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THE USE OF SUBLETHAL CRITERIA FOR TOXICITY TESTS WITH THE FRESHWATER ROTIFER *BRACHIONUS CALYCIFLORUS* (PALLAS)

HET GEBRUIK VAN SUBLETALE CRITERIA VOOR TOXICITEITSTESTEN MET DE ZOETWATERROTIFEER *BRACHIONUS CALYCIFLORUS* (PALLAS)

Colin JANSSEN

Thesis submitted in fulfillment of the requirements
for the degree of Doctor in Environmental Sanitation

Proefschrift voorgedragen tot het bekomen van de
graad van Doctor in de Milieusanering

op gezag van
Rector : Prof. Dr. L. DE MEYER

Decaan :
Prof. Dr. ir. L. MARTENS

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Colin
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LIST OF ABBREVIATIONS AND SYMBOLS

a.o.	= among others
ASTM	= American Society for Testing and Materials
cf.	= confer (compare)
C_o	= (algae) concentration at time 0
C_t	= (algae) concentration at time t
DCA	= 3,4-dichloroaniline
e.g.	= exempli gratia (for example)
EC_{50}	= efffect concentration 50 % ; i.e. concentration at which 50 % of the test organisms are affected
Ecu	= European currency unit
e_o	= life expectancy at hatching
EPA	= Environmental Protection Agency (USA)
et. al.	= et alii (and other people)
F	= filtration rate
F.T.	= flow-through
fem.	= female
F_x	= x^{th} generation
h	= hour
I	= ingestion rate
K	= carrying capacity
l	= liter
LC_{50}	= lethal concentration 50 % ; i.e. concentration at which 50 % of the test organisms are killed
ln	= natural logarithm
LOEC	= lowest observed (adverse) effect concentration

log	= logarithm
L.T.	= life table
l_x	= age-specific survival
max.	= maximum
m.s.	= mean survival
mg	= milligram
min.	= minimum
min.	= minutes (in chapter 3 only)
ml	= milliliter
mm	= millimeter
m_x	= age-specific fertility
n	= number
N_o	= population density at time 0
NOEC	= no obsevered (adverse) effect concentration
N_t	= population density at time t
offspr.	= offspring
p	= probability
P	= parental generation
PCP	= pentachlorophenol
P.G.	= population growth
pH	= minus logarithm, base 10, of H^+ activity
r_m	= intrinsic rate of natural increase
R_o	= net reproductive rate
S.D.	= standard deviation
sec	= seconds

sq.	= squares
t	= time
T	= generation time
T	= time lag (in chapter 8 only)
v	= volume
x	= geometric mean
x	= at time x
°C	= degrees Celsius
$	= dollar (USA)
±	= plus minus
>	= smaller than
>	= greater than
%	= percentage
µg	= microgram
µl	= microliter

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INTRODUCTION

Ecotoxicology in an interdisciplinary science that deals with the adverse effects of chemicals on biological systems. A well-balanced assessment of these adverse effects of pollutants in the environment should therefore be based on studies integrating analytical, toxicological and ecological information. This study will focus on the latter two aspects of the hazard assessment of chemicals in aquatic environments.

Biological indicators can be used to assess either the actual or the potential impact of xenobiotic substances on aquatic ecosystem and can thus be applied in two different ways : (a) for a-posteriori assessment - to monitor the actual effects in nature, or (b) for a-priori assessment - to predict the impact of a substance prior to its release (Calow, 1989). This predictive ecotoxicological information is usually obtained from laboratory studies with a (limited) number of experimental organisms in the so-called toxicity tests or bioassays. In an ecotoxicological context a bioassay can be defined as a procedure that uses living material to estimate the effects of pollutants. Ideally, bioassays should predict the levels of chemicals that produce no-observable effects on biological systems at the population, community, and ecosystem level, and should identify the biological resources at risk (Cairns and Pratt, 1989). However, because of the complexity of natural systems and the resulting poor understanding of the structure and function of these systems, aquatic toxicologists in practice mostly have to restrict their investigations to the testing of the chemical compounds under (very) limited conditions.

Most of the toxicity testing to date has been performed with laboratory test systems. In a recent review on the applications of bioassay techniques, Maltby and Calow (1989) reported that 90 % of all studies classified as predictive bioassays were single species laboratory tests. These authors also showed that the most commonly used test organisms were invertebrates (74.8 %), mostly cladoceran crustaceans. Fish, with fathead minnow (Pimephales promelas) and rainbow trout (Salmo gairdneri) as the major representatives, accounted for 23.9 % of the test species. Cladocerans and fish are in fact the only categories of aquatic animals which are prescribed by the various national and international regulatory and standardization organizations in their standard toxicity test methods. Compared to the complexity of natural systems these standard bioassays are, however, so

surprisingly simple, that one can be concerned about the ecological relevance and predictive capacity of these types of bioassays. More complex multi-species tests, which have a higher degree of realism, have been suggested as a means to increase the predictive capacity of toxicity assessments. But these bioassays are more expensive to run in terms of time, effort and resources, not to mention the problems of reproducibility and standardization. Moreover, there is no concrete scientific basis, nor conclusive evidence that these types of testing provide better information in terms of predictive capacity (Cairns, 1986).

As already mentioned, bioassays are usually carried out to determine (or extrapolate) the no-adverse biological effect concentration of a chemical in the environment. This is done with the assumption that the test systems and organisms are surrogates for all organisms occurring in natural ecosystems. If this assumption is correct then bioassays should accurately predict the effects of pollutants on complex natural systems. However, in practice, the predictive capacity of these types of bioassays has been shown to be very limited (Cairns and Pratt, 1989 ; Geisy and Graney, 1989 ; Maltby and Calow, 1989).

There are literally millions of chemicals documented in the American Chemical Society's Chemical Abstracts, 63,000 of which are in common use in quantities large enough to be of environmental concern (Geisy and Graney, 1989). It has been estimated that only 5 to 10 % of the known chemicals have been tested for toxicity, and that less than 1 % of the 50,000 compounds produced in the USA have been tested for their adverse effects on aquatic organisms (Martell et al., 1988). Faced with this enormous and urgent task to screen these products for potential hazard and since there are no "perfect" predictive bioassays (cf. above), the aquatic toxicologist will need to rely, at least for the time being, on information obtained from some of the well-established, standard toxicity assays.

Routine toxicity evaluations (even the simplest of acute bioassays) are hampered by the prohibitive costs of the tests. Indeed, even the two most widely used bioassays, i.e. the acute fish- and invertebrate toxicity tests, cost in excess of $ 700 (Blondin et al., 1989). Persoone and Van de Vel (1987), in cooperation with 47 profit and non-profit laboratories, made an in-depth study of the costs involved in the various phases (preparation, performance and data-processing) of 5 standard aquatic toxicity tests. These authors reported that the costs of acute toxicity tests such as the 48 hour Daphnia tests and the 96 hour fish test range from 239 to 2283 Ecu (1 Ecu= approximately $ 1.2), depending on the

frequency of testing and the type of testing facility (profit- or non-profit organization) performing the bioassays. A breakdown of these costs into the three main phases revealed that the recruitment and maintenance or the continuous culturing of the test organisms make up 34 % to 81 % of the total costs. From this, the authors concluded that the two main factors hampering the routine use of aquatic toxicity tests are: (a) the costs of year-round recruitment, maintenance or mass culturing of the test organisms, and (b) the difficulties and high level of expertise needed to culture test organisms and to maintain them in a healthy state in the laboratory for a long time.

To bypass these technological, biological and especially financial handicaps in routine toxicity testing, an alternative approach has been developed during recent years, based on the following biological facts. Several groups of invertebrates are capable of shifting their "normal" mode of reproduction to the production of resting stages (- eggs). The production of these resting eggs - often called cysts - is usually triggered by a change in the environmental conditions and is one of the major survival mechanisms for certain invertebrate populations inhabiting unstable biotopes. In nature, these resting eggs, which are in fact dormant embryos encapsulated by a thick shell, can survive adverse conditions for long periods and hatch when environmental conditions become favourable again (Persoone et al., 1990). The use of resting eggs of several species of aquatic invertebrates as biological starting material for toxicity tests has been suggested by several authors (Vanhaecke and Persoone, 1984 ; Persoone et al., 1990 ; Snell and Persoone, 1989 a&b ; Persoone, 1991 ; Snell et al., 1991 a&b ; Centeno et al., in press ; Persoone and Janssen, in press ; Van Steertegem et al, in press). Indeed, these cysts can be hatched at will, thus providing test organisms which are in uniform physiological condition and originate from genetically defined stocks. Cyst-based bioassays have a number of advantages over conventional tests (Persoone et al., 1990) :

- as cysts can be stored for long periods the uncertainty about test animal availability is eliminated.
- as the need for culturing and/or recruitment of test organisms is eliminated, the total cost of the bioassay is greatly reduced.
- as cysts can be produced in the laboratory from genetically well-defined cultures, the potential for standardization of the test organisms and thus the precision of the test method, are substantially enhanced.

To date, 4 cyst-based screening bioassays with aquatic invertebrates have been developed. For freshwater environments, 24 h LC_{50} tests have been described with the rotifers Brachionus rubens (Snell and Persoone, 1989b) and B. calyciflorus (Snell et al., 1991b) and with the anostracan crustacean Streptocephalus proboscideus (Centeno et al., in press). Similarly bioassays for the marine environment have been developed with the rotifer Brachionus plicatilis (Snell and Persoone, 1989a ; Snell et al., 1991a) and with the anostracan Artemia salina (Vanhaecke and Persoone, 1984 ; Van Steertegem and Persoone, in press). To make these tests available for routine use, these cyst-based acute bioassays have recently been modified into a kit form. The principle of the so-called "Toxkits" is the incorporation into a kit of all the key materials necessary to perform an acute toxicity test, i.e. the cysts, the hatching- and test containers and the medium to hatch the cysts and to prepare the toxicant dilutions (Persoone, 1991). The practicability and precision of three of the Toxkits have been evaluated in a round-robin test, the results of which indicated that these test methods are suitable for routine toxicity testing and that their precision and standardization is similar to that of conventional, long-established acute tests (Persoone et al., in press). Because cyst-based bioassays eliminate the need for costly and often difficult culturing of test organisms, this concept seems to be very promising for routine, cost-effective screening of chemicals and effluents. As with any new methodology, however, further research is needed to establish their full potential and limitations.

Considering these important advantages associated with the use of invertebrate resting eggs as biological starting material for aquatic toxicity tests, the "cyst-producing" freshwater rotifer Brachionus calyciflorus was selected as test organism for this study. In addition, there are several other important reasons which warrant its selection :

- compared to cladocerans (mainly Daphnia and Ceriodaphnia), rotifers have been rarely used in toxicity studies (chapter 2). However, they represent, one of the three main zooplankton groups and play an important role in several ecological processes (chapter 2).
- many members of the genus Brachionus are cosmopolitan and are common in mesotrophic and eutrophic ponds and lakes.
- B. calyciflorus is relatively easy to culture in the laboratory and through its small size, toxicity tests with this species require little bench space.
- B. calyciflorus reproduces quickly and has a short generation time which makes this species particulary attractive for chronic reproduction tests.

The primary goal of this study was to examine the potential use of the freshwater rotifer Brachionus calyciflorus for the development of sublethal laboratory toxicity tests. To achieve this, the effects of 4 model chemicals (copper, pentachlorophenol, 3,4-dichloroaniline and lindane) on several aspects of the ecology of this rotifer were examined. The choice of the processes and parameters studied was based on practical as well as fundamental considerations. Indeed, the experimental techniques involved were relatively simple and are readily adaptable for routine toxicity assessment applications. Additionally, the ecological relevance of the selected test parameters and bioassay procedures were addressed. The increasing importance of this "symbiosis" between fundamental and applied research in aquatic toxicology was summarized by Calow and Silby (1990) as follows :

> " Environmental toxicologists often want to use bioassays that can be carried out quickly, easily and hence inexpensively on individual animals, to make predictions about long-term impacts of toxicants at an ecological level. More fundamentally, it is of interest for the population dynamicist to understand to what extent processes within individuals, as compared to interactions between them contribute to population changes."

The thesis is composed of 8 distinct chapters, each of which covers a specific topic :

In chapter 1, the morphology, systematics and some aspects of the biology of B. calyciflorus will be presented.

In chapter 2, a brief overview of the ecology of rotifers will be presented and the literature on the use of these organisms in toxicity assessment studies will be summarized. A conceptual framework for the development of laboratory toxicity tests with B. calyciflorus will be given and illustrated by examples of some of the aspects of the rotifer ecology examined in this study. This chapter is an introduction to the methods and the concepts which will be developed in detail in the following chapters.

In chapter 3, the effects of the four model chemicals : copper, pentachlorophenol, 3,4-dichloroaniline and lindane, on the swimming behaviour of B. calyciflorus will be examined. A "swimming behaviour toxicity test", based on the rotifers' swimming rate as they swim over a grid, will be described. The results of these swimming activity assays will be compared to those of acute and chronic toxicity tests. Finally, the potential use and

relevance of this behaviour bioassay for routine toxicity assessments will be evaluated.

In chapter 4, the effects of the 4 selected model chemicals on the feeding rate of B. calyciflorus will be studied. Preliminary experiments examining the influence of the rotifer density, exposure time, rotation of the test vessels on the feeding behaviour of the rotifers will be presented. A simple test procedure for assessing the effects of xenobiotics on the feeding rate of B. calyciflorus will be provided.

In chapter 5, the development of two short chronic toxicity tests with B.calyciflorus : a 4-day static renewal test and a 3-day static test, will be presented. The toxicity of the four models toxicants will be assessed using the described methods. The potential use and the advantages of the test methods will be discussed and compared to (short) chronic bioassays with other test species.

In chapter 6, some fundamental aspects of the life history characteristics of B. calyciflorus will be examined and described. The influence of temperature and food-availability on the demographic parameters of this rotifer species will be studied. Additionally, the effect of food-availability on the results of chronic toxicity tests (with copper and pentachlorophenol) will be investigated and the implications of the findings for routine toxicity testing will be discussed.

In chapter 7, the effects of copper and pentachlorophenol on the life history characteristics of four consecutive generations of rotifers will be analyzed. Multi-generation life table tests, in which the offspring of the initial experimental generation are again introduced into a toxicity test (and so on until F_3), will be described. Additionally, the effects of the maternal age of a rotifer cohort exposed to copper and pentachlorophenol, on the susceptibility of the F_1 offspring will be investigated. Here, the demographic parameters of the mother generation and of its first and fourth offspring will be compared. The observations made in these experiments and the consequences of these findings will be discussed. The work that will be presented here represents a logical link between the single generation life table studies described in chapter 5 and 6 and the population studies that will be described in chapter 8.

In chapter 8, the changes in the dynamics of large rotifer populations exposed to

copper, pentachlorophenol, 3,4-dichloroaniline and lindane will be studied. A flow-through bioassay system for conducting population studies with rotifers will be described. Finally, the results of these long-term toxicity tests will be compared to those of the short-term tests obtained in the previous chapters.

CHAPTER I.

The test organism : Brachionus calyciflorus.

I.1. Morphology and systematic classification

The Rotifera have been included as a class in the Phylum Aschelminthes by some authors (e.g. Koste, 1978), but have also been considered as a distinct phylum by others (Edmondson, 1959 ; Nogrady, 1982). In this study the Rotifera will be treated as a phylum. In this context, the accepted taxonomic classification of the rotifer species used is :

Phylum : Rotifera
Class : Monogonata
Order : Ploimida
Family : Brachionidae
Genus : Brachionus
Species : Brachionus calyciflorus Pallas, 1766

Approximately 1800 to 2000 species of rotifers have been described, the majority of which inhabit freshwater environments (Starkweather, 1987 ; Wallace and Snell, 1991). Most rotifers are between 0.1 and 1 mm long and have an elongated or saccate body which is relatively cylindrical. The rotifer body can be divided into 3 main regions (Fig. I.1). The short anterior region bears a ciliated organ called the corona, which is characteristic of all members of the phylum. The major part of the body is formed by a large trunk, covered by a distinct cuticle (lorica) which may be ringed, sculptured or ornamented in various ways. The terminal part of the body is formed by the foot, which is considerably narrower than the trunk region (Barnes, 1987).

The division into classes is based on the structure of the female genitalia, while the lower subdivisions are based on the structure of the corona and the differentiated pharynx called mastax. Three classes of rotifers can be distinguished. The smallest and least known group, the Seisonidea (containing only one genus, Seison), is sexual and dioecious, with

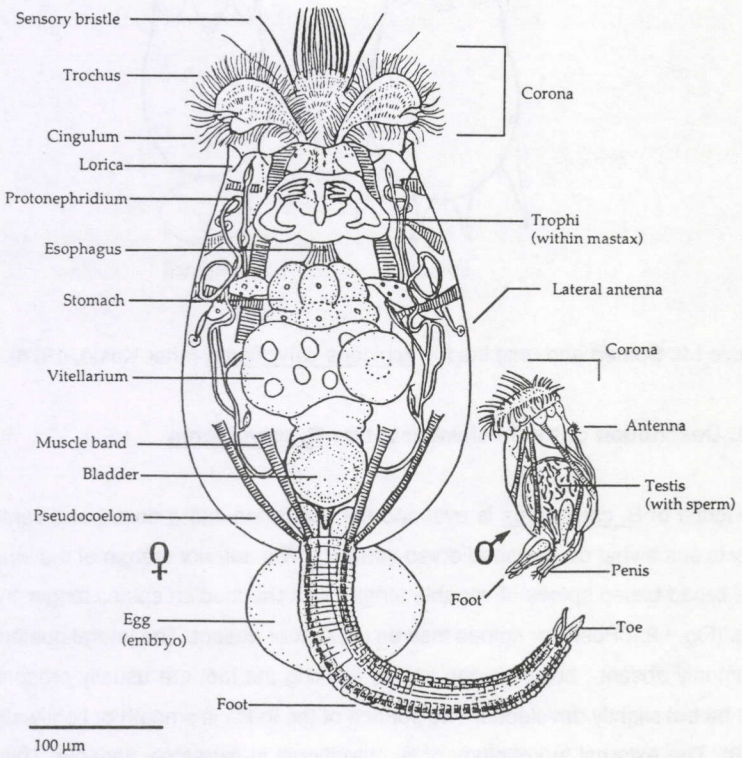


Figure I.1. Morphology of a member of the genus Brachionus, female and male (after Pourriot, 1986).

males similar in size and morphology to females. This class is marine and parasitic on certain planktonic crustaceans. The second class of rotifers, the Bdelloidea, is as far as is known, entirely parthenogenetic. Members of this group are often found in temporary habitats and have a diapause-like state in which the adult animal retracts both the corona and the foot to form a resilient, ellipsoidal "tun". The largest and most well studied class of rotifers is that of the Monogononta, represented by the most common inhabitants of lakes and ponds in both littoral and planktonic environments. Details on the reproductive biology of this class are given in paragraph I.2.

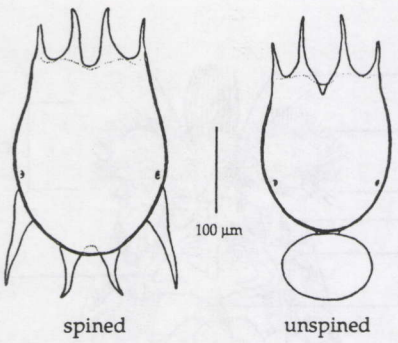


Figure I.2. Spined and unspined Brachionus calyciflorus (after Koste, 1976).

I.1.1. Description of the freshwater rotifer B. calyciflorus

The lorica of B. calyciflorus is oval and not separated into a dorsal and ventral plate. The body is somewhat compressed dorso-ventrally. The anterior margin of the lorica possesses four, broad-based spines of variable length, with the median spines longer than the lateral ones (Fig. I.2.). Posterior spines may be present or absent. The lateral posterior spines are commonly absent ; however, the spines flanking the foot are usually present though they may be but slightly developed. The surface of the lorica is smooth or lightly stippled (Koste, 1978). The external morphology of B. calyciflorus is extremely variable. This is especially true for their size, length of the occipital spines, and the presence and length of the posterior spines (Koste, 1978).

Body length : 220-400 µm (females)
Distribution : cosmopolitan
Habitat : mostly eutrophic, alkaline waters

I.2. Reproduction

The life history of monogonont rotifers is complex with principal periods of parthenogenetic reproduction interrupted by episodes of facultative sexuality. Figure I.3

shows some of the unique aspects of the life history pattern of this group. As indicated, the cycle of diploid parthenogenesis by amictic females is interrupted in response to one or several environmental stimuli (Gilbert, 1974 ; 1977), leading to the production of females that usually produce haploid eggs through conventional meiotic processes. Such haploid stages, if unfertilized, develop directly into free-swimming males. Males are usually smaller than females, are generally without functional guts, are rapid swimmers and have a very short life

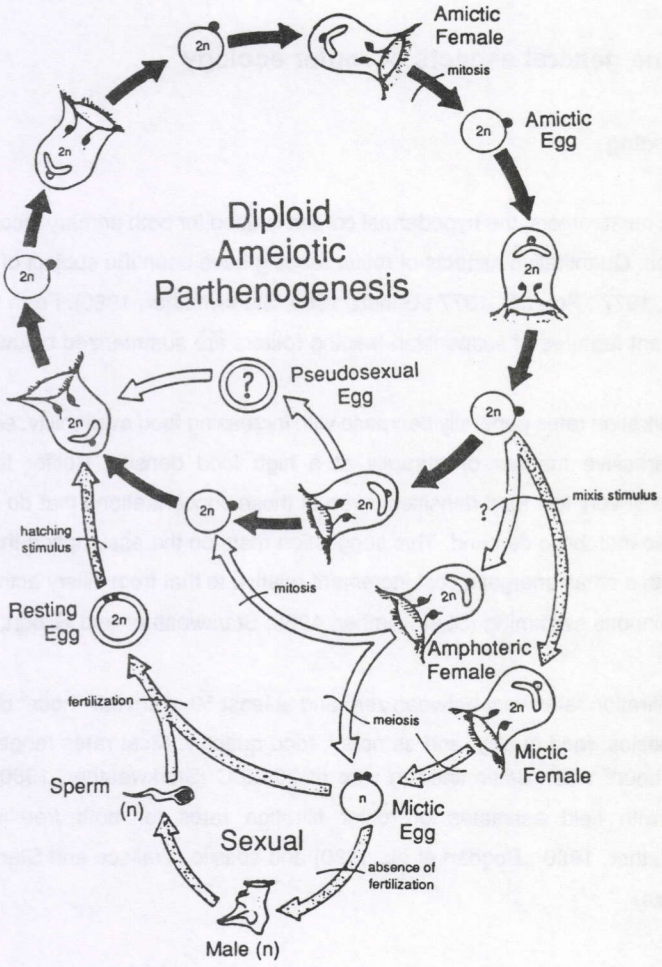


Figure I.3. Generalized life cycle of the Monogononta (after King and Snell, 1977).

span compared to conspecific females (Snell, 1977). With fertilization, the 1n eggs are transformed into zygotes, which, after partial development, may form true diapausing embryos, commonly referred to as "resting eggs" (Pourriot and Snell, 1983).
It is this ability of the monogonont rotifer B. calyciflorus which has lead to the development of the" cyst-based acute toxicity tests" (mentioned in the introduction) and which forms the basis for the more in-depth investigations in the present study.

I.3. Some general aspects of rotifer ecology

I.3.1. Feeding

In most rotifers, the hypodermal corona is used for both primary locomotion and food acquisition. Quantitative aspects of rotifer feeding have been the subject of several reviews (Dumont, 1977 ; Pourriot, 1977 ; Gilbert, 1980, Starkweather, 1980). From these, a number of important features of suspension-feeding rotifers are summarized below.

Filtration rates generally decrease with increasing food availability, either in a gradual and progressive manner or abruptly at a high food density. Rotifer feeding probably continues at very low food densities, even at those concentrations that do not compensate for chronic metabolic demand. This suggestion rests on the assumption that food handling represents a small energetic cost increment relative to that from ciliary activities associated with continuous swimming (Starkweather, 1980; Starkweather and Gilbert, 1977).

Filtration rates vary between zero and at least 50 µl animal^{-1} hour^{-1} depending on the rotifer species, food quality, and as noted, food quantity. Most rates range from 1 to 10 µl animal^{-1} hour^{-1} when determined in vitro at 20-25°C (Starkweather, 1980). These values overlap with field estimates of rotifer filtration rates for both free-living, planktonic (Starkweather, 1980 ; Bogdan et al., 1980) and sessile (Wallace and Starkweather, 1983, 1985) taxa.

Ingestion rates are directly proportional to food density at low and moderate food levels. At higher densities, ingestion rates generally plateau ; ingestion becoming density independent in a gradual curvilinear pattern. As for filtration estimates, relative ingestion

rates for rotifers are very high. For B. calyciflorus, for instance, one adult (0.2 µg dry weight) may consume an amount of food equivalent to its body weight every 2 hours or at least 10 times dry weight biomass per day (Starkweather, 1980).

Rotifers may be highly selective in choosing particular diets from an array of suspended foods. Although some taxa (such as e.g. B. calyciflorus) have broad nutritional latitude (Halbach and Halbach-Keup, 1974), others appear to be highly restricted in dietary choice (Starkweather, 1980). It is clear from field studies, for instance, that certain sympatric rotifer species may show sufficient feeding specialization to effectively reduce resource-based niche overlap (Bogdan et al., 1980).

I.3.2. Swimming

Planktonic rotifers swim more or less continuously, usually in a helical pattern reminiscent of other ciliated metazoans and protists (Viaud, 1940, 1951 ; Clément, 1977). Swimming speeds of rotifers tend to cluster around a value of 1 mm sec^{-1}, at least at temperatures between 17 and 23°C (Starkweather, 1987). Several environmental variables influence the swimming speed : light intensity and wavelength (Viaud, 1943a,b ; Clément, 1977), medium osmolarity (Epp and Winston, 1978), temperature and food density (Epp and Lewis, 1980, 1984). For this last variable, the swimming activity of B. calyciflorus is uniform through a range of low to medium algae cell densities, dropping to near zero at about 500 µg cell dry weight per ml. Due to the low mechanical efficiencies of ciliary motion (Epp and Lewis, 1979), the energetic cost of rotifer swimming is high (Epp and Lewis, 1984).

I.3.3. Growth and production

Postembryonic individual growth in rotifers is limited to a moderate tissue expansion, with no proliferation of cells beyond the number fixed during the establishment of the organ primordia (Hymann, 1951). This pattern of individual growth limitation, however, is not apparent from the growth performance of rotifer populations. Reflecting the overall metabolism of the group, individual rotifer reproductive rates may be very high. Population growth is subject to both biotic influences, principally nutrition (reviewed in Dumont, 1977) and abiotic influences, principally temperature (reviewed in Hofmann, 1977). Rotifers are capable of achieving very high rates of population growth compared to other freshwater

zooplankton (Allan, 1976), but this is not necessarily translated in a sustained competitive advantage for a given species (Gilbert, 1985). In certain temperate planktonic systems, the rotifer component of pelagic secondary productivity (Edmondson, 1974, 1977) rivals that of sympatric microcrustaceans, despite the general higher standing biomass of the latter group (Makarewicz and Likens, 1979).

I.4. The use of rotifers in aquatic toxicology

A brief literature overview on the use of rotifers for toxicity studies is given in chapter 2.

CHAPTER II.

An introduction to the potential use of the freshwater rotifer Brachionus calyciflorus for ecotoxicological applications.

II.1. Introduction

Historically daphnids have been the most extensively used test organisms in freshwater aquatic toxicology. The two other main invertebrate zooplankton groups present in freshwater communities : rotifers and copepods, have been largely ignored by the applied aquatic toxicologist.

Rotifers, however, are very attractive test organisms for aquatic toxicity assessment for several reasons. In freshwater environments, many rotifer species have a major impact on several important ecological processes (ASTM, 1991). As filter-feeders on phytoplankton and bacteria, many rotifers exert substantial grazing pressure that at times exceeds that of the larger crustacean zooplankton (Gilbert and Bogdan, 1984 ; Bogdan and Gilbert, 1987). Rotifer grazing on phytoplankton can be highly selective (Pourriot, 1977 ; Starkweather, 1980 ; Bogdan and Gilbert, 1987) and can influence phytoplankton composition, the coexistence of competitors and overall water quality (Diner et al., 1986). The contribution of rotifers to the secondary production of many aquatic communities is substantial (Edmondson, 1974 ; Bogdan and Gilbert, 1982a ; Hernroth, 1983 ; Heinbokel et al., 1988). Freshwater rotifers also often account for the major fraction of zooplankton biomass at certain times of the year (Makarewicz and Likens, 1979 ; Pace and Orcutt, 1981). Rotifers are, together with other zooplankton species, a significant food source for many larval fish, planktivorous adult fish (O'Brien, 1979 ; Evans, 1986) and several invertebrate predators (Williamson, 1983 ; Stemberger, 1985 ; Egloff, 1988). The high metabolic rates of rotifers contribute to their role in nutrient cycling, which may be more important than crustaceans in certain communities (Ejsmont-Karabin, 1983, 1984).

In addition to their important ecological role in aquatic communities, rotifers are attractive organisms for ecotoxicological studies because an extensive data base exists on

the basic biology of this group. Techniques have been published for the culture of many rotifer species (Pourriot, 1977 ; Stemberger, 1981). The rotifer life cycle is well defined (Birky and Gilbert, 1971 ; King and Snell, 1977) and the factors regulating it are reasonably well understood (Gilbert, 1980b, 1983 ; Snell, 1986 ; Snell and Boyer, 1988). Several aspects of rotifer behaviour have been closely examined (Gilbert, 1963 ; Snell and Hawkinson, 1983 ; Clement et al., 1983 ; Snell et al., 1987). The biogeography of many rotifer species has been characterized (Pejler, 1977 ; Dumont, 1983) and the taxonomy of the group is well described (Ruttner-Kolisko, 1974 ; Koste, 1978).

The species Brachionus calyciflorus is particularly useful for environmental toxicology because of its rapid reproduction, short generation time, sensitivity and the commercial availability of dormant eggs (cysts) (Halbach et al., 1983 ; Snell and Persoone, 1989 a & b ; Snell et al., 1991 a & b). The genus Brachionus has a cosmopolitan distribution that spans the six continents (Dumont, 1983).

Quite a number of rotifer species have been used in toxicity assessment studies of xenobiotics. A few studies have reported on the effects of contaminants on natural rotifer populations (a.o. Ramadan et al., 1963 ; Yan and Geiling, 1985). However, the bulk of the literature dealing with the use of rotifers in environmental toxicology are laboratory studies. Buikema et al. (1974) suggested Philodina acutiformis as test organism in acute bioassays for heavy metals, and Schaefer and Pipes (1973) used P. roseola in short-term mortality and long-term reproduction tests. Rotifers of the genus Brachionus have been frequently used in toxicity tests. Capuzzo (1979a) studied the effects of chlorinated cooling waters on the estuarine rotifer B. plicatilis. Using the same species the adverse effects of acrylamide on the neuromuscular activity were observed by Kleinow (1986) and Echeverria (1980) studied the bioaccumulation of benzene and related compounds in this species. A standardized 24 h LC_{50} (mortality) test with B. plicatilis using resting eggs as biological starting material to obtain test organisms is described by Snell and Persoone (1989a). Acute toxicity testing methods have been developed with B. rubens (Halbach et al., 1983 ; Snell and Persoone, 1989b) and B. calyciflorus (Snell et al. 1991b). Couillard (1987) and Couillard et al. (1989) evaluated the acute toxicity of lake sediments and heavy metals with this species. Changes in the demographic characteristics of B. patulus (Rao and Sarma, 1986), B. rubens (Halbach et al. 1983) and B. calyciflorus (Ferrando et al., in press, Snell and Moffat, in press) have been used as sensitive indicators of toxic stress for a wide variety of chemical compounds.

Halbach et al. (1983) proposed a standardized toxicity test with large populations of B. rubens.

For many years, much of the work carried out under the general heading of "ecotoxicology" consisted of short-term tests in which organisms were exposed to different concentrations of chemicals and their mortality recorded. Although such tests have been invaluable in the development of aquatic toxicology as a science and are still useful in the initial screening of chemicals and effluents, there has been a growing awareness that mortality is a very crude endpoint from which to predict "safe" environmental concentrations. It is only in the last 10 to 15 years that efforts have been directed towards the development of toxicity tests which use more sensitive, sublethal, effect measurements such as activity, growth and reproduction. Although a number of standardized test methods using these sublethal test criteria are now being used routinely and many studies have been performed on the effects of chemicals on a large variety of behavioural, physiological and demographic parameters, there have been very few attempts to elucidate the relationships or the degree of correlation between these different end criteria. If the ultimate goal of aquatic toxicology is to protect the structure and function of the aquatic ecosystem, then the understanding of how toxicants affect the various biological systems at different levels of biological organization and how those different levels interact, is a prerequisite for reaching that goal.

