereas less sensitive ones may be more relevant (see section 9.1.3).

2) Further disadvantages of mesocosm studies are that results are often difficult to interpret. It is widely acknowledged that inter and intra-specific differences in toxic effects can be found due to eg. age, developmental stage, sex and surface-to-volume ratio. Causal interpretation of the toxic impact on eg. freshwater communities may require intensive studies on the demographic structure of at least the most prominent populations. And then to gain a proper insight this information needs to be combined with laboratory life-cycle tests performed with these species (Brock and Budde 1994; Sherratt et al. 1999).

Mesocosm population level responses are difficult to interpret and to link with single-species test results.The present proposal aims to bridge the gap between single-species toxicity data and population level effects. With the envisaged protocol it will be possible to obtain rapidly population level effects of species with divergent strategies based on just a few life-cycle data and results from single-species toxicity tests.

9.1.3 Protocol design: reproductive strategies and life-cycle diversity

Reproductive strategy

Apart from fishes and algae many of the invertebrate toxicity test species are notable for their similarity in asexual reproductive strategies among the different species. Under normal and recommended test conditions the widely used aquatic crustaceans *Daphnia magna*, and *Ceriodaphnia spp* (Persoone and Janssen 1998) are parthenogenetic even as the soil springtail *Folsomia candida* (Van Gestel and Van Straalen 1994), the soil nematode *Plectus acuminatus* (Kammenga et al. 1996), the aquatic rotifer *Brachionus plicatilis* (Carmona et al. 1995) and the oribatid mite *Platynothrus peltifer* (Van Gestel and van Straalen 1994). The earthworm *Eisenia fetida* (OECD 1994) is a hermaphrodite. Within the framework of risk assessment it must be realized that these species were selected with the aim to develop rapid, reproducible test systems which are cost effective and robust. However in the "real" world the asexual reproductive system is not a dominant feature among different organisms. For instance in soil micro-arthropod communities only two out of twelve strategies were recorded as asexual (Siepel, 1994). Moreover it was noted that in heavily polluted areas the proportion of parthenogenetic Collembola species increased with decreasing distance from the pollution source (Siepel, 1994).

It has long been acknowledged by ecologists that the reproductive strategy is of great importance in determining the responses of individuals and populations to pollution stress (Grassle and Grassle 1974; Gray 1979). It was suggested that the presence of a species in a polluted area depended more on the reproductive strategy than the degree of tolerance (Gray 1979). Moreover Forbes and Depledge (1992) stipulated the importance of reproductive strategy for ecotoxicological risk assessment by underlining the significance of sexual reproduction in terms of population recovery following toxic stress.

Life-cycle diversity

Although the importance of life-cycle diversity is acknowledged within the ecotoxicological scientific community this aspect has received little attention in terms of a quantitative approach. Like the bias toward asexual reproductive test species, there is a bias toward short living and fast reproducing species for pragmatic and cost effective reasons. The main reason being to assess a (sub)lethal effect within a short time span. Yet the type of life-cycle such as short vs. long living, iteroparous vs. semelparous or the shape of the survival curves strongly determine the effects at the population level (Postma et al. 1995; Kammenga et al. 1997; Groenendijk et al. 1999; Forbes and Calow, 1999).

The susceptibility of organisms to chemical stress however is not necessarily determined by the effect on sensitive life-cycle traits. Life-cycle analyzes have pointed out that the impact of a chemical depends on the effect on the rate of population increase (Van Straalen and Kammenga 1998). It was shown that apparently small effects on less sensitive traits may impair the population growth rate of organisms to a greater extent than large effects on more susceptible traits. The argument is illustrated in Figure 9.1. On the right-hand side of the figure, a normal concentration-response relationship is shown for two different species 1 and 2. The toxicant effects are expressed in terms of some life-cycle trait (e.g. reproduction, growth, survival). At a certain concentration species 2 is affected greater than species 1. On the left-hand side, graphs are shown that relate population growth rate "r" to the trait considered. The theoretical example was chosen such that the r-value of the species 1 reacts much more strongly to a decrease in the life-cycle trait than the r-value of species 2. The result is that r of species 1 is affected to a greater extent than r of species 2, even though both species are exposed to the same concentration and species 2 is more sensitive with respect to the trait considered (Van Straalen and Kammenga 1998).

Figure 9.1. Theoretical response curves for the effect of a toxicant on a life-cycle trait (right) and the influence of this trait on population growth rate "r" (left). Curves are shown for two species, 1 and 2, that are assumed to differ in sensitivity

Also annual iteroparous species with *equal* juvenile and adult periods appeared to be vulnerable to toxicant induced adult mortality when the juvenile survival was low. In contrast, species with an iteroparous life cycle and with *unequal* juvenile and adult periods appeared to be susceptible to toxicant induced adult mortality when the reproductive rate was low (Kammenga et al. 1997). In a comparative study between two nematode species with different life cycles it was shown that the effect of copper on population grwoth rate was the same despite a difference of a factor 3 between the EC_{20} for reproduction (Kammenga and Riksen 1996). Similar conclusions were drawn for comparing sensitivity between life-cycle traits in terms of population growth rate (Kammenga et al. 1997). A similar argument was given by Calow et al. (1997).

