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"Analysis of toxicity tests on fish growth"

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ANALYSIS OF TOXICITY TESTS ON FISH GROWTH

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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 judged with the profile likelihood function. The method is illustrated with applications to several data-sets for body size versus concentration of toxicant.

Key words—NEC; bioassays; growth; tolerance concentration; profile likelihood

INTRODUCTION

This article is one in a series that aims to analyse the full set of routine aquatic toxicity experiments (Bedaux and Kooijman, 1994; Kooijman and Bedaux, 1996a,b; 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 because of the statistical problems with this characteristic (Kooijman, 1981, 1996a; 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 by toxic chemicals directly, or indirectly via effects on feeding or maintenance, because these processes are intimately linked to each other. 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 and Bedaux, 1996b). Satisfying analyses of toxicity data for growth must be consistent with that of the indirect effects on reproduction. This is why the analyses of effects on growth and reproduction are linked.

In this article, 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 only simple 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 it with the standard analysis. Since we tie 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 the large fast growing fish (Weatherley and Gill, 1984) that 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 and 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 and 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 and

Fletcher, 1984; Steele *et al.*, 1991; Lucas and Priede, 1992) and the trout (Brown, 1946; Phillips, 1989) and do affect uptake kinetics and effects of the toxicant (Arthur and 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 full discussion of the DEB theory is outside the scope of this article, 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 (Staples and 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 and Yousef, 1976), they are remarkably conservative (Weatherley and Gill, 1983). The shape of the organism during growth is taken to be constant in this article, 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 from the size of the organism and the food density (Staples and Nomura, 1976).

Material derived from food is added to the reserves, which are rich in fat (Denton and Yousef, 1976; Atherton, 1975). The reserve density (i.e. the reserves per structural bio-volume) 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 article 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, because of 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, because of the assumption of the shape that 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 the von Bertalanffy growth equation

$$\frac{\mathrm{d}}{\mathrm{d}t} V = 3\dot{\gamma}(V^{2/3}V_{\mathrm{m}}^{1/3} - V) \tag{1}$$

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 $V_{\mathrm{m}}^{1/3}$ 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

$$\dot{\gamma} = \frac{1}{3}\frac{\dot{m}g}{1+g} \tag{2}$$

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 $\dot{\gamma}V_{\mathrm{m}}^{1/3}$ is independent from 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 is frequently observed empirically (Galliucci and Quinn, 1979; Xiao, 1994).

Since weight is proportional to structural volume at abundant food (Kooijman, 1993), (1) 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

$$\frac{\mathrm{d}}{\mathrm{d}t} W = 3\dot{\gamma}(W^{2/3}W_{\mathrm{m}}^{1/3} - W) \tag{3}$$

$$\frac{\mathrm{d}}{\mathrm{d}t} L = \dot{\gamma}(L_{\mathrm{m}} - L) \tag{4}$$

The von Bertalanffy growth equation has been fitted frequently to fish data and usually fits quite well (Chen *et al.*, 1992; Hearn and Leigh, 1994). Figure 1 gives a test for model (4) against experimental data for zebrafish and rainbow trout. To simplify the discussion that follows, we only use length measures

as in equation (4), but cubic roots of weights can always be substituted for lengths. Length measurements have the advantage above weight measurements that the fish need less handling, which allows repeated length measurements during growth of particular individuals. However, 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 fix 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 and 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 energetic perspective. The direct elimination is assumed to be proportional to the surface area again 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 assumed to be instantaneous again. The uptake/elimination kinetics reduces to

$$\frac{d}{dt}[Q] = cP_{vd}\dot{k}_a L_m/L - [Q]\left(\dot{k}_a L_m/L + \frac{d}{dt}\ln(L/L_m)^3\right) \qquad (5)$$

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 equation (5) accounts for the dilution by growth. This correction has empirically as well as numerically