The aim of the present study is to construct and test a conceptual framework for laboratory toxicity testing based on the assumption that events observed at one level of biological organization are determined by elements of the lower level. This type of research, i.e. connecting pure demography with the lower levels of complexity such as physiology and behaviour, has in recent year gained popularity (Gatto et al. 1989). Central to most of these approaches is the organism's energy allocation. Indeed, the energy acquired from food can be used for foraging activities and maintenance (and storage) and for reproduction. From this type of model it is clear that if of one of these processes is affected this change should be reflected in one or more of the others at the same or at a higher level of organization (Gatto et al., 1989). Toxic stress can be such an "affecting" factor. The framework is illustrated in Figure II.1, in which some of the causal links between the different hierarchal levels : ecological factors, physiological characteristics, life history parameters, population dynamics and community structure, are presented. The objective of this introductory chapter is to examine the effects of toxic stress on several of the processes presented in Figure II.1 and

to illustrate some of the ideas and methods that will be developed in this study. To that end, the toxic effects of copper, on the important "energy-related" processes : locomotion and feeding, and the consequences of these physiological changes for the life history characteristics of the rotifers were examined. Details of the results obtained with each of the experimental procedures described here, are presented in chapters 3 through 7.

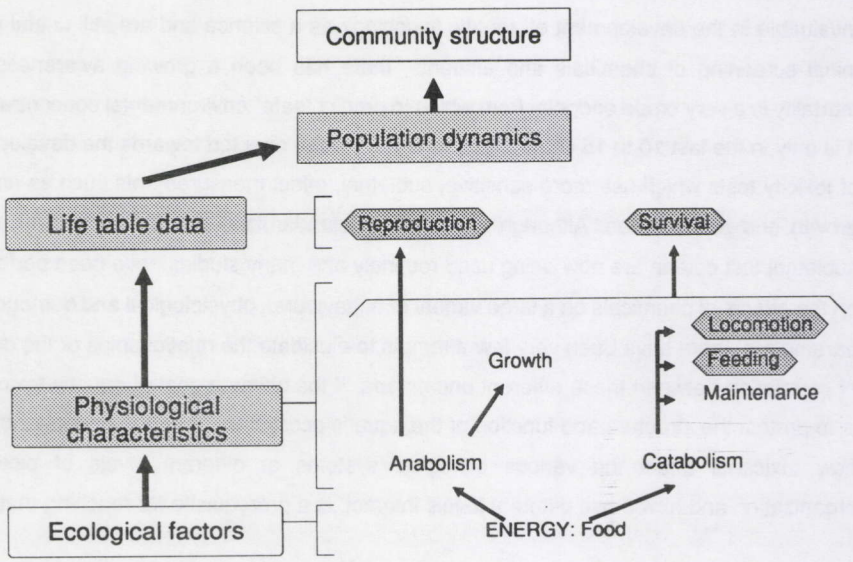


Figure II.1. Conceptual framework illustrating the links between the different levels of biological organization (left) and some of the links between the different processes within these levels (right). The parameters measured in the present study are indicated with a <box>.

II.2. Materials and Methods

II.2.1. General experimental procedures

All experiments were performed with the freshwater rotifer Brachionus calyciflorus Pallas, using a strain originally collected in Gainesville, Florida, USA (Snell et al. 1991b). Test animals were obtained by hatching cysts which were produced under controlled

conditions in laboratory cultures by Dr. T.W. Snell (Georgia Institute of Technology, Atlanta, Georgia, USA). Cysts were hatched at 25°C in light (6000 lux) in synthetic freshwater. Neonates were collected and used in the experiments, 16-18 hours after the initiation of the hatching. A moderately hard synthetic freshwater medium (EPA water) was used as culture and dilution medium in all experiments (Horning and Weber, 1985). This medium consists of 96 mg $NaHCO_3$, 60 mg $CaSO_4.2H_2O$, 60 mg $MgSO_4$ and 4 mg KCl in one liter of deionized water, adjusted to pH 7.8. All chemicals used were of analytical grade and obtained from UCB Industries, Belgium. Feeding behaviour and life table tests were incubated in complete darkness (to avoid growth of algae) and all experiments were conducted at 25 $\pm$ 1°C.

Nannochloris oculata Droop, which was used as food in all experiments, was cultured in Bolds Basal Medium (BBM) under constant illumination (Nichols, 1973). The log phase algae were harvested, centrifuged, washed, and resuspended in EPA water. The algae were counted with the aid of a hematocytometer, stored in the refrigerator and used for 3 days, after which they were discarded and the whole process repeated. Unless otherwise indicated the algal density used in all experiments was $5x10^5$ cells/ml. Prior to the definitive toxicity tests, range-finding tests were performed to determine the Cu concentrations at which the swimming-, feeding- and demographic parameters were affected. Stock solutions of 100 mg/l Cu ($CuSO_4.5H_2O$, analytical grade, UCB Industries, Belgium) were prepared in deionized water and the actual test solutions were made up in EPA water. The Cu concentration ranges used in each of the experiments are reported below. The EC_{50}'s (the concentration of the toxicant that reduces the test parameter to 50 %) were calculated using linear regression analysis.

To determine statistically significant differences between groups a one-way analysis of variance was used. Mean separation was accomplished using Duncan's multiple range test, at a significance level of $p<0.05$.

II.2.2. Swimming behaviour

The swimming activity of B. calyciflorus was measured using the (modified) method proposed by Snell et al. (1987). Tests were initiated by transferring a single female into a shallow, circular polystyrene chamber (diameter 23.5 mm ; depth 1.2 mm) containing 0.7 ml

of the test solution. Under the chamber a grid with 1 mm squares was placed, so that as the rotifer swam, the number of squares entered could be recorded. Observations of the swimming activity were performed under a binocular microscope at 12x magnification. Microscope light intensity and position were carefully standardized and kept constant in all experiments. Swimming activity was recorded as the number of 1 mm squares entered in 30 seconds (sq./30sec). For every treatment 10 isolated females were observed.

A series of preliminary experiments were conducted to identify the influence of rotifer age (<2 and 24 hour old) and food concentration (range : 0-5x10^7 cells/ml of N. oculata) on the swimming activity of B. calyciflorus. The experimental conditions used in the toxicity tests were: 0-2 hour old neonates exposed (without food) to 6, 12, 25, 60, 120, and 250 µg/l of Cu for periods ranging from 5 minutes to 5 hours. The results of the swimming activity experiments are presented as mean values ± S.D.(n=10). Details of the experimental procedures are given in chapter 3.

II.2.3. Feeding behaviour

Filtration and ingestion rates were used as measures of the feeding behaviour . Feeding experiments were performed in 8 ml glass vials containing 5 ml of the treatment solution with 30 rotifers/ml and an initial food concentration of 5x10^5 cells/ml of N. oculata. The neonates (0-2 h old) were allowed to feed for 5 hours, after which the final food concentration was measured using a haematocytometer. Details of the development of the experimental procedures are given in chapter 4.

In the toxicity experiments, the rotifers were exposed to 12, 20, 25, and 60 µg/l of Cu. Each treatment consisted of 5 replicates. Filtration and ingestion rates were calculated using the equations defined by Gauld (1951) which are given in chapter 4. Results of the feeding experiments are presented as mean values (± S.D.) of the filtration- and ingestion rates (n=5).

II.2.4. Life history characteristics

To obtain life table data, 0-2 hour neonates (hatched from resting eggs) were cultured

individually and observed throughout their life. All experiments were conducted in 24-well polystyrene plates, which were used once and then discarded. Each experiment was initiated by introducing one neonate into each of the wells containing 1 ml of test solution. To allow statistical analysis, the rotifers were randomly distributed into 4 replicates of 12 test organisms, each treatment thus consisted of a cohort of 48 neonates. Each rotifer was checked every 12 hours and the number of attached eggs, offspring, and mortality recorded. The parental female was transferred into fresh medium every 24 hours. The food density in all treatments was $5x10^5$ cells/ml N. oculata. Tests were terminated when the last individual of every cohort had died.

At every age x, the survivorship (l_x) and fertility (m_x) tables were constructed using standard methods (Poole, 1974 ; Southwood, 1976) and the following demographic parameters were calculated : net reproductive rate R_o, generation time T, life expectancy e_o and the intrinsic rate of natural increase r_m. Details of these methods are given in chapter 5. Based on the results of a 24 h LC_{50} toxicity test conducted using the protocol described by Snell and Persoone (1989b), four Cu concentrations : 1.2, 2.5, 5.0, and 10 µg/l were tested in the life table experiments.

II.3. Results

II.3.1. Swimming behaviour

The results of the combined effects of rotifer age and food concentration on the swimming activity of B. calyciflorus are presented in Figure II.2. The swimming activity of <2 hour old rotifers is not significantly affected by the presence of different food concentrations in the range of 0 to $5x10^6$ cells/ml.

At the highest food concentration the swimming rate was reduced to 11.0 (± 1.4) sq./30sec, i.e. significantly lower than that observed at all the other food concentrations. The swimming activity of older rotifers (24 hours) exposed to food concentrations of $5x10^4$, $5x10^5$, and $5x10^6$ cells/ml were almost identical : 14.0 (± 0.8), 14.6 (± 1.6) and 13.4 (± 1.7), respectively. Rotifers fed at $5x10^3$ cells/ml and those without food, however, swam significantly slower : 8.6 (± 1.2) and 10.0 (± 1.2), respectively. From these preliminary experiments it can be concluded that the swimming activity of < 2 hour old non-fed neonates was a good indicator

of that of an "average" rotifer. All toxicity experiments reported on below were performed using these test conditions.

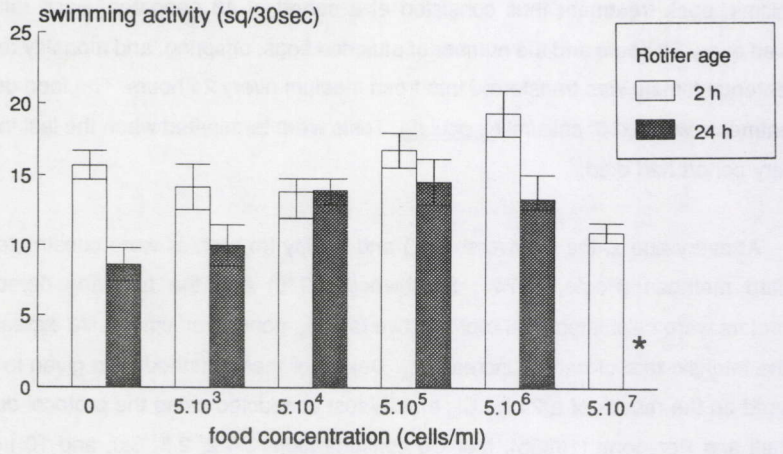


Figure II.2. The effect of the presence of different food concentrations on the swimming behaviour of two age classes of B. calyciflorus. (*) no data.

B. calyciflorus neonates were exposed to different Cu concentrations for periods ranging from 5 minutes to 5 hours, after which the swimming speed was measured. Exposure to copper caused a reduction in the rotifers' swimming speed, the magnitude of which was a function of both Cu concentration and the exposure period (Fig. II.3). An exposure period of 5 minutes did not cause a significant reduction (compared to the controls) in swimming activity up to Cu concentrations of 60 µg/l. At the two highest (120 and 250 µg/l) concentrations however, it was significantly reduced by 39 % and 49 %, respectively. The lowest observed effect concentration (LOEC, lowest concentration at which there is a statistically significant difference with the control values) after a 30 minute exposure was 25 µg/l Cu and the rotifers had completely stopped swimming at 120 and 250 µg/l. With increasing exposure periods the swimming activity continued to decrease, and the dose-response curves became steeper.

The EC_{50}'s, the concentration of copper that reduces swimming activity to 50 % of the control, for the different exposure times are presented in Figure II.4. The EC_{50}'s for the

5- and 10 minute exposures are very similar but prolonged exposure to copper reduced the EC_{50}. This value, however, remained relatively constant from 2 hours onwards.

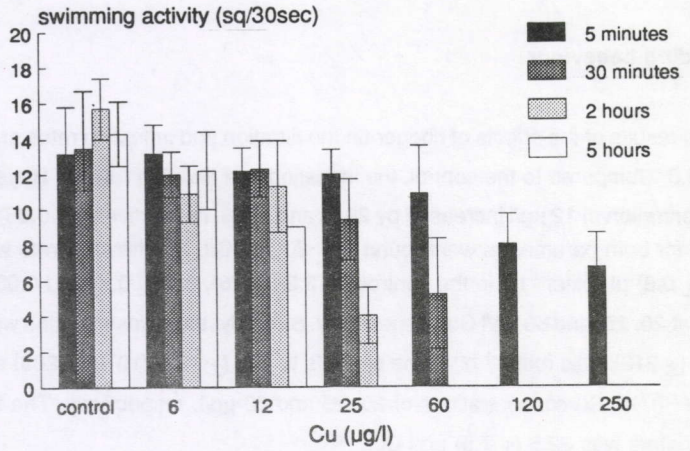


Figure II.3. The effect of copper on the swimming activity of B. calyciflorus (0-2 hour old) neonates in function of the exposure period (10, 20 and 180 min. exposures are not shown) Vertical lines indicate ± S.D.

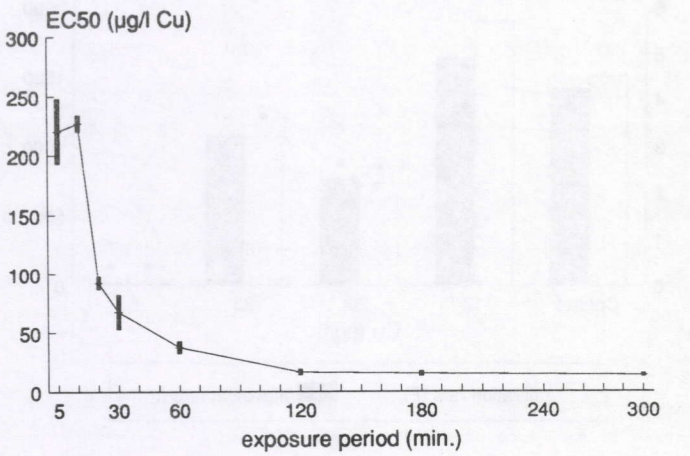


Figure II.4. The relationship between the swimming activity EC_{50}'s and the exposure time. Vertical lines indicate ± S.D.

For that reason the 2 h EC_{50} values were used in the further discussion of the swimming activity results.

II.3.2. Feeding behaviour

The results of the effects of copper on the filtration and ingestion rates are presented in Figure II.5. Compared to the control, the ingestion and filtration rates of B. calyciflorus at a Cu concentration of 12 µg/l increased by 26 % and 16 %, respectively ($p>0.05$). Significant decreases for both parameters were found at ≥ 20 µg/l Cu. The filtration rate was reduced from 4.5 (± 0.8) µl rotifer^{-1} h^{-1} in the controls to 2.2 (± 0.6), 3.2 (± 0.8) and 0.03 (± 0.01) µl rotifer^{-1} h^{-1} at 20, 25, and 60 µg/l Cu, respectively. Similarly, the ingestion rates were reduced from 1416 (± 210) cells rotifer^{-1} h^{-1} in the controls to 772 (± 199), 1076 (± 289) and 20 (± 7) cells rotifer^{-1} h^{-1} at Cu concentrations of 20, 25 and 60 µg/l, respectively. The 5 h EC_{50} for both parameters was 32.5 (± 7.5) µg/l Cu.

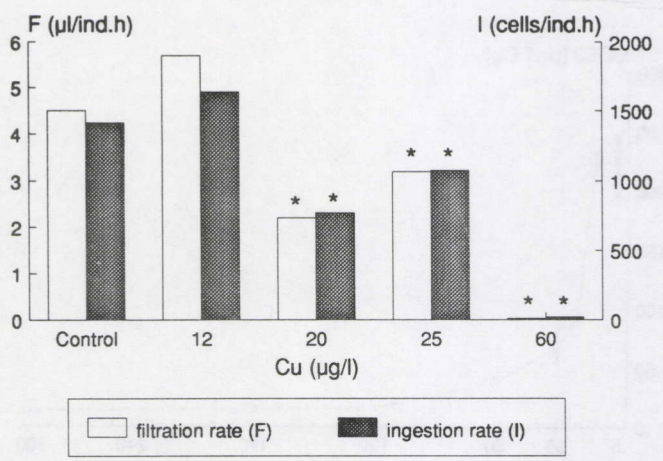


Figure II.5. The effect of copper on the filtration- (F) and ingestion rate (I) of B. calyciflorus. (*) Significantly different from the control ($p<0.05$).

II.3.3. Life history characteristics

The survivorship (l_x) of the control and that of the animals exposed to 1.2 µg/l Cu were very similar (Figure II.6). At all other concentrations the survivorship decreased with increasing Cu concentrations. This is especially clear at the two highest concentrations tested (5.0 and 10 µg/l), where the number of surviving rotifers after 4 days was reduced to 77 % and 22 %, respectively. The values for the mean survival, the time required for the test population to decrease to 50 %, are presented in Table II.1.

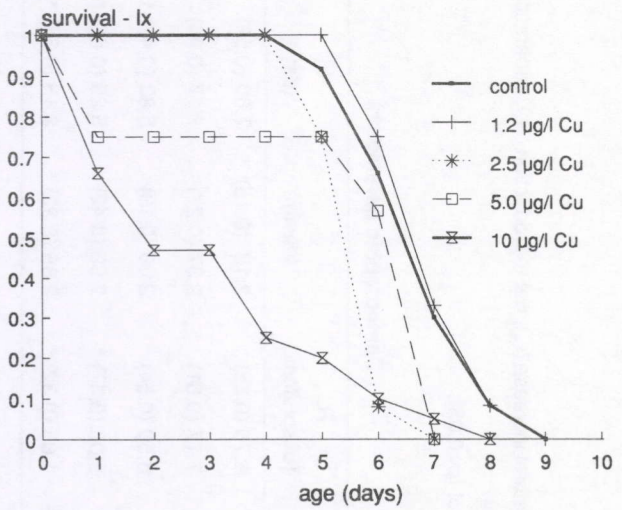


Figure II.6. The effect of copper on the age specific survival (l_x) of B. calyciflorus.

A reduction in fertility (m_x) with increasing Cu concentrations was observed during the first 2 days of the life tables (Figure II.7). After one day the fertility of the rotifers exposed to 2.5 µg/l was slightly higher than that in the controls (1.25 eggs/female), while for those exposed to Cu concentrations of 1.2 and 5 µg/l the m_x values were clearly lower (1.08 and 0.89 eggs/female). Most rotifers exposed to 10 µg/l Cu had not produced any eggs (m_x=0.10 eggs/female) at day one. On day 2, the rotifers exhibited a maximal reproductive output of 2.17 eggs/female in the control, while the m_x values for the 1.2, 2.5, 5.0 and 10 µg/l Cu treatments were 1.83, 1.58, 1.88 and 1.11 eggs/ female, respectively. The reproduction of the rotifers exposed to 10 µg/l was clearly reduced and delayed with its maximum of 1.4

Table II.1. Effect of copper on the intrinsic rate of natural increase (r_m), net-reproduction (R_o), generation time (T),life expectancy at hatching (e_o) and mean survival of B. calyciflorus.
(*) Significantly different from the control ($p<0.05$).

Cu treatment (µg/l)	Demographic parameters				
	r_m	R_o (offspr./fem.)	T (days)	e_o (days)	mean survival (days)
control	0.898 (0.074)	6.75 (0.85)	2.12 (0.15)	7.00 (0.59)	6.5 (0.62)
1.2	0.830 (0.094)	7.17 (0.61)	2.37 (0.27)	7.17 (0.94)	6.6 (0.86)
2.5	0.910 (0.101)	6.50 (0.54)	2.06 (0.19)	5.83 (0.48) *	5.37 (0.61)
5.0	0.640 (0.085) *	5.00 (0.47) *	2.52 (0.42)	5.25 (0.55) *	6.00 (0.75)
10	0.176 (0.034) *	1.65 (0.32) *	2.86 (0.22) *	3.15 (0.34) *	1.84 (0.21) *

eggs/female being reached after only 3 days. Compared to the controls, 5.0 and 10 µg/l copper significantly reduced the R_o values (Table II.1). Similarly the intrinsic rate of natural increase was significantly lower at these two highest Cu concentrations. The life expectancy at hatching (e_o) however, was already significantly lower than that of the controls at a Cu concentration of 2.5 µg/l. The generation time on the other hand, exhibited a clear increase at the highest Cu concentration. The EC_{50}'s (i.e. the Cu concentration that reduces the value of that parameter to 50 %) for the demographic parameters : R_o, e_o, and r_m were 7.4, 9.2 and 6.9 µg/l Cu, respectively.

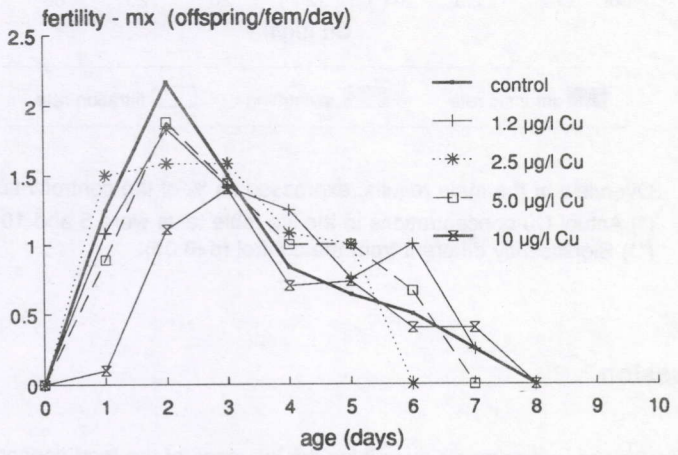


Figure II.7. The effect of copper on the age specific fertility (m_x) of B. calyciflorus.

An overview of the main results obtained in this study is given in Figure II.8., in which the relative reductions of the three test criteria (swimming activity, filtration rate and the intrinsic rate of natural increase) are plotted as a function of increasing copper concentrations. Statistically significant reductions in the filtration rate (5 hour exposure) and the swimming speed (2 hour exposure) are observed at > 20 µg/l and > 6 µg/l (lowest concentration tested), respectively. In the life table experiments the LOEC for the intrinsic rate of natural increase is 5.0 µg/l.

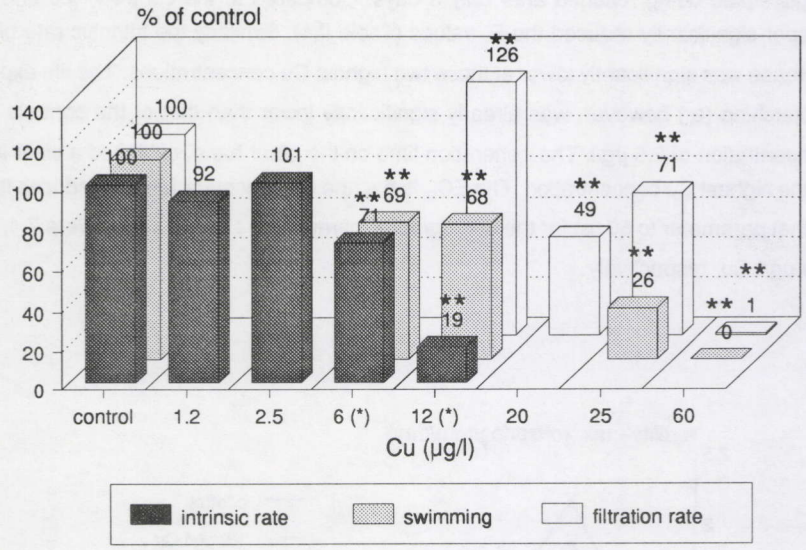


Figure II.8. Overview of the main results, expressed as % of the control value.
(*) Actual Cu concentrations in the life table tests were 5 and 10 µg/l.
(**) Significantly different from the control ($p<0.05$).

II.4. Discussion

The preliminary experiments examining the influence of the food concentration and the age of the rotifers on the swimming behaviour were aimed at developing a method reflecting the "average" swimming activity of B. calyciflorus. The swimming activity of young B. calyciflorus females (0-2 h old) without food was not significantly different from that of the neonates swimming in the presence of food. At a concentration of $5x10^7$ cells/ml of N. oculata the swimming activity was significantly lower than that of the other treatments, suggesting a stress situation caused by the dense algal suspension. The swimming activity of older females kept for 24 hours without food, and those exposed to food for 2 hours was lower than that of the 0-2 hour old neonates. This starvation effect was significant when the 24 h old females were exposed to very low food concentrations ($5x10^3$ cells/ml). At higher food concentrations the rotifers had apparently recovered from the starvation effects as they had been able to feed during the 2 hour exposure time prior to the activity measurement.

Snell et al. (1987) used similar techniques to measure the swimming activity of different age classes of Brachionus plicatilis and found that the activity peaked in one day old females and declined linearly through day four. They also showed that neonate females exhibited only 50 % and 5-6 day old females only 20 %, of the swimming activity of one day old animals. B. plicatilis (48 h old) fed at different Dunaliella concentrations exhibited an increase in swimming activity with increasing food concentrations (Snell et al. 1987). Although the same trend can be noted in the present study with B. calyciflorus, a comparison of both studies is not completely valid as in our work the influence of the presence of food on the swimming activity of non-fed animals was examined and not the influence of the nutritional status of the rotifers on the swimming activity.
When exposed to copper, the swimming activity of B. calyciflorus decreased with increasing concentrations, and within the same Cu treatment decreased with increasing exposure periods. Snell et al. (1987) studied the effect of un-ionized ammonia on the swimming activity of B.plicatilis and found that a 10-minute exposure was sufficient to produce a maximum decrease in the swimming activity response. Indeed the time required to reach the maximal response in the swimming activity criterion (and for that matter in any test criterion) will be dependent on the mode of action of the chemical.

Although quite a few studies on the rotifer swimming behaviour have been published (see hereunder), no reports on the effects of xenobiotics on this important aspect of the biology of rotifers were found. Changes in the swimming activity of B.plicatilis, for example, have been shown to be a very sensitive indicator of pH, un-ionized ammonia, starvation and temperature effects (Snell et al. 1987). Beauvais and Enesco (1985) showed that the swimming activity gradually declined with age in the rotifer Asplanchna brightwelli, but that treatment with very low doses of curare could slow down this activity loss. Snell and Garmann (1986) described how changes in swimming speed affect male-female encounter probabilities. Coulon et al. (1983) reported on the development of a computerized automatic tracking system to study several aspects of a rotifer's swimming behaviour . Clement (1987) has used this system to correlate some behaviourial aspects with the ultrastructure of rotifers. Swimming activity measurements have also been used to determine the cost, speed and efficiency of locomotion of B. plicatilis and Asplanchna sielboldi (Epp and Lewis, 1979, 1984). They found that the actual cost of locomotion in B. plicatilis males accounted for a large portion of its total metabolism, which might explain the high sensitivity of the swimming activity to environmental stress.

The filtration rate of the B. calyciflorus females in the controls was 4.5 µl rotifer^{-1} h^{-1}, which is similar to the values obtained by Bogdan and Gilbert (1982b) for the same rotifer species fed on radioactive labelled Rhodotorula and Aerobacter aerogenes. In his review of the feeding behaviour of suspension feeding rotifers, Starkweather (1980) concluded that most published filtration rates are between 1 and 10 µl rotifer^{-1} h^{-1} (in the 20-25°C temperature range). The results of the toxicity tests indicate that the feeding rate (F and I values) decrease with increasing Cu concentrations. However, at the lowest Cu concentration tested an increase in the filtration rate can be observed ($p>0.05$). In toxicity testing this phenomenon of "hormosis", i.e. low concentrations of a toxicant having a stimulatory effect on the test parameter, is well known and will be discussed in the following chapters (Rand and Petrocelli, 1985 ; Gentile et al., 1982). The mean F and I values at 25 µg/l Cu are slightly higher than at 20 µg/l ($p>0.05$). Feeding has almost completely stopped at 60 µg/l Cu. While the variability among the replicates in the control is acceptable (coefficient of variation : 18 %), it is high in all Cu treatments (C.V.'s ranging from 25 to 32 %). This could possibly be attributed to small differences in Cu concentration among the replicates of the same treatment and/or the inaccuracy of the algal counting method to detect the small algal density changes caused by the reduced feeding activity of the rotifers in the toxicant treatments.

Despite the vast amount of literature on the feeding behaviour of rotifers (Starkweather, 1980, Bogdan and Gilbert, 1982a&b ; Gilbert and Bogdan, 1984), only one paper was found reporting the effects of toxicant stress on the feeding rate (Capuzzo, 1979b). This author found that the filtration rate of B. plicatilis was reduced by approximately 50 % after a 24 hour exposure to 1 mg/l of the halogen toxicants : free chlorine and chloramine. The effects of xenobiotics on the filtration rates of other zooplankters have been investigated by several workers (Cooley, 1977 ; Geiger and Buikema, 1981 ; Kersting and van der Honing, 1981 ; Flickinger et al., 1982 ; Day and Kaushik, 1987 a&b).

The survivorship- and fertility curves of B. calyciflorus in the controls in this study are very similar to those reported by Halbach (1970) for the same species, and to those for Brachionus rubens (Halbach et al. 1983). The survivorship curves become steeper with increasing Cu concentrations (>2.5 µg/l), reflecting toxicant inflicted mortality. The fertility of the rotifers under Cu stress is only slightly reduced in all treatments up to 5 µg/l ; at the highest concentration the decrease in m_x is more pronounced. The timing of the reproduction

is not affected by Cu concentrations < 5µg/l, with m_x values reaching a maximum at day 2. At 10 µg/l this reproduction peak is clearly delayed and reaches a maximum at day 3. A similar delay was observed for Brachionus patulus under DDT stress (Rao and Sarma, 1986). Separate measures of age-specific survival and fertility rates can be integrated in the population parameter r_m, the intrinsic rate of natural increase. The value of r_m is affected by age at first reproduction, reproductive period, clutch size (Allan, 1976 ; Streans, 1976) and survival. In this study r_m was significantly reduced at the two highest Cu concentrations. The effect of the delayed reproduction combined with the reduced survival is clearly reflected in the value of r_m at 10 µg/l Cu. Mean survival, e_o and R_o all exhibited a similar trend : a decrease with increasing toxicants concentrations. A reduction in these parameters as a consequence of chronic exposure to a toxicant was also observed in Brachionus plicatilis (Capuzzo, 1979b), B. rubens (Halbach et al., 1983), B. patulus (Rao and Sarma, 1986) and B. calyciflorus (Ferrando et al., in press). The delay in reproduction under Cu stress is also reflected in the significantly higher generation time at 10 µg/l.

The use of life table techniques to study the effects of chronic exposure to xenobiotics has been demonstrated with several invertebrates (Daniels and Allan, 1981 ; Gentile et al., 1982 ; van Leeuwen et al., 1985a&b). All these authors advocate the use of demographic parameters (especially r_m) in chronic toxicity testing since these parameters incorporate all important aspects of an organism's life history, which ultimately determine the population dynamics.

The objectives of the present study were to examine the effects of copper on some important functions of B. calyciflorus with the aim of developing new and ecologically relevant test methods. Significant reductions in the filtration rate, the swimming activity and the demographic parameter r_m were observed at 20, 6 and 5 µg/l, respectively. In ecotoxicological terms these differences are relatively small, considering the amount of uncertainty that is associated with all other aspects of chemical toxicity evaluations. These results not only confirm that toxicant-induced changes at one level of organization can be detected at the next higher level of biological organization at approximately the same concentration, they also offer some interesting possibilities for the field of applied (routine) aquatic toxicology. If similar relationships between physiological, behaviour al and demographic parameters could be established for other classes of chemical compounds, the time needed to perform routine chronic toxicity tests could be dramatically reduced while still

retaining its ecological relevance.

II.5. Summary

The potential use of several aspects of the biology of the freshwater rotifer Brachionus calyciflorus as test endpoints in ecotoxicological studies was examined. Changes in the feeding-, swimming behaviour - and demographic characteristics of the rotifers under toxic stress (copper) were studied and the relationships between the different test parameters and their ecological relevance evaluated.

Relatively simple short-term bioassay methods were developed to measure the swimming activity (2 hours) and the filtration (5 hours) rate. Life-table experiments were performed to assess the long-term effects of Cu on the rotifers. The LOEC's obtained in the filtration rate, and the chronic toxicity tests (r_m) were 20 and 5 µg/l Cu, respectively. The swimming activity of the rotifers had significantly decreased at the lowest Cu concentration tested (6 µg/l). The implications and the possible ecotoxicological applications of the results were discussed.

CHAPTER III.

The effects of xenobiotics on the swimming behaviour of Brachionus calyciflorus.

III.1. Introduction

The development and use of aquatic toxicity tests using behavioural responses as test endpoints has significantly increased during the last few years. This is not surprising since behavioural criteria integrate many cellular processes vital to an organism's survival and reproduction, thus reflecting both biochemical and ecological consequences of toxic insult. Consequently, toxicity tests using behavioural test criteria may possibly be excellent tools for evaluating the toxicity of chemicals and effluents.

Several aspects of changes in the swimming behaviour of fish caused by sublethal exposure to xenobiotics have been used as sensitive indicators of toxic stress (Diamond et al., 1990 ; Little and Finger, 1990). Such behavioural modifications not only provide an index of sublethal toxicity, but may also reflect the potential for subsequent mortality and the possible impairment of food uptake, predator avoidance and reproduction. Besides these important ecological features, the use of behavioural test criteria also offers another advantage important to many types of toxicity assessment : rapidity. Indeed, most behavioural changes caused by toxic insult become "visible" much more quickly than conventional test criteria such as survival, reproduction and growth.