From these findings it can be concluded that both life-cycle diversity and reproductive strategy are of paramount importance for determining the effect of toxic insults on the population level.

9.1.4 Why nematodes as a blue print model?

The phylum Nematoda offers a perfect model to meet the objectives of this project, in particular free-living species which are those not associated with plant roots. Most free-living nematodes can be easily reared in the laboratory and life-cycle observations offers no difficulties (Kammenga et al. 1996). Table 9.1 shows the species which have been selected for this research on the basis of life-cycle differences and ability to culture them. A division was made between relatively short and long living species with different reproductive strategies, i.e. i) hermaphroditic, ii) sexual and iii) parthenogenetic. Hermaphroditic nematodes have both male and female reproductive organs, parthenogenetic species have only female organs and produce parthenogenetic offspring.

These three strategies cover the largest part of reproductive strategies within the animal kingdom. We use nematodes as a blue print because a major advantage over many other invertebrates is the absolute difference between short and long living species ranging from 3 days to more than 12 months while they can still be reared under the same, small scale, conditions. This would be impossible for other invertebrates, arthropods or fish species because their life-cycle is far too long with respect to complete life-cycle experimentation. It is envisaged that parthenogenetic strategies of another species can be matched to the nematode ones just by changing parameter values or time between life-cycle events within the DEB model.

Table 9.1 Selected free-living nematodes: Caenorhabditis elegans, Panagrellus redivivus, Acrobeloides nanus, Aphelenchus avenae, Tobrilus gracilis, Dorylaimus stagnalis, Aporcelaimellus sp., Prionchulus punctatus.

Short living (max. 7 days)			long living (max. 12 months)	
Hermaphrodite	sexual reprod.	parthenogenetic	sexual reprod.	parthenogenetic
-	*P. redivivus*	*A. nanus*	*T. gracilis*	*Aporcelaimellus*
C. elegans	*C. elegans*[*]	*A. avenae*[**]	*D. stagnalis*	*P. punctatus*

[*] C. elegans can be reared sexually by increasing temperature for 3 hours at 37° C. Hermaphrodites produce approx. 300 eggs while sexually reproductive ones produce approx. 1000 eggs.
[**] A. avenae is parthenogenetic at 20° C. At higher temperatures sexual reproduction occurs.

All selected nematode species are well described in terms of taxonomy and can be identified easily. Moreover, they are very well culturable in agar Petridishes each with different food sources (see 9.1.7).

9.1.5 The DEB approach

General background

The Dynamic Energy Budget (DEB) theory aims to understand and describe the processes of substrate (food) uptake and use for the processes of maintenance, growth, development, and reproduction as it changes during the full life cycle of an individual (Figure 9.2). A lot of effort has been invested to test the theory against experimental data (Kooijman, 1993, http://www.bio.vu.nl /thb/deb, downloadable), and found to be in excellent agreement when due attention is paid to life-cycle peculiarities. It has been developed by the group of Kooijman to evaluate how toxic substances, with various modes of action, change resource allocation patterns, and how these changes work out at the population level. During the last twenty years, the mathematical theory of physiologically structured population dynamics matured (e.g. Metz and Diekmann 1986; Ebenman and Persson 1988; Goldstein et al. 1991; Tuljapurkar and Caswell 1996), and provides the machinery for this evaluation.

The DEB theory is simple enough to be of use to quantify effects of toxicants, when the description is decomposed into physiological parameters, as identified by the DEB theory, and the translation of effects on these parameters to endpoints, such as survival and reproduction. This approach not only allowed new characterizations of toxic effects that are independent of exposure time, but also the estimation of No-Effect Concentrations (NECs) as model parameters, including likelihood based confidence intervals. The powerful method of profile likelihoods can be used to solve all statistical testing problems.

The DEBtox programme will soon be extended with a module comprising variable toxicant levels and mixture toxicity. Because the international ecotoxicological and legislative as well as industrial community has shown increased interest it is expected that the utilisation of DEBtox will expand quite rapidly and will decrease costs of empirical research. Already new developments along this line can be viewed on http://www.bio.vu.nl/thb/users/bas/.

DEB and nematodes

We here plan to apply the DEB theory to quantify toxic effects on life-cycle traits of nematodes, and to translate these effects into effects on the steady state population growth rate ie. at constant food levels. The DEB approach has already been successfully applied to different nematode species (Van Haren 1995) including two species which we plan to use, ie. *A. avenae* and *Caenorhabditis spp.*. Besides it was shown that body growth obeys Von Bertalanffy growth equations (Van Haren 1995; Boon 1992). From these observations it is estimated that also other DEB approximations will apply to nematodes. Possible effects include effects on maintenance, growth, maturation and reproduction. The application of the DEB theory to nematodes with different reproduction strategies treat males and females as `different species', that is: all parameters can differ, in principle. In practice most parameters will turn out to be (almost) identical, such as the specific maintenance costs, and the specific costs for growth, and, probably, parameters involved in the feeding and digestion process.