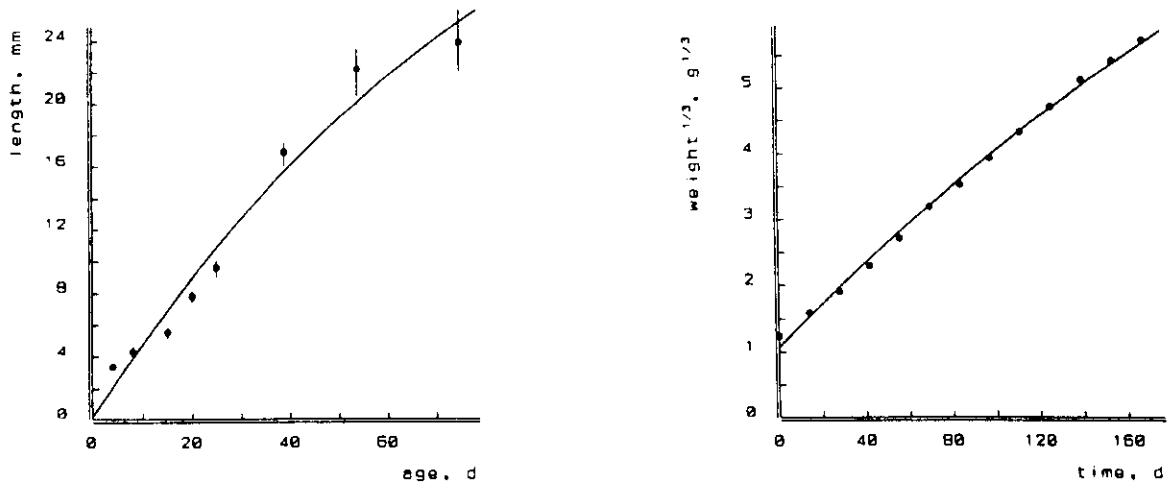


Fig. 1. Length-at-age for the zebrafish *Brachydanio rerio* at 25.5 C (left) and weight-at-age for the rainbow trout *Oncorhynchus mykiss* at 12 C (right). Data from Eaton and Farley (1974) and Weatherley and Gill (1983). The fitted curves are the von Bertalanffy growth curve (4). The parameter estimates (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 $\dot{\gamma} = 2.36 \times 10^{-3}$ (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.

been found to be essential (Borgmann and 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

$$\frac{\mathrm{d}}{\mathrm{d}t} c_q = c\dot{k}_a L_m/L - c_q\left(\dot{k}_a L_m/L + \frac{\mathrm{d}}{\mathrm{d}t}\ln(L/L_m)^3\right) \quad (6)$$

$$= \dot{k}_a\left(c - c_q - \frac{3c_q}{\dot{k}_a L_m}\frac{\mathrm{d}}{\mathrm{d}t}L\right)\frac{L_m}{L} \quad (7)$$

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

$$c_0(t) = \frac{c_0}{\dot{k}_a}\frac{\exp\{-3\dot{\gamma}t + (3\dot{\gamma} + \dot{k}_a)\int_0^t \Gamma(t_1)\,\mathrm{d}t_1\}}{\int_0^t \exp\{-3\dot{\gamma}t_1 + (3\dot{\gamma} + \dot{k}_a)\dot{k}_a\int_0^{t_1}\Gamma(t_2)\,\mathrm{d}t_2\}\Gamma(t_1)\,\mathrm{d}t_1} \overset{t\to\infty}{\longrightarrow} c_0 \quad (8)$$

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_q 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 $c_q \leqslant c_0$. 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 (4) leads to

$$\frac{\mathrm{d}}{\mathrm{d}t} L = \dot{\gamma}(L_m - L)\frac{1 + g}{1 + g(1 + s(c_q))} \quad (9)$$

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.