Despite the growing interest in the use of behavioural responses in toxicity testing with fish, very little has been done to develop behavioural indices with invertebrates. Even with the freshwater cladoceran Daphnia, the long time favourite for toxicity screening, little is known about the influence of pollutants on the behaviour of this crustacean and only a few studies have examined such changes. The reduction of the feeding rate of Daphnia magna under toxic stress, for example, has been studied by Kersting and van der Honing (1981). Flickinger et al. (1982) and more recently Goodrich and Lech (1990) have suggested the phototactic behaviour of this cladoceran as a sublethal indicator of toxic stress. These

studies clearly show that it is possible to develop short-term toxicity test methods with invertebrates using behavioural responses as test endpoints.

In this chapter the changes in the swimming behaviour of the freshwater rotifer Brachionus calyciflorus under toxic stress were examined. The objectives of the present study were to examine the utility of the rotifer's swimming behaviour as an ecotoxicological test endpoint and to compare the "sensitivity" of this behavioural response with the conventional acute and sub-lethal (long-term) test criteria such as longevity and reproduction.

III.2. Materials and methods

III.2.1. General experimental procedures

All experiments were performed with the freshwater rotifer Brachionus calyciflorus Pallas, using a strain originally collected in Gainesville, Florida, USA (Snell et al., 1991b). Details of the cyst hatching procedure are given in chapters 2 and 5.

III.2.2. Swimming activity tests

The rotifers' swimming activity was measured using the (modified) method described by Snell et al. (1987). Tests were initiated by transferring a single rotifer into a shallow, circular polystyrene chamber (diameter 23.5 mm ; depth 1.2 mm ; Carolina Biological, USA) containing 0.7 ml of the test solutions. Under the chamber a grid with 1 mm squares was placed, so that as the rotifer swam, the number of squares entered could be recorded. Observations of the swimming activity were performed under a binocular microscope at 12x magnification. Microscope light intensity (halogen light at 400 lux) and position (at an angle of 45° lateral to the microscope stage) were carefully standardized and kept constant for all experiments. Swimming activity was recorded as the number of squares entered in a 30 second observation period (sq./30sec). To examine the influence of the toxicant exposure period on the swimming activity of B. calyciflorus, observations were made after 5, 10, 20, 30, 60, 120, 180 and 300 minutes. The rotifers were kept in the observation chamber throughout the exposure period. For each toxicant, a control and 5 to 7 concentrations were tested and for each toxicant concentration 10 isolated neonates were observed. The terms

"swimming activity" and "activity count" are used interchangeably and denote the number of squares entered by the rotifer during 30 seconds.

III.2.3. Acute toxicity tests

Acute 24 hour LC_{50} tests were conducted using the protocol described by Snell & Persoone (1989b), which basically consists of exposing (in 24-well polystyrene plates) 3 replicates of 10 rotifers to 5 toxicant concentrations for 24 hours after which the mortality is recorded. Details of the methods and results for Cu and PCP are reported in Janssen et al. (in press) and in Snell et al. (1991b), and for DCA and lindane in Ferrando and Andreu (in press).

III.2.4. Chronic toxicity tests

Details of the experimental procedures for conducting life table experiments are given in chapters 2 and 5. The methods for culturing the microalga Nannochloris oculata, which was used as food for the rotifers, are given in chapters 2 and 4.

III.2.5. Test compounds and statistical analysis

Four chemicals were tested : copper (Cu), sodium pentachlorophenol (PCP), 3,4-dichloroaniline (DCA) and lindane. All chemicals were analytical grade ; Cu was obtained from UCB Industries, Belgium, the three other compounds were obtained from Shell Research Centre, Sittingbourne, UK. Acetone, at a concentration of 0.25 ml/l, was used as carrier for DCA and lindane.

The EC_{50}'s, the concentration that reduces the rotifer swimming activity to 50 % of that in the controls were obtained by linear regression analysis. The 24 h LC_{50} from the acute tests were calculated using probit analysis. (Finney, 1971). Significant differences between the controls and the treatments were determined using a one-way ANOVA, followed by Duncan's multiple range test ($p<0.05$).

III.3. Results

Exposure to copper caused a clear decrease in the swimming activity of the Brachionus calyciflorus neonates. An exposure period of only 5 minutes significantly decreased the rotifers' swimming ability by 39 % and 49 % at 120 and 250 µg/l Cu, respectively. The swimming activity continued to decrease with increasing exposure periods (Fig. III.1A). Thirty minute, 1 hour and 5 hour exposures, for example, caused significant reductions in the rotifers' swimming activity at Cu concentrations of ≥ 60, 25, and 12 µg/l, respectively. The EC_{50}'s for the different exposure periods are presented in Figure III.1B. The EC_{50} values after 5 and 10 minute exposures were very similar, but sharply decreased to 42 % (of the 5 minute value) after 20 minutes. The EC_{50}'s further decreased gradually with increasing exposure periods to reach an incipient value after 2 hours. The ratio between the 5 minute and 5 h EC_{50} is 16, i.e. a difference of more than one order of magnitude.

For pentachlorophenol (PCP) a dose-response similar to that found for Cu was observed (Fig. III.2A). The rotifers' swimming behaviour was not affected at 0.5 mg/l PCP, except for those exposed for only 5 minutes, which was significantly higher. Similarly, significant increases were observed at 1 mg/l for rotifers exposed to the toxicant for one hour or less. At this PCP concentration the swimming activity was 30, 60, 50, 31 and 26 % higher than that of the controls after 5, 10, 20, 30, and 60 minute exposures, respectively, while the swimming activity was significantly lower after 3 and 5 hours. At 2 mg/l and higher the rotifers exhibited a reduced activity with increasing exposure periods. Unlike for Cu (for which the EC_{50}'s sharply decreased during the first 30 minutes of the exposure period) the EC_{50}'s for PCP gradually decreased with increasing exposure periods in an almost linear fashion. The EC_{50} values (± S.D.) ranged from 7.0 (± 0.23) mg/l after 5 minutes to 1.5 mg/l (± 0.09) mg/l PCP, after a 5 hour exposure (Fig. III.2B).

Acetone was used as carrier for DCA and lindane. No significant differences between the swimming activity of the B. calyciflorus neonates in the controls and in the control + acetone were observed. At the lowest DCA concentration tested (60 mg/l) significant reductions in the activity count were observed after an exposure of 2 hours (Fig. III.3A). At 80 mg/l, the rotifer's swimming activity had decreased by 40 % after only 30 minutes. The two highest concentrations (100 and 150 mg/l) had already adversely affected the swimming capacity after 5 minutes. In Figure III.3B, a gradual decrease of the EC_{50}'s in function of

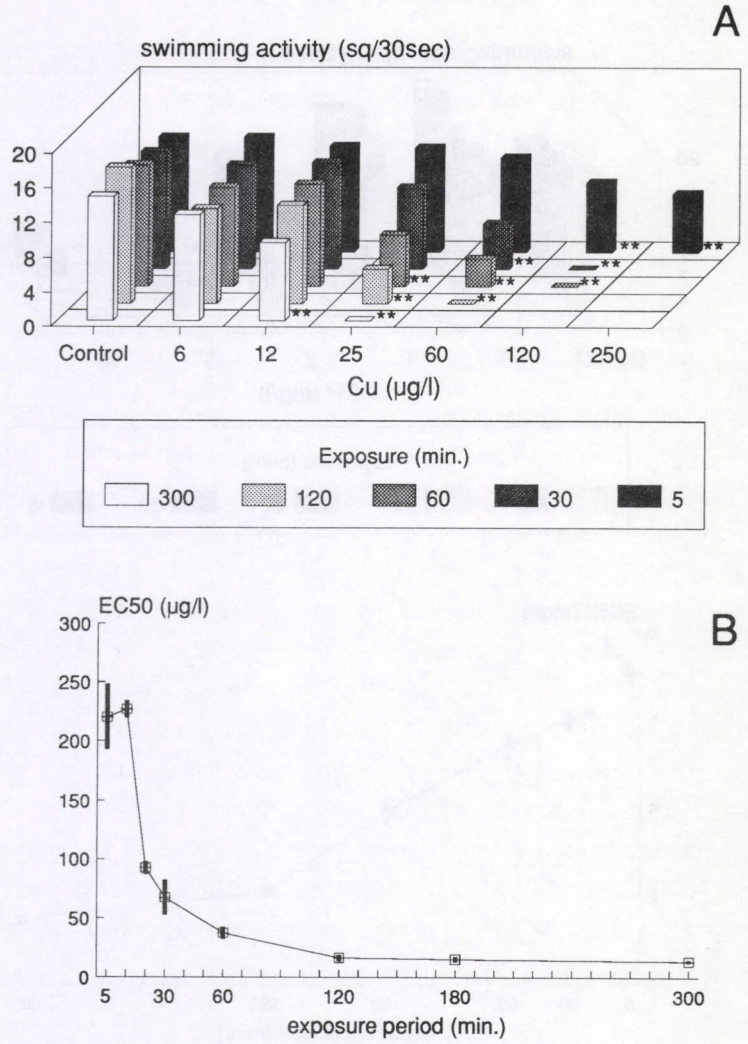


Figure III.1. The effect of copper on the swimming activity of B. calyciflorus (0-2 h old) A : The swimming activity (squares/30 seconds) in function of the exposure time and copper concentration (10, 20 and 180 min. exposures are not shown) ; (**) : significantly different from the control, $p<0.05$). B : The relationship between the EC_{50}'s and the exposure time (Vertical lines indicate $\pm$ S.D.).

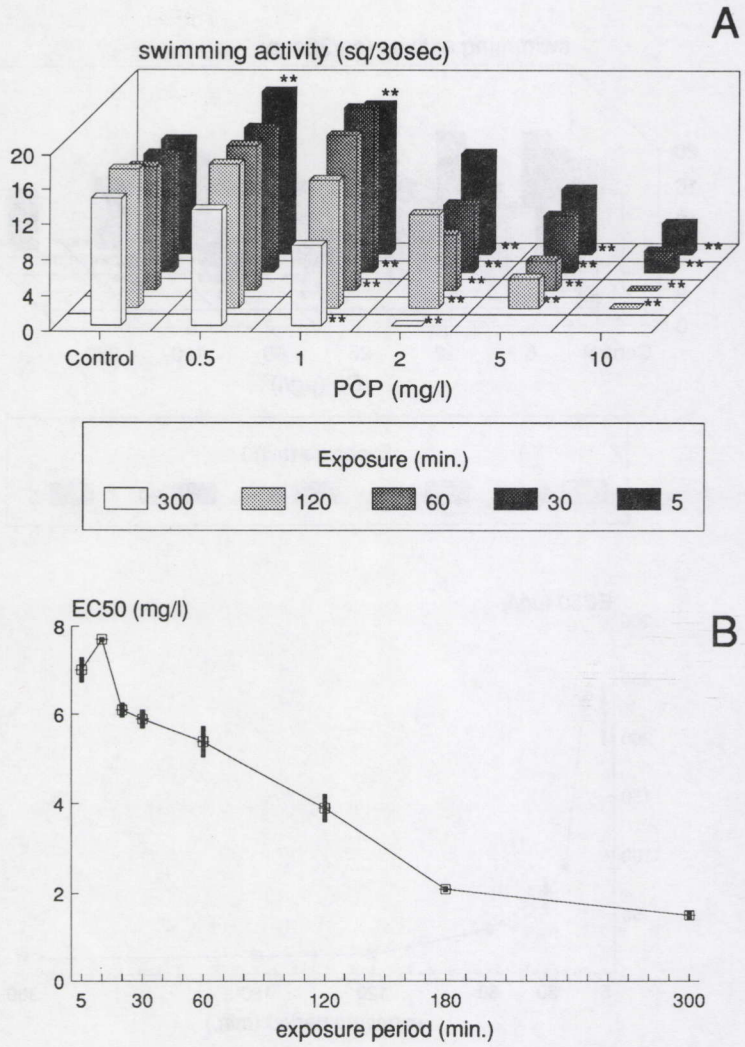


Figure III.2. The effect of pentachlorophenol on the swimming activity of B. calyciflorus (0-2 h old). A : The swimming activity (squares/30 seconds) in function of the exposure time and pentachlorophenol concentration (10, 20 and 180 min. exposures are not shown) ; (**) : significantly different from the control, $p<0.05$). B : The relationship between the EC_{50}'s and the exposure time (Vertical lines indicate $\pm$ S.D.).

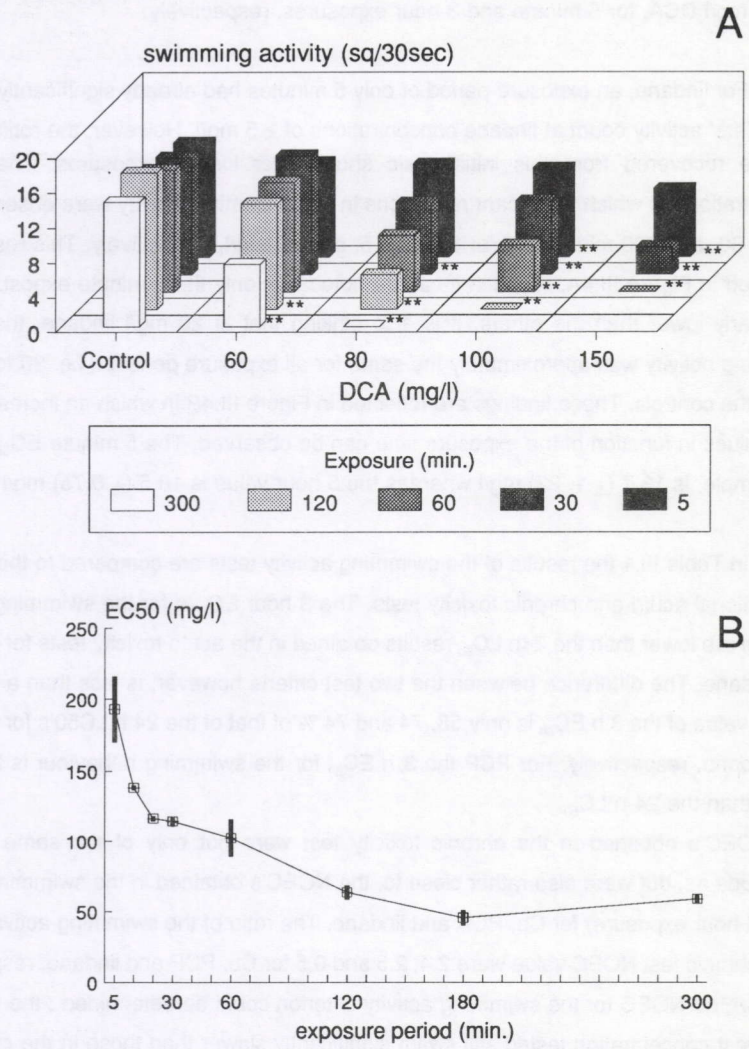


Figure III.3. The effect of 3,4-dichloroaniline on the swimming activity of B. calyciflorus (0-2 h old). A : The swimming activity (squares/30 seconds) in function of the exposure time and 3,4-dichloroaniline concentration (10, 20 and 180 min. exposures are not shown) ; (**) : significantly different from the control, $p<0.05$). B : The relationship between the EC_{50}'s and the exposure time (Vertical lines indicate $\pm$ S.D.).

exposure time is observed, with a highest value of 193 mg/l (± 22.7) and a lowest of 45.5 (± 5.3) mg/l DCA, for 5 minute and 3 hour exposures, respectively.

For lindane, an exposure period of only 5 minutes had already significantly reduced the rotifers' activity count at lindane concentrations of ≥ 5 mg/l. However, the rotifers seem to have recovered from this initial toxic shock after longer exposures. The lindane concentrations at which significant reductions in the swimming activity were observed after 10, 30, 60, and 300 minutes were: 10, 15, 15, and 20 mg/l, respectively. This response is illustrated in Figure III.4A, in which it can be noted that only the 5 minute exposure "bars" are clearly lower than the others. It is also striking that at 20 mg/l lindane, the rotifers' swimming activity was approximately the same for all exposure periods ; i.e. 30 to 44 % of that of the controls. These findings are reflected in Figure III.4B in which an increase of the EC_{50} values in function of the exposure time can be observed. The 5 minute EC_{50} (±S.D.), for example, is 13.7 (± 1. 22) mg/l whereas the 5 hour value is 18.5 (± 0.75) mg/l lindane.

In Table III.1 the results of the swimming activity tests are compared to those of the conventional acute and chronic toxicity tests. The 3 hour EC_{50}'s for the swimming activity-criterion are lower than the 24h LC_{50} results obtained in the acute toxicity tests for Cu, DCA and lindane. The difference between the two test criteria however, is less than a factor 2 ; i.e. the value of the 3 h EC_{50} is only 58, 74 and 74 % of that of the 24 h LC50's for Cu, DCA and lindane, respectively. For PCP the 3 h EC_{50} for the swimming behaviour is 2.3 times higher than the 24 h LC_{50}.
The NOEC's obtained in the chronic toxicity test were not only of the same order of magnitude as, but were also rather close to, the NOEC's obtained in the swimming activity tests (3 hour exposure) for Cu, PCP and lindane. The ratio of the swimming activity NOEC to the chronic test NOEC value were 2.4, 2.5 and 0.5 for Cu, PCP and lindane, respectively. For DCA, no NOEC for the swimming activity criterion could be determined ; the rotifers in the lowest concentration tested still swam significantly slower than those in the controls.

The variability of the swimming activity counts between the different individual rotifers (exposed to the same treatment) was rather low. In the controls the coefficient of variation of the mean swimming activity count for the different exposure periods ranged from 11 % to 24 %. The variability of this criterion is illustrated in Figure III.1-4 B in which the EC_{50}'s ± S.D. are presented.

Table III.1. Comparison of the results obtained in acute-, swimming activity- and chronic toxicity tests for the 4 model chemicals.

	Acute test	**Swimming activity test** 3 hour exposure			**Chronic test**[1] life-cycle	
Test criterion:	24 h LC50	3 h EC50	LOEC	NOEC	LOEC	NOEC
Cu (µg/l)	26	15	12	6.0	5.0	2.5
PCP (mg/l)	0.92	2.1	1.0	0.5	0.4	0.2
DCA (mg/l)	61.5	45.5	-[2]	-[2]	5.0	2.5
Lindane (mg/l)	22.5	16.7	10.0	5.0	15.0	10.0

[1] Details of the chronic toxicity tests results are presented in chapter 5.

[2] LOEC and NOEC could not be derived.

III.4. Discussion

The major drawback associated with the swimming toxicity test, as described in this paper, is that it was rather labour intensive. One test with 5 toxicant treatments and a control, each consisting of a minimum of 5 rotifers, requires at least 3 hours to complete. An alternative to this quantitative evaluation of the decrease of the rotifer's swimming activity, could be a simple mobility - immobility observation. To explore this possibility, the toxicant concentrations at which the rotifers had completely stopped swimming (hereafter indicated as the EC_{100} or the "immobility" criterion) are summarized in Table III.2. The ratio's of the EC_{100} to the EC_{50} (for the same exposure period) ranged from 1.3 to 4 for Cu, from 1.3 to 2.6 for PCP and from 1.4 to 2.2 for DCA. The EC_{100} for the 5 hour exposure period is very similar to the 24 h LC_{50}'s reported in Table III.1 for Cu, PCP, and DCA. No comparison of the different test endpoints could be made for lindane, as the rotifers did not exhibit immobility.

Table III.2. Comparison of the toxicant concentrations that reduced the rotifers' swimming activity to 50% of the control value (EC_{50}) and the concentrations that caused complete immobility (EC_{100}).

		Swimming activity - exposure period (minutes)							
		5	10	20	30	60	120	180	300
Cu (µg/l)	EC_{100}	-	-	120	120	120	60	60	25
	EC_{50}	220	228	92	67	37	16	15	14
PCP (mg/l)	EC_{100}	-	-	-	-	10.0	10.0	5.0	2.0
	EC_{50}	7.0	7.7	6.1	5.9	5.4	3.9	2.1	1.5
DCA (mg/l)	EC_{100}	-	-	-	-	150	100	100	80
	EC_{50}	192	138	116	114	102	63.5	45.5	58.7
Lindane (mg/l)	EC_{100}	-	-	-	-	-	-	-	-
	EC_{50}	13.7	14.1	15.1	15.7	16.5	15.0	16.7	18.5

-: no immobility recorded.

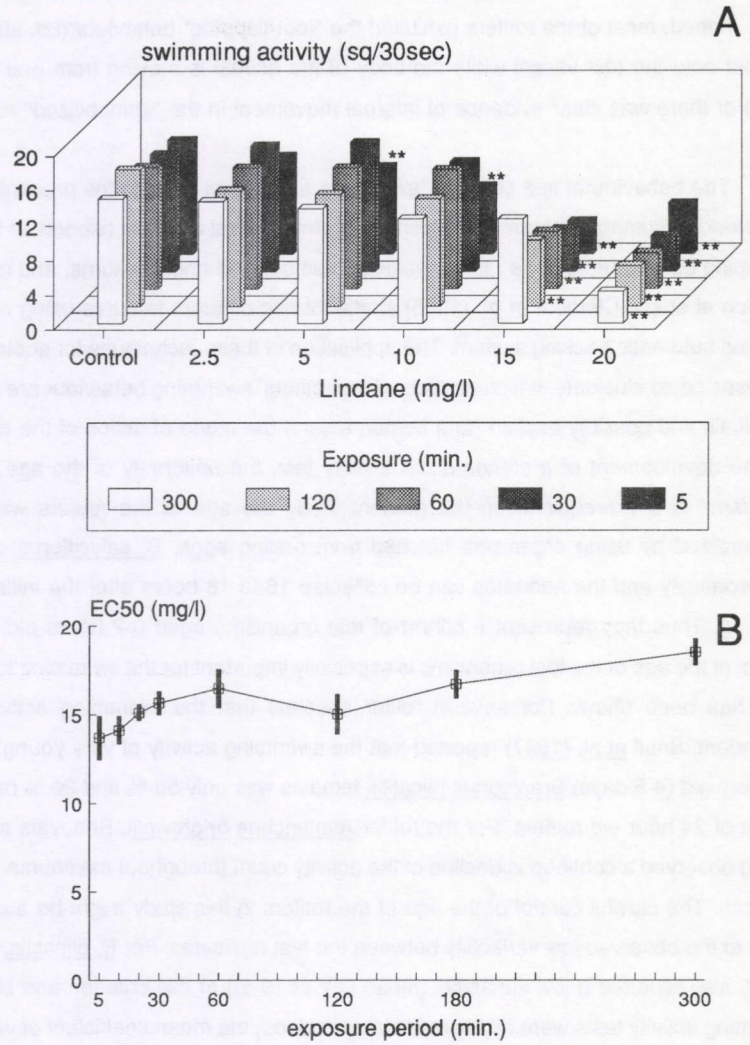


Figure III.4. The effect of lindane on the swimming activity of B. calyciflorus (0-2 h old). A : The swimming activity (squares/30 seconds) in function of the exposure time and lindane concentration (10, 20 and 180 min. exposures are not shown) ; (**) : significantly different from the control, $p<0.05$). B : The relationship between the EC_{50}'s and the exposure time (Vertical lines indicate $\pm$ S.D.).

It must be noted that at the EC_{100} reported in Table III.2, the majority of the rotifers were not dead. Indeed, most of the rotifers exhibited the "foot-flapping" behaviour (i.e. attached with the foot onto the test vessel while the body of the animal is moving from one side to the other) or there was clear evidence of internal movement in the "immobilized" rotifers.

The behavioural test criterion "swimming activity" as used in the present study is in fact a kind of summary parameter which integrates several different aspects of the rotifers' swimming behaviour such as : linear velocity, number and angle of turns, and number and duration of stops. Clément et al. (1985) studied these different features using a computer-assisted automatic tracking system. The application of these techniques for ecotoxicological purposes could elucidate which aspects of the rotifers' swimming behaviour are affected by chemicals and possibly explain (to a certain extent) the mode of action of the toxicants.
For the development of a standardized toxicity test, the uniformity of the age of the test organisms is a prerequisite. In the present study the age of the rotifers was carefully standardized by using organisms hatched from resting eggs. B. calyciflorus cysts hatch synchronously and the neonates can be collected 16 to 18 hours after the initiation of the hatching. Thus they represent a cohort of test organisms aged 0-2 hours old. This strict control of the age of the test organisms is especially important for the swimming toxicity tests as it has been shown (for several rotifer species) that the swimming activity is age-dependent. Snell et al. (1987) reported that the swimming activity of very young (0-2 h old) and very old (4-5 days) Brachionus plicatilis females was only 50 % and 20 % respectively, of that of 24 hour old rotifers. For the rotifer Asplanchna brightwelli, Beauvais and Enesco (1985) observed a continuous decline of the activity count throughout the course of its 5-day life-span. The careful control of the age of the rotifers in this study might be a contributing factor to the observed low variability between the test replicates. For B. plicatilis, Snell et al. (1987) also reported a low variability (mean C.V.of 19 %) of this criterion and showed that swimming activity tests were highly repeatable. Indeed, the mean coefficient of variation, for the average swimming activity counts of seven tests repeated in time was 4.5 %.

Several fundamental aspects of the swimming behaviour of rotifers have been studied. Snell et al. (1987) used similar techniques as those applied in the present study to examine the effects of aging and the presence of food on the swimming behaviour of the marine rotifer B. plicatilis. They also found that the swimming behaviour of this rotifer was a very sensitive indicator of environmental variables such as pH, water quality, temperature

and food availability. Un-ionized ammonia at a concentration of 0.32 mg/l for example, reduced the swimming capacity of B. plicatilis by 17 % after an exposure period of only one hour. Other authors have described the swimming activity of several rotifer species in relation to the sexual reproduction (Snell and Garmann, 1986), aging (Beauvais and Enesco, 1985) and the ultrastructure (Clément, 1987) of rotifers. Swimming activity measurements have also been used to determine the cost, speed, and efficiency of locomotion of B. plicatilis and the predatory rotifer Asplanchna sielboldi (Epp and Lewis, 1979, 1984). These authors found that the actual cost of locomotion of B. plicatilis accounts for a large part of its total metabolism and suggest that this may be one of the reasons why rotifer swimming behaviour is a sensitive indicator of environmental stress.

Behavioural changes under toxic stress of several other species of aquatic invertebrates have been reported. The phototactic behaviour of Artemia salina has been suggested as a sublethal test endpoint by Saunders et al. (1985) and Dojmi di Delupis and Rotondo (1988). Flickinger et al. (1982) have studied the influence of copper on the phototactic behaviour of Daphnia magna and compared this test criterion to more conventional chronic test endpoints such as survival and reproduction. They found that animals exposed to 10 µg/l Cu exhibited a reduction in filtration rate and negative phototaxis ; survival, on the other hand was only affected at copper concentrations ≥ 20 µg/l. Recently, Goodrich and Lech (1990) have developed a short-term behavioural assay with D. magna. This method, which is based on the interference of directional migration of the D. magna neonates under stress conditions, was found to be very sensitive to lindane and produced effects on the daphnid's orientation at concentration of 50 µg/l.

Another important aspect of the behaviour of aquatic invertebrates, which has a potential use for sublethal toxicity testing, is feeding. The effects of xenobiotics on the filtration rates of aquatic crustaceans (mainly daphnids) has been investigated by several workers (Cooley, 1977 ; Reeve et al. 1977 ; Geiger and Buikema, 1981 ; Kersting and van der Honing, 1981 ; Flickinger et al., 1982 ; Day and Kaushik, 1987a ; Day et al., 1987). These aspects of invertebrate behaviour modified by toxic stress will be addressed in chapter 4.

The present study has shown that the swimming behaviour of B. calyciflorus is a sensitive indicator of sublethal concentrations of toxic compounds. The results obtained with

the (relatively simple) swimming activity test, as described in this chapter, showed that the swimming behaviour of the rotifers, after short exposures to the stressors, is affected at toxicant concentrations which affect survival and reproduction after much longer exposures. If the relationship between behavioural- and more conventional test criteria such as survival, reproduction and growth are further examined for B. calyciflorus and other invertebrates alike, we believe that standardized behavioural toxicity tests can have an important future in the first tier of hazard assessment schemes.

II.5. Summary

The swimming behaviour of the freshwater rotifer Brachionus calyciflorus exposed to copper (Cu), pentachlorophenol (PCP), 3,4 dichloroaniline (DCA) and lindane, for periods ranging from 5 minutes to 5 hours, was examined. A swimming behaviour test, based on the rotifers' movement rate as they swim over a grid, is described. For all 4 toxicants a clear dose-response was observed, with the swimming activity decreasing with increasing toxicant concentrations. For Cu the EC_{50}'s, the concentration that reduced the swimming activity to 50 % of that of the control value, sharply decreased from 0.22 mg/l after an exposure of 5 minutes to 0.068, 0.038 and 0.014 mg/l after exposures of 30, 60 and 300 minutes, respectively. PCP affected the rotifers' swimming behaviour more gradually, with EC_{50}'s decreasing from 7.0 mg/l after an exposure of 5 minutes to 5.9, 5.4, and 1.5 mg/l after 30, 60 and 300 minutes, respectively. A similar pattern was found for DCA with EC_{50}'s ranging from 193 to 45.5 mg/l for the 5 minute and 3 hour exposures, respectively. Exposed to lindane however, B. calyciflorus swimming activity exhibited a different response, the EC_{50}'s gradually increased from 13.7 mg/l after an exposure of 5 minutes to significantly higher values of 16.4 and 18.5 mg/l after periods of 1 and 5 hours, respectively. The results of the swimming activity assays were compared to those of acute and chronic toxicity tests performed with the same test species. The potential use and relevance of this behavioural test criterion were evaluated and discussed.

CHAPTER IV.

The effects of xenobiotics on the feeding behaviour of Brachionus calyciflorus.

IV.1. Introduction

Several researchers have suggested that changes in the physiology and/or behaviour of aquatic organisms (e.g. respiration, feeding and swimming behaviour) could be used as rapid and sensitive indicators of toxic stress (Berman and Heinle, 1980 ; Harding et al. 1980 ; Geiger and Buikema, 1981 ; Hirata et al. 1984 ; Day and Kaushik, 1987 a&b ; Janssen et al., in press). Indeed, such changes may be the initial responses of an organism to environmental perturbation and might help to explain other observations such as reduced survival, growth or reproduction (Flickinger et al. 1982).

Behavioural changes have been used successfully as sensitive sublethal indicators of toxic stress in fish (Webb and Brett, 1973 ; Bengtsson, 1974 ; Little and Finger, 1990) but little has been done to develop behavioural indices with zooplankton. A few studies have reported on toxicant-induced changes in the feeding behaviour of various species of zooplankton under laboratory conditions (Reeve et al. 1977 ; Berman and Heinle, 1980 ; Day and Kaushik, 1987 a&b). Cooley (1977) studied the food-uptake of Daphnia retrocurva in the field and found that the filtration rate of this daphnid was much lower near a pulp mill effluent discharge than that of organisms found in open water away from human impact.

Although two aspects of rotifer behaviour i.e. swimming activity and food uptake have been intensively investigated (a.o. Starkweather et al. 1979 ; Coulon et al. 1983 ; Yufera and Pascual, 1985), these aspects of rotifer biology have rarely been used as indices of toxic stress (Geiger and Buikema, 1981 ; Snell et al. 1987 ; Janssen et al., in press).

The objective of this study was to examine the effects of xenobiotics on the feeding behaviour of the freshwater rotifer Brachionus calyciflorus and explore the possibility of developing a short-term toxicity screening test using this sublethal test criterion. To that end,

the filtration- and ingestion rates of rotifers exposed to copper, pentachlorophenol, 3,4-dichloroaniline and lindane were measured using simple laboratory techniques.

IV.2. Materials and methods

IV.2.1. General experimental conditions

Test organisms were obtained by hatching cysts which were produced in laboratory cultures under rigorously controlled conditions (Snell et al., 1991b). Details of the storage conditions and the hatching procedure are given in chapters 2 and 5.

The microalgae Nannochloris oculata, which was used as food in all experiments, was cultured in BBM medium (Nichols, 1973). The log phase algae were harvested, centrifuged, washed, and resuspended in EPA water. The concentrated algae (stock) were stored in the refrigerator (4°C) and used for 3 days (after harvesting). The Nannochloris density chosen for all the experiments was $5x10^5$ cells/ml and was obtained by diluting the concentrated algae stock with EPA water.

IV.2.2. Food uptake experiments

Filtration and ingestion rates were used as measures of feeding behaviour. To determine suitable (practical) test conditions for the toxicity experiments, a series of preliminary experiments were performed to examine the effects of the following test variables on the rotifers' feeding : rotation of the exposure vessels (on a modified plankton wheel in vertical or horizontal positions at 0.25 rotations/minute ; and non-rotated) ; and rotifer density (10, 20, 30, 50 rotifers/ml). The feeding experiments were performed in 8 ml glass vials containing 5 ml of the test solution. Based on the results of the preliminary experiments, which will be discussed in this chapter, the test conditions selected for the toxicity tests were : 30 rotifers/ml exposed in non-rotating, vials, in darkness. Rotifers were exposed to the test solution containing food for 5 hours, after which the algae were fixed (with formaldehyde) and the final food concentration was measured using a hematocytometer.