(Differences between sexes usually relate to allocation to development plus reproduction versus maintenance plus growth.) Direct effects on reproduction in sexual species can be classified as a reduction of the gamete production or gamete survival rate, or a reduction of fertilization efficiency. Particular stages, such as the embryo, can turn out to be the most sensitive. The problem of dilution by growth needs special attention, because this phenomenon modifies the level of internal toxicant, to which effects are to be related.

Figure 9.2. Dynamic Energy Budget theory aims to quantify the energetics of individuals as it changes during life history. The key processes are feeding, digestion, storage, maintenance, growth, development, reproduction and aging. The theory amounts to a set of simple rules, and a wealth of consequences for physiological organization and population dynamics

9.1.6 Why toxicity tests will be performed in this project

In this project complete life-cycle toxicity experiments will be performed with all nematode species. The reason for doing this is to compare differences or similarities in toxic responses among species with different strategies. In a theoretical paper it was shown that species with different strategies may respond in a similar way to a toxic insult. For instance, a decrease in reproduction due to cadmium may lead to increased sensitivity of the population growth rate to effects on adult mortality (Kammenga et al. 1997). In order to assess these kinds of relationships and their generality we need to perform life time exposure experiments.

9.1.7 Methodology and time schedule

Table 9.3 shows the time schedule and planned tasks.

Part 1. Blue print design of the risk assessment protocol - selection of test compounds
In the first part, different nematode species will be exposed to various pesticide concentrations during their whole life span. To obtain comparable exposure conditions and to make observations easy we propose to rear populations in water agar. We propose to test compounds of which the mode of action and toxicology are well known, but differ between the different compounds, as well their environmental behaviour. We plan to test two pesticides and cadmium.
Pesticides: At present we have much information on the aforementioned aspects for the fungicide carbendazim and within the framework of the EU-funded MIXTOX project (see 9.2). At this stage results indicate that long-term exposure to this pesticide have significant effects on various nematode species, thus indicating that the selection of these compounds for the present project is promising. Carbendazim, a benzimidazole, is a commonly and widely used systemic fungicide that is relatively easy to analyse in soil samples by liquid chromatography. Benzimidazoles are known for their interference with DNA synthesis and they inhibit cellular development.

We also plan to study an insecticide of which a great deal of information is available with respect to population level effect and environmental fate. Chloropyrifos is an organophosphate compound and is widely studied for many invertebrate species with diverging life cycles and in different test systems ranging from single-species tests to more complex mesocosm setups (Brock and Budde 1994). Since nematodes have nerve tissue it is expected that long term effects will become visible during the complete life-cycle tests.

It must be noted that although some nematode species are known for their relative insensitivity to **acute** effects of pesticides it has been shown that in complete life-cycle exposure or long term population level experiments they are very often **sublethally** affected (Kammenga et al. 1997; Parmelee et al. 1997).

Cadmium was selected because of its well known effects on nematodes and its ease of application in these kinds of tests systems (Kammenga 1995).

Chemical analysis: The pesticides will be added to the agar and analysed by the group of Dr M. Leistra at SC-DLO, Wageningen, The Netherlands. This group has a long standing record in measuring low levels of various pesticides in different substrates. The group is also involved in the MIXTOX project (see 9.2). Methods will be updated and tested for chemical analysis of the pesticides in agar. Furthermore the rate of transformation in agar will be studied in an incubation study. The concentration of cadmium in agar and inside the nematodes will be measured by the Central Laboratory of the sub-department of Soil Science and Plant Nutrition of Wageningen University. Uptake of metals in nematodes can be measured in a bulk analysis of several hundreds of individuals which are rinsed with 0.05 M HCL for 20 minutes.

Life-cycle toxicity experiments: For each species the following traits will be studied according to the DEB approach: Body volume (using Andrassy's formula (Freckman 1982) for nematode size) and Von Bertalanffy growth curve, storage, reproduction and survival. Additonally time between life-cycle events will be recorded. All traits will be measured under different toxicant concentrations to obtain different effect levels. For this purpose the nematodes will be kept individually (with approx. 15-20 replicates) in small Petri-dishes or multi-compartment plates (see Kammenga et al. 1996). Using a stereo-microscope, effects on various traits will be observed and recorded. The food level is kept relatively constant by transferring nematodes at regular intervals of 3 days to fresh agar plates with new food. Table 9.2 shows the food source for each species and the optimal rearing temperature. In case of predatory nematodes, the food level of the prey can be maintained constant by offering full grown populations of *C. elegans.* Since rearing of this species is very easy we do not expect any problems in prey density or availability.

During the life-cycle experiments, toxicity endpoints such as the "no effect concentration" (NEC) will be estimated for various life-cycle traits during relatively short time spans (see Part 2). These data will be used as single-species toxicity data which are the input data for the DEBtox risk assessment module.