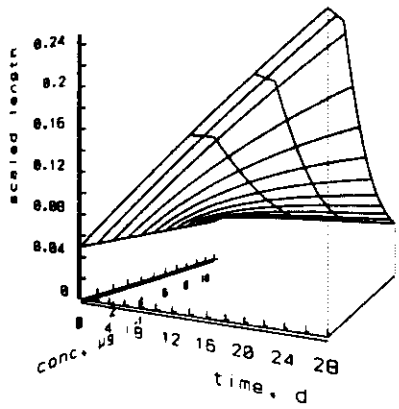
Maintenance. Many toxic compounds are likely to affect maintenance requirements, which translates into an increase in the maintenance costs. Because maintenance has priority over growth in the DEB theory, such an increase leads to a reduction of the growth rate. Since the feeding rate depends on body 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 (4) leads to

$$\frac{\mathrm{d}}{\mathrm{d}t} L = \dot{\gamma}(L_m - L(1 + s(c_q))) \quad (10)$$

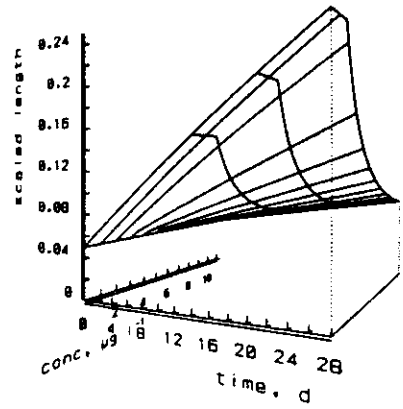
where $\dot{\gamma}$ is the von Bertalanffy growth rate in the control and L_m is the maximum length in the control.

Assimilation. If assimilation is affected, i.e. the incoming energy is reduced, growth is effected 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

maint., $c_M = 0.6\ \mu g\ l^{-1}$



growth, $c_G = 0.8\ \mu g\ l^{-1}$



assim., $c_A = 10\ \mu g\ l^{-1}$

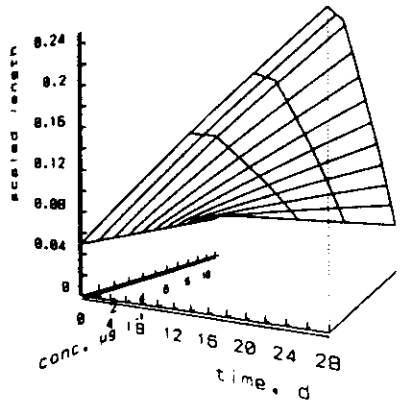


Fig. 2. Direct and indirect effects on growth. The von Bertalanffy growth rate is chosen $\dot{\gamma} = 0.008\ d^{-1}$, which is typical for zebrafish at 26°C. The elimination rate is $\dot{k}_e = 0.1\ d^{-1}$ and the no-effect concentration is $c_0 = 1.5\ \mu g\ l^{-1}$. The tolerance concentrations for maintenance, growth and assimilation are chosen 0.6, 0.8 and 10 $\mu g\ l^{-1}$, respectively, to produce similar response levels.

concentration for assimilation and c_0 the no-effect concentration. Notice that the stress function appears with a negative sign, rather than a positive one, to model adverse effects on assimilation. The consequence is that $c_q < c_A + c_0$ 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 a bit 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 (4) leads to

$$\frac{\mathrm{d}}{\mathrm{d}t} L = \dot{\gamma}(L_m(1 - s(c_q)) - L) \qquad (11)$$

The model for direct effects on growth and the two indirect ones are illustrated as response surfaces above the exposure time-concentration plane in Fig. 2. 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 large with respect to the von Bertalanffy growth rate (Hawker & Connell, 1985), so $\dot{k}_e \gg \dot{\gamma}$, equation (7) reduces to $c_q = c$ and equation (4) leads to the explicit growth curve

$$L(t) = L_{m,c} - (L_{m,c} - L_0)\exp\{-\dot{\gamma}_c t\} \qquad (12)$$

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	(9)	$L_{m,0}$	$\dot{\gamma}_0 \frac{1+g}{1+g(1+s(c))}$
Maintenance	(10)	$L_{m,0}(1+s(c))^{-1}$	$\dot{\gamma}_0(1+s(c))$
Assimilation	(11)	$L_{m,0}(1-s(c))$	$\dot{\gamma}_0$