The filtration rate (F) is defined as the volume swept clear per unit of time and the

ingestion rate (I) as the number of cells consumed by an animal in an interval of time. For the calculation of average filtration- (µl rotifer^{-1} h^{-1}) and ingestion rate (cells rotifer^{-1} h^{-1}) the following equations were used (Gauld, 1951) :

$$F=\frac{v}{n}\left(\frac{lnC_o-lnC_t}{t}-A\right)$$

$$A=\frac{\ln C_o-lnC_t'}{t}$$

$$I=F\sqrt{C_o.C_t}$$

where C_o and C_t are initial and final food concentrations (cells µl^{-1}), t is time (duration of the experiment in hours), and n is the number of rotifers in volume v (µl). A : is a correction factor for changes in the algal density in the control with final concentration C_t' after time t. The expression $\sqrt{C_o . C_t}$ represents the geometric mean of food concentration during time t.

Prior to the feeding behaviour experiments, acute 24 hour lethality tests were performed to establish the range of concentrations to be used in the feeding tests. Details of the acute toxicity tests are reported in Snell and Persoone (1989b), Janssen et al. (in press) and Ferrando and Andreu (in press). In the feeding toxicity tests the rotifers were exposed to four toxicant concentrations and a control for each compound. Each treatment consisted of 5 replicates. All experiments were performed in a temperature controlled room at 25 $\pm$ 1 °C.

IV.2.4. Test compounds and statistical analysis

Details of the preparation of the chemical dilutions and the statistical methods are given in chapter 3. The EC_{50}'s were calculated using linear regression analysis.

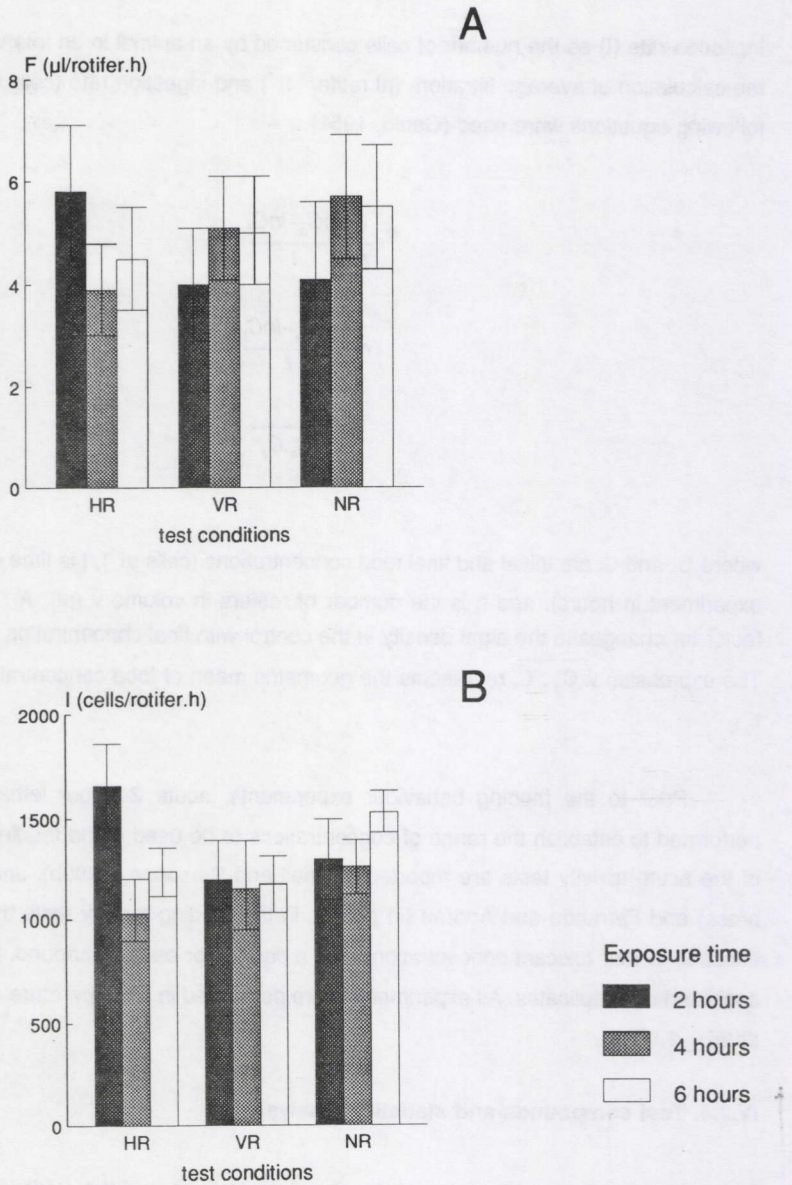


Figure IV.1. The effect of the rotation of the test vessels (horizontal:HR; vertical:VR and static:NR) on A : the filtration (F in µl rotifer^{-1} h^{-1}) and B : the ingestion rates (I in cells rotifer^{-1} h^{-1}) of B. calyciflorus after 2, 4 and 6 hours.

IV. Results

In Figure VI.1 the effect of the rotation of the test vessels on the filtration (F)- and ingestion (I) rates of B. calyciflorus are presented (rotifer density : 10/ml). The F-rate after 2 hours was 5.8 (± 1.3), 4.0 (± 1.1) and 4.1 (± 1.5) µl rotifer^{-1} h^{-1} for the horizontally rotated-, vertically rotated-, and non-rotated test vessels, respectively. The filtration rates of the rotifers in all three treatments were not significantly affected by the duration of the experiment. The ingestion rates exhibited a similar trend (Fig. IV.1). In Figure IV.2. the influence of the rotifer density on the mean filtration- and ingestion rates are shown (5 hour experiments). No significant differences in F- and I rates were observed between the tests with rotifer densities of 10, 20 and 30 rotifers/ml. The mean filtration rate at these respective rotifer densities was 6.6 (± 2.1), 7.7 (± 2.2) and 6.7 (± 0.2) µl rotifer^{-1} h^{-1}, which is close to the maximum F-values under the given test conditions (F_{max}= 7.9 (± 1.3) ; unpublished data). In the tests with 50 rotifers/ml, however, the filtration- and ingestion rate were significantly reduced (compared to the 30 rot./ml), which indicates that after 5 hours the rotifers did not forage at their maximum capacity.

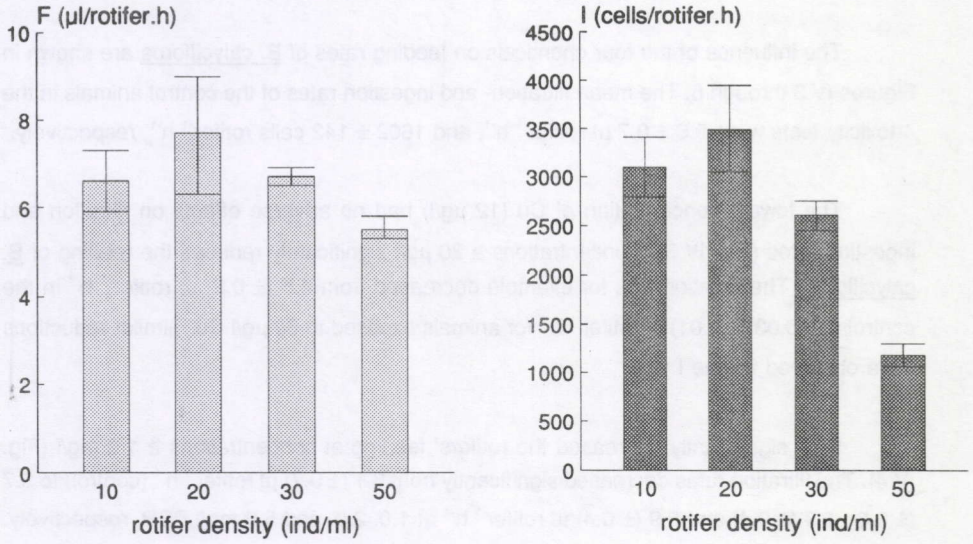


Figure IV.2. The influence of the rotifer density on the mean filtration (F) - and ingestion (I) rates of B. calyciflorus after an exposure period of 5 hours. Error bars indicate ± S.D.

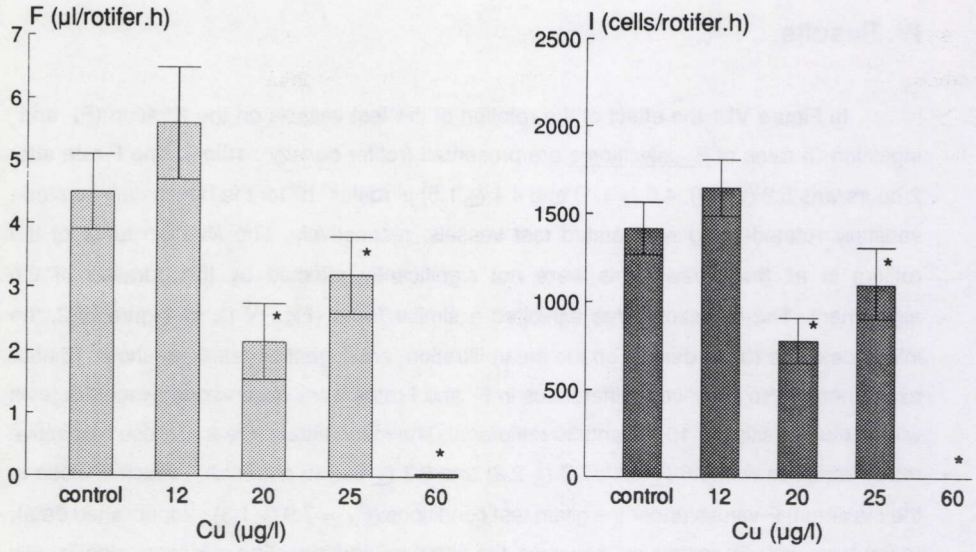


Figure IV.3. The effect of Cu on the filtration (F) - and ingestion (I) rates of B. calyciflorus after an exposure period of 5 hours. Error bars indicate ± S.D. ; (*) = significantly different with the controls ($p<0.05$).

The influence of the four chemicals on feeding rates of B. calyciflorus are shown in Figures IV.3 through 6. The mean filtration- and ingestion rates of the control animals in the 4 toxicity tests were 5.6 ± 0.7 µl rotifer^{-1} h^{-1}, and 1602 ± 142 cells rotifer^{-1} h^{-1}, respectively.

The lowest concentration of Cu (12 µg/l) had no adverse effects on filtration and ingestion rates (Fig. IV.3). Concentrations ≥ 20 µg/l significantly reduced the feeding of B. calyciflorus. The filtration rate for example decreased from 4.5 (± 0.8) µl rotifer^{-1} h^{-1} in the controls, to 0.03 (± 0.01) µl rotifer^{-1} h^{-1} for animals exposed to 60 µg/l Cu. Similar reductions were observed for the I rate.

PCP significantly decreased the rotifers' feeding at concentrations ≥ 1.0 mg/l (Fig. IV.4). The filtration rates decreased significantly from 6.1 (± 0.7) µl rotifer^{-1} h^{-1} (control) to 3.7 (± 0.8), 1.3 (± 0.4) and 0.9 (± 0.4) µl rotifer^{-1} h^{-1} at 1.0, 2.0, and 5.0 mg/l PCP, respectively. Similarly, the ingestion rates were reduced ($p<0.05$) from 1687 (± 247) cells rotifer^{-1} h^{-1} (control) to 1175 (± 240), 536 (± 201) and 405 (± 190) cells rotifer^{-1} h^{-1} at the same PCP concentrations.

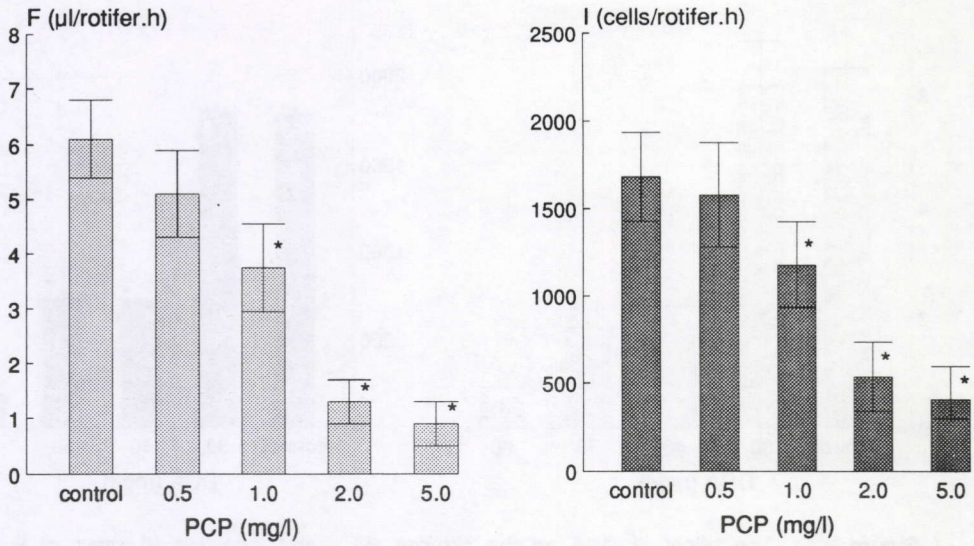


Figure IV.4. The effect of PCP on the filtration (F) - and ingestion (I) rates of B. calyciflorus after an exposure period of 5 hours. Error bars indicate ± S.D. ; (*) = significantly different with the controls ($p<0.05$).

Acetone, which was used as solvent for DCA and lindane, did not affect the feeding behaviour in the acetone control treatments ; consequently the results of these treatments are not included in Figures IV.5 and IV.6.

3,4-dichloroaniline concentrations of ≥ 40 mg/l caused significant decreases in the filtration- and ingestion rates (Fig. IV.5). The filtration rate was reduced from 6.5 (± 0.86) µl rotifer^{-1} h^{-1} (control) to 1.9 (± 0.56), 1.8 (± 0.34) and 0.5 (± 0.27) µl rotifer^{-1} h^{-1} which are reductions ($p<0.05$) of 71, 72 and 92 %, at DCA concentrations of 40, 50 and 60 mg/l, respectively. Similarly, the ingestion rates were reduced ($p<0.05$) from 1784 (± 251) cells rotifer^{-1} h^{-1} in the controls to 725 (± 210), 740 (± 179) and 198 (± 109) cells rotifer^{-1} h^{-1} at DCA concentrations of 40, 50 and 60 mg/l, respectively. Note that at 60 mg/l DCA, a concentration close to the 24 h LC50 (61.5 mg/l), had almost completely stopped feeding. Microscopic examination confirmed that these animals were still alive.

Lindane concentrations of ≥ 5.0 mg/l significantly reduced the feeding of B. calyciflorus (Fig. IV.6). At the highest concentration (15.0 mg/l) the filtration rate was still

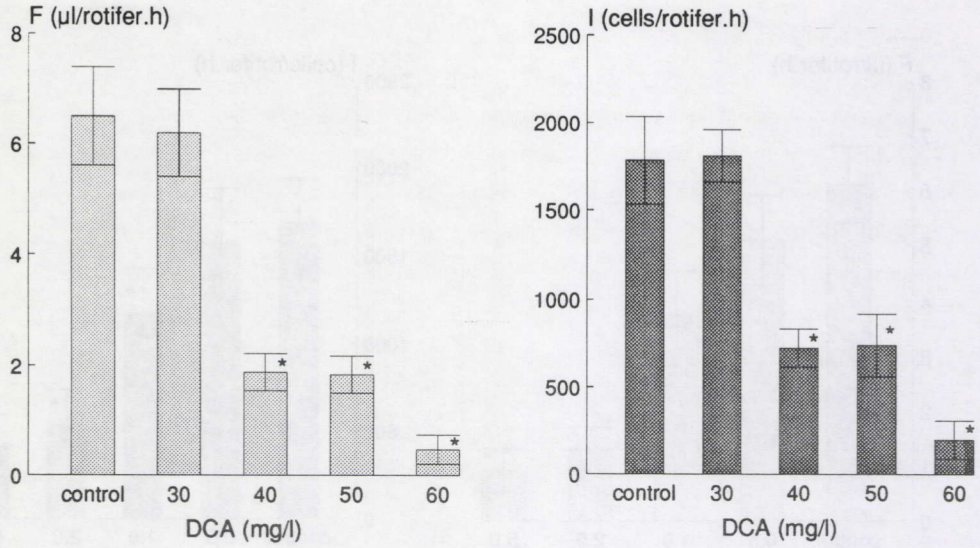


Figure IV.5. The effect of DCA on the filtration (F) - and ingestion (I) rates of B. calyciflorus after an exposure period of 5 hours. Error bars indicate ± S.D. ; (*) = significantly different with the controls ($p<0.05$).

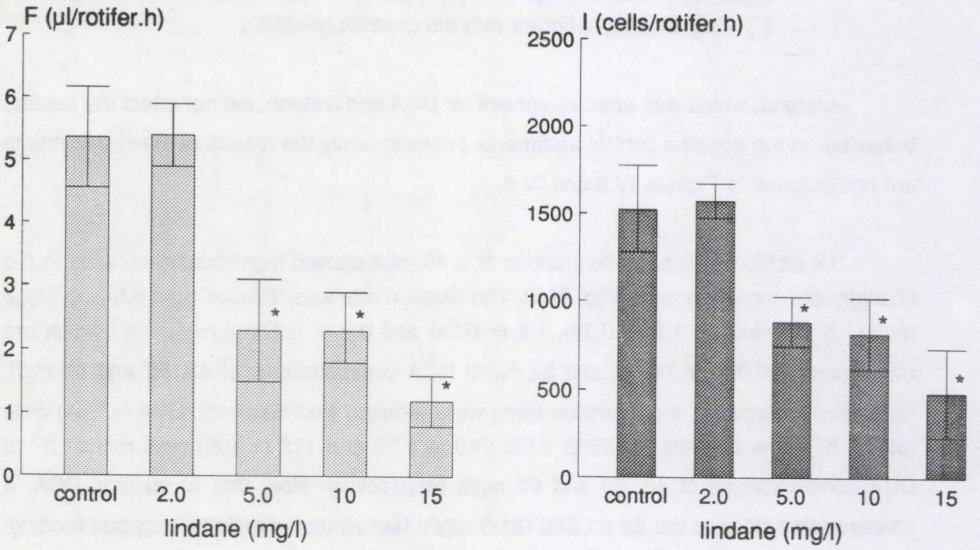


Figure IV.6. The effect of lindane on the filtration (F) - and ingestion (I) rates of B. calyciflorus after an exposure period of 5 hours. Error bars indicate ± S.D. ; (*) = significantly different with the controls ($p<0.05$).

approximately 23 % of that of the controls.

A useful, quantitative parameter analogous to the LC_{50} derived in acute toxicity tests, is the EC_{50}, the median effective concentration of the toxicant at which the value of a given parameter is reduced to 50 % of that in controls. The 5 h EC_{50} values for DCA and lindane (Table IV.1), 41.2 and 8.5 mg/l, respectively, were lower than the 24 h LC_{50} values for both toxicants (61.5 mg/l for DCA, and 22.5 mg/l for lindane). Thus, in both cases, toxicant concentrations below the 24 h LC_{50} values are sufficient to reduce the feeding rate of B. calyciflorus to 50 % in 5 hours. On the other hand, the 5 h EC_{50}'s for PCP and Cu, 1.85 mg/l and 32 µg/l, respectively (Table IV.1) are slightly higher than the 24 h LC_{50}'s (1.2 and 0.026 mg/l, respectively). The differences between the 24 h LC50's and the 5 h EC_{50}'s however are smaller than a factor 2 for 3 of the 4 toxicants tested.

The variability among replicates within one toxicity test was considerably higher in the toxicant treatments than in the controls. For Cu, for example the coefficients of variation for the mean filtration rates ranged from 18 % in the controls to 33 % in the highest Cu concentration tested. For PCP, DCA and lindane the ranges for the among-replicate variability were 11-44 %, 20-50 % and 13-57 %, respectively.

Table IV.1. Comparison of the 5 h EC_{50}'s (± S.D.) obtained in the food uptake tests (filtration rate) and the 24 h LC_{50}'s from acute toxicity tests.

	Food uptake test (5 h EC_{50} ± S.D.)	**Acute toxicity test**[1] (24 h LC_{50})
Cu (µg/l)	32 (3.0)	26
PCP (mg/l)	1.85 (0.45)	0.92
DCA (mg/l)	41.2 (2.6)	61.5
Lindane (mg/l)	8.5 (2.5)	22.5

[1]: data from chapter 3

IV.4. Discussion

The preliminary experiments on the feeding behaviour of B. calyciflorus, aimed at developing an appropriate test method, showed that filtration (F) and ingestion (I) rates in static experiments (vials not rotated) were very similar to those in the test with rotating vials (Fig. IV.1). However, in feeding experiments with Brachionus plicatilis fed on Dunaliella sp. Schlosser and Anger (1982) found that a lack of rotation reduced the filtration rate by 40 %. The fact that a similar reduction was not found in the present study might be attributed to the very small size of the microalgae used which "settles" very slowly, thus, remaining available for the feeding rotifers.

The test protocol used for the food uptake experiments should be considered as preliminary. Indeed the experiments performed to define a set of "standard" toxicity test conditions were specifically designed to develop a relatively rapid (< 1 day) and practical method. The considerable among-replicate variability indicates that additional test development is required. Additionally, the test procedure as described in this study does not consider the rotifers' "gut passage time". Indeed, under the reported experimental conditions the time required for the food the pass through the rotifers' alimentary track is approximately 10 minutes (T.W. Snell, personal communication). Consequently, during the 5 hour exposure period, some algal cells ingested by the rotifers could have passed through the gut intact (several times), which might possibly have resulted in an underestimation of the rotifers' food uptake. This weakness in the experimental design can be resolved by determining the algal density twice : i.e. at t_x and at t_x +10 minutes (with x = exposure time to the toxicant).

In the present study a mean F-rate of 5.6 ($\pm$ 0.7) µl rotifer^{-1} h^{-1} was found. However, a comparison of these feeding rates to those reported in literature is difficult because of the differences in experimental procedures. Starkweather and Gilbert (1977) reported rates of filtration between 0.5 and 50 µl rotifer^{-1} h^{-1}, and ingestion rates between 100 and 5000 cells rotifer^{-1} h^{-1} for B. calyciflorus feeding on different densities of the yeast Rhodotorula glutinis. Pennington (1941) observed mean rates of filtration of 0.5 µl rotifer^{-1} h^{-1} feeding on Diogenes rotunda while Halbach and Halbach-Keup (1974) reported F-rates of 3.4 µl rotifer^{-1} h^{-1} for the same rotifer species. For Brachionus rubens feeding on Chlorella vulgaris, Pilarska (1977) reported values of 11.3 µl rotifer^{-1} h^{-1}. Filtration rates ranging from 0.18 to 6.0 µl rotifer^{-1} h^{-1} were found for the estuarine rotifer B. plicatilis feeding on Chlorella (Hirayama and Ogama,

1972).

Despite the vast amount of literature on the fundamental aspects of rotifer feeding (a.o. Starkweather, 1980 ; Bogdan and Gilbert, 1982a&b ; Gilbert and Bogdan, 1984), only two papers reported on the effects of toxicant stress on the feeding rate. Capuzzo (1979b) found that the filtration rate of Brachionus plicatilis was reduced by approximately 50% after a 24 hour exposure to 1.0 mg/l of the halogen toxicants : free chlorine and chloramine. No significant differences were obtained in the feeding rate of B. plicatilis exposed to 0.3 mg/l of the herbicide benthiocarb by Hirata et al. (1984).

The effects of xenobiotics on the filtration rates of other zooplankters have been investigated by several workers (Cooley, 1977 ; Reeve et al. 1977 ; Geiger and Buikema, 1981 ; Day et al. 1987). Day and Kaushik (1987a) showed that the filtration rate of the cladocerans Daphnia galeata mendotae and Ceriodaphnia lacustris, and of the calanoid Diaptomus oregonesis were adversely affected by sublethal concentrations (0.01-0.1 µg/l) of the synthetic pyrethroid fenvalerate. They observed that fenvalerate induced the adhesion of particulate material (algae and detritus) onto the various appendages of D. galeata mendotae, and such clumping impeded the daphnids' mobility, thus affecting the food uptake. Flickinger et al. (1982) studied the influence of copper on the filtration rate of Daphnia magna and compared the sensitivity of this test criterion to more conventional, chronic test endpoints such as survival and reproduction. They found that animals exposed to 10 µg/l Cu exhibited significant reductions in the filtration rate and the phototactic behaviour of the exposed organisms as well as in the body length of their offspring. Survival, on the other hand was only affected at copper concentrations of ≥ 20 µg/l.

The results from this study suggest that the feeding behaviour of the freshwater rotifer Brachionus calyciflorus might be an interesting and valuable new test endpoint for rapid toxicity screening of chemicals. The results from the "feeding" toxicity test, as described in this study, also showed that behavioural indices (can) reflect the potential for subsequent mortality (acute mortality tests) at approximately the same toxicant concentrations. Considering the ecological importance of food-uptake and the ease and rapidity by which it can be evaluated, standardized toxicity tests with invertebrates using feeding behaviour as test endpoints could be a valuable new approach in the first tier of hazard assessment schemes.

IV.5. Summary

The changes in the feeding behaviour of the freshwater rotifer Brachionus calyciflorus exposed to sublethal levels of the 4 model chemicals were examined. The development of short-term toxicity test using the rotifers' filtration- and ingestion rate was described. The 5 h EC_{50}'s for copper, pentachlorophenol, 3,4-dichloroaniline and lindane were 0.032, 1.85, 41.2 and 8.5 mg/l. The potential use of the feeding behaviour as a test criterion for toxicity screening tests with aquatic invertebrates was discussed.

CHAPTER V.

The effects of xenobiotics on the life history characteristics of Brachionus calyciflorus : life table and short-term population experiments.

V. Introduction

Invertebrate short-term chronic toxicity tests have become an accepted and in many cases essential part of the toxicological base used to evaluate the hazard of toxic substances. The two main freshwater chronic bioassays with invertebrates, which are recommended by several standardization and regulatory organizations and used routinely (Persoone and Janssen, 1991), are the 21-day Daphnia and the 7-day Ceriodaphnia survival and reproduction tests (Mount and Norberg, 1984 ; ASTM, 1987). The selection and acceptance of these test organisms is well justified since daphnids have many advantages important for routine toxicity testing, e.g. sensitivity to toxicants, parthenogenetic reproduction and short reproductive cycle (Buikema et al., 1980). However, from an ecological point of view these test organisms represent only one of the 3 main groups of pelagic invertebrates important to the structure and function of freshwater communities (a.o. Herzig, 1987). The two other groups : copepods and rotifers, have largely been ignored by the aquatic toxicologist.

In this chapter the potential use of the freshwater rotifer Brachionus calyciflorus as a test organism for short chronic toxicity tests will be examined. The ecological relevance and the advantages associated with using this species as test organism for ecotoxicological studies have been reported in chapters 1 and 2, and are summarized below. Indeed, factors contributing to their ecological relevance include their cosmopolitan distribution (Pejler, 1977 ; Dumont, 1983), and their importance as grazers on phytoplankton (Bogdan and Gilbert, 1987) and as prey for larval fish and invertebrates ('O Brien, 1979 ; Williamson, 1983 ; Evans, 1986). Furthermore, their rapid reproduction and short generation time (Halbach, 1970 ; Halbach and Halbach-Keup, 1974) are particularly attractive for ecotoxicological applications.

However, the main advantage of using this species for routine ecotoxicological applications is the ability of Brachionus to produce cysts (resting eggs) which can be stored for months and hatched upon demand by simple manipulations. In practical terms, this means that the use of these cysts as biological starting material for obtaining test organisms, completely eliminates one of the main bottle-necks in routine aquatic toxicology : the need for the continuous culture and maintenance of test organisms in sufficient numbers. This concept has recently led to the development of a 24 h LC_{50} test with the freshwater rotifer B. calyciflorus (Snell et al., 1991b) and its marine counterpart B. plicatilis (Snell and Persoone, 1989a ; Snell et al. 1991a) and with the crustaceans Streptocephalus proboscideus (Centeno et al., in press) and Artemia salina (Persoone, 1991 ; Van Steertegem et al., in press ; Persoone et al., in press) for freshwater and marine toxicity testing, respectively.

The objective of the present study was to develop a rationale for short chronic toxicity testing with B. calyciflorus incorporating the practical and financial advantages of using eggs. The effects of copper, pentachlorophenol, 3,4-dichloroaniline and lindane on the demographic parameters of this rotifer were studied and the results evaluated in order to determine a set of standardized test conditions. Additionally, these results were compared to those of a series of simple toxicity tests in which the population growth of B. calyciflorus under toxic stress was examined.

V.2. Materials and Methods

V.2.1. General experimental procedures

The B. calyciflorus strain used in the toxicity tests was originally collected in Gainesville, Fl., USA, and maintained in laboratory cultures by T.W. Snell, Georgia Institute of Technology, Georgia, Atlanta, USA. Cysts were produced in mass cultures under rigorously controlled conditions, collected, dried and stored at 6°C in the dark. Cysts stored under these conditions remain viable for at least one year. Hatching is initiated by transferring the cysts to a 10 ml glass petridish containing 5 ml of a synthetic freshwater medium (EPA-medium, see below), followed by incubation at 25 $\pm$ 1°C, in light (6000 lux). Under these conditions hatching is synchronous and rapid. A cohort of 0-2 hour old neonates

can be collected and used in the toxicity tests, 16 to 18 hours after the initiation of the hatching. Transfer of the neonates was done under a dissecting microscope (12x) with the aid of a small plastic pipet (bore ∅ = 0.4 mm). A simple, moderately hard synthetic, freshwater medium consisting of 96 mg $NaHCO_3$, 60 mg $CaSO_4.2H_2O$, 60 mg $MgSO_4$, and 4 mg KCl in one liter of deionized water was used as hatching- and dilution medium (Horning and Weber, 1985). Details of the cysts storage of B. rubens, a rotifer reproductively and ecologically closely related to B. calyciflorus, are given in Snell and Persoone (1989b).

The microalga , Nannochloris oculata, which was used as rotifer food in the life table- as well as in the population growth experiments, was cultured in BBM medium under constant illumination (Nichols, 1973). The 2 liter algae (batch) cultures were harvested when the log phase was reached and then centrifuged, washed and resuspended in 100 ml of EPA water. This algae concentrate was stored in the refrigerator (4°C) and used as food for 3 days after which it was discarded.

V.2.2. Life table experiments

Survival and reproduction data were obtained by exposing isolated rotifer neonates to the toxicant and recording their life history characteristics at regular intervals throughout their life. All toxicity tests were conducted in sterile, 24-well polystyrene plates (multidish Nunclon Delta Si). Each toxicity test was started by introducing one neonate into each of the wells containing 1 ml of test solution and $1x10^5$ cells/ml of N. oculata. The rotifers were checked every 12 hours and the number of attached eggs, offspring and mortality recorded. The parent female was transferred into freshly prepared test solution (plus food) every 24 hours and the tests were terminated when the last individual had died. All life-table experiments were conducted at 25 ± 1°C, in darkness.
For each chemical, a control and 3 toxicant concentrations were tested, each consisting of 4 replicates of 12 rotifers. Survivorship- (l_x) and fertility- (m_x) tables were constructed for each cohort (replicate) using conventional life table techniques (Poole, 1974). The intrinsic rate of natural increase r_m was calculated by successive approximation of the following equation (Lotka, 1913) :

$$\sum_{x=0}^{\infty} l_x.m_x.e^{-r_m x}=1$$

The other demographic parameters were derived as follows :

Net productive rate :

$$R_o = \sum_{x=0}^{\infty} l_x . m_x$$

Generation time :

$$T = \frac{\sum_{x=0}^{\infty} l_x . m_x . x}{R_o}$$

Life expectancy :

$$e_x = \frac{\sum_{x}^{\infty} L_x}{n_x}$$

with L_x = the number of rotifers alive during the age interval x to x+1 and n_x = the number of rotifers (Krebs, 1985).

The mean survival (0.5 l_x), the time required to decrease the population by 50 %, was calculated using linear regression analysis.

V.2.3. Population growth experiments

The population growth experiments were started with a cohort of 0-2 hour old neonates, cultured in 24-well plates. Five neonates were placed into each well containing 1 ml of medium. For both chemicals a control and 4 toxicant concentrations were tested and for each treatment 12 replicates were used. Two definitive tests were performed with Cu and PCP (test A & B) ; and one with DCA and lindane. In a preliminary experiment with PCP, the rotifer density was counted every 24 hours until day 4. Based on these results, the population growth of the rotifers in all other tests was measured on day 3. The medium which contained 1×10^6 cells/ml of N. oculata, was not renewed throughout the exposure period.

The intrinsic rate of natural increase in the population growth experiment was calculated using the equation describing the population growth in an unlimited environment (Krebs, 1985) :

$$r_m = \frac{\ln N_t - \ln N_o}{t}$$

with N_o and N_t representing the population size at t=0 and t= 3, respectively.

V.2.4. Chemical compounds and statistical analysis

For copper ($CuSO_4.5H_2O$) and sodium pentachlorophenol (NaPCP), stock solutions of 100 mg/l were prepared in deionized water and actual test concentrations were made up in EPA water. Chemical concentrations in the stock solutions were measured using atomic absorption spectrophotometry (for Cu) and the spectrophotometric method of Haskins (1951) for NaPCP. The chemical concentrations in the actual test solutions were tested at regular intervals. Acetone was used as solvent for 3,4 dichloroaniline (DCA) and lindane. Actual test concentrations were prepared in EPA water. The lindane and DCA concentrations were checked using gas chromatography and high pressure liquid chromatography, respectively. The maximum acetone concentration in the test solutions was 0.1 ml/l. Control treatments containing this concentration of acetone were performed concurrently with the other treatments.