Table 9.2 Nematode food source and optimal temperature

nematode species	food source	Optimal temperature (° C)	Reference
Caenorhabditis elegans	*Escherichia coli (OP50)*	20	Wood (1988)
Panagrellus redivivus	*Escherichia coli (OP50)*	20	Duggal (1978)
Acrobeloides nanus	*Acinetobacter johnsonion*	20	(Sohlenius 1973)
Dorylaimus stagnalis	*C. elegans*	20	Shafqat et al. (1987)
Aporcelaimellus obtusicaudatus.	*Haematococcus sp. or Protosiphon* (algae)	18	Wood (1973)
Prionchulus punctatus	*C. elegans*	22	Maertens (1975)
Aphelenchus avenae	*Rhizoctonia solani* (train AG4)	20	Bonnel and Camporota (1989)
Tobrilus gracilis	Organic material	18	Schiemer and Duncan (1974); Tahseen et al. (1993)

Culturing of nematodes in Petridishes (9 cm diameter)

- *C. elegans*: (soil species) stock cultures are already available in the laboratory of Nematology and its developmental biology is extremely well known (Wood 1988). Rearing of populations is standardized on NGM agar with *E. coli* food source (Wood 1988). Two reproductive strategies will be used: hermaphroditic and sexual. Self-fertilizing hermaphrodites and males can easily be distinghuised on the basis of their morphology. Males arise spontaneously at low frequency (0.7%) but higher ratios can be obtained by heat shock treatment of the population (Wood 1988). To avoid overcrowed plates, Petridishes can be stored for months at 4° C.

- *A. avenae*: (soil species) stock cultures are already available in the laboratory of Nematology. Its devlopmental biology is well known. Populations are kept on malt agar (1.5%) inoculated with the fungus *R. solani*. To avoid overcrowed plates, Petridishes can be stored for months at 4° C.
- *A*. nanus.: (soil species) stock cultures are already available in the laboratory of Nematology. For detailed info on agar cultures, developmental biology and life cycle see Sohlenius (1973).
- *P. redivivus*: (freshwater species) stock cultures are already available in the laboratory of Nematology. Its biology is well described (Duggall 1978). Populations are kept on NGM agar (1.5%) inoculated with the bacteria *E. coli*. To avoid overcrowed plates, Petridishes can be stored for months at 4° C.
- *A. obtusicaudatus*: (soil and sediment species) will be extracted from a small patch of loamy sand in Wageningen, The Netherlands (Kammenga et al. 1994). Populations will be kept in soil extract agar (1.0%) supporting populations of algae (see Wood 1973, also for its developmental biology).
- *D. stagnalis*: (soil and sediment species) will be extracted from a small patch of loamy sand in Wageningen, The Netherlands (Kammenga et al. 1994). Populations will be kept in proteose pepton agar using *C. elegans* as prey and *E. coli* as food source for *C. elegans* (according to Shafqat et al. 1987).
- *P. punctatus*: (soil species) will be extracted from a small patch of forest in Wageningen, The Netherlands. Populations will be kept in 1.0% bacto-agar and 10% humus extract which will be inoculated with *C. elegans* (see Maertens 1975 also for developmental biology).
- *T. gracilis*: (freshwater species) to our knowledge this species has not been reared before. However it has a sexual strategy and its developmental biology is well described (Tahseen et al. 1993). It is believed to feed on organic material.

*Part 2. Derivation of a population level risk assessment protoco*l

The most efficient method to produce a userfriendly software package, is to extend the already existing DEBtox package with an extra module that allows for:

- the simultaneous analysis of survival, growth and reproduction data since particular parameters can occur in different data-sets at the same time (this kind of analysis is not possible with the present version of DEBtox). This increase in parameter space makes the problem of finding initial guesses for the parameter estimates a demanding job, as well as tests on the local versus global extremes of the likelihood function. Since DEBtox is meant to be a user-friendly software package, these complex tasks are taken away from the user, and many checks on local extremes of the likelihood function are performed in the background. Just to mention an example: for infinitely small or large elimination rates, the models reduce to different degenerated cases, which are always evaluated and compared with the non-degenerated one. This is less easy than it seems, because effects on growth and reproduction are decribed in terms of differential equations, that must be evaluated numerically during the parameter estimation procedure. Depending on the parameter values, these equations can be stiff (i.e. they contain both slow and fast kinetics), which means that special care must be taken to avoid large numerical deviations.

- data extension. With the extension of the analysis from one data-set to more data sets, it also will become possible to use DEBtox for the analysis of multi-sample data. It will be possible, for instance, to select survival data related to compound 1 and survival data related to compound 2, and test statistically the difference in toxicity between compound 1 and 2. The way to do this test is first assume that the toxicity parameters are identical, and obtain the likehood values base on this assumption; then assume that these parameters differ, and obtain a new likelihood value, that can be compared with the earlier one. The likelood ratio test can be used to convert the difference in likelihood to probabilities and test the hypothesis of equal toxicity statistically. Although these applications do not directly relate to the population growth of nematodes, the possibility to use the planned extended version of DEBtox results naturally from this research.
- the translation of effects on survival and reproduction to effects on population growth. The power of the approach is that the responses in terms of survival, growth and reproduction, are all considered as functions of the concentration as well as the exposure time. This means that response surfaces are analysed, and the results are independent of the usually arbitrarily chosen length of the (standardized) exposure period. It not only allows the evaluation of effects on populations, but also means that chronic toxicity can be estimated from acute toxicity, given the validity of the assumptions on which the models are based. Although the value of such extrapolations is always limited, because we can never be certain that the models are `true', it is frequently the best that can be done in absence of other knowledge. The many graphs that can be extracted from DEBtox can be used to judge the validity of the model. Many years of experience so far learns that the fits are frequently very good.
- the calculation of NEC and ECx values, including confidence intervals for data on individuals and for the population growth rate.