(13)

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_{xd}, 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 $P_{xd} \to \infty$ such that $\dot{k}_a P_{xd}$ is constant. The stress function is in fact a function of the tissue-concentration, so we define a new stress function $S(\mathscr{C}_q) = \mathscr{C}_*^{-1}(\mathscr{C}_q - \mathscr{C}_0)_+$ for $* \in \{G, M, A\}$, where $\mathscr{C}_q \equiv c_q/\dot{k}_a$, $\mathscr{C}_0 \equiv c_0/\dot{k}_a$ and $\mathscr{C}_* \equiv c_*/\dot{k}_a$. 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 $\mathscr{C}_*$. The dimension of the $\mathscr{C}$'s is concentration times time. We will refer to $\mathscr{C}_0$ as the no-effect concentration-time. Notice that $S(\mathscr{C}_q) = s(c_q)$. The equations (7) and (9), (10), (11) now become

$$\frac{\mathrm{d}}{\mathrm{d}t}\mathscr{C}_q = cL_m/L - \mathscr{C}_q \frac{\mathrm{d}}{\mathrm{d}t}\ln(L/L_m)^3 \tag{14}$$

$$\text{growth} \quad \frac{\mathrm{d}}{\mathrm{d}t}L = \dot{\gamma}(L_m - L)\frac{1+g}{1+g(1+S(\mathscr{C}_q))}$$

$$\text{for } S(\mathscr{C}_q) = \mathscr{C}_G^{-1}(\mathscr{C}_q - \mathscr{C}_0)_+ \tag{15}$$

$$\text{maint.} \quad \frac{\mathrm{d}}{\mathrm{d}t}L = \dot{\gamma}(L_m - L(1+S(\mathscr{C}_q)))$$

$$\text{for } S(\mathscr{C}_q) = \mathscr{C}_M^{-1}(\mathscr{C}_q - \mathscr{C}_0)_+ \tag{16}$$

$$\text{assim.} \quad \frac{\mathrm{d}}{\mathrm{d}t}L = \dot{\gamma}(L_m(1 - S(\mathscr{C}_q)) - L)$$

$$\text{for } S(\mathscr{C}_q) = \mathscr{C}_A^{-1}(\mathscr{C}_q - \mathscr{C}_0)_+ \tag{17}$$

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 $\{t_1, t_2, \ldots, t_r\}$ and test concentrations $\{c_1, c_2, \ldots, c_k\}$, 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, p. 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 it 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. McCullag and 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 likelihood function $p(c_0)$ for the no-effect concentration c_0 is in this case of a normally distributed 'error' given by

$$p(c_0) = n \ln \sigma(c_0)/\sigma_{\min} \tag{18}$$

where n is the number of observations on data points; $\sigma(c_0)$ is the mean residual deviation $\sqrt{n^{-1}\Sigma_i(L_i - \mu_i(c_0))^2}$, 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 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 are exposed rather than young fish, 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 a 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 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 work out to be very similar (see Table 1).

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 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 Fig. 4), but the

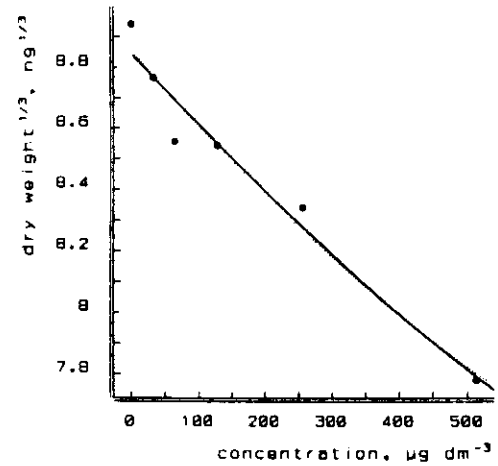


Fig. 3. Effects of seven days of exposure of the fathead minnow *Pimephales promelas* to sodium pentachlorophenol on growth. Data from Weber *et al.* (1989), as given by Bruce and Versteeg (1992). The curves represent model expectations for direct effects on growth while the elimination rate is zero (drawn curve) or infinitely large (stippled 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 1.