The concentrations chosen for the life table and population growth experiments were based on the results of 24 h LC_{50} tests conducted using the protocol described by Snell and Persoone (1989b). Statistically significant differences between the treatments were determined using an one-way ANOVA. Mean separation was accomplished with the Duncan's multiple range test, the significance level was set at $p<0.05$.

V. Results

The effects of PCP on the age-specific survival (l_x) and fertility (m_x) are presented in Figure V.1. The survivorship curves of the control and that of the rotifer cohort exposed to 0.1 mg/l were very similar. At 0.2 mg/l, toxicant inflicted mortality became apparent after 4 days. The effect of PCP was very dramatic at 0.4 mg/l, with the survival decreasing to 50 % and 30 % after 2 and 4 days, respectively. This cohort had completely died out after 6 days. The values for the mean survival, the time required to decrease the population to 50 %, are presented in Table V.1. The age-specific fertility (m_x) of the rotifers exposed to 0.2 and 0.4 mg/l PCP was lower than that of the control cohort. Maximal reproduction in the controls occurred at day 3 (1.50 eggs/female), while in the 0.1 mg/l treatment a first peak in m_x (1.25

eggs/female) already appeared at day 1 (Fig. V.1B). The consequences of this earlier reproduction on the intrinsic rate of natural increase are discussed below.

Although, the survival curves in the controls of the Cu test and those of the PCP test are very similar in shape, it can be noted that the onset of mortality occurred a day earlier

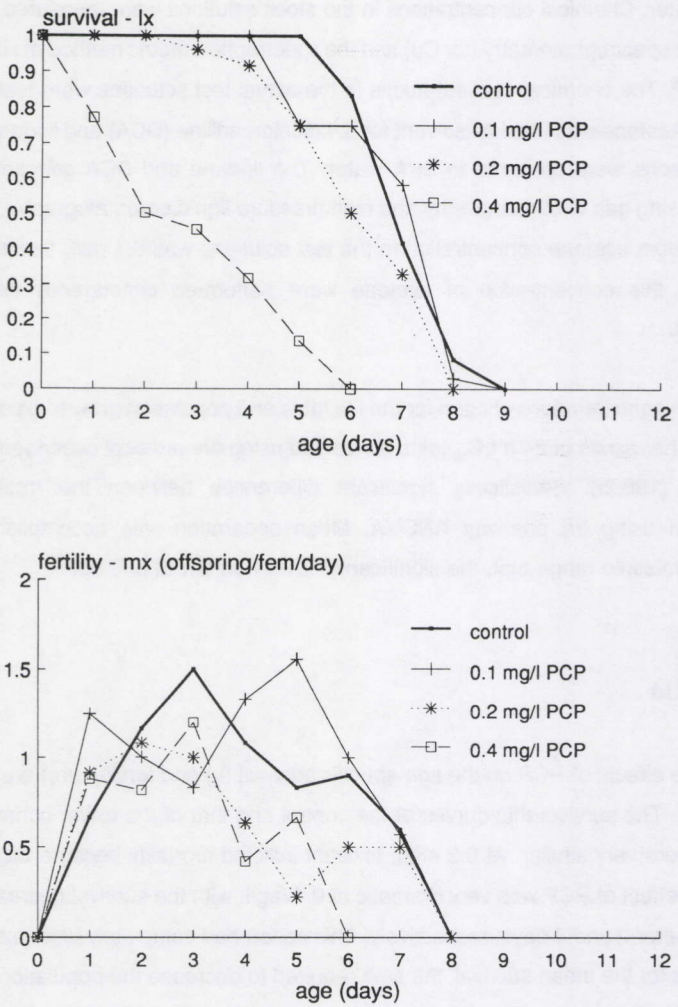


Figure V.1. The effect of pentachlorophenol on the age-specific survivorship (A) and fertility (B) of B. calyciflorus.

Table V.1. Life table experiments with B. calyciflorus. The effect of pentachlorophenol (PCP) and copper (Cu) on the intrinsic rate of natural increase (r_m), net-reproduction (R_o), generation time (T), life expectancy at hatching (e_o) and the mean survival (m.s.). Mean values (± S.D.) ; (*) = significantly different from the controls ($p<0.05$).

	PCP (mg/l)			
	control	0.1	0.2	0.4
Demographic parameter				
r_m	0.654 (0.069)	0.753 (0.052)	0.627 (0.105)	0.305 (0.073) *
R_o (offspr./fem.)	6.25 (0.91)	6.67 (0.47)	4.08 (0.62) *	1.86 (0.32) *
e_o (days)	7.23 (0.63)	7.42 (0.51)	6.41 (0.72)	3.15 (0.25) *
T (days)	2.80 (0.23)	2.52 (0.32)	2.24 (0.30)	2.03 (0.19)
m.s. (days)	6.40 (0.49)	7.33 (0.99)	6.00 (0.59)	2.00 (0.25) *

	Cu (µg/l)			
	control	1.2	2.5	5.0
Demographic parameter				
r_m	0.803 (0.075)	0.785 (0.110)	0.850 (0.064)	0.423 (0.084) *
R_o (offspr./fem.)	6.18 (0.81)	6.16 (0.68)	4.80 (0.41) *	2.52 (0.36) *
e_o (days)	6.80 (0.57)	6.42 (0.82)	4.99 (0.39) *	3.76 (0.51) *
T (days)	2.27 (0.29)	2.32 (0.34)	1.83 (0.16) *	2.18 (0.19)
m.s. (days)	6.48 (0.89)	5.83 (0.60)	4.45 (0.32) *	3.67 (0.39) *

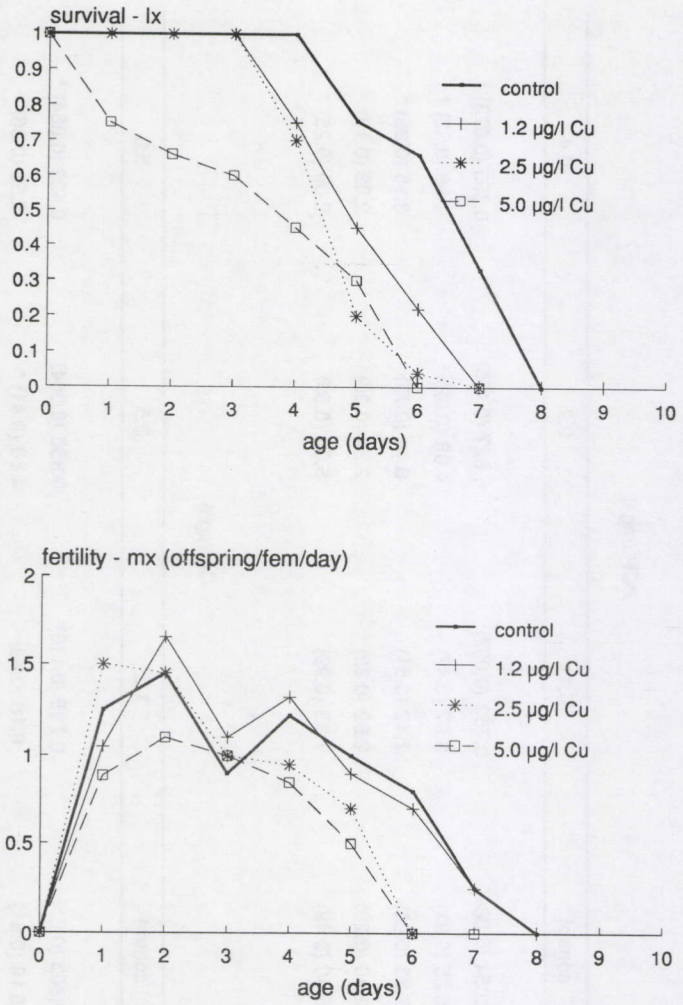


Figure V.2. The effect of copper on the age-specific survivorship (A) and fertility of B. calyciflorus.

in the Cu test (Fig. V.2A). The age-specific survival was clearly adversely affected by Cu at all concentrations tested. At 1.2 and 2.5 µg/l, toxicant-inflicted mortality was observed from day 4 onwards and the survival of the animals exposed to 5 µg/l Cu exhibited a linear decline with time. The age-specific fertility of the rotifers in the 1.2 and 2.5 µg/l Cu

treatments was higher than that of the control animals on day 2 and 3 (Fig. V.2B). Additionally, a similar response as that observed at the lowest PCP concentration was noted in the 2.5 µg/l Cu treatment : peak reproduction occurred on day 1, i.e. the onset of reproduction in this treatment was earlier than in the controls.

The effects of Cu and PCP on the net-reproduction R_o, intrinsic rate of natural increase r_m, life expectancy at hatching e_o, and the generation time T are presented in Table V.1. All demographic parameters of the rotifers treated with 0.4 mg/l PCP were, with the exception of T, significantly different from those of the controls. Exposure to 0.2 mg/l significantly decreased the value of R_o. Exposure to 2.5 µg/l Cu did not significantly affect r_m, while all other demographic parameters were, compared to the controls, significantly lower. All parameters were significantly reduced at 5 µg/l Cu, except T.

Similarly, the effects of DCA and lindane on the demographic parameters are presented in Table V.2. As the toxicity tests were conducted concurrently, the same control and control+acetone treatments were used for both tests. Acetone did not adversely affect r_m, R_o, e_o, T, and the mean survival, although the latter was reduced by 18 % ($p>0.05$). The values of all demographic parameters of the rotifers exposed to 20 mg/l lindane (LOEC) were significantly lower than those of the controls. No adverse effects were noted at $\leq$ 10 mg/l lindane.
DCA clearly reduced the survival and reproduction of the rotifers at $\geq$ 2.5 mg/l, which is reflected in the low R_o, e_o and mean survival. The intrinsic rate of natural increase at 2.5 mg/l is, however, not affected. Noteworthy, is that this parameter attained a negative value at the highest DCA concentration tested.

The relative progress of r_m in function of time in the life-table experiment with Cu is shown in Figure V.3. These "incomplete r_m's" were calculated using the Lotka equation (see materials and methods) on the l_x and m_x data obtained after 1, 2, ..., x days. In the controls 50.9, 86.8, 96.9 and 99.2 % of the value of r_m was attained after 1, 2, 3 and 4 days, respectively. In the Cu treatments the progress of r_m in function of time was somewhat slower, however, 98.3, 99.2 and 96.3 % of the r_m value for the 1.2, 2.5 and 5 µg/l exposures were reached after 4 days. The implications of these findings will be discussed below.

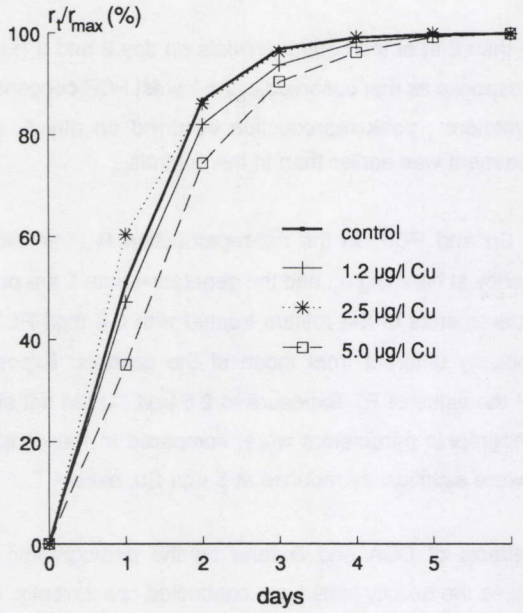


Figure V.3. Temporal changes in the intrinsic rate of natural increase (r_m) of B. calyciflorus exposed to copper. Data are expressed as percentages of r_m calculated for the entire life table.

As a possible alternative to the labour-intensive life table experiments, a series of simple, static, toxicity tests were performed to determine the effects of Cu, PCP, DCA and lindane on the population growth of small cohorts of rotifers. The effect of PCP on the population growth in function of time is presented in Figure V.4. The control populations increased from 5 rotifers (day 0) to 8.2, 22.8, and 30.2 after 1, 2 and 3 days exposure, respectively. On day 4, however the rotifer density decreased drastically and this can be attributed to food depletion. Based on these results a 3-day exposure was chosen for all further population growth tests. The results of the 3-day chronic tests are summarized in Table V.3. Compared to the controls, r_m was significantly reduced at 0.8 mg/ PCP in both tests (A&B). Exposed to copper, the r_m was reduced ($p>0.05$) by 10 % at 2.5 µg/l (NOEC) and 28 % at 5 µg/l (LOEC). The lowest lindane and DCA concentrations which adversely affected ($p<0.05$) the population growth were : 10 and 20 mg/l, respectively.

Table V.2. Life table experiments with B. calyciflorus. The effect of lindane and 3,4-dichloroaniline (DCA) on the intrinsic rate of natural increase (r_m), net-reproduction (R_o), generation time (T), life expectancy at hatching (e_o) and the mean survival (m.s.). Mean values (± S.D.) ; (*) = significantly different from the controls ($p<0.05$).

	lindane (mg/l)				
	control	C + acetone	5.0	10	20
Demographic parameter					
r_m	0.782 (0.051)	0.752 (0.081)	0.841 (0.046)	0.805 (0.072)	0.217 (0.069) *
R_o (offspr./fem.)	6.74 (0.45)	7.09 (0.59)	6.23 (0.37)	6.67 (0.58)	1.92 (0.29) *
e_o (days)	5.54 (0.32)	4.82 (0.45)	4.91 (0.55)	5.08 (0.69)	2.23 (0.31) *
T (days)	2.24 (0.31)	2.69 (0.42)	2.50 (0.32)	2.60 (0.35)	1.53 (0.24) *
m.s. (days)	5.30 (0.36)	4.32 (0.51)	4.72 (0.75)	4.52 (0.49)	1.95 (0.35) *

	DCA (mg/l)				
	control	C + acetone	2.5	5.0	10
Demographic parameter					
r_m	0.782 (0.051)	0.752 (0.081)	0.675 (0.095)	0.192 (0.066) *	-0.090 (0.087) *
R_o (offspr./fem.)	6.74 (0.45)	7.09 (0.59)	4.34 (0.58) *	1.37 (0.52) *	1.20 (0.29) *
e_o (days)	5.54 (0.32)	4.82 (0.45)	3.51 (0.32) *	2.71 (0.41) *	2.65 (0.55) *
T (days)	2.24 (0.31)	2.69 (0.42)	2.19 (0.45)	1.89 (0.32)	2.01 (0.22)
m.s. (days)	5.30 (0.36)	4.32 (0.51)	3.40 (0.41) *	2.60 (0.39) *	2.25 (0.45) *

Table V.3. Population growth (3-day) tests with B. calyciflorus. The effect of pentachlorophenol (PCP), copper (Cu), lindane and 3,4-dichloroaniline (DCA) on the intrinsic rate of natural increase (r_m). Mean values (± S.D.); (*) = significantly different from the control ($p<0.05$). Two tests were performed for PCP and Cu and one test for DCA and lindane.

	PCP (mg/l)				
	control	0.1	0.2	0.4	0.8
r_m (test A)	0.599 (0.066)	0.598 (0.095)	0.562 (0.025)	0.577 (0.054)	0.368 (0.085) *
r_m (test B)	0.658 (0.078)	0.629 (0.105)	0.597 (0.045)	0.575 (0.095)	0.321 (0.096) *

	Cu (µg/l)				
	control	1.2	2.5	5.0	10
r_m (test A)	0.558 (0.044)	0.522 (0.035)	0.489 (0.020)	0.409 (0.038) *	0.231 (0.037) *
r_m (test B)	0.592 (0.056)	0.581 (0.024)	0.547 (0.069)	0.420 (0.087) *	0.315 (0.055) *

	lindane (mg/l)					
	control	C + acetone	2.5	5.0	10	20
r_m	0.520 (0.051)	0.501 (0.084)	0.443 (0.037)	0.443 (0.065)	0.351 (0.044) *	0.182 (0.092) *

	DCA (mg/l)					
	control	C + acetone	2.5	5.0	10	20
r_m	0.520 (0.051)	0.501 (0.084)	0.475 (0.055)	0.490 (0.035)	0.515 (0.075)	0.050 (0.015) *

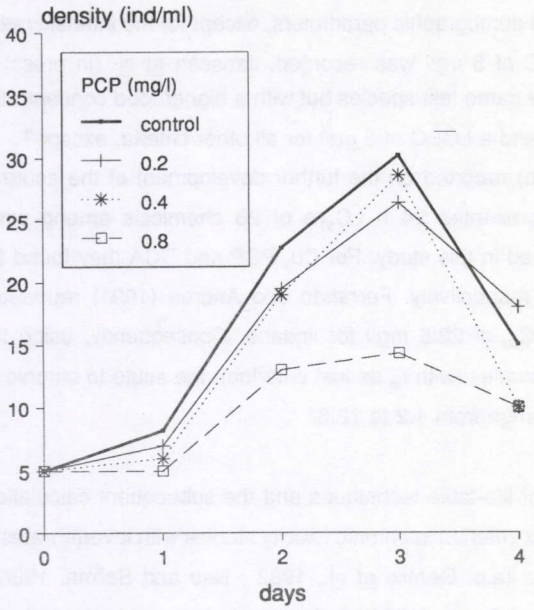


Figure V.4. The influence of PCP on the rotifer density in the short term population growth experiments.

V. Discussion

A brief overview of the effects of sublethal concentrations of chemicals on the life-history characteristics of several species of rotifers is given in Janssen et al. (in press). In their life table experiments with the freshwater rotifer Brachionus rubens exposed to PCP (0.05 - 0.3 mg/l), Halbach et al. (1983) found survival curves which are very similar to those observed in the present study with B. calyciflorus. However, the reproduction of B. rubens had almost completely stopped at 0.3 mg/l PCP (Halbach et al., 1983), while the m_x values for B. calyciflorus in this study were still quite high at the highest PCP concentration tested (0.4 mg/l). In the present study the LOEC for the net-reproductive rate (0.2 mg/l) was lower than that of the other demographic parameters (0.4 mg/l). The values for the generation time in any of the PCP treatments were not significantly different from that of the controls. These

values are similar to those found by Halbach et al. (1983) for B. rubens. For Cu the LOEC was 2.5 µg/l for all demographic parameters, except for the intrinsic rate of natural increase for which a LOEC of 5 µg/l was recorded. Janssen et al. (in press) in similar life table experiments at the same test species but with a higher food concentration, found the same LOEC for the e_o, and a LOEC of 5 µg/l for all other criteria, except T.
Snell et al. (1991b) reported on the further development of the acute toxicity test with B. calyciflorus and presented 24 h LC_{50}'s of 28 chemicals among which are 3 of the 4 chemicals examined in this study. For Cu, PCP and DCA they found 24h LC_{50}'s of 0.026, 1.2 and 62 mg/l, respectively. Ferrando and Andreu (1991) reported for the same test species a 24 h LC_{50} of 22.5 mg/l for lindane. Consequently, using the results from the chronic life table studies (with r_m as test criterion), the acute to chronic ratios for these four test compounds range from 1.2 to 12.6.

The use of life-table techniques and the subsequent calculation of the population statistic r_m as effect criterion in chronic toxicity studies with invertebrates has been proposed by several authors (a.o. Gentile et al., 1982 ; Rao and Sarma, 1986 ; Day and Kausik, 1987a ; Boyum and Brooks, 1988). Indeed r_m, is from an ecological point of view, a more realistic criterion to study than the currently used separate measures of survival and reproduction, as it integrates age-specific survival and several aspects of the reproductive process : (first) time of reproduction, reproductive frequency, brood or clutch size and reproductive longevity. However, the present study has shown that r_m is not always the most sensitive parameter. Because of the observed shift towards earlier reproduction at the lower concentrations of both Cu and PCP, the adverse effects of the toxicants on the total survival and/or reproduction were masked in the integrative parameter r_m. Indeed, several authors have stressed the importance of the time of first reproduction as one of the main factors determining r_m (a.o. Daniels and Allan, 1981 ; van Leeuwen et al., 1985a&b). Although time and rate of reproduction are negatively affected by most toxicants, stimulatory effects of very low concentrations of heavy metals have been reported before (Gentile et al., 1982).

The calculation of the progress of r_m in function of time showed that at least 95 % of the r_m calculated for the entire life table was reached after only 4 days for all Cu treatments. Similar results were obtained for the 3 other compounds (data not shown). Consequently, the correlation between the 4-day r_m and the values based on the entire life table test was very good (r^2=0.98, linear regression analysis). Similar good correlations between r_m values

after 21 days and from entire life tables was found for Daphnia pulex (Daniels and Allan, 1981) and for D. magna (van Leeuwen et al., 1985b). These findings have practical consequences for the use of life table techniques in routine aquatic toxicity testing. Indeed, the exposure period for chronic toxicity tests with B. calyciflorus can be reduced to only 3 to 4 days without loss of information on the value of the test parameter r_m. A semi-static short chronic test with rotifers which can be performed within one work week could be an interesting alternative to the existing bioassays with crustaceans.

A drawback for the routine use of the experimental design used in the present study is the fact that the rotifers have to be checked every 12 hours. As all experiments were performed at 25°C the rotifers exhibited a very rapid developmental rate which meant that 12 hourly observations were necessary to avoid difficulties in distinguishing between the parent females and its offspring. However, the results of a subsequent study, in which the changes of the demographic parameters of B. calyciflorus in life table experiments conducted at different temperatures and food concentrations were studied, showed that in life table test performed at 20°C the rotifers should only be checked every 24 hours. The among-replicate variation for this test parameter was rather low with a mean coefficient of variation of 13.6 % (min.-max.: 6.2-23.9). The values of the test parameters in the controls in the toxicity tests with both compounds were not significantly different, indicating that the results of life table tests performed under these conditions are quite reproducible.
In summary, a short chronic toxicity test with B. calyciflorus can be proposed with the following test conditions : 4 day exposure, daily renewal of the test solutions, performed at 20°C and with the r_m as test criterion.

The LOEC's obtained with the 3-day population growth tests were similar to those obtained in the life table tests. This static population growth test, which only requires the initial set-up of the test and the final counting of the total number of living rotifers after 3 days, could be a simple alternative to the more labour-intensive life table tests. Besides their simplicity and short duration, population growth tests with B.calyciflorus have another advantage over existing short chronic tests : test organisms of at least 2 successive generations are exposed to the toxicant (Snell and Moffat, in press). In this context, van Leeuwen et al. (1985b, 1987), while comparing life table bioassay techniques to toxicity tests with whole populations of daphnids, suggested that chronic bioassays started with neonates from previously unexposed parents may neglect an important part of the test organisms' life

cycle (oogenesis and early embryogenesis). They concluded that tests with F_1 generations from pre-exposed animals would probably give a more realistic figure for chronic toxicity. The among-replicate variation in the controls was very low with a mean coefficient of variation of 10 % (min.-max.: 7.9-12.8). The LOEC's obtained in the repeated tests (test A&B) for both Cu and PCP were identical, indicating that the 3-day population growth test was quite reproducible.

In summary, the 3-day population growth test may be considered as a valid and practical alternative to life table tests, producing reproducible results and detecting adverse effects of chemical stress at approximately the same concentrations as the life table tests. Moreover, a semi-static test design with the daily transfer of the rotifers to freshly prepared toxicant concentrations could possibly further improve the static design used in the present study, thus avoiding possible problems with biodegradable substances.

A comparison of the results obtained in the short-chronic tests with those of the 21-day Daphnia survival and reproduction test indicate that B. calyciflorus is as sensitive as D. magna for Cu and PCP. Indeed, Adema and Vink (1981) reported a 21-day D.magna NOEC of 0.34 mg/l for PCP, while Enserink et al. (1991) noted a LOEC of 0.11 mg/l for Cu. However, B. calyciflorus seems to be rather insensitive to the 2 other compounds, with 21-day D. magna NOEC's of 10 and 6.5 µg/l for DCA and lindane, respectively (Adema and Vink, 1981 ; Crossland and Hillaby, 1985). In assessing the comparative sensitivity of the acute toxicity test with B. calyciflorus however, Snell et al. (1991b) concluded that this rotifer species is at least as sensitive as Daphnia and fathead minnows to many compounds of current concern in aquatic environments. Recently, Snell and Moffat (1992) proposed a chronic toxicity test with B. calyciflorus which is very similar to the 3-day population growth test reported upon in this paper. The two major differences in the two test designs are : the time of exposure (2 days), the type and density of the food ($3x10^6$ cells/ml of Nannochloris from agar plates) and the type of test vessel (test tubes on a rotator). They reported NOEC values of 20 µg/l and 0.11 mg/l for Cu and PCP, respectively. This is approximately 4 x higher and 7 x lower than the NOEC's observed in the 3-day population growth experiments (present study). These authors also compared the sensitivity of the B. calyciflorus 2-day chronic test to that of the 7-day Ceriodaphnia test and found that for 4 (Cu, phenol, Cu and 2,4 dimethyl phenol) of the 5 compounds tested the NOEC's were within factor 6 of one another. For Cd, however, Ceriodaphnia was 80 times more sensitive than Brachionus. In

conclusion, Snell and Moffat (in press) state that the main advantages of the 2-day chronic test are : the small test volume required (important to many types of toxicity testing where sample volumes are limited), the excellent reproducibility of the test (C.V.'s <10 %) and perhaps most importantly, the ease and rapidity by which the test can be performed (70 % less labour effort than the 7-day Ceriodaphnia or fathead minnow test). Additionally, in this bioassay two consecutive generations of test organisms are exposed to the toxic compound.

Short-chronic tests with B. calyciflorus could be convenient tools for chronic toxicity assessments in freshwater environments. The two tests developed in this study have two major advantages over the presently used chronic test methods : the relatively short exposure time and the use of cysts to obtain test organisms. Both methods can be completed within one work week, thus greatly enhancing their cost-effectiveness. Obtaining test organisms by hatching cysts not only ensures physiological uniformity of the animals which makes the tests potentially highly reproducible but also eliminates stock culturing and maintenance of the organisms, thus further reducing the total cost of the bioassays (Persoone, 1991).

V. Summary

The development and potential use of a 4-day static renewal test (4-day Life Table test) and a 3-day static test (3-day Population Growth test) with the freshwater rotifer Brachionus calyciflorus are described. For both bioassays, test animals are obtained by hatching cysts which eliminates the need for the culturing and maintenance of the organisms.

The toxicity of copper (Cu), pentachlorophenol (PCP), 3,4-dichloroaniline (DCA) and lindane was assessed using the developed methods. The NOEC's, based on the test endpoint r_m, obtained with the 4-day L.T. test were 0.0025, 0.4, 5 and 20 mg/l for Cu, PCP, DCA and lindane, respectively. Similar results were obtained with the 3-day P.G. tests for which NOEC's of 0.005 mg/l, 0.8, 20 and 10 mg/l, respectively, were recorded. The mean C.V. between replicated 3-day P.G. tests was 10 %, indicating a good intra-laboratory reproducibility of the test results. For Cu and PCP, the sensitivity of B. calyciflorus compared favourably to chronic toxicity tests with Daphnia magna while for the other 2 compounds B. calyciflorus, proved to be rather insensitive. Considering the increasing need for relatively

short toxicity tests, the 2 described short-chronic bioassays could be valuable new tools for routine toxicity evaluations. The major advantages associated with these tests are: they are less labour-intensive than existing chronic tests, they can be completed within one work week and do not require stock culturing of test organisms.

CHAPTER VI.

The effects of xenobiotics on the life history characteristics of Brachionus calyciflorus : temperature and food effects on the demographic parameters.

VI.1. Introduction

In recent years, there has been a growing awareness among ecotoxicologists that the currently used standard methods for assessing the potential toxicity of chemicals do not always permit direct extrapolation of the toxicological data to natural populations, communities and ecosystems (Buikema et al., 1980 ; Cairns, 1984, 1986 ; Moore and Winner, 1989 ; Winner et al., 1990). The common criticism is that there is a lack of ecological realism in the test methods used. Multispecies toxicity testing - the holistic approach - which has been proposed as a possible alternative to the currently used single species methods, has been shown to have limited applicability owing to their inherent complexity, scatter of data and prohibitive costs. On the other hand, a reductionist approach to aquatic toxicity testing, i.e. trying to understand to what extent processes within individuals and interactions between them contribute to population changes (Metz and Diekman, 1986) and ultimately to ecosystem changes, could be one way to improve the understanding and ecological relevance of single species toxicity test results. This trend is clearly visible in scientific literature in which more and more papers examine the consequences of the physiological changes in stressed animals on the population dynamics of these organisms (a.o. Kooijman and Metz, 1984 ; Kooijman et al., 1989 ; Barber et al., 1990 ; Brouwer et al., 1990). Moreover, this type of research not only contributes to the fundamental knowledge of the science of ecotoxicology, but it also has spin-offs for practical applications. Indeed, such studies may contribute to the development of bioassays that can be carried out quickly, easily and hence inexpensively, yet have the potential to predict long-term effects at an ecological level (Maltby and Calow, 1990).

In an attempt to bridge the gap between individual-based and ecosystem-based approaches to environmental toxicology, the use of the population dynamics of stressed

populations as ecotoxicological assessment tools has increased in recent years (Halbach et al., 1983 ; Vinegar, 1983 ; Halbach, 1984 ; van Leeuwen et al., 1986, 1987 ; Barnthouse et al., 1989). Additionally, the ecological realism of aquatic toxicity tests has been criticized by a number of authors who have remarked that in currently used laboratory tests systems the food level which is offered to the test organisms is unrealistically high and does not reflect natural food levels (Buikema et al., 1980 ; van Leeuwen et al., 1985a ; Rao and Sarma, 1986 ; Lanno et al., 1989). Based on both model predictions and experimental data, Kooijman and Metz (1984) have shown that the impact on the population growth rate of effects which chemicals exert on filtration rate, digestion, basal metabolism and survival, strongly depends on food availability. Consequently, in natural populations, in which food shortages are very common, it is likely that the chemical stress to which populations and eventually an ecosystem is subject, will be much higher than in laboratory systems.

That food availability can change the results of toxicity tests can be easily understood based on theoretical considerations on energy budgets (Kooijman and Metz, 1984). However, the extent to which food availability can change the toxic thresholds of chronic toxicity tests (and thus the decisions based on those results) has not been adequately addressed nor tested by critical experimentation.

In this study the effects of copper and pentachlorophenol on the population dynamics of the rotifer Brachionus calyciflorus were examined. The main objectives of this investigation were (1) to examine the effects of temperature and food availability on the demographic parameters of this rotifer, with the aim of developing a practical chronic toxicity test and, (2) to examine the effects of high and low food concentrations on the results of chronic toxicity tests in order to evaluate the possible consequences of these findings for practical ecotoxicological applications.

VI.2. Materials and methods

VI.2.2. General experimental procedures

Details of the origin of the rotifer cysts and the cyst hatching procedure are given in chapter 5.

VI.2.2. Life table experiments

The experimental procedures for conducting life table experiments are described in chapter 5. Details of the culturing conditions of the micro alga Nannochloris oculata, are given in chapters 2 and 4.

Each life table experiment consisted of 4 replicates of 12 rotifers. At every age x, the survivorship (l_x) and fertility (m_x) tables were constructed using standard methods (Poole, 1974 ; Southwood, 1976) and the following parameters were calculated : intrinsic rate of natural increase (r_m), net reproductive rate (R_o), generation time (T) and the life expectancy at hatching (e_o). Details of the calculation methods are given in chapter 5.

From the life table data, the stable age distribution of the rotifer populations under toxic stress were also calculated using the following formula :

$$c_x = \frac{l_x . e^{-r_m x}}{\sum l_x . e^{-r_m x}}$$

Derivations of these equations can be found in Roughgarden (1979) and Pielou (1977).

In the first part of this study the combined effects of food density and temperature on the life history characteristics of the rotifers were examined. A factorial test design with 3 food concentrations: $5x10^4$, 10^5, and 10^6 cells/ml of N. oculata and 3 temperatures: 15, 20 and 25°C, was used. Preliminary experiments had shown that $5x10^4$ cells/ml was the lowest food level which supported rotifer reproduction. The other two food densities were chosen to represent moderately low and moderately high food levels.

In the second part, the effect of food availability on the demographic parameters of the rotifers under copper (Cu) and pentachlorophenol (PCP) stress was studied. For Cu, 3 x 4 life table experiments were conducted, representing 3 food concentrations and 4 toxicant treatments (i.e. control + 3 Cu concentrations). However, at the highest food level 4 Cu concentrations were tested. For PCP, 3 x 5 life table experiments were performed: 3 food levels x 5 treatments (i.e. control + 4 PCP concentrations). All toxicity tests performed in the second part of this study were conducted at 25° $\pm$ 1°C.

VI.2.3. Test compounds and statistical analysis

Details of the preparation of the toxicant dilutions, the chemical analysis techniques and the statistical methods are given in chapter 5.