The DEBtox program extracts information form the standardized toxcicity test results which can than be used to estimate the effects at the population level.. The theory of DEBtox allows for the population evaluation of important steering factors such as food availability in the field. Obviously, population level effects can be actually measured for some species but this seems to be vary laborious and tedious work for many different food conditions thus illustrating the value of the model approach.

Table 9.3 Time schedule and tasks to be carried out.

	2000	2001	2002	2003
Part 1 PhD, WAU	⇓1			
Setting up stock cultures				
Pilot toxicity experiments				
Life-cycle toxicity experiments				
Develop nematode DEB models			⇓2	
Part 2 Post-doc, VU				
Simultaneous DEB analysis of traits				
Develop population growth models			⇓3	
Setup userfriendly Windows based protocol				⇓4

Deliverables: 1) set of stock cultures available for research and other interested parties, 2) Complete life-cycle blue-print DEB model for nematodes, 3) Population growth model applicable to ther spacies based on the nematode blue-print model, 4) user friendly Windows based risk assessment software package.

9.1.8 Translation to other species

The DEB theory implies rules to translate parameter values from one species to another, with different (maximum) body sizes. Given the restrictions and reservations that are always involved in extrapolation procedures, this allows to evaluate expected effects in species that cannot be included in experimental protocols, but species that are still relevant to be protected. (Body size scaling relations for physiological parameters are discussed in Kooijman 1993). Since toxic effects are related to internal toxicant levels, physical chemical information about toxicants (such as octanol-water partition coefficients and ionization coefficients) that can be used to infer about toxicants levels, can also be used to infer about effects (Kooijman and Bedaux 1996). This can be used to translate information about one toxicant into that of another one, with the same mode of action. Environmental risk assessment is always in great need for methods that supplement limited available information, and our approach offers extrapolation methods both across species and compounds. For instance it was reported that toxicokinetics and population level consequences in earthworms were comparable to fish (C. Klok, ALTERRA, Wageningen, The Netherlands pers. comm.).

Since many other species than nematodes live for more than one or two years their life cycle very often is adapted to the year seasons. Starting of with nematode life cycles we will integrate parthenogentic and sexual strategies with multiple breeding periods in order to tailor these life cycles to other species. This can easily be done by introducing a non-reproductive stage in the nematode life-cycle model.

9.1.9 Application to other compounds

Within this project we will focus on pesticides and a metal. We selected pesticides mainly because mesocosms are particularly used within the tiered risk assessment approach of these compounds. Moreover we have selected compounds which can be analysed quite well, have a well know mode of action, toxicology and degradation profile. However the end product, *the risk assesment protocol*, can also be used for other compounds since the input data require just NEC-values of any compound and not just pesticides.

The applicability of the protocol is not restricted to pesticides alone. It can be used for any other potentially toxic compound as long as toxicity data are available.

9.2 How does the project fit in the research programme of the department?

Research of the Kooijman group has focused on the DEB approach for many years. A userfriendly (Windows and Unix compatible) software package "DEBtox" has been written to evaluate the results of toxicity tests, as standardized by the OECD, which allows technicians to apply the theory practically. This method to estimate NECs is under review by the OECD, as alternative for the widely critized concept of No-Observed Effects Concentrations (NOECs), which suffers from serious statistical and methodological problems (Kooijman 1996; Laskowski 1995). Research is presently focusing on the development of a similar package "DEBdeg" for the analysis of the results of standardized bioassays for biodegradation of compounds. Since environmental risk assessment is primarily based on the combination of exposure and effects, the combined application of DEBtox and DEBdeg can be really useful. Drs. Luger (VU) will be involved in the project because of his expertise in writing the programme DEBtox.

Research of the Kammenga group has been dedicated to the use of nematodes as indicators for environmental pollution. Fundamental life-cycle research was carried out over the last 8 years to unravel underlying mechanisms of population consequences of toxic stress. Nematodes are used as a model because of their ease of culturing and short life cycles. At present two large EU projects are coordinated, one focusing on biomarkers for toxic stress in soil invertebrates (BIOPRINT-II) and one on conceptual development of mixture toxicity in soil organisms (MIXTOX). Furthermore a joint research project is carried out with the Institute of Terrestrial Ecology, Monks Wood UK on fitness consequences of toxic stress in earthworm populations. Recently Kammenga organized an international workshop in Krakow, Poland, on Demographic Ecotoxicology together with Dr. R. Laskowski.

9.3 The scientific relevance of the project vis-à-vis related research

The project may have value for ecological research focusing on life-cycle responses to stress. The parallel comparison of species with divergent life cycles to different stress factors may reveal specific rules of how species deal with abiotic stress factors. Furthermore ongoing research in the field of non-linear population dynamics will benefit from the present project.