differences in 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 2. 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 $\%_0 = 0$ for the assimilation model is 0.6, which corresponds with a tail probability of 0.27. The no-effect concentration-time $\%_0$ 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 Fig. 5). The parameter estimates are given in Table 3.

Table 1. 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 Fig. 3

Parameter	Growth		Maintenance		Assimilation	
	$k_a = 0$	$k_a = \infty$	$k_a = 0$	$k_a = \infty$	$k_a = 0$	$k_a = \infty$
$\%_0$, 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)
$\%_e$, c_e (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)
$W_0^{1/3}$ ($\mu g^{1/3}$)	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 ($\mu g^{1/3}$)	0.0737	0.0732	0.077	0.0742	0.0793	0.0755

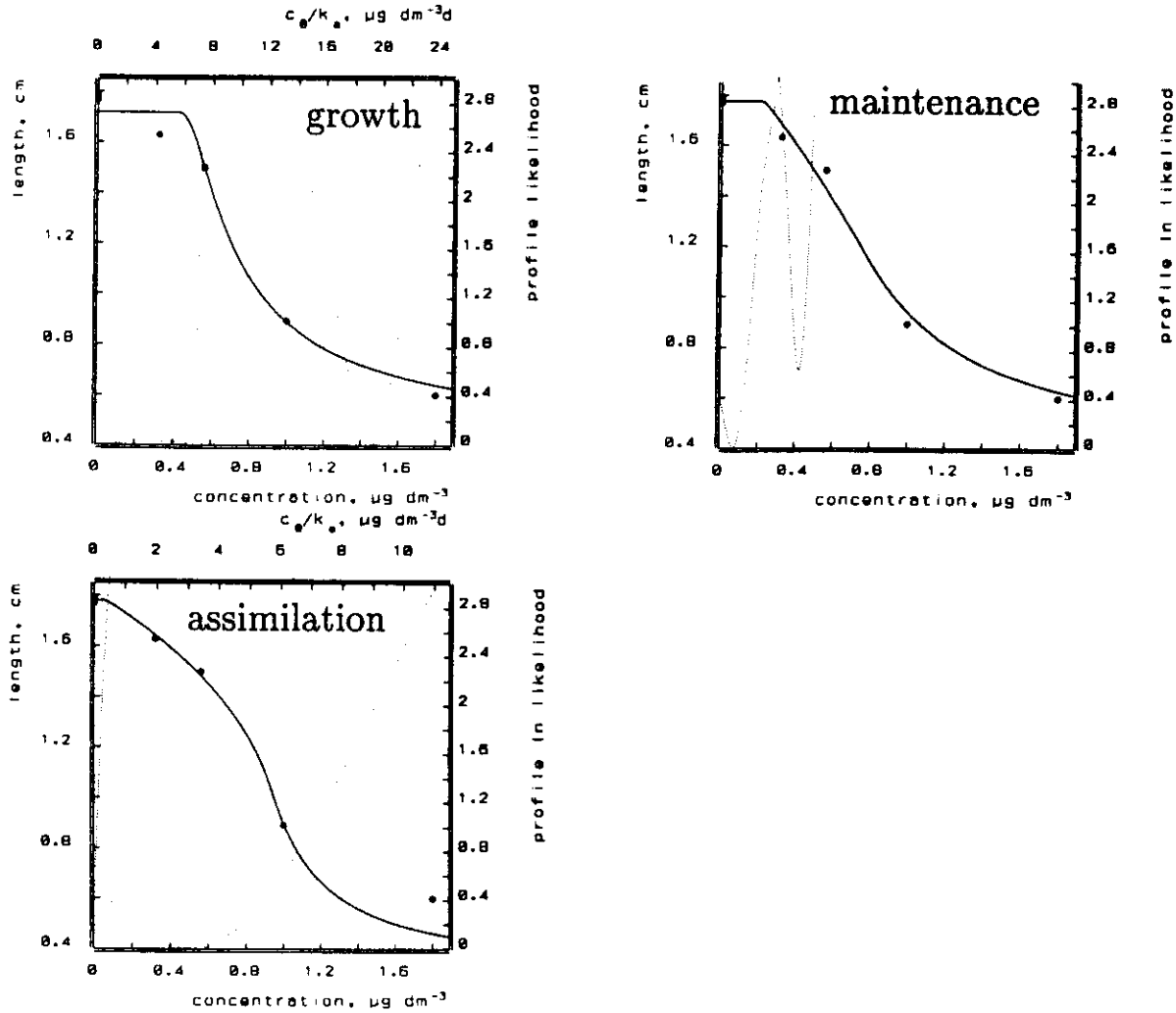


Fig. 4. Effects of 37 days of exposure of the zebrafish to benzo(k)-fluoranthene on growth. Data from Hooftman and Evers-de Ruiter (1992). The curves represent model expectations for direct effects on growth (upper left), maintenance (above) and assimilation (left). The stippled curves represent the profile ln likelihood functions for no-effect concentrations c_0. The coarsely stippled curve in the graph for effects on assimilation is the profile likelihood for $\mathscr{C}_0$. 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 2.