V.3. Results

Survivorship (l_x) patterns as a function of temperature and food concentration are shown in Figure VI.1. At all food levels tested, the survivorship curves became steeper with increasing temperatures. This is reflected in the fact that the demographic parameter e_o (life expectancy at birth) decreased with increasing temperatures (Fig. VI.2A). The e_o values for the rotifers fed $5x10^4$ cells/ml N. oculata for example, were 9.39 and 8.58 days at 15 and 20°C respectively, and decreased significantly to 4.95 days at 25°C (Fig. VI.2A). Similar trends, although not statistically significant, were observed at 10^5 and 10^6 cells/ml. At 15 and 20°C, increasing food availability did not significantly affect e_o. For example, at 20°C the e_o values were 8.58, 8.83 and 8.39 days for $5x10^4$, 10^5, and 10^6 cells/ml, respectively. However, at 25°C the e_o value at a food density of $5x10^4$ cells/ml (4.95 days) was significantly lower than that of the two other algal levels tested : 7.29 and 6.38 days for 10^5 and 10^6 cells/ml, respectively.

The fertility (m_x) curves of rotifers cultured in the various temperature-food combinations are presented in Figure VI.1. At the same temperature, the general shape of the m_x curves and the magnitude of the m_x values were very similar for the two lowest food concentrations tested. Irrespective of the temperature, rotifers at these food levels typically produced between 0.5 and 1.5 eggs/day throughout their life. At the highest food level however, the m_x values were much higher with maximal number of eggs/female of 2.58, 4.58, and 2.78 for rotifers cultured at 15, 20 and 25°C, respectively.
The onset of reproduction was clearly temperature dependent and not affected by the food ration. For all food concentrations reproduction started after 2, 2 and 1 day(s) at 15, 20 and 25°C, respectively. Independent of the temperature the net-reproductive rate reached a maximum at the highest food level (Fig. VI.2B). At the lowest food concentration for example the R_o values were 3.83, 5.25 and 3.43 eggs/female /day, while those at the highest food level were 12.16, 12.53 and 9.91 eggs/female/day, both for rotifers cultured at 15, 20 and

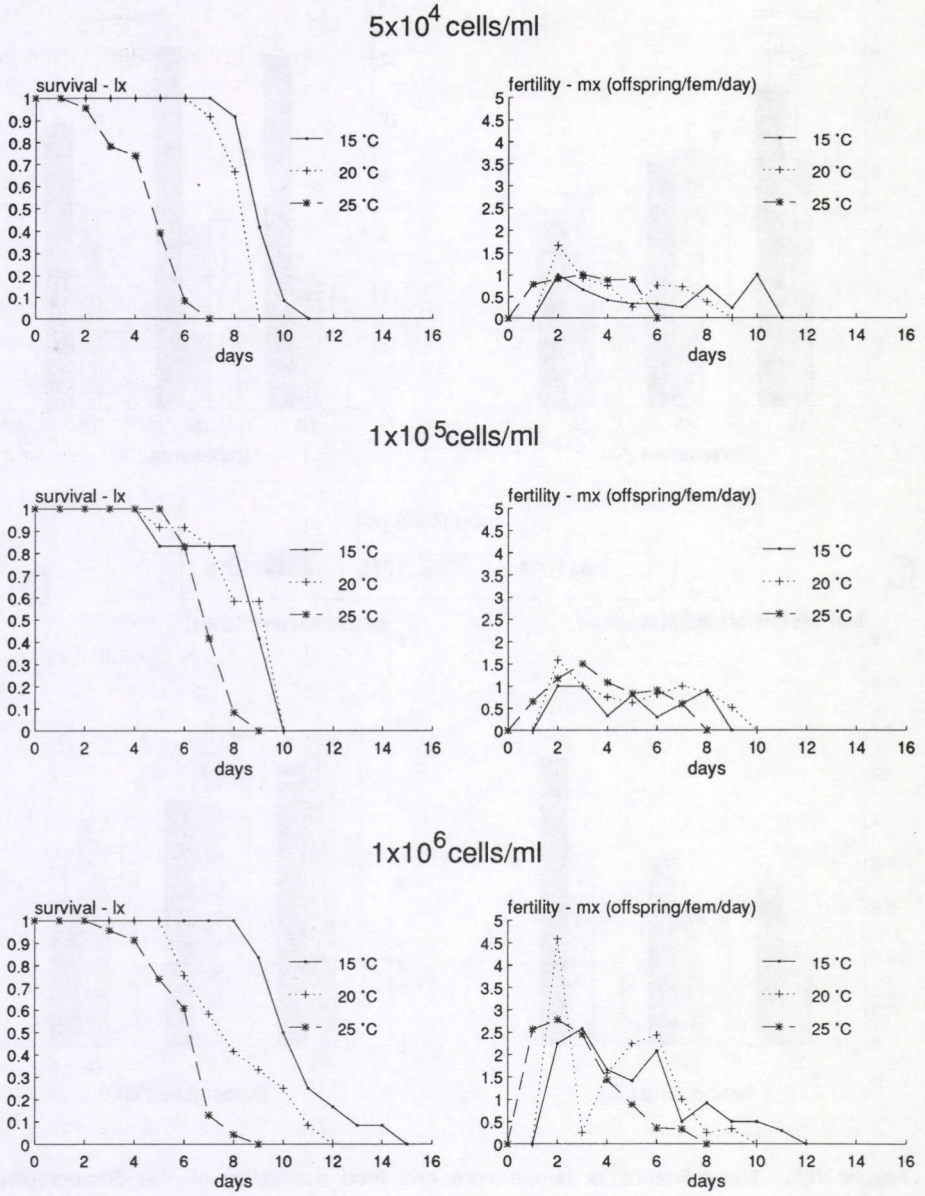


Figure VI.1. The influence of temperature and food-availability on the age specific survival (l_x) and fertility (m_x) of B. calyciflorus.

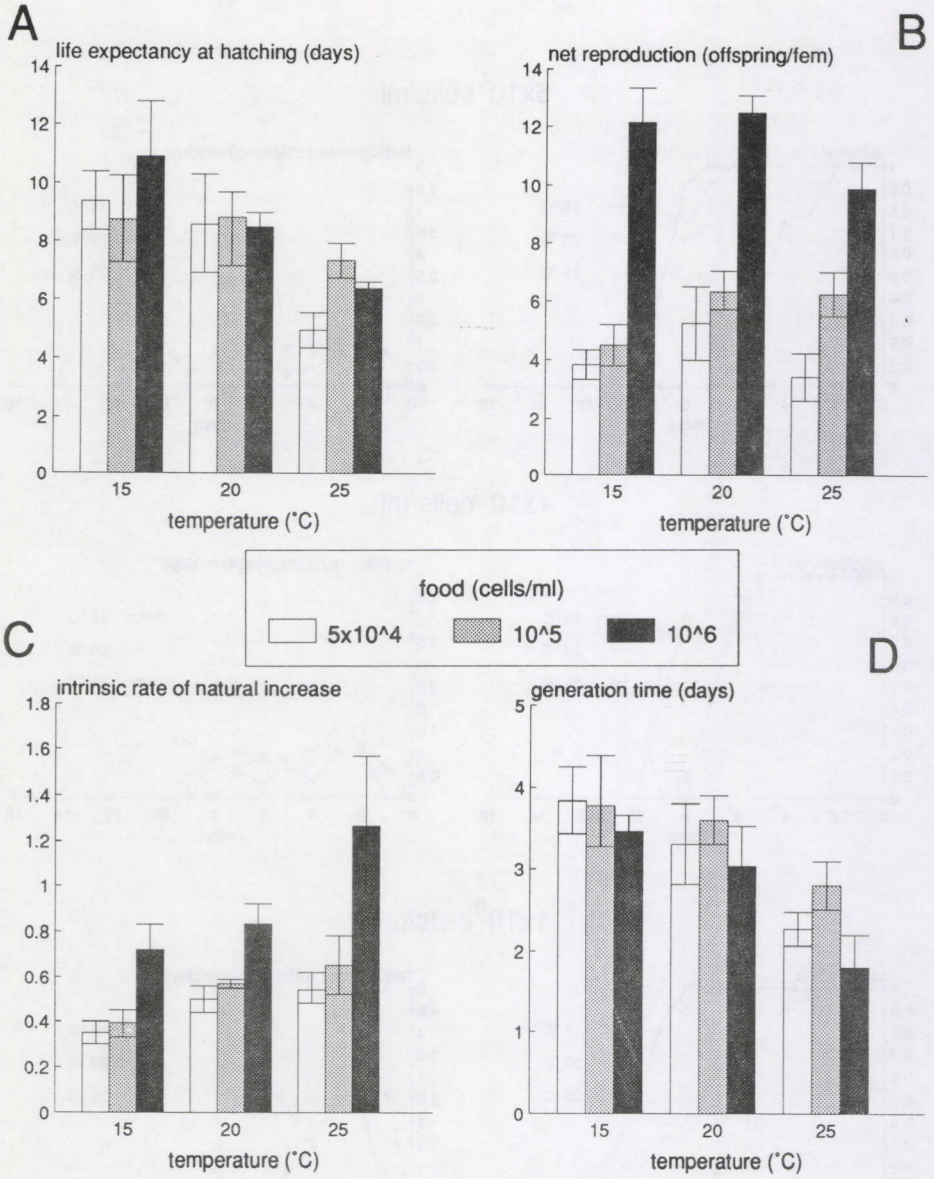


Figure VI.2. The influence of temperature and food availability on the demographic parameters of B. calyciflorus. A : life expectancy at hatching (e_o) ; B net reproduction (R_o) ; C : intrinsic rate of natural increase (r_m) ; D : generation time (T).

25°C, respectively.

Survival and reproduction data may be integrated into the demographic parameter r_m : the intrinsic rate of natural increase. In Figure VI.2C the r_m is plotted as a function of the food concentration and temperature. The r_m value at 15°C and 10^6 cells/ml of food was 0.719 and increased by 78 % at 25°C ($p<0.05$). Similar significant increases in the r_m were observed at the two lower food levels. The r_m values were also positively correlated with the food level. At 25°C for example, the intrinsic rate increased from 0.542 to 0.654 and 1.271 ($p<0.05$) with increasing concentrations of N. oculata. It can thus be stated that for the test conditions used in this study, the intrinsic rate of natural increase of this rotifer species was maximized when both food and temperature levels were high. The generation time (T) decreased with increasing temperatures (Fig. VI.2D). At all food levels the T values at 25°C were significantly lower those than of the 15° C treatments. No significant effect of the food ration on the value of T was observed. The shortest T recorded in the present investigation was 1.81 days for rotifers cultured at 25°C and with 10^6 cells/ml of N. oculata.

The results of the toxicity experiments performed at different food levels are summarized in Tables VI.1 (Cu) and VI.2 (PCP). In the life tables with the lowest food density, 2.5 µg/l Cu caused a significant decrease in the R_o and the r_m. Toxicity experiments conducted with a moderately low food level (10^5 cells/ml) gave similar LOEC's for R_o, e_o and T, while r_m was only adversely affected at 5 µg/l Cu. At moderately high food concentrations (10^6 cells/ml) the LOEC's for Ro and r_m were 10 µg/l. It is noteworthy, that significant reductions of the R_o and the e_o of the rotifers exposed to Cu were not always reflected in a significant decrease of the integrative parameter r_m. This phenomenon was observed in the life table 10^5 cells/ml - 5.0 µg/l Cu.
A similar response, i.e. an increase in the value of the LOEC's with increasing food concentration, was observed for PCP. Indeed, using r_m as test criterion, the LOEC increased from 0.1 mg/l at the lowest food level to 0.4 mg/l PCP for the experiments performed with the 2 highest food rations.

Finally, the consequences of toxic stress on the stable age distribution of the rotifer population were analyzed. In Figure VI.3 the relative importance of each age-class in function of the food level and toxicant concentration are presented. Comparing the controls it is clear that the proportion of young individuals in the population increases with increasing

Table VI.1. The effect of Cu on the demographic parameters of B. calyciflorus cultured at 3 food levels. Demographic parameters (mean ± S.D.) : r_m, intrinsic rate of natural increase ; R_o, net reproduction ; T, generation time ; e_o, life expectancy at hatching. (*) : significantly different from the control ($p<0.05$). At the two lowest food levels the 10 µg/l Cu life table test was not performed.

Food concentration (cells/ml)	Parameter	Cu (µg/l)				
		Control	1.2	2.5	5	10
$5x10^4$	r_m	0.649 (0.075)	0.630 (0.052)	0.244 (0.061) *	0.458 (0.041) *	-
	R_o (offspr./fem.)	4.43 (0.51)	5.16 (0.61)	1.83 (0.29) *	2.91 (0.0.32) *	-
	T (days)	2.29 (0.19)	2.59 (0.25)	2.46 (0.34)	2.33 (0.28)	-
	e_o (days)	4.16 (0.082)	6.41 (0.105)	3.50 (0.41)	4.33 (0.61)	-
$1x10^5$	r_m	0.803 (0.075)	0.785 (0.110)	0.850 (0.064)	0.423 (0.084) *	-
	R_o (offspr./fem.)	6.18 (0.81)	6.16 (0.68)	4.80 (0.41) *	2.52 (0.36) *	-
	T (days)	2.27 (0.29)	2.32 (0.34)	1.83 (0.16) *	2.18 (0.19)	-
	e_o (days)	6.80 (0.57)	6.42 (0.82)	4.99 (0.39) *	3.67 (0.51) *	-
$1x10^6$	r_m	1.470 (0.120)	1.324 (0.134)	1.450 (0.192)	1.361 (0.123)	1.174 (0.119) *
	R_o (offspr./fem.)	13.66 (2.12)	12.81 (1.45)	13.45 (2.50)	14.54 (2.03)	9.14 (1.11) *
	T (days)	1.77 (0.13)	1.93 (0.22)	1.79 (0.25)	1.97 (0.18)	1.88 (0.21)
	e_o (days)	6.23 (0.79)	6.72 (0.56)	6.64 (0.88)	7.27 (0.71)	5.65 (0.45)

Table VI.2. The effect of PCP on the demographic parameters of B. calyciflorus cultured at 3 food levels. Demographic parameters (mean ± S.D.) : r_m, intrinsic rate of natural increase ; R_o, net reproduction ; T, generation time ; e_o, life expectancy at hatching. (*) : significantly different from the control ($p<0.05$).

Food concentration (cells/ml)	Parameter	PCP (mg/l)				
		Control	0.05	0.1	0.2	0.4
$5x10^4$	r_m	0.481 (0.032)	0.391 (0.021)	0.335 (0.045) *	0.352 (0.054) *	0.205 (0.032) *
	R_o (offspr./fem)	4.50 (0.51)	3.30 (0.37) *	3.16 (0.51) *	3.33 (0.46) *	0.58 (0.19) *
	T (days)	3.12 (0.29)	3.07 (0.19)	3.43 (0.41)	3.42 (0.39)	2.63 (0.31) *
	e_o (days)	6.00 (0.65)	5.41 (0.37)	6.25 (0.71)	6.08 (0.41)	2.50 (0.32) *
$1x10^5$	r_m	0.654 (0.069)	0.616 (0.072)	0.753 (0.052)	0.627 (0.105)	0.305 (0.073) *
	R_o (offspr./fem.)	6.25 (0.91)	5.83 (0.64)	6.67 (0.47)	4.08 (0.62) *	1.86 (0.32) *
	T (days)	2.80 (0.23)	2.86 (0.30)	2.52 (0.32)	2.24 (0.30)	2.03 (0.19) *
	e_o (days)	7.23 (0.63)	6.25 (0.49)	7.42 (0.51)	6.41 (0.72)	3.15 (0.25) *
$1x10^6$	r_m	1.542 (0.152)	1.591 (0.192)	1.470 (0.121)	1.514 (0.203)	1.208 (0.109) *
	R_o (offspr./fem.)	17.99 (2.45)	17.33 (3.14)	15.99 (2.61)	16.17 (2.15)	11.74 (1.10) *
	T (days)	1.87 (0.15)	1.79 (0.20)	1.88 (0.21)	1.84 (0.23)	1.91 (0.16)
	e_o (days)	6.06 (0.65)	6.42 (0.85)	6.37 (0.91)	6.23 (0.78)	5.75 (0.49)

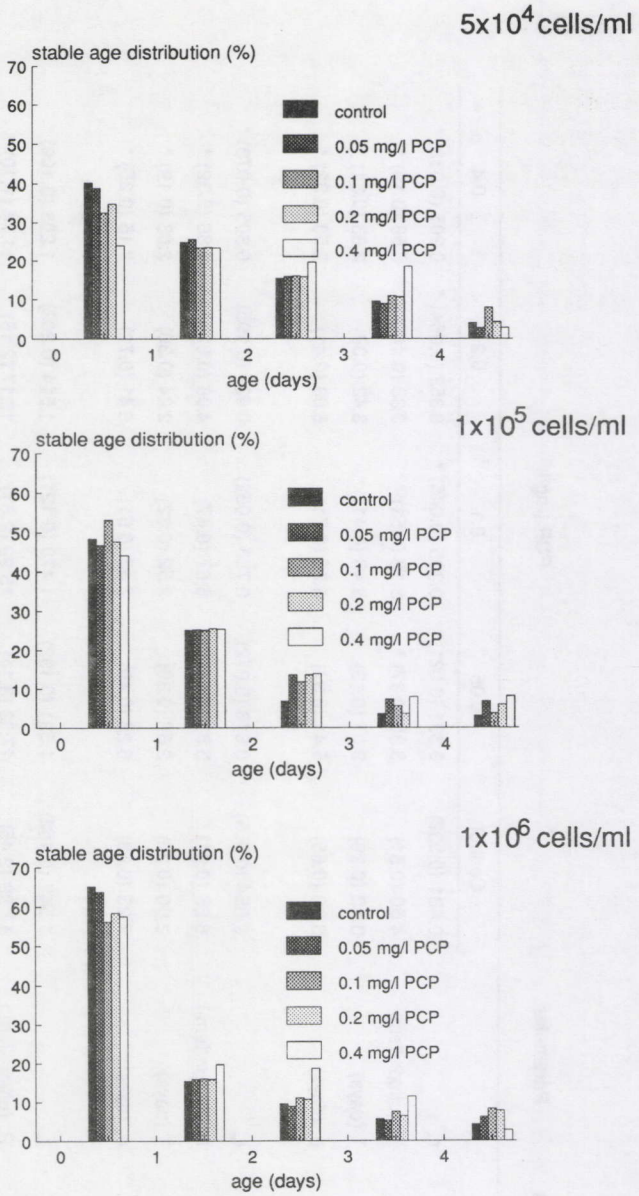


Figure VI.3. The effect of PCP on the stable age distribution of B. calyciflorus cultured at 3 food levels : $5x10^4$, 10^5, 10^6 cells/ml N. oculata.

food concentrations. For example, animals less than 1.5 days old represented 65, 74, and 95 % of the total population at food concentrations of $5x10^4$, 10^5 and 10^6 cells/ml of N. oculata, respectively. Under toxic stress there is a significant change in the age distribution of the rotifer population fed at low food levels. Indeed, the relative contribution of the youngest age classes (<1.5 days) to the population composition is significantly reduced from 65 % (control) to 42 % in the highest PCP treatment, while the contribution of the older classes (≥2.5 days) increased from to 19 % to 32 %. In populations fed with 10^5 and 10^6 cells/ml the observed differences were statistically insignificant.

VI.4. Discussion

The influence of temperature on the population dynamics of B. calyciflorus has been studied in depth by Halbach (1970). His observations are similar to the results obtained in the present study : i.e. an increase in the r_m with increasing temperatures. Halbach furthermore reported that the duration of all developmental stages decreased with increasing temperatures. This was also found in the present study in which a decrease of T was observed as a function of increasing temperatures. Miracle and Serra (1989), in their excellent review of temperature (and salinity) effects on the life history characteristics of rotifers, came to a similar conclusion : an increase in temperature - within the natural environmental range - produced an exponential increase of the r_m. This response is mainly a consequence of the changes in the individual rates of development and reproductive timing. The effect of temperature on the total number of offspring per individual life time was, however, negligible. This latter observation is partly confirmed in the present study, in which we noted relatively small differences in the R_o values of the rotifers kept at different temperatures. Several other studies have come to similar conclusions (Galkovskaya, 1963 ; Vinberg and Galkovskaya, 1979 ; Duncan, 1983 ; Galkovskaya, 1983 ; Herzig, 1983 ; Snell, 1986 ; Galkovskaya, 1987). Increasing food densities also resulted in higher r_m values, but here the effect was clearly a consequence of corresponding differences in the net-reproduction rather than from changes in the survivorship or reproductive timing (developmental rates). Several authors came to similar conclusions for various other rotifer species: Brachionus plicatilis (Snell, 1986) ; Euchlanis dilata (King, 1966), and Brachionus patulus (Rao and Sarma, 1986 ; Sarma, 1986).

The first part of this study was designed to examine the changes in the life history characteristics of B. calyciflorus cultured in specific test conditions, i.e. temperature and food concentration, but also to evaluate other variables such as test medium, type of test vessel, and the semi-static test design. This was mainly aimed at the development of a practical chronic bioassay method with rotifers. The implications of the influence of temperature on the demographic parameters of B. calyciflorus are very important for routine toxicity testing with this test organism. In the life table experiments performed at 25°C, the rotifers exhibited a very rapid developmental rate which meant that 12 hourly observations were necessary in order to distinguish the parent female from its offspring. As shown, the rate of development and reproduction is considerably lower at 20 and 15°C. At these temperatures the rotifers only need to be checked every 24 hours, which considerably reduces the labour time involved in performing the life table bioassays. The life tables typically took between 8 to 12 days to complete. However, the minimal length of a chronic toxicity test could be considerably shortened when using the r_m as test criterion. Indeed, in chapter 5 it was shown that at least 95 % of the r_m value calculated from a complete life table experiment (8-12 days) was reached after only 4 days, so that a chronic toxicity with an exposure period of only 4 days provides as much information on the value of r_m as a complete life cycle test. For the other variables tested it can be concluded that: (a) the test medium supports good rotifer growth and reproduction, (b) the 24-well culture plates are very practical for performing life-table studies with B. calyciflorus, (c) the microalgae N. oculata is a convenient rotifer food which supports healthy rotifer cultures. The food concentration to be used in the toxicity tests should be moderate ($\geq 10^5$ cells/ml of N. oculata) to allow for sufficient reproduction of the rotifers, so that possible differences due to toxic stress can be detected.

In the second part of this study the influence of the food concentration on the results of chronic toxicity tests with B. calyciflorus was examined. Snell and Moffat (in press) reported on a 2 day chronic toxicity test with B. calyciflorus and found LOEC's (for the population growth of the rotifer) of 30 µg/l and 0.19 mg/l for Cu and PCP, respectively. Although this LOEC value for PCP is quite similar to that found in our study, the LOEC for Cu is 6x higher than what we observed. This difference could be attributed to differences in test design, exposure period and possibly to the very high food concentration these authors used, which might have affected the bioavailability of Cu. For the 2 chemicals tested, B. calyciflorus exhibited a sensitivity which is comparable to that of Daphnia. Details on the comparative sensitivity of this test species are given in chapter 5.

Several authors have suggested that the food levels used in current toxicity methods are unrealistically high and that results of such tests consequently will not reflect the "real" chronic toxicity of the compound. Rao and Sarma (1986), for example, examined the effects of low and high food concentrations on the demographic parameters of B. patulus exposed to DDT. They noted a significant DDT toxicity - food level interaction: rotifers fed at low food concentration were more susceptible to DDT than those fed at high food levels. A closer examination of their results, however, shows that the differences between the EC_{50}'s (concentration at which the parameter value is reduced to 50 % of that of the controls) obtained in the life table experiments performed at high and low food levels were rather small. Indeed, the ratio's between the EC_{50}'s of experiments performed at high to those performed at low food concentrations were 1.4, 1.5 and 3.2 for the e_o, R_o and r_m, respectively. These findings are comparable to the results obtained in this study for B. calyciflorus, in which the ratio between the NOEC's at high and low food levels was 4 for both Cu and PCP.

Concerns about the high food levels used in conventional (life table type) chronic toxicity tests have led some workers to explore the use of the population dynamics of invertebrates under food limited conditions (a.o. van Leeuwen et al., 1985, 1987 ; Enserink et al., 1991). In this type of toxicity tests, populations of test organisms are usually exposed to the toxicants in a flow-through bioassay system with a constant food supply, so that as the population grows, the organisms eventually encounter a food-limited situation. van Leeuwen et al. (1987) explored the use of this approach and tested the toxicity of 10 chemicals with the life-table and population toxicity tests concurrently. From the comparison of the LOEC's obtained in the life table experiments with the EC_{10}'s (concentration which reduced the carrying capacity of the population by 10 %) from the population studies, these authors concluded that the bioassays performed with "food-limited" populations were more sensitive than the life table tests. Indeed, the life table LOEC's were higher than the population EC_{10}, for 9 of the 10 compounds . However, when examining the degree of increase in resolution, it can be noted that for 7 of those 9 chemicals the difference is less than 0.5 of an order of magnitude. For the two other compounds, PCP and Cd the ratio of the life table - LOEC to the population - EC_{10} was 7.4 and 91, respectively. Similar work was performed by Enserik et al. (1991) for 8 metals. Omitting the results of 2 chemicals which were included in the work discussed above (van Leeuwen et al., 1987), the ratio's here are less than 5, for 4 of the 6 metals. Based on the results reported in the 2 above mentioned

papers, the difference in the toxicity levels found with the life table approach (ad-libitum feeding) and the population approach (food-limited) is less than 0.5 of an order of magnitude for 75 % of the chemicals tested.

The fact that logistic growth and especially the yield or carrying capacity (population studies) is usually a more sensitive parameter than exponential growth (life tables) can be due to following reasons. The first, and perhaps the most important, is that in experiments with populations the test organisms inevitably arrive at a food-limited situation. This food stress means that the organisms have to filter more intensively to obtain sufficient food which may result in less energy being available for processes such as growth and reproduction. The second explanation for the observed differences in the results obtained with the two methods might be that in life table experiments organisms are not stressed during oogenesis and embryogenesis. Such stress does occur for the first and subsequent generations in the population toxicity studies. The effects of toxic stress on the life history characteristics of several generations of B. calyciflorus will be addressed in chapter 7.

The changes in the stable age distribution of the organisms exposed to PCP illustrated the possible effects of toxic substances at the population level. Indeed in chapter 5, we concluded that "old" females (> 4 days old) do not effectively contribute to the natural increase of the population ; this may eventually lead to the extinction of the population.

In this series of experiments we have examined the effects of temperature and food availability on the life history characteristics of B. calyciflorus with the aim of selecting a set of practical test conditions for the development of a chronic toxicity test with this species. This research aspect, i.e. short-chronic bioassays with B. calyciflorus for routine toxicity testing has been described in detail in chapter 5. This part of the study has also shown that food availability affects the results of chronic toxicity tests. However, based on the results obtained with B. calyciflorus and those of other workers with D. magna, it is clear that the consequences of these findings for practical aquatic toxicology should be put in a proper perspective. We have indeed shown that the differences in observed effect levels between laboratory chronic toxicity tests performed in food-limited and food-unlimited environments are usually rather small (see above). Taking into consideration the increased variability inherently associated with more complicated bioassay systems (Enserink et al., 1991), the considerable inter-laboratory variability of even the most standardized chronic toxicity tests

(Buikema et al., 1980 ; Persoone and Janssen, in press) and the difficulties in extrapolating single species laboratory test results to real world situations, we believe that the issue of food-availability in toxicity test should not be one of the major concerns for routine chronic toxicity testing. From a fundamental point of view, however, attempts at increasing the ecological realism of bioassays should be encouraged since this type of research should contribute to the understanding of how individuals, populations and eventually communities are impacted by xenobiotics.

VI.5. Summary

The influence of temperature and food-availability on the demographic characteristics of the freshwater rotifer Brachionus calyciflorus were studied with the aim of developing a chronic toxicity test. Life table experiments were performed at three temperatures (15, 20, 25°C) and three food levels ($5x10^4$, 10^5, and 10^6 cells/ml of Nannochloris oculata). The intrinsic rate of natural increase (r_m) of the animals was positively correlated with the temperature, and this was mainly a consequence of the response of the individual rates of development and reproductive timing. Similarly, increasing food concentrations also resulted in higher r_m values, which was due to increased net-reproductive rates. Based on these results, a set of practical experimental conditions is proposed for conducting life table toxicity tests with B. calyciflorus.

Additionally, the effects of food-availability on the results of chronic toxicity tests were investigated. In three series of life table tests, the rotifers were cultured at low, moderate and high food levels and exposed to copper and pentachlorophenol. For both toxicants the LOEC's based on the demographic parameters increased with increasing food concentrations. The ratio between the LOEC's obtained in the toxicity tests performed at high and low food levels was 4, for both toxicants.

The implications of these findings for routine aquatic toxicity testing were discussed.

CHAPTER VII.

The effects of xenobiotics on the life history characteristics of Brachionus calyciflorus : multi-generation life table experiments.

VII.1. Introduction

As the principal objective of aquatic toxicology is to determine concentrations of pollutants which, when released into the environment, will not impact natural populations and the community integrity, parameters should be studied which cover these aspects (Gentile et al., 1982). To reach that goal, several types of multispecies tests have been proposed (Taub, 1985 ; Cairns, 1986). However, none of these tests have been accepted as a standardized, reproducible toxicity test method for regulatory purposes. Consequently, to date, most aquatic toxicologists mainly use single species bioassays for evaluating the potential toxicity of xenobiotics. It is generally accepted that single species tests certainly have a place in the evaluation of toxic effects of xenobiotics, provided that relevant parameters are studied (van Leeuwen et al., 1986). Analysis of the effects of toxicants on the longevity and reproductive rate of cohorts of test organisms for example, can be integrated by life table calculations from which important demographic parameters such as the intrinsic rate of natural increase (r_m) can be derived. Indeed, r_m is an ecologically more relevant parameter than the total number of offspring produced in a fixed time interval, a procedure frequently used in the currently recommended chronic toxicity tests with invertebrates. The use of life table toxicity tests with Brachionus calyciflorus has been described in detail in chapters 5 and 6.

Although the life table approach for toxicity testing purposes has gained popularity in the last decade (Daniels and Allan, 1981 ; Gentile et al., 1982 ; van Leeuwen et al., 1985a, 1986, 1987 ; Rao and Sarma, 1986 ; Day and Kaushik, 1987 ; Bodar et al., 1988 ; Boyum and Brooks, 1988 ; Bridgham, 1988 ; Barbour et al., 1989 ; Coniglio and Baudo, 1989 ; Münzinger and Moncelli, 1991), its capability for predicting chronic toxicity effects at the population level has been questioned by several authors (Halbach et al., 1983 ; van Leeuwen et al, 1986, 1987 ; Bodar et al., 1988 ; Enserink et al., 1991). One of the reported

potential weaknesses in single generation life table tests is that the test organisms are not exposed to the pollutant during an important part of their life history, namely oogenesis and embryogenesis. Additionally, in life table studies with Daphnia magna and other invertebrates only living young are scored and generally no attempt is made to investigate if the F_1 generation shows disturbed development, growth or reproduction (van Leeuwen et al., 1987). In this context, it could be hypothesized that sublethal concentrations of certain types of xenobiotics might not directly affect the maternal (P) generation test animals, but that through accumulation and transfer through the egg yolk, the detrimental effects of the toxicant only become apparent in the subsequent generations, thus underestimating the "overall" toxicity of the pollutant. This phenomenon has indeed been suggested by Halbach et al. (1983) who, in their population studies with B. rubens exposed to sub-lethal concentrations of 4-chloroaniline, noted an accumulation of chronic effects over many generations. For pentachlorophenol, on the other hand, these authors reported a long-term adaptation of the rotifer population, in the sense that the carrying capacity of the population returned to normal (control) levels after an initial significant decrease.

The objective of this part of our study was to evaluate the above mentioned possible "weaknesses" in the currently used life table approach to chronic toxicity testing with invertebrates. The effects of copper and pentachlorophenol on the life history characteristics of several generations of B. calyciflorus continuously exposed to the same chemical concentrations were examined. Additionally, the effects of the maternal age on the susceptibility of the F_1 offspring was investigated. This study represents a logical link between the single generation life table studies described in chapters 5 and 6 and the population studies performed in chapter 8.

VII.2. Materials and methods

VII.2.1. General experimental procedures

Test organisms were obtained by hatching cysts which were produced in laboratory cultures under rigorously controlled conditions (Snell et al., 1991b). Details of the storage conditions and hatching procedure are given in chapter 5.
Details of the culturing methods of the microalga Nannochloris oculata (log phase), which

was used as food for the rotifers, are given in chapters 2 and 4.

VII.2.2. Multi-generation life tables

The experimental procedures for performing the life table studies and the calculation of the demographic parameters are described in chapters 5. The food concentration used in this study was $5x10^5$ cells/ml of N. oculata.

In the first part of this study the effects of the toxicants on the life history characteristics of 4 consecutive generations continuously exposed to the same chemical concentrations were examined. To that end, the first born of each female in the initial life table experiment (P) were introduced into a new life table (F_1) with the same test conditions. Similarly, the first born of the F_1 generation were transferred to a new life table (F_2) and finally this procedure was repeated and a F_3 experiment was set up with the F_2 offspring. For all 4 life tables (P, F_1, F_2 and F_3) the same observations, manipulations and calculations as described in chapters 5 and 6 were performed. Similarly, the described test design: 4 treatments (per chemical), each consisting of 4 replicates of 12 rotifers, was adhered to for the 4 generations. Consequently for each chemical, a total of 768 rotifers were followed throughout their lifespan. In practice, however, the number of offspring available to initiate the F_3 life table test was usually somewhat lower (between 8 and 11 rotifer per replicate, instead of 12).