The use of profile likelihood functions in the statistical analysis of multi-samples is new, as far as we know, and might provide a powerful method in the case that the large sample theory of likelihood ratios does not apply. (which is ususally the case with small samples). This allows for a statistically sound analysis based on small samples.

9.4 New equipment

No request for large new equipment is included.

9.5 Relations to other research groups in related areas in The Netherlands

The project complements to research carried out by the department of Ecology and Ecotoxicology of the Vrije Universiteit (Prof. Van Straalen) in Amsterdam. Both participating groups in this project have close connections and combined projects with the group of Van Straalen. Moreover the EU research carried out by the Van Straalen group on Terrestrial Micro Ecosystems (MTE) may benefit from this project. On the topic of toxicity testing we have connections with the company Aquasense (ir. A. Derksen) in Amsterdam which is specialized in ecotoxicity testing of new chemicals and environmental samples according to GLP. Furthermore there are links with TNO-Den Helder within the framework of the Knowledge Soil Centre.

9.6 Related research outside The Netherlands

The group of Kammenga has collaborates with the Institute of Terrestrial Ecology, Monks Wood UK (Dr. J.M. Weeks) on fitness consequences of toxic stress in earthworm populations. Furthermore the group has working contacts with the Department of Pure and Applied Zoology (Prof. R.M. Sibly) on life-cycle approaches and toxic stress.

10. Utilisation and protocol validation

10.1 Identification of users

It is foreseen that many companies and legislative authorities focusing on ecotoxicological risk assessment procedures and and testing of chemicals will benefit from the proposed protocol. The neccessity of the current project is reflected by the external amount of financial support dedicated to the project which can estimated to a total of Dfl 42,500. The following companies, institutes and ministry will benefit from the project outcomes:

Aquasense: AquaSense carries out biological, ecological, chemical and toxicological analyses which are, for example applied in the determination of the effects of chemical substances, effluents, sediments and soils on organisms. These results can be used to procure discharge licences for industrial clients, to set priorities for remedial and gathering of information on the toxicity of chemicals for humans and the environment.

In the laboratory research, standard toxicity tests are executed with freshwater and marine water samples, effluents and soil samples, in accordance to national or international protocols. Examples are tests with algae, bacteria, Microtox®, *Chironomus*, *Daphnia*, various types of fish, oyster larvae, amphipodes, sea urchins, springtails, plant seeds and nematodes. The outcomes of this project will add value to their products in terms of ecological relevance.

Support: a minimum of Dfl. 5,000 or twice this amount.

contact person: Dr J. Postma, Laboratory for Ecotoxicology, tel: +31 020 5922244, fax: +31 020 5922249, e-mail: JPostma@aquasense.com.

NOTOX: Within the section Ecotoxicology tests are carried out mainly for industry aiming to assess the potential ecological effects of newly developed materials. Next to vertebrate testing, many tests involve sublethal tests with a range of aquatic invertebrates. In order to cost-effectively assess population level effects for these species, the software protocol may prove to be very useful.

Support: Dfl. 2,500

Contact person: Dr R.I. Bogers, Head Ecotoxicology, tel: +31 073 6419575, fax: + 31 073 6418543, e-mail: Ribo@compuserve.com.

TNO: MEP-TNO in Den Helder performs ecotoxicological impact studies of pesticides for third parties such as industry and government. Research is focused on the effects at both the invertebrate population and ecosystem level in aquatic mesocosms. The interpretation of population level effects requires insight into underlying effects at the individual level and the envisaged protocol will enable MEP-TNO to reach this goal.

Support: Dfl 25,000

contact person: Drs. J.M. Brils, tel: +31 0223 638805, fax: +31 0223 630687, e-mail: J.m.brils@mep.tno.nl.

VROM: The Ministry of Housing, Spatial Planning and the Environment seeks to continue to utilise environmental functions in a sustainable manner thus providing clear water air, soil and succours the crops. A clean environment allows to live safely in green surrounding and sustainable development requires that the environment is clean and free of pollution. The Netherlands government has espoused the objective of sustainable development in various agreements, including the Convention on Biological Diversity. The results achieved at the climate conference at Kyoto also illustrate the international importance attached to the environment.

With regard to the present project the Directorate Soil, Soil Protection and Soil quality, supports the initiative.

contact person: Dr. G.H. Crommentuijn, tel: +31 070 3394296, e-mail: Trudie.Crommentuyn@dbo.dgm.minvrom.nl

P&G: At the Environmental Science Department, ecological risk assessment compares an ingredient's ecological effects to its exposure concentration in the environment. Reducing uncertainty of ecological risk estimates is a central focus of study. As new product technologies are developed, a tiered process of risk assessment delivers increasingly accurate safety information. In this way, the company directs more of its resources toward the product improvements that deliver the greatest benefits to consumers. In the early tiers of the process, tests are simple, conservative, and relatively inexpensive. Many substances used in consumer products may be judged safe at the first tier of the process. If no definitive answer results, however, and the ingredient warrants further development, successive tiers of testing follow. At this stage the putcomes of the project will prove to be valuable since it provides the link between the lower tier and higher tier level testing. In higher-tier testing, fate and effects testing methods are more sophisticated and realistic and the level of uncertainty is reduced.