The high control value is not included in the estimation. The points estimate for the no-effect concentration is zero. The model for the effect on assimilation again fits best, but the differences in 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 Fig. 6). 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 4. The model for the direct effect on growth fits best, but the differences in 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 and Nelder, 1989, p. 255); they give some indications at best. The application of profile likelihood functions to obtain confidence sets is less sensitive for 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

Table 2. 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 Fig. 4

Parameter	Growth	Maintenance	Assimilation
$\%_0$, c_0, $\%_0$ (d μg/l, μg/l, d μg/l)	19 (1.33)	0.07 (0.17)	2.49 (2.49)
$\%_G$, c_M, $\%_A$ (d μg/l, μg/l, d μg/l)	8.33 (1.63)	0.121 (0.264)	116.6 (5.96)
k_d (d^{-1})		0.0105 (0.0357)	
L_t (cm)	4.67 (0.098)	4.85 (0.118)	4.86 (0.071)
σ (cm)	0.0547	0.0509	0.0307

Table 3. 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 Fig. 5

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)
k_d (d^{-1})	1.77 (65) 10^{-5}	1.84 (25) 10^{-3}	0.00595 (0.0245)
L_t (cm)	2.819 (0.023)	2.88 (0.047)	2.88 (0.045)
σ (cm)	0.0159	0.0148	0.0139

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 and Takizawa (1994) discussed multiple comparison methods in logistic growth models. Bruce and Versteeg (1992) applied a log-probit model, where the weights at the end of the exposure experiment are taken to be proportional to the survivor function of the normal distribution when plotted against the