In the second part of this study, the effect of the maternal age of rotifers under toxic stress on the susceptibility of the F_1 generation under the same stress was examined using life table techniques. Here the first and the fourth (or fifth) offspring of the P generation exposed to the chemical were introduced into new life table experiments: F_{11} and F_{14}, respectively. The number of treatments, replicates and number of organisms per replicate were the same as described above. In the highest concentration of both toxicants, however, the reproduction of the P generation was reduced to such an extent that the cohort of 12 x 4 F_{14} test organisms could not be completed. Usually, each replicate of this cohort consisted of only 7 to 11 rotifers.

Finally, the life history characteristics of the neonates hatched from cysts (sexual - mictic reproduction) and neonates originating from asexual mothers (asexual - amictic eggs),

were compared. The latter test organisms were obtained by isolating 150 to 200 ovigerous amictic rotifers (originating from a rotifer mass culture which is described in detail in chapter 8) in a glass petridish followed by incubation at 25 $\pm$ 1°C, in light. Two hours later the hatched offspring were collected and introduced into a life table test. Concurrently, a life table experiment with the 0-2 hour neonates hatched from cysts was performed. The experimental conditions used in these tests were identical to those described above.

VII.2.3. Test compounds and statistical analysis

Details of the preparation of the toxicant dilutions, the chemical analysis techniques and the statistical methods are given in chapter 5.

VII.3. Results

The values of the demographic parameters (calculated from the survivorship and fertility data) of the 4 generations of B. calyciflorus exposed to copper are presented in Table VII.1. In the P generation significant reductions (LOEC's) of the intrinsic rate of natural increase (r_m) and the net reproductive rate (R_o) of the rotifers exposed to 10 µg/l were noted, while the value for the generation time (T) was significantly higher. At this concentration no clear difference ($p>0.05$) with the control value was observed for the life expectancy at hatching (e_o) ; at 5 µg/l Cu, however, this parameter was significantly higher. The observed general trend, namely significant differences (as compared to the control) of the R_o and the r_m of the P cohort exposed to 10 µg/l was also noted for the rotifers of the 2 subsequent generations (F_1 and F_2). Indeed, in those life tables all demographic parameters were significantly different from the controls. In the F_3 life table, however, the demographic parameters of the rotifers exposed to 10 µg/l were not statistically different from the control values, while an increase in the r_m and the R_o was noted at 5 µg/l ($p<0.05$).

A cross-comparison of the demographic parameters in the control treatments of the 4 generations shows that the r_m, the R_o, and the T in the first 3 generations are rather similar ($p>0.05$). In the F_3 generation however, the r_m and the R_o decreased ($p<0.05$) by 46 and 35 %, respectively in comparison to the P values. The generation time of the F_3 cohort on the other hand, had increased significantly by 45 %. The values of the life expectancy in the

Table VII.1. The demographic parameters (mean ± S.D.) : intrinsic rate of natural increase (r_m), net reproduction (R_o), generation time (T) and the life expectancy at hatching (e_o) of 4 generations of B. calyciflorus continuously exposed to Cu. (*) : significantly different from the control ($p<0.05$). underlined values are significantly different from the corresponding value in the P generation ($p<0.05$).

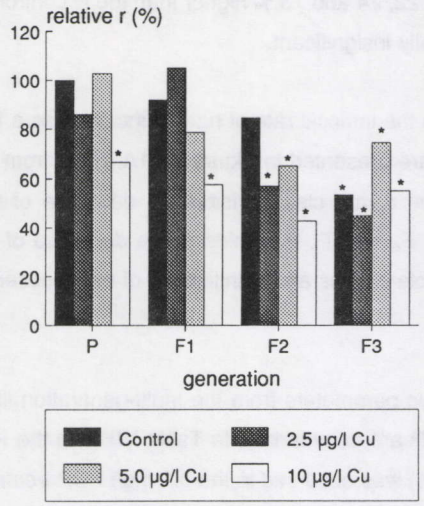
Generation	Parameter	Cu (µg/l)			
		Control	2.5	5.0	10
P	r_m	0.848 (0.094)	0.730 (0.045)	0.871 (0.100)	0.565 (0.037) *
	R_o (offspr./fem.)	7.03 (0.85)	6.55 (0.32)	7.95 (1.12)	5.92 (0.45) *
	T (days)	2.30 (0.31)	2.57 (0.58)	2.38 (0.29)	3.14 (0.28) *
	e_o (days)	5.90 (0.067)	6.44 (0.50)	7.47 (0.75) *	5.81 (0.52)
F_1	r_m	0.780 (0.052)	0.895 (0.119)	0.668 (0.068)	0.490 (0.034) *
	R_o (offspr./fem.)	6.79 (0.92)	6.71 (0.98)	6.08 (0.54)	4.47 (0.28) *
	T (days)	2.45 (0.32)	2.13 (0.19)	2.70 (0.24)	3.06 (0.12) *
	e_o (days)	7.19 (0.90)	6.59 (0.82)	6.55 (0.65)	4.93 (0.52) *
F_2	r_m	0.719 (0.102)	0.485 (0.57) *	0.554 (0.064) *	0.364 (0.029) *
	R_o (offspr./fem.)	6.38 (0.46)	6.18 (0.72)	5.83 (0.95)	3.99 (0.28) *
	T (days)	2.58 (0.34)	3.75 (0.41) *	3.18 (0.18) *	3.80 (0.28) *
	e_o (days)	7.30 (0.85)	7.68 (1.1)	7.70 (0.67)	4.31 (0.57) *
F_3	r_m	0.454 (0.071)	0.383 (0.046)	0.632 (0.052) *	0.468 (0.058)
	R_o (offspr./fem.)	4.56 (0.69)	4.74 (0.60)	6.59 (0.039) *	3.86 (0.73)
	T (days)	3.34 (0.52)	4.06 (0.56)	2.98 (0.45)	2.89 (0.31)
	e_o (days	6.66 (0.95)	7.84 (0.51)	7.93 (1.24)	5.72 (0.64)

F_1, F_2, F_3 controls were 22, 24 and 13 % higher than the P control value ; these differences were however statistically insignificant.

The relative changes in the intrinsic rate of natural increase as a function of the generation and Cu concentration are presented in Figure VII.1A. (data from Table VII.1). Here, the 2 reported changes in the r_m are clearly visible : a decrease of the r_m at the highest Cu concentration in the P, F_1, and F_2 life tables and a decrease of the r_m values across the generations. The possible causes and significance of these observations will be discussed below.

The demographic parameters from the multi-generation life table experiments with rotifers exposed to PCP are summarized in Table VII.2. In the P generation a significant increase of the r_m (33 %) was observed in the 0.1 mg/l PCP treatment, while at the highest PCP concentration this parameter decreased to 75 % of that of the control value ($p<0.05$). The changes of the other demographic parameters were statistically insignificant. All demographic parameters of the rotifers in the F_1 life table data were significantly affected by 0.4 mg/l PCP, except T. The same trend, a decrease ($p<0.05$) in the r_m, the R_o and the e_o, was observed for the F_2 rotifers exposed to 0.4 mg/l. PCP did not affect any of the parameters in the F_3 life tables, except for the r_m and the R_o in the 0.1 mg/l PCP treatment which were significantly higher than the control value. In Figure VII.1B the values of the r_m relative to the P control are plotted per generation and as a function of increasing PCP concentrations. No significant differences between the r_m of the 4 control life tables were observed. Furthermore, the decrease of the r_m in the 0.4 mg/l PCP treatments of the first 3 life tables (P, F_1, and F_2) is clearly visible in this figure. As mentioned above, no such trend was present in the F_3 experiments.

In the second part of this study, the effect of the maternal age (P) on the susceptibility of the first generation offspring (F_1) to Cu and PCP was examined. The demographic parameters of the maternal generation organisms and those of its first offspring (F_{11}) and fourth offspring (F_{14}) under Cu stress are presented in Table VII.3. In the P life table treated with 10 µg/l the r_m and the R_o were reduced ($p<0.05$) to 72 and 82 %, respectively, of that of the control value. The generation time of this cohort had increased by 26 % to 2.45 days ($p<0.05$). A similar significant reduction of the r_m (to 64 % of its control) was noted in the life table experiments performed with the first offspring of the



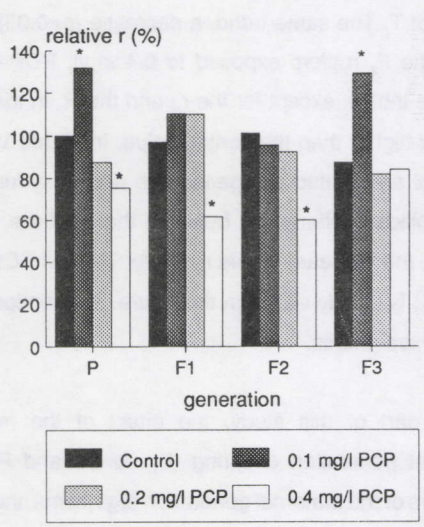


Figure VII.1. The effect of Cu (A) and PCP (B) on the intrinsic rate of natural increase (r_m) of 4 consecutive generations of B. calyciflorus. The values of r_m are expressed as % of the maternal generation (P) control value. (*) : significantly different from the P control value ($p<0.05$).

Table VII.2. The demographic parameters (mean ± S.D.) : intrinsic rate of natural increase (r_m), net reproduction (R_o), generation time (T) and the life expectancy at hatching (e_o) of 4 generations of B. calyciflorus continuously exposed to PCP. (*) : significantly different from the control ($p<0.05$). underlined values are significantly different from the corresponding value in the P generation ($p<0.05$).

Generation	Parameter	PCP (mg/l)			
		Control	0.1	0.2	0.4
P	r_m	0.930 (0.095)	1.224 (0.078) *	0.815 (0.055)	0.703 (0.065) *
	R_o (offspr./fem.)	8.34 (1.10)	8.42 (0.85)	6.18 (0.99)	7.24 (0.75)
	T (days)	2.28 (0.32)	1.88 (0.21)	2.23 (0.29)	2.59 (0.24)
	e_o (days)	5.14 (0.65)	6.13 (0.71)	5.73 (0.75)	6.15 (0.60)
F_1	r_m	0.904 (0.112)	1.028 (0.084)	1.024 (0.091)	0.573 (0.073) *
	R_o (offspr./fem.)	7.43 (0.85)	8.45 (1.20)	6.36 (0.41)	3.84 (0.41) *
	T (days)	1.92 (0.11)	1.88 (0.25)	1.65 (0.15)	2.17 (0.29)
	e_o (days)	6.34 (0.75)	6.03 (0.99)	5.42 (0.38)	3.45 (0.51) *
F_2	r_m	0.941 (0.061)	0.891 (0.075)	0.861 (0.101)	0.573 (0.066) *
	R_o (offspr./fem.)	7.01 (0.65)	5.97 (0.81)	7.05 (0.45)	3.48 (0.42) *
	T (days)	2.07 (0.15)	2.00 (0.23)	2.27 (0.39)	2.18 0.19)
	e_o (days)	5.26 (0.43)	5.11 (0.59)	5.79 (1.11)	3.45 (0.40) *
F_3	r_m	0.815 (0.065)	1.200 (0.173) *	0.763 (0.091)	0.784 (0.064)
	R_o (offspr./fem.)	6.18 (0.45)	7.37 (0.51) *	7.24 (0.84)	5.47 (0.65)
	T (days)	2.23 (0.31)	1.66 (0.19)	2.59 (0.31)	2.17 (0.19)
	e_o (days	5.73 (0.68)	5.84 (0.98)	6.16 (1.01)	5.23 (0.49)

maternal generation (F_{11}). Except for the significant increases in T and R_o at 5 µg/l Cu, the offspring of "old" mothers (F_{14}) were not affected by any of the Cu treatments. As in the first part of the study, the relative values of the most important population parameter - the r_m - were plotted as a function of the Cu concentrations in Figure VII.2A. Compared to the control of the maternal cohort, the value of the r_m decreased by 28 and 52 % in the F_{11} and F_{14} control treatments, respectively. The second main conclusion that can be drawn from this figure is that the LOEC's (r_m as test criterium) obtained for the P and the F_{11} life tables were identical : 10 µg/l Cu. No LOEC, however, could be derived from the F_{14} life table.

The effects of PCP on the demographic parameters obtained in the P, F_{11} and F_{14} experiments are shown in Table VII.4. In the P cohort, two parameters were significantly affected (LOEC) at the highest PCP concentration tested: the R_o and the T. The lowest PCP concentrations did not affect the demographic parameters. In the life tables performed with the first offspring (F_{11}) of the maternal generation, the value of the R_o was higher ($p<0.05$) than the control value in all PCP treatments, while the generation time had significantly increased at 0.4 mg/l PCP. The rotifers in the F_{14} life table exhibited (compared to control R_o) a higher net reproductive rate in all PCP treatments and an increased e_o in the 2 highest treatments. The highest value for the r_m in the control treatments was observed in the maternal generation (P). The relative values of r_m in function of PCP concentrations are presented in Figure VII.2.

The survivorship - (l_x) and fertility (m_x) curves of the rotifers hatched from cysts and those hatched from parthenogenetic eggs are compared in Figure VII.3. The fact that both the l_x and m_x data were almost identical is reflected in the values of the derived demographic parameters (Table VII.5).

VII.4. Discussion

The results from the experiments in the first as well as those from the second part confirm the observations reported in the previous chapters, namely that despite its integrative character the r_m, is not always the most sensitive test endpoint in toxicity tests with B. calyciflorus. The possible reasons for this have been discussed in detail in chapter 6. van Leeuwen et al. (1985a) and Bridgham (1988) came to a similar conclusion for life table

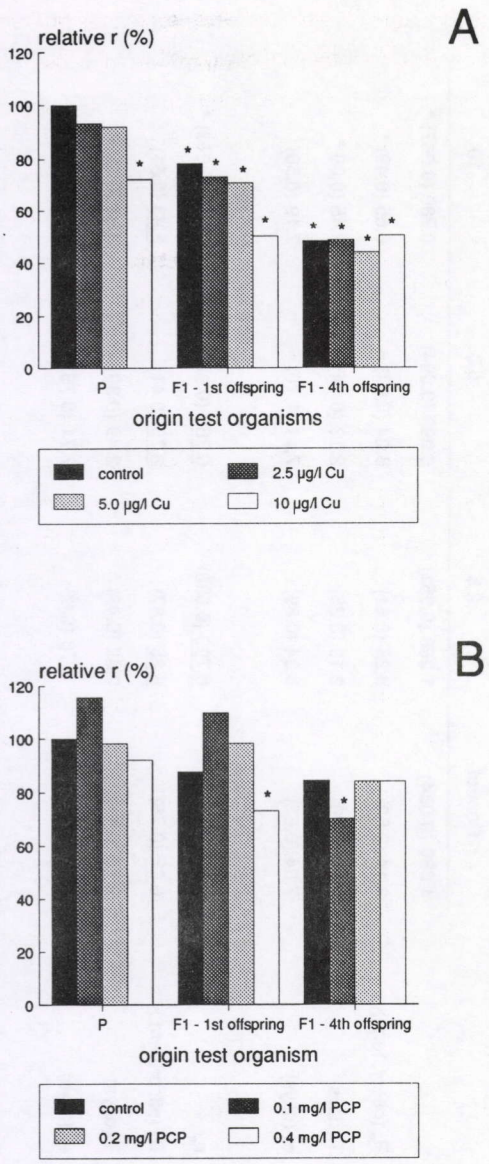


Figure VII.2. The effect of Cu (A) and (PCP) (B) on the intrinsic rate of natural increase of B. calyciflorus females (P) and its first (F_{11}) and fourth offspring (F_{14}). The values of r_m are expressed as % of the maternal generation (P) control value. (*) : significantly different from the P generation control value ($p<0.05$).

Table VII.3. The influence of the age of B. calyciflorus females exposed to Cu, on the susceptibility of its offspring.
Demographic parameters (mean ± S.D.) : intrinsic rate of natural increase (r_m), net reproduction (R_o), generation time (T) and the life expectancy at hatching (e_o). Origin test organisms : maternal generation (P) ; 1st offspring of P (F_{11}) and 4th offspring of P (F_{14}). (*) : significantly different from the control ($p<0.05$). underlined values are significantly different from the corresponding value in the P generation ($p<0.05$).

Origin test organisms	Parameter	Cu (µg/l)			
		Control	2.5	5.0	10
P	r_m	1.080 (0.084)	1.008 (0.051)	0.993 (0.094)	0.780 (0.067) *
	R_o (offspr./fem.)	8.14 (0.52)	8.35 (0.61)	8.24 (0.67)	6.65 (0.49) *
	T (days)	1.94 (0.20)	2.10 (0.25)	2.12 (0.14)	2.45 (0.09 *
	e_o (days)	7.04 (0.62)	6.94 (0.89)	7.41 (1.10)	7.19 (0.58)
F_{11}	r_m	0.845 (0.051)	0.790 (0.086)	0.756 (0.91)	0.540 (0.063) *
	R_o (offspr./fem.)	6.05 (0.75)	6.25 (0.87)	6.75 (0.41)	5.83 (0.61)
	T (days)	2.13 (0.11)	2.32 (0.43)	2.49 (0.30)	3.18 (0.25) *
	e_o (days)	6.92 (1.05)	6.78 (0.68)	7.71 (0.75)	7.70 (0.59)
F_{14}	r_m	0.520 (0.031)	0.526 (0.065)	0.473 (0.073)	0.542 (0.050)
	R_o (offspr./fem.)	4.38 (0.067)	4.76 (0.042)	4.69 (0.059)	4.24 (0.042)
	T (days)	2.84 (0.12)	2.97 (0.20)	3.27 (0.12) *	2.66 (0.19)
	e_o (days)	5.99 (0.43)	6.71 (0.48)	7.09 (0.39) *	6.42 (0.60)

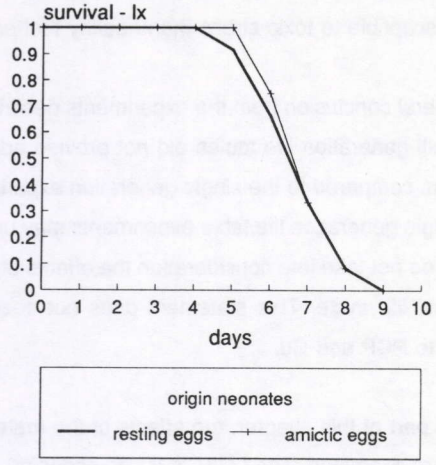
Table VII.4. The influence of the age of B. calyciflorus females exposed to PCP, on the susceptibility of its offspring. Demographic parameters (mean ± S.D.) : intrinsic rate of natural increase (r_m), net reproduction (R_o), generation time (T) and the life expectancy at hatching (e_o). Origin test organisms : maternal generation (P) ; 1st offspring of P (F_{11}) and 4th offspring of P (F_{14}). (*) : significantly different from the control ($p<0.05$). underlined values are significantly different from the corresponding value in the P generation ($p<0.05$).

Origin test organisms	Parameter	PCP (mg/l)			
		Control	0.1	0.2	0.4
P	r_m	1.029 (0.114)	1.190 (0.098)	1.012 (0.115)	0.947 (0.076)
	R_o (offspr./fem.)	8.49 (1.02)	9.87 (0.80)	7.77 (0.64)	6.11 (0.49) *
	T (days)	2.08 (0.24)	1.92 (0.12)	2.03 (0.30)	1.19 (0.09) *
	e_o (days)	6.67 (0.71)	6.35 (0.59)	6.21 (0.73)	6.25 (0.45)
F_{11}	r_m	0.902 (0.075)	1.130 (0.153)	0.866 (0.108)	0.752 (0.079)
	R_o (offspr./fem.)	5.53 (0.63)	8.41 (0.67) *	7.10 (0.48) *	7.61 (0.70) *
	T (days)	1.89 (0.21)	1.88 (0.15)	2.26 (0.32)	2.70 (0.19) *
	e_o (days)	6.10 (0.45)	5.93 (0.64)	6.33 (0.57)	6.16 (0.37)
F_{14}	r_m	0.869 (0.091)	0.723 (0.093)	0.865 (0.067)	0.866 (0.082)
	R_o (offspr./fem.)	5.85 (0.45)	7.07 (0.39) *	7.86 (0.81) *	7.10 (0.49) *
	T (days)	2.03 (0.23)	2.71 (0.43)	2.38 (0.19)	2.26 (0.20)
	e_o (days)	4.77 (0.50)	5.80 (0.87)	6.12 (0.48) *	6.32 (0.61) *

Table VI.5. The demographic parameters (mean ± S.D.) of B. calyciflorus neonates originating from cysts and amictic eggs. r_m : intrinsic rate of natural increase ; R_o : net reproduction ; T : generation time ; e_o : life expectancy at hatching.

Parameter	**Origin rotifer neonates**	
	cysts	amictic eggs
r_m	0.899 (0.091)	0.830 (0.102)
R_o (offsr./fem)	6.75 (0.81)	7.17 (0.90)
T (days)	2.12 (0.32)	2.37 (0.22)
e_o (days)	7.00 (0.45)	7.17 (0.67)

toxicity studies performed with daphnids. Copper concentrations of 10 µg/l clearly affected the life history parameters of B. calyciflorus in the P, F_1 and F_2 life tables. This LOEC is similar to those found in the single generation experiments discussed in chapter 6. However, no significant reductions in the demographic parameters of the F_3 generation were observed. This "decrease in sensitivity" of the organisms of this generation cannot be easily explained. In the multi-generation experiments with PCP a similar phenomenon was noted : identical LOEC's (0.4 mg/l for r_m) for the P, F_1 and the F_2 generation, while no significant decrease of the r_m was noted in the F_3 experiment. Although the observed "decrease in sensitivity" was statistically significant, some caution for the interpretation of this phenomenon is warranted. Halbach et al. (1983) reported similar "adaptation effects" over several generations in their population studies with B. rubens exposed to PCP, however, these authors were not willing to speculate on the possible mechanisms involved in this (apparent) decrease in the sensitivity of the organisms. Possibly, an increased induction of stress proteins and/or the production of other detoxifying proteins after several generations might occur (T.W. Snell, personal communication), which could explain this phenomenon. This is however speculation as no evidence for this hypothesis is presented. The reasons for the observed decrease of the r_m in the control treatments in each consecutive generation (which is very obvious in the Cu experiment) are not very clear either. In principle, this trend should be due to weaknesses in the experimental procedures ; indeed all test conditions such as toxicant concentrations, algae culture procedures and food level used, dilution medium and temperature were carefully standardized and checked at regular intervals throughout the



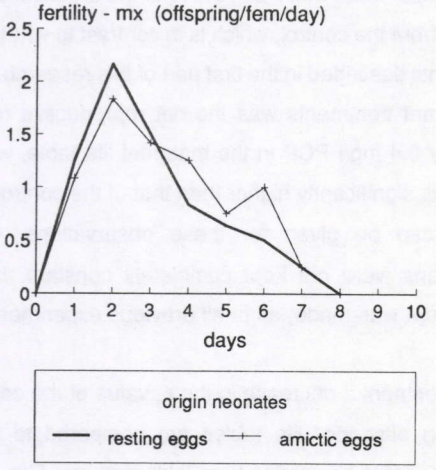


Figure VII.3. The age specific survival (l_x) and fertility (m_x) of B. calyciflorus hatched from amictic eggs and from resting eggs.

multi-generation experiments.
Moreover, the reduced overall fitness of the F_3 generation, cannot explain the observed "insensitivity" of the F_3 organisms, since it is generally accepted that stressed, unhealthy animals are more susceptible to toxic stress than healthy test animals.

The main general conclusion from the experiments described above, is that (at least for Cu and PCP) multi-generation life tables did not provide additional information on the effects of toxicants, as compared to the single generation experiments. van Leeuwen et al. (1986) stated that single generation life table experiments may underestimate the toxicity of pollutants since they do not take into consideration the effects of the toxicants on important part of the organisms' life cycle. This statement does not seem to valid, at least for B. calyciflorus exposed to PCP and Cu.

In the second part of this chapter, the effects of the maternal age on the sensitivity of the F_1 offspring were examined. The LOEC's (r_m as endpoint) obtained for the P and the F_1 life tables was 10 µg/l Cu for both tests. This value is identical to that obtained in the multi-generation tests described in the first part of this study. No LOEC could be derived from the life table studies conducted with the offspring originating from "old" females (F_{14}). In the maternal age experiment with PCP, the r_m of the organisms exposed to PCP were not significantly different from the control, which is in contrast to what was observed in the multi-generation experiments described in the first part of this research. The parameter which was affected by the toxicant treatments was the net reproductive rate. However, the R_o was adversely affected by 0.4 mg/l PCP in the maternal life table, while in the F_{11} and the F_{14} experiment the R_o was significantly higher than that of the control in all PCP treatments. No logical explanation can be given for these observations, except perhaps that the experimental conditions were not kept completely constant throughout this experiment (although every attempt was made, as in all previous experiments).

In the Cu experiment a decrease in the r_m value of the controls was noted when the results of the "young offspring" life tables are compared to those of the experiments performed with the neonates originating from "old" mothers. This observation might possibly be attributed to the "Lansing effect" (Lansing, 1942a ; 1942b ; 1947 ; 1948 ; 1954). This worker established isogenic lines of the rotifer Euchlanis dilatata (orthoclones) differing only in maternal age, and found in orthoclones originating from young females expanding patterns

of survivorship, whereas the lifespan of old orthoclones decreased until extinction occurred. Lansing's conclusions on these maternal effects were confirmed and extended by King (1966), who found that aging patterns could be modified by both the food quantity and quality and that the fecundity of young orthoclones was significantly higher than that of old orthoclones. Although the experiments in the present study were not designed to examine these effects and only one generation of young and old "orthoclones" were observed, the differences in e_o could possibly be explained by this "Lansing phenomenon".

Elendt and Bias (1990) used multi-generation life table techniques to examine the adequacy of different media for Daphnia magna culturing and Winner (1990) applied a similar approach to evaluate several diets and culture media for Ceriodaphnia dubia. However, despite a rather extensive literature search, only one paper was found which examined the possible adverse effects of toxicants using multi-generation life table experiments. Indeed, Bridgham (1988) exposed 3 consecutive generations of Daphnia puliceria to the PCB 2,2'-dichlorobiphenyl and found no differences between the results of the first 2 generations. The data of the toxicity tests with the third generation daphnids are, however, difficult to compare with the former, as the experimental conditions were changed during the test. Noteworthy is that the values of the demographic parameters of the control treatments of the F_1 life tables were much lower than those of the maternal life tables. As mentioned above, a similar trend was noted in our experiments with B. calyciflorus.

The values of the r_m in the control treatments of the P and F_1 life tables were not significantly different in 3 of the 4 experiments performed (multi-generation Cu + PCP and maternal age Cu + PCP). Only in the maternal age experiment with Cu was the r_m (and R_o) in the P generation significantly lower than that of the F_1 generation. Although unlikely (as this was not noted in the 3 other experiments, see above) this could have been caused by possible differences in life history characteristics of rotifers hatched from asexually versus sexual (cysts) eggs. However, in concurrently conducted life tables with rotifers hatched from cysts and with rotifers hatched from amictic eggs, no significant differences of the demographic parameters were noted, indicating that the observed differences in demographic parameters were not caused by the different "origin" of the test organisms. A comparison of the demographic parameters in the P and F_1 generations of the PCP (multi-generation and maternal age) and Cu (multi-generation and maternal age) experiments, respectively, reveals that although the same general trends can be noted, some (significant)

differences occur in the values of the parameters. This can be due to experimental "noise", i.e. small differences in the test conditions of the respective experiments. It is clear that these observations should be taken into consideration when assessing the data presented in this study.

The potential use of single-generation life table toxicity tests with B. calyciflorus has been discussed in the previous chapters. The multi-generation approach as described in the present study, does not seem to provide more information on the toxicity of chemicals (at least for Cu and PCP). From a strictly pragmatic point of view, this is rather fortunate ; indeed such experiments are very labour intensive and consequently are not suited for routine applications. Nevertheless, for particular substances this approach might give a better insight into possible "accumulation" or "adaptation" effects. The observations on the influence of the maternal age on the susceptibility of the offspring might be a contribution to the understanding of the effects of xenobiotics on the age-structure of populations.

VII.5. Summary

Multi-generation life table toxicity tests were performed to assess the effects of copper and pentachlorophenol on the life history characteristics of four generations of the freshwater B. calyciflorus, continuously exposed to the toxicants. Additionally, the effects of the maternal age on the susceptibility of F_1 offspring to these chemicals was investigated.
For both chemicals the LOEC's (based on demographic parameters) observed in the first and second generation life tables were similar to those noted in the initial maternal life cycle experiments. No LOEC, however, could be derived (within the range of toxicant concentrations tested) from the results of the toxicity tests performed with the third generation rotifers.
In experiments investigating the effects of the maternal age, the LOEC's derived from the life table tests with the maternal generation and those from the experiments with the offspring originating from young mothers were identical for both Cu and PCP. Rotifer neonates originating from old mothers seem to be less susceptible to toxic stress and no LOEC could be established (within the concentration range tested).
The consequences of these findings for practical ecotoxicology are discussed.

CHAPTER VIII.

The effects of xenobiotics on the population dynamics of Brachionus calyciflorus : long-term toxicity tests with populations.

VIII.1. Introduction

Although the ultimate concern of ecotoxicologists may be the effects of pollutants on ecosystems, the standardized methods imposed by regulators to assess the toxicity of pollutants, are still laboratory single species tests (Maltby and Naylor, 1990). In the last decade, methods have been developed which examine the effects of xenobiotics on a spectrum of biological systems encompassing the subcellular- the whole organism- and the community level (Sheehan, 1984). Moving up through this hierarchy of biological organization, the ecological relevance and realism of the bioassay systems increase, but the repeatability and the potential for standardization decrease. Increased realism usually usually means longer exposure- and response periods resulting in higher costs of such bioassay systems. As a result, the bulk of the research aimed at the development of ecotoxicological methods has tended to concentrate on bioassays using effect criteria at the sub-organismic and organismic level of biological organization. However, this still leaves the problem of determining the ecological consequences of the data obtained from such tests and ultimately, of relating these "laboratory" results to the natural environment. Indeed, if the ecological realism and the predictive capacity of the currently used and the newly developed bioassays is to be improved, one of the main research areas in aquatic toxicology may prove to be the need to resolve the issue of the extrapolation of responses from one level of biological organization to another (Geisy and Odum, 1980 ; Geisy, 1985 ; Geisy and Alfred, 1985 ; Cairns and Pratt, 1989).

Most chronic laboratory test methods with invertebrates expose individual organisms (or cohorts of identical organisms) to xenobiotics for a certain part of their life cycle, and use the organisms' survival, reproduction and growth as test criteria. Although this (partial) life cycle approach has been used with various invertebrate species (see chapters 5, 6 and 7), some authors have suggested that toxicity tests with populations might be an ecologically

more realistic and more sensitive way to detect the effects of chemicals (Halbach et al., 1983 ; van Leeuwen et al, 1986, 1987 ; Bodar et al., 1988 ; Enserink et al., 1991). Indeed, there are two fundamental differences between the life table and the population approaches. Firstly, in the life table tests the organisms are, in most experiments, only exposed to the chemical for a certain part of their life cycle and possible adverse effects on the life history characteristics of the subsequent generations are not taken into consideration. The second and probably more important difference, is the amount of food available for consumption. Indeed, in life table studies the organisms are fed ad-libitum, while in population tests the organisms inevitably arrive at a food-limited situation. As pointed out by Kooijman and Metz (1984), the effects of chemicals on the behaviour of populations is strongly dependent on the food availability. Consequently toxicity tests performed in a food-limited environment might enhance the toxicant-induced effects, thus producing more sensitive endpoints and/or more ecologically relevant information. A similar view was expressed by Halbach et al. (1983) who stated that changes in the dynamics of large populations of organisms can be used as a "magnifying glass" to detect small effects of environmental pollutants which can not be detected in tests with individual organisms.

In this chapter the effects of the 4 chemicals, used in the previous chapters of this study, on the population dynamics of B. calyciflorus were examined. The main objective of this investigation was to establish NOEC and LOEC values for these chemicals using test criteria at the highest level of biological organization possible within single-species toxicity testing namely, the population. The results from this study will be used to evaluate the (ecological) relevance of the results obtained in life table studies described in chapters 5, 6 and 7.

VIII.2. Materials and Methods

VIII.2.1. Population experiments

The population studies with B. calyciflorus were started with rotifers obtained from a laboratory stock culture, maintained under conditions which were analogous to those used for the control treatments of the toxicity experiments described below. The test populations taken from this stock culture, to initiate the toxicity tests, had reached a stable-age

distribution (log phase). Details on the origin of the B. calyciflorus strain are given in chapter 5. The procedures used to culture the microalga Nannochloris oculata, which was used as food in all experiments, are given in chapters 2 and 4. The rotifer culture medium was a moderately hard synthetic freshwater medium (EPA medium) described by Horning and Weber (1985) ; details on the composition of this medium are given in chapters 2 and 5.