Support: USD 5,000

contact person: Dr J. Jaworska, tel: +32 2 4562845, fax: +32 2 456 2076, e-mail: Jaworska.j@pg.com.

ALTERRA: Part of the research programme underpins government policy on pesticides and the environment. For that purpose, the authorization tools are improved with respect to surface water load, hazards to aquatic organisms at higher organisation levels such as the community and ecosystem, and leaching to groundwater. Finally, methods, techniques and formulations are developed and tested in order to adequately assess the biological impact. At this stage the outcomes of this proposal may be useful in calculation population level effects.
contact person: Dr. T.C.M. Brock, tel: +31 317 474661, fax: +31 317 424812.

The persons mentioned above may also be part of the "gebruikerscommissie" or users committee. We have close contacts with these companies in terms of mutual research interest in ecotoxicological risk assessment procedures. Collaborative contacts exist with Aquasense on the use of nematodes in single-species toxicity test systems.

10.2 Application of the protocol by end-users
At present the testing of potentially hazardous compounds (eg. pesticides) involves a multi-tiered approach comprising mesocosm or population level studies as a final step toward ecosystem effect assessment. Mesocosms require a huge amount of financial resources and the proposed life-cycle scenario risk assessment protocol will greatly increase the cost-effectiveness of these systems by aiding to interpret the population level outcomes. Reported costs for mesocosms range from USD 6,000 - 30,000 for small ones and USD 300,000 - 1,000,000 for large scale setups (Cairns and Cherry 1998). It is estimated that the envisaged protocol including required man-hours for obtaining toxicity and life-cycle data (either from literature or experimental) will cost about USD 1000 - 2000. We think that the outcomes of this project may substantially contribute current risk assessment procedures in a cost-effective and ecological relevant way.

Once the protocol has been developed it is suggested to validate it for its general application to a range of aquatic species, because at present mesocosms are part of the tiered test procedure only for aquatic environments. The validation process will be conducted in close collaboration with the abovementioned companies and institutes. It is envisaged that following the validation process, if successful, we will seek endorsement by the EU for the protocol to be included as an explanatory and versatile tool linking single-species toxicity tests mesocosm test systems. Previously the DEBtox version has already been adopted by the OECD (Kooijman SALM, Bedaux JJM, Gerritsen AAM, Oldersma H and Hanstveit AO (1998) Dynamic measures for ecotoxicity. In: Report of the OECD Workshop on Statistical Analysis of Aquatic Toxicity Data, OECD, OECD Environmental Health and Safety Publications 10: 64-90.)

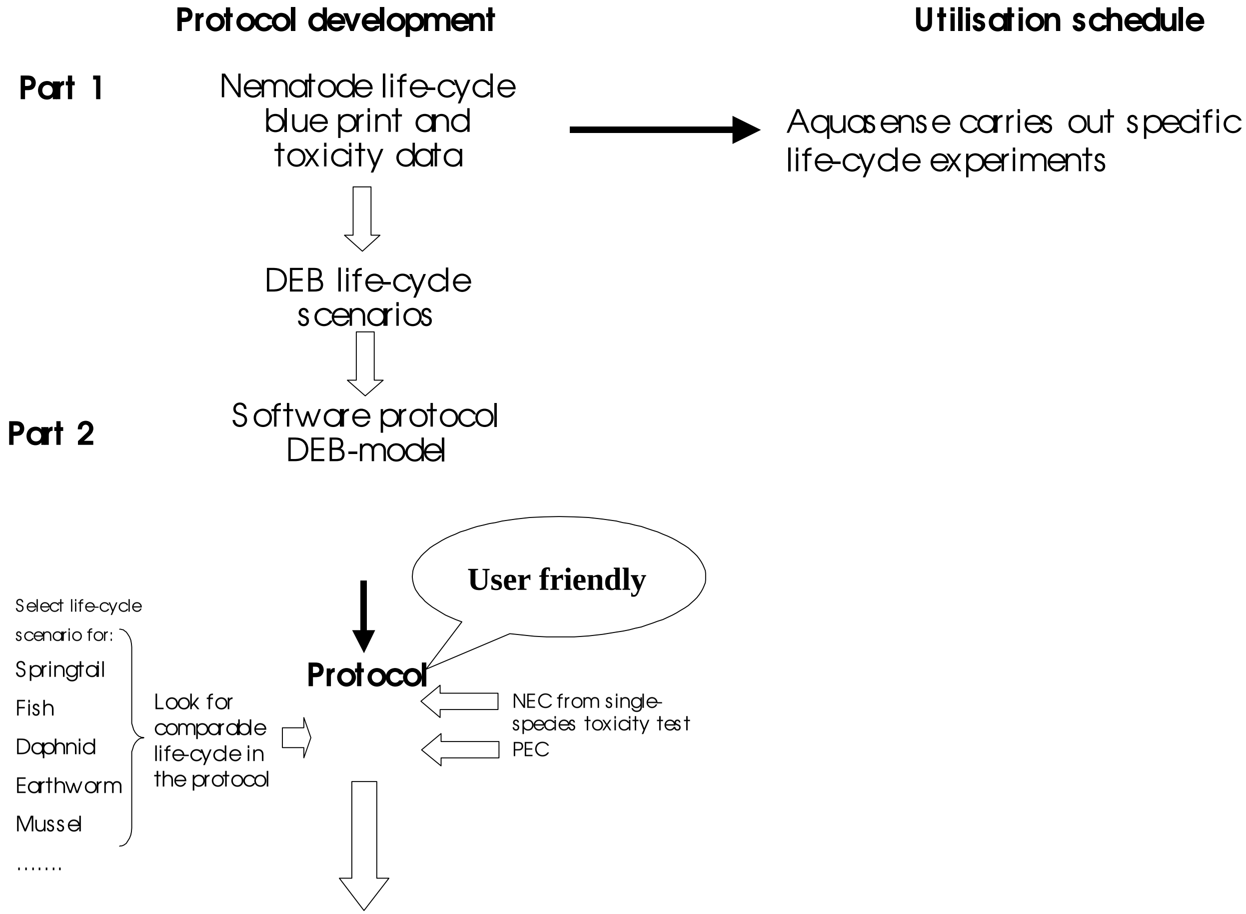
10.3 Product outcomes and utilisation scheme
Figure 10.1 shows the outcome of the project. The project will produce a software package which will be connected to the DEBtox package. To perform a risk analysis the following parameters need to be entered into the programme:

INPUT: - Predicted environmental concentration of the toxicant.
- The type of life cycle ie. estimated life-span, reproductive strategy and reproductive output. (For many wild species occurring in the Netherlands these data are known)
- LC_{50} or NEC concentrations for given life-cycle traits obtained from toxicity tests.

The output of the protocol will be:
OUTPUT: - Population growth rate at the PEC and the risk of extinction.

Example: a risk-assessor from industry has to perform a final tier mesocosm test for pesticide X (EU 1996). In order to interpret population level effects in these mesocosms he uses the *DEBtox module protocol for risk-assessment*. He therefore 1) selects some representative species (Daphnid, snail, fish and midge) which are normally present in the mesocosms and determines the type of life cycle and reproductive strategy. (This information was already stored once in a database). He 2) determines the PEC based on previous chemical analyses and looks for 3) NEC values obtained during previous short-term toxicity experiments. He looks in the DEBtox protocol for comparative life cycles and feeds the data into the population model. The model predicts the effect on the population growth rate at the PEC and indicates a risk of extinction. The whole analysis will take one person 3 days.

Figure 10.1. Software protocol (as a DEBtox module) outcome and utilisation scheme of a risk assessment of species having different life-cycle types and the risk of extinction for each type estimated from its predicted population growth rate at the Predicted Environmental Concentration (PEC)



Life cycle type	Population growth rate at PEC	Risk of extinction (for example)
Springtail	1.2	0.1 (low)
Fish	0.8	0.3 (intermediate)
Daphnid	-0.1	0.7 (high)

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10.4 Product limitations

The present proposal aims to predict effects of toxicants at the population level which can be used to forecast mesocosm population level responses. However mesocosm experiments allow also for studies at higher organisation levels such as species interactions and ecosystem functions. Besides, indirect effects (those effects that follow a reduction or elimination of sensitive species) are not included. They greatly enhance the ecological relevance of toxicity testing *per se* by focussing on long term behaviour of both the compound as well the population dynamics of various species. The envisaged protocol does not include these higher level effects. Also it does not comprise any temporal and spatial variations due to migration of organisms which may play an important part in field effects. These limitations should be kept in mind while progressing with the interpretation of population responses in mesocosms.

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Van Straalen NM and Kammenga JE (1998) Assessment of ecotoxicity at the population level using demographic parameters. In: Ecotoxicology (G Schüürmann and B Markert, eds.), John Wiley & Sons, New York, USA, pp. 621-644.

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13. Previous developed products by the participating groups

The Kooijman group:

- With financial support of the ministry of VROM, a userfriendly (Windows and Unix compatible) software package "DEBtox" has been written to evaluate the results of toxicity tests, as standardized by the OECD, which allows technicians the apply the theory practically. This method to estimate NECs is under review by the OECD, as alternative for the widely critized concept of No-Observed Effects Concentrations (NOECs), which suffers from serious statistical and methodological problems (Kooijman, 1996).
- In collaboration with TNO, STW-funded research is presently executed to develop a similar package "DEBdeg" for the analysis of the results of standardized bioassays for biodegradation of compounds. The is to find realistic characterizations for the rate of the decomposition process, to improve the prediction of exposure levels. Since environmental risk assessment is primarily based on the combination of exposure and effects, the combined application of DEBtox and DEBdeg will be promising.

The Kammenga group:

- A nematode soil toxicity test was developed supported by the Stimulation Programme for Soil Biology. The test is based on a life-cycle approach and is currently under review for standardisation by the International Standardisation Organisation (ISO).
- Funded by the EU, a battery of biomarkers (biochemical indicators for toxic exposure and effect) is under development within the BIOPRINT-I and BIOPRINT-II projects.
- A soil toxicity test was developed within the framework of the EU project SECOFASE based on the competition for food between two bacterivorous nematode species.

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