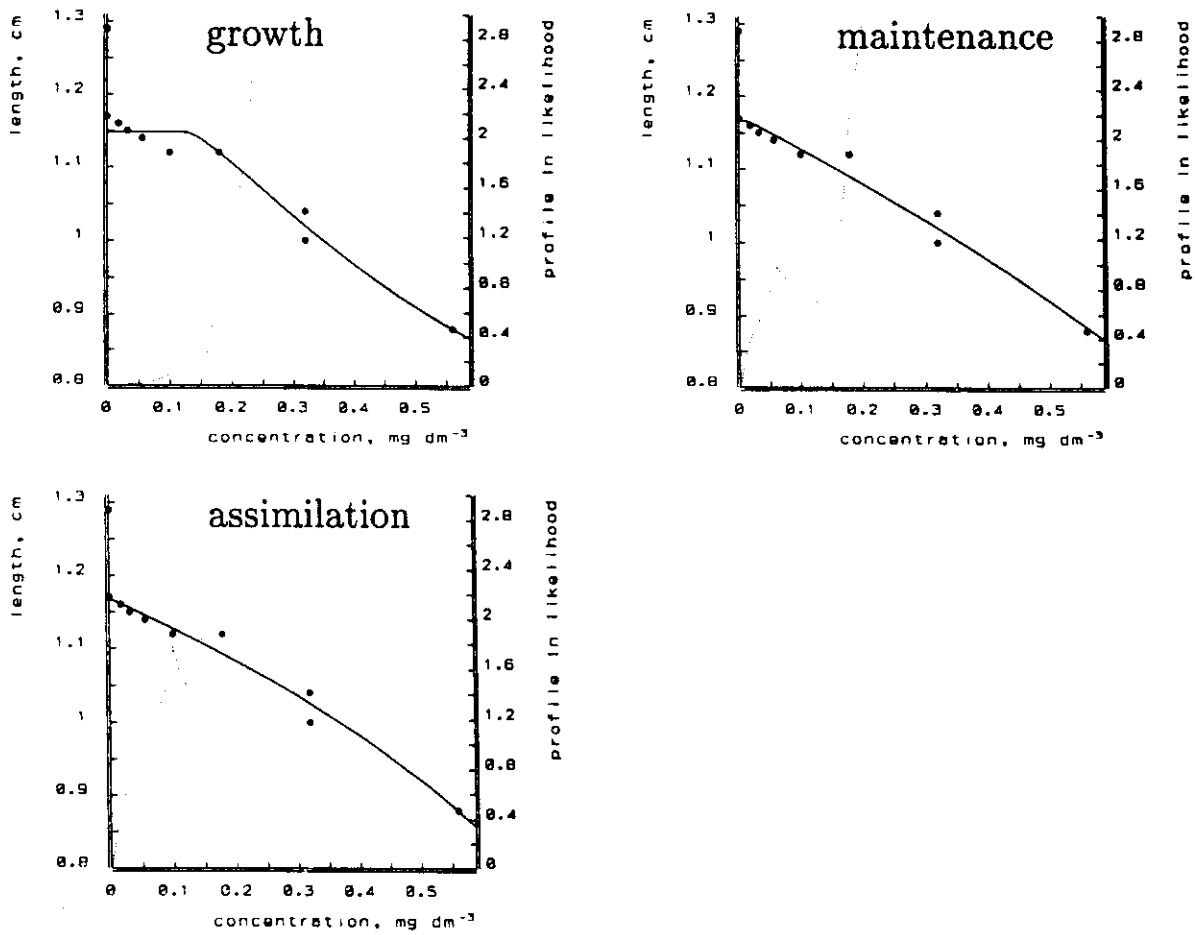


Fig. 5. Effects of 37 days of exposure of the zebrafish to phenanthrene on growth. Data from Hooftman and de Ruiter (1991). The curves represent model expectations for direct effects on growth (upper left), maintenance (above) and assimilation (left). The stippled curves represent the profile ln likelihood 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.

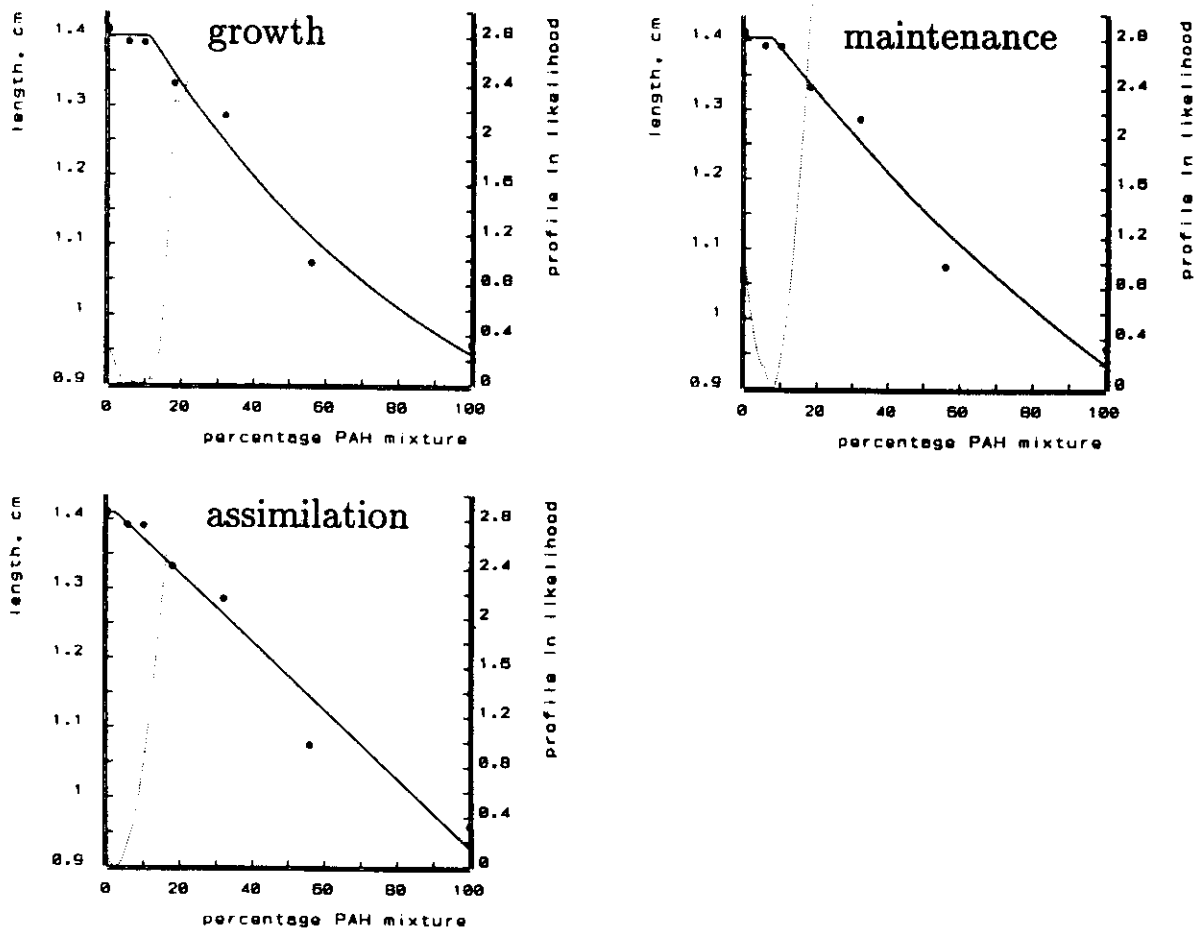


Fig. 6. Effects of 37 days of exposure of the zebrafish to a mixture of polycyclic aromatic hydrocarbons on growth. Data from Hooftman *et al.* (1993). The curves represent model expectations for direct effects on growth (upper left), maintenance (above) and assimilation (left). The stippled curves represent 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 4.

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. Borgmann and Whittle, 1992; Madenjian *et al.*, 1993). 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 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 and Bedaux (1995c) and Kooijman (1995) 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'

Table 4. 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 Fig. 6

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)
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

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 for such changes. The details of effects of changes in lipid content have been worked out (Kooijman and van Haren, 1990; Kooijman, 1993). Because the present description of effects of compounds is on the basis of the tissue-concentrations, variations in time can be taken into account. Such variations include metabolic transformation of the compound.

The software package DEBtox, as provided in Kooijman and Bedaux (1996), can be used to do all computations for the application of the models that are discussed in this article.

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