The toxicity tests were conducted in a flow-through bioassay system which consisted of 12 to 16 test containers (680 ml), a peristaltic multichannel pump (Watson & Marlow, Falmouth, U.K.) and 12 to 16 supply vessels (1000 ml) containing toxicant dilutions and food. The cylindrical test containers, which were modified chemostat vessels (Cell-Lift, Ventrex, Portland, USA), were equipped with an inlet-tube at the bottom of the test vessel and an outlet overflow tube, fitted with a 20 µm screen (to retain the rotifers), at the top of the vessels (Fig. VIII.1). The test solutions, i.e. the toxicant dilutions and the algae ($5x10^6$ cells/ml of N. oculata) were pumped from the supply vessels into the test containers at a constant rate of 340 ml/day (± 5ml).

The population experiments were initiated by introducing 680 ± 20 rotifers into each test vessel with the required test conditions. At 24 ± 2 hour intervals, the population density was measured by taking 5 replicates of 1 ml aliquot samples from each test vessel and

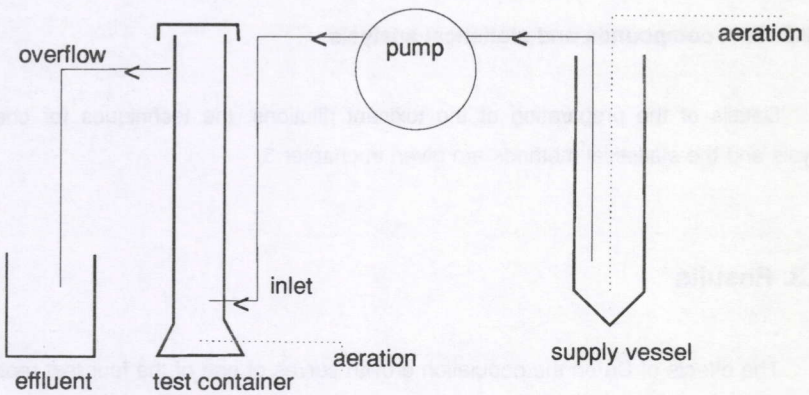


Figure VIII.1. Diagram of the test design used in the flow-through toxicity tests with B. calyciflorus populations.

recording the number of rotifers and the egg-ratio (mean number of eggs per female) under a dissection microscope. For each test substance, two concentrations of the chemical and one control were tested. To enable statistical treatment of the data, each treatment was replicated 4 times. The tests with Cu and PCP and those with DCA and lindane were performed concurrently. All experiments were conducted in a temperature controlled room at 24 $\pm$ 1 °C, in darkness.

The data from each replicate were fitted to the logistic model (with time lag) describing population growth in a limited environment and the carrying capacity (K) of the test population was calculated (Halbach et al., 1983) :

$$\frac{dN_t}{dt} = r_m N_t \frac{K - N_{(t-T)}}{K}$$

where r_m = the intrinsic rate of natural increase and N_t = the population density at time t. The time lag T was estimated by iterative calculations based on the population data. Estimates of the intrinsic rate were obtained by using the standard equation (Poole, 1974) :

$$r_m = \frac{\ln N_t - \ln N_o}{t}$$

VIII.2.2. Test compounds and statistical analysis

Details of the preparation of the toxicant dilutions, the techniques for chemical analysis and the statistical methods are given in chapter 5.

VIII.3. Results

The effects of Cu on the population growth curves of one of the four test replicates is presented in Figure VIII.2. The rotifer populations exhibited sigmoid growth curves with subsequent oscillations around the equilibrium density - the carrying capacity. In the control populations the 3 phases of a typical logistic growth curve can be distinguished : the lag-

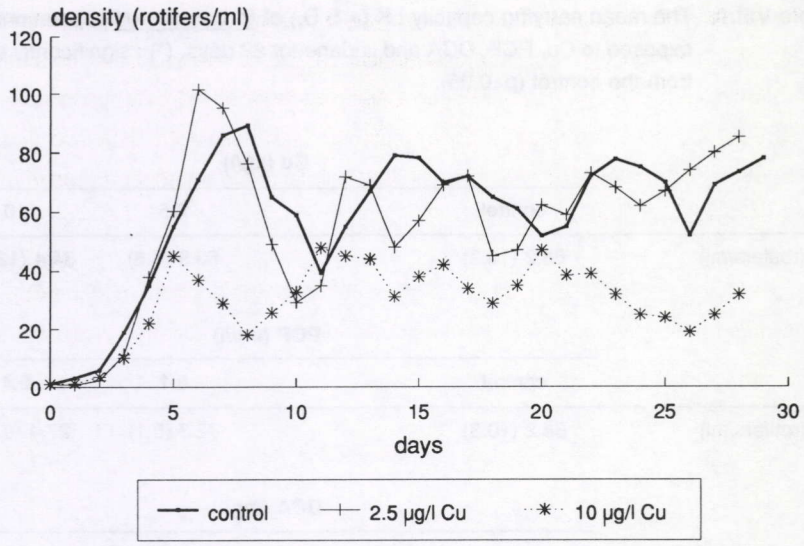


Figure VIII.2. Population curves of B. calyciflorus exposed to Cu.

phase (day 0-2), the exponential growth phase (day 3-8) and the equilibrium phase (from day 9 onwards). The periodical oscillations around the carrying capacity, will be discussed furtheron. In the control population the highest population density was reached on day 8. Rotifers exposed to Cu exhibited similar population growth curves although the frequency and amplitude of the oscillations were somewhat different. The populations exposed to 2.5 µg/l Cu for example, reached the first density peak after 6 days, while in those treated with 10 µg/l Cu, this peak was reached after only 5 days. From the logistic growth equation the ecologically important parameter: the carrying capacity (K), was calculated. No significant differences were noted between the mean K (n=4) of the control populations and that of rotifers exposed to 2.5 µg/l Cu (Table VIII.1). The carrying capacity of the population treated with 10 µg/l Cu, however, was reduced ($p<0.05$) to 50 % of that of the control populations.

The mean number of eggs per female (egg-ratio) noted in this experiment are given in Figure VIII.3. Similar trends were observed in all treatments : high egg numbers (per female) in the first 7 (to 10) days and relatively low egg-ratios during the rest of the observation period. Because of the scatter of the data and the absence of clear differences between the control and the toxicant treatments, this parameter will not be used in the further discussion of the results.

Table VIII.1. The mean carrying capacity : K (± S.D.) of B. calyciflorus populations (n=4), exposed to Cu, PCP, DCA and lindane for 28 days. (*) : significantly different from the control ($p<0.05$).

	Cu (µg/l)		
	control	2.5	10
K (rotifers/ml)	68.2 (10.3)	63.9 (8.6)	34.4 (12.3) *

	PCP (mg/l)		
	control	0.1	0.4
K (rotifers/ml)	68.2 (10.3)	72.3 (5.1)	27.4 (9.7) *

	DCA (mg/l)			
	control	control+acetone	2.5	10
K (rotifers/ml)	72.5 (8.1)	69.5 (9.0)	64.3 (6.6)	extinction

	lindane (mg/l)			
	control	control+acetone	10	20
K (rotifers/ml)	72.5 (8.1)	69.5 (9.0)	38.5 12.9) *	24.8 (7.2) *

Similar population growth to that observed in the Cu experiment was found in the PCP test (Fig. VIII.4). Here, the carrying capacity of the test populations exposed to 0.4 mg/l PCP decreased to 40 % of that of the control K, while the rotifers treated with 0.1 mg/l PCP were not adversely affected.

In the DCA test the general shape of the population curves was similar to those described above. The maximum population density (111rot./ml on day 8) in the control population was, however, somewhat higher than that observed in the Cu and PCP experiments. Acetone, which was used as a solvent for DCA and lindane, did not adversely affect the carrying capacity of the test populations. The rotifer populations exposed to 2.5 mg/l DCA exhibited the following characteristics : a delayed start of the exponential

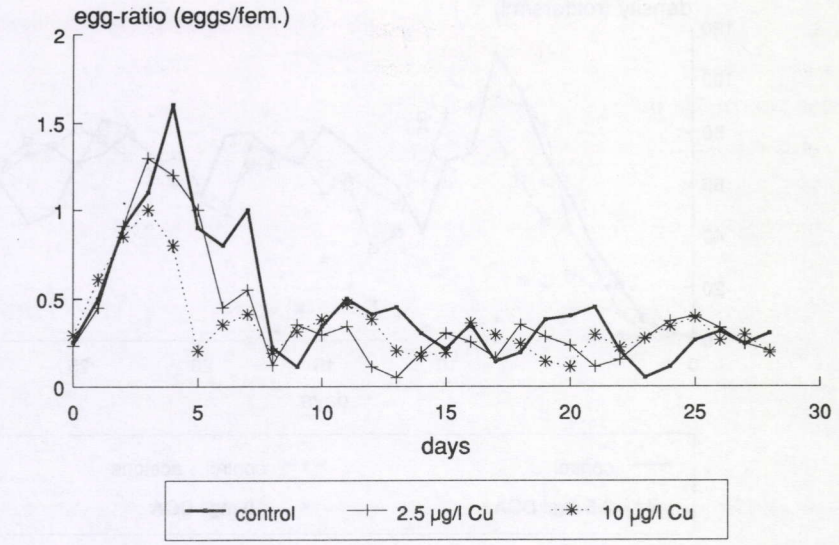


Figure VIII.3. The egg-ratio of B. calyciflorus populations exposed to Cu.

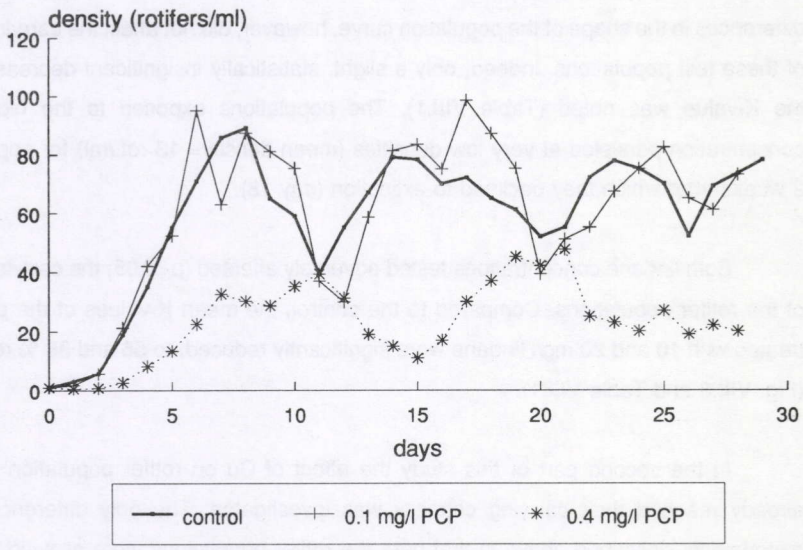


Figure VIII.4. Population curves of B. calyciflorus exposed to PCP.

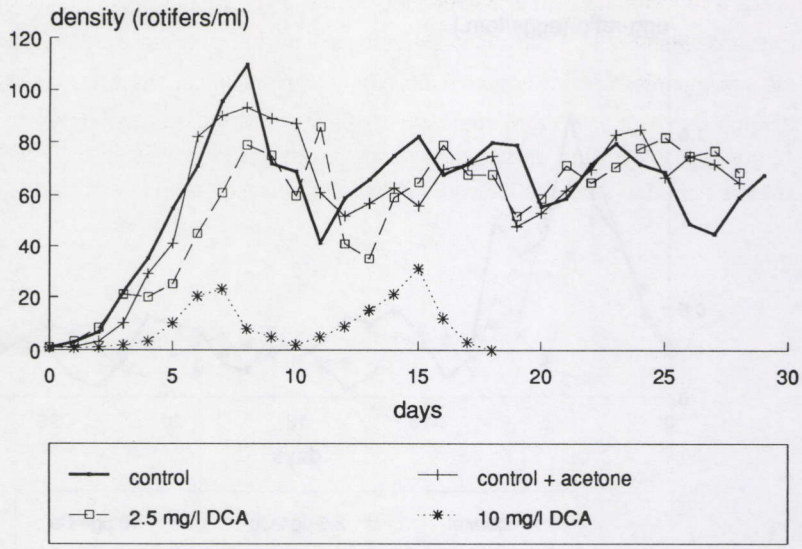


Figure VIII.5. Population curves of B. calyciflorus exposed to DCA.

phase of the population growth, a lower maximum density (85 rot./ml on day 11) and the absence of the typical population "overshoot" observed in the control treatments. These differences in the shape of the population curve, however, did not affect the carrying capacity of these test populations. Indeed, only a slight, statistically insignificant decrease (7 %) in the K-value was noted (Table VIII.1). The populations exposed to the highest DCA concentration persisted at very low densities (mean density= 13 rot./ml) for approximately 2 weeks after which they declined to extinction (day 18).

Both lindane concentrations tested adversely affected ($p<0.05$) the carrying capacity of the rotifer populations. Compared to the control, the mean K-values of the populations treated with 10 and 20 mg/l lindane were significantly reduced, to 56 and 36 % respectively (Fig. VIII.6 and Table VIII.1).

In the second part of this study the effect of Cu on rotifer population which had already reached their carrying capacity was investigated. The only difference with the experiments described above is that here the rotifer populations were allowed to develop "normally" for 8 days after which the test- and supply vessels were spiked with the required

toxicant concentration. An example of the population growth curves of the 3 test treatments is given in Figure VIII.7. The exponential phase of the three curves is rather similar, although the maximum rotifer densities were attained at different times. The populations treated with 2.5 µg/l Cu (from day 8 onwards) did not seem to be affected, while in those exposed to 10 µg/l Cu, the rotifers persisted at rather low densities for 4 to 5 days after the initial spiking. However, both the 2.5 and the 10 µg/l Cu treatments did not have a permanent

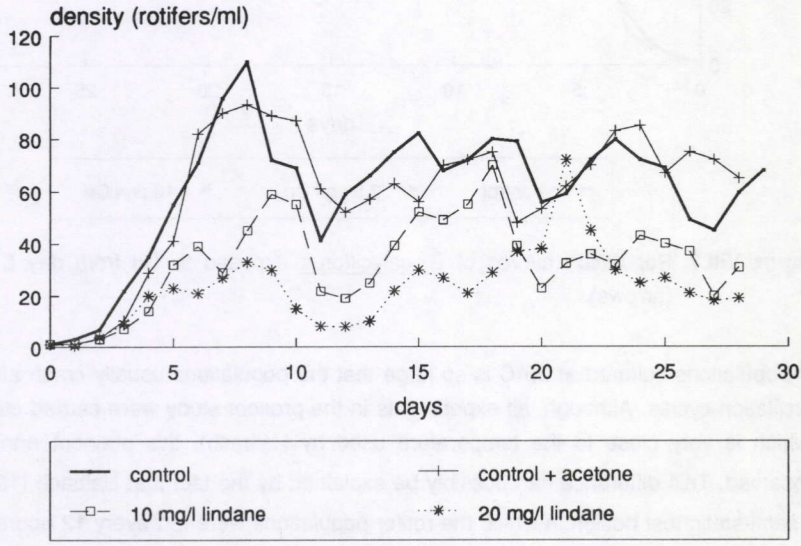


Figure VIII.6. Population curves of B. calyciflorus exposed to lindane.

effect on the rotifer populations. Indeed, the mean carrying capacities of the test populations at the end of the test were not significantly different : 75 (± 10.5), 66.5 (± 12.3) and 63.7 (± 20.4) rot./ml for the control, 2.5 µg/l and 10 µg/l Cu populations, respectively.

VIII.4. Discussion

The general shape of the (control) population curves obtained in the present investigation are very similar to those reported by Halbach (1970, 1973, 1978 a&b) for the same test species. This author, however, reported that the amplitude of the oscillations

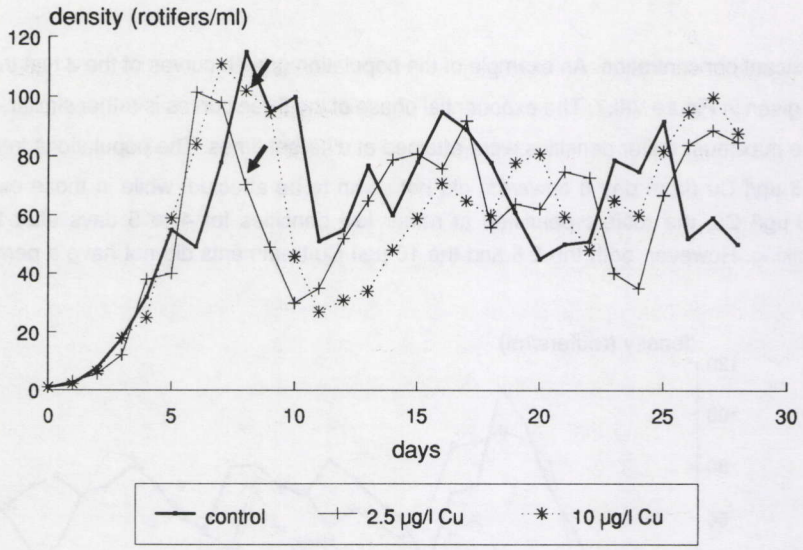


Figure VIII.7. Population curves of B. calyciflorus exposed to Cu from day 8 onwards (arrows).

of populations cultured at 25°C is so large that the populations usually crash after 2 to 3 oscillation cycles. Although, all experiments in the present study were carried out at 24°C (which is very close to the temperature used by Halbach), this phenomenon was not observed. This difference can possibly be explained by the fact that Halbach (1970) used a semi-static test design in which the rotifer populations were fed every 12 hours, while in the present study the rotifers were kept in a flow-through system with a constant food supply. Indeed, the sudden changes in food density in Halbach's semi-static experiment (large differences in food density between the start and the end of the 12- hour periods) might have led to large "overshoots" in population density which in turn, may have resulted in the extinction of the population.

As illustrated by the rather large standard deviations of the mean K-values, the population curves exhibited some variance in average density as well as in the frequency and timing of the oscillations. Consequently, averaging the data of the replicates would have resulted in the loss of the typical oscillating patterns on the populations dynamics. In this context, Halbach et al. (1983) suggested that the use of cross-correlations, followed by auto-correlation analysis on the resulting generalized curve, is a statistically better procedure than averaging, to analyze population data from replicate populations. As the main objective of

our research was to detect significant differences between the treatments, this type of analysis was not performed. Instead, the logistic growth model was fitted to data of each replicate separately and the resulting K-values were compared using analysis of variance.

There are very few studies which have examined the effects of chemicals on the dynamics of invertebrate populations exposed for several consecutive generations. Marshall (1978) showed that cadmium reduced the carrying capacity of Daphnia galeata mendotae populations to such an extent that no threshold (NOEC) concentration for this parameter could be established. This author also reported numerical oscillations, as observed in the present study, around the equilibrium density. van Leeuwen et al. (1987) examined the effects of 10 chemicals on the population parameters of Daphnia magna and compared two test methods : semi-static life table experiments and tests with small expanding populations. Details of the main results obtained in that study are given in chapter 6. These authors found that for most toxicants the carrying capacity of the populations was a more sensitive test parameter than the demographic parameters obtained in the life-table experiments, and attributed this to the fact that in the population studies the organisms encounter a food-limited situation and are thus more susceptible to toxic stress. The magnitude of this increase in sensitivity and the consequences for practical ecotoxicology are discussed in detail in chapter 6.

However, this conclusion does not hold true when the results obtained in the present population studies are compared to those of the life table studies. Indeed, the NOEC's obtained in the life table experiments (with r_m as test criterion) for Cu, PCP and DCA were very similar to those obtained in the population studies. For Cu for example, a reduction of 47 % of the r_m (life-table) was found at 5 µg/l, while in the population test the K had decreased to 50 % in the 10 µg/l treatments. Similarly, in the 0.4 mg/l PCP treatments, the r_m was reduced by 53 % and the carrying capacity by 60 %, for the life table and population tests, respectively. The rotifer populations exposed to 10 mg/l DCA became extinct after 18 days, which is in close agreement with the observation in the life-table test : i.e. the r_m value at this concentration was negative. In the population tests with lindane the lowest concentration tested (10 mg/l) significantly reduced the K while this concentration did not affect the r_m value in the life-table tests. The carrying capacity of the populations exposed to 20 mg/l lindane was reduced to 67 %, while the same concentration in the life table experiments resulted in a 76 % decrease of the r_m value.

Based on these observations it may be concluded that the NOEC's obtained in life table

toxicity tests with B. calyciflorus are very similar to those obtained in experiments with large populations of rotifers. However, in their population studies with the freshwater rotifer B. rubens, Halbach et al. (1983) stated that the use of the "population dynamics" as indicator of toxic stress may be a more sensitive tool than life table toxicity tests. These authors indeed argued that the population dynamics works like a "magnifying glass" which "integrates the slight reductions of the organisms vitality (which can hardly be detected in isolated animals) from thousands of animals into the next higher level of biological organization, thus facilitating detection". Although it is difficult to interpret their results, since no significance testing was performed, we feel that their experimental data do not support these statements. For PCP for example, the rotifers exposed to 0.15 mg/l in the life table tests exhibited a clear reduction in longevity and reproduction while in the population studies, reductions in r_m and K were noted at approximately the same toxicant levels (0.1-0.2 mg/l PCP). Similar conclusions can be drawn from the toxicity studies with the 3 other chemical compounds. In fairness to these authors, it should be mentioned that this excellent study was performed mainly to develop mathematical models which describe the population dynamics of rotifers under toxic stress, however, we feel that the phrase "magnifying glass" is an overstatement and inappropriate. Since its introduction, several authors have used the term "magnifying glass" in connection with the potential use of toxicity studies with populations. In this context the work of van Leeuwen et al. (1987) and Enserink et al. (1991) is discussed at length in chapter 6.

Rao and Sarma (1986) examined the effects of DDT on the life history characteristics of B. patulus in a series of life table experiments, and in a more recent paper (Rao and Sarma, 1990) these authors reported on the effects of this chemical on the population growth of B. patulus in a food limited environment. In both studies they found that DDT toxicity was inversely correlated with the amount of food available. However, comparing the results from the life table tests with those of the population studies it can be concluded that both approaches yielded similar NOEC's (20-30 µg/l DDT).

From this discussion and the results obtained in the first part of this chapter it may be argued that the life table approach (with r_m as test endpoint) and the population approach both provide information on the toxicity of xenobiotics at approximately the same toxicant levels. However, the complexity of assessing the toxicity of chemical substances even at the single species level under laboratory conditions, is illustrated by the population experiments

conducted in the second part of this study. The addition of Cu to rotifer populations after they had reached their first density peak, did not affect the carrying capacity. These Cu concentrations had, however, adversely affected the exponentionally growing populations (see above). A clear explanation for these observation cannot be given. No other laboratory studies have as yet, addressed this issue of the timing of toxicant introduction in relation to the demographic state of the test population.

The population studies in this chapter have shown that within the constraints of the laboratory toxicity test systems, the currently used life table approach provides ecologically relevant information which can be predictive of events occurring at the population level. However, as illustrated in the second part of this study, more work is needed on the effects of chemicals on the various processes at the individual and population level is needed before one can begin to understand or predict, with any degree of certainty, the effects of xenobiotics on communities and ecosystems.

VII.5. Summary

The effects of copper, pentachlorophenol, 3,4-dichloroaniline and lindane on the population dynamics of the freshwater rotifer Brachionus calyciflorus were studied. The toxicity tests, which were conducted in a flow-through bioassay system, were started with small expanding rotifer populations and followed for 28 days. Most test populations exhibited typical sigmoid growth curves with subsequent oscillations around the equilibrium density : the carrying capacity (K). The mean K values for the control populations was around 70 rotifers/ml. For Cu and PCP, the carrying capacity of the test populations were adversely affected (with reductions of 50 and 40 % in the K-value) at 0.01 and 0.4 mg/l, respectively. Rotifer populations exposed to 10 mg/l DCA became extinct after 18 days while those treated with 2.5 mg/l were not affected. Both lindane concentrations (10 and 20 mg/l) tested produced significant reductions in the carrying capacity of the test populations.
The results of these long-term toxicity experiments are compared to those of short-term chronic tests and consequences of this study for routine toxicity testing is discussed.

SUMMARY

In this study the potential use of the freshwater rotifer Brachionus calyciflorus as a test organism for sublethal toxicity testing was examined. Rotifers of the genus Brachionus, especially B. calyciflorus are ideal biological test models because of their small size (little bench space needed), short generation time (reproduction test on several generations), ease of culturing in the laboratory and the availability of resting eggs from which test organisms can be hatched "on demand". In addition to these important advantages (compared to other aquatic test organisms) the selection of B. calyciflorus as test species is ecologically well justified since this cosmopolitan species is not only very abundant but also plays an important role in several ecological important processes in freshwater communities.
Several aspects of the rotifer's ecology under toxic stress were examined with the aim of developing guidelines for conducting sublethal toxicity tests with this organism.

In chapter 1, the morphology, systematics and biology of Brachionus calyciflorus was presented.

In chapter 2, a brief overview of the role of rotifers in freshwater ecosystems was presented. Additionally, the literature on the use of rotifers in aquatic toxicity studies was reviewed. The effects of copper on the feeding, the swimming behaviour and the life history characteristics were studied and the relationships between the different test parameters evaluated. This chapter was an introduction to the methods and the concepts which were developed in detail in the subsequent chapters.

In chapter 3, the swimming behaviour of B. calyciflorus exposed to four model chemicals (copper (Cu), pentachlorophenol (PCP), 3,4 dichloroaniline (DCA) and lindane) was examined. A swimming behaviour test, based on the rotifers' movement rate as they swim over a grid, was described. For 3 toxicants a clear dose-response was observed, with the swimming activity decreasing with increasing toxicant concentrations. For Cu the EC_{50}'s (the concentration which reduces the swimming activity to 50 % of that of the control) decreased from 220 µg/l after an exposure of 5 minutes to 68, 38 and 14 µg/l after exposures of 30, 60 and 300 minutes, respectively. Similar patterns were found for PCP and DCA. The swimming activity of rotifers exposed to lindane however, exhibited a different

response : the EC_{50}'s gradually increased from 13.7 mg/l after an exposure of 5 minutes to significantly higher values of 16.4 and 18.5 mg/l after periods of 60 and 300 minutes, respectively. The results of the swimming activity assays (3 h EC_{50}'s) were found to be similar to those obtained in acute toxicity tests (24 h LC_{50}'s). The potential use and the relevance of this behavioural test criterium for ecotoxicological applications were evaluated.

In chapter 4, the changes in the feeding behaviour of rotifers exposed to sublethal levels of the four chemicals, were examined. A set of standard test conditions was determined in a series of preliminary experiments. A simple bioassay in which the filtration rate of a cohort of rotifers (exposed to the toxic substance for 5 hours) is determined, was presented. The 5 h EC_{50}'s for copper, pentachlorophenol, 3,4-dichloroaniline and lindane were 0.032, 1.85, 41.2 and 8.5 mg/l, respectively. Considering the ecological importance of this criterion and the ease and rapidity by which it can be assessed, it was suggested that a standard toxicity assay, using the rotifers' feeding rate as test endpoint, could be a valuable new approach for rapid toxicity evaluations.

In chapter 5, the development and potential use of a 4-day static renewal test (4-day Life Table test) and a 3-day static test (3-day Population Growth test) with B. calyciflorus were described and the toxicity of the 4 model chemicals was evaluated. The NOEC's, based on the demographic parameter r_m (intrinsic rate of increase), obtained with the 4-day L.T. test were 0.0025, 0.4, 5 and 20 mg/l for Cu, PCP, DCA and lindane, respectively. Similar results were obtained with the 3-day P.G., tests for which NOEC's of 0.005 mg/l, 0.8, 20 and 10 mg/l, respectively, were recorded. The mean C.V. between replicated 3-day P.G. tests was 10 %, indicating a good intra-laboratory reproducibility of the test results. For Cu and PCP, the sensitivity of B. calyciflorus compared favourably to chronic toxicity tests with Daphnia magna while for the other 2 compounds B. calyciflorus, proved to be insensitive. Considering the increasing need for relatively short toxicity tests, it is suggested that the 2 short-chronic bioassays described here, could be attractive new tools for routine toxicity evaluations. The major advantages associated with these tests are : (a) they can be completed within 1 working week, (b) since the test organisms are obtained by hatching cysts, no stock culturing of test organisms is required.

In chapter 6, the influence of temperature and food-availability on the demographic characteristics of the test organism were studied. Life table experiments were performed at

3 temperatures and 3 food levels. The intrinsic rate of natural increase (r_m) of the animals was positively correlated with the temperature, which was mainly a consequence of the response of the individual rates of development and reproductive timing. Similarly, increasing food concentrations also resulted in higher r_m values, but here this was due to increased net-reproductive rates. Based on these results, a set of practical experimental conditions for conducting life table toxicity tests with B. calyciflorus were proposed.
Additionally, the effects of the food-availability on the results of chronic toxicity tests were investigated. In 3 series of life table tests, the rotifers were cultured at low, moderate and high food levels and exposed to copper and pentachlorophenol. The LOEC's based on the demographic parameters increased with increasing food concentrations for both Cu and PCP. For both toxicants, the ratio between the LOEC's obtained in the toxicity tests performed at high and low food levels was 4. The implications of these findings for routine aquatic toxicity testing were discussed.

In chapter 7, the effects of copper and pentachlorophenol on the life history characteristics of four consecutive generations of rotifers were assessed in multi-generation life table experiments. In a second series of experiments the effects of the maternal age on the susceptibility of F_1 offspring to these chemicals was investigated.
For both chemicals, the LOEC's (based on the intrinsic rate of increase) observed in the first and second generation life tables were similar to those noted in the initial (maternal) life cycle experiments. No LOEC's, however, could be derived (within the range of toxicant concentrations tested) from the results of the toxicity tests performed with the third generation rotifers.
In experiments investigating the effects of the maternal age, the LOEC's derived from the life table tests with the maternal generation and those from the experiments with the offspring originating from young mothers were identical for both Cu and PCP. Rotifer neonates originating from "old" mothers were less susceptible to toxic stress and no LOEC could be established (within the concentration range tested). Compared to the single generation bioassays described in chapters 5 and 6, the multi-generation toxicity tests did not reveal additional information on the toxicity of Cu and PCP. Several aspects on the design of chronic toxicity tests were discussed in the context of the results obtained and the consequences of these findings for applications in ecotoxicology were evaluated.

In chapter 8, the effects of the four chemicals on the dynamics of large rotifer populations were studied. A flow-through bioassay system for conducting this type of tests was presented. The toxicity tests were initiated with small expanding rotifer populations and observed for 28 days. Most test populations exhibited typical sigmoid growth curves with subsequent oscillations around the equilibrium density : the carrying capacity (K). The mean K values for the control populations was around 70 rotifers/ml. For Cu and PCP, the carrying capacity of the test populations were adversely affected (with reductions of 50 and 40 % in the K-value) at 0.01 and 0.4 mg/l, respectively. Rotifer populations exposed to 10 mg/l DCA became extinct after 18 days while those treated with 2.5 mg/l were not affected. Both lindane concentrations (10 and 20 mg/l) tested produced significant reductions in the carrying capacity of the test populations.
The NOEC's observed in these long-term toxicity experiments were similar to those obtained with the short-term chronic tests described in chapter 5.

One of the main objectives of the research presented in this thesis was to examine the degree of correlation of the results obtained with different test methods, with various exposure periods and with different test criteria. The differences between the NOEC's obtained with the simplest of the toxicity tests, namely the 3-day population growth test (P.G.) and the most elaborate test, namely the 28-day flow-through test (F.T.) were, for all compounds tested, relatively small. Indeed, the ratios of the NOEC's from the 3-day P.G. to those of the 28-day F.T. tests were only 4, 1 and 4 for Cu, PCP and lindane, respectively. For DCA no NOEC 28-day F.T. could be established within the concentration range tested. From this comparison it can be concluded that very little information on the level of toxicity of the chemical is gained by increasing the exposure time and/or the experimental complexity, at least for this set of chemical compounds and methodologies used. Thus, the relatively simple short chronic toxicity test, such as the 3-day P.G. and the 4-day rotifer life cycle tests seem to be good predictors of adverse effects occurring at the population level.

The two short toxicity tests using the rotifers' swimming behaviour and food-uptake as test criteria, also produced interesting results. The ratios between the 3 h EC_{50}'s obtained with the swimming assays and the 24 h LC_{50}'s obtained with the acute toxicity tests were : 1.2, 2, 0.67 and 0.38 for Cu, PCP, DCA and lindane, respectively. The same comparison of the 5 h food uptake test (EC_{50}) and the 24 h mortality (LC_{50}) test produced the following ratios : 0.58, 2.3, 0.74 and 0.74 for Cu, PCP, DCA and lindane, respectively. It can thus be

concluded that the "very short" (a few hours) toxicity tests, using behavioural and physiological parameters as test criteria, were very good indicators of acute toxicity levels, and have a good potential for rapid hazard prediction of xenobiotics.

GENERAL CONCLUSIONS and PERSPECTIVES

The only aquatic invertebrates which are used in a regulatory framework to assess the hazard of chemicals to biological communities in the pelagic part of aquatic ecosystems are cladoceran crustaceans. From an ecological point of view, however, this practice is disturbing, since the other two main freshwater zooplankton groups (copepod crustaceans and rotifers) important to the structure and function of freshwater communities are neglected. In order to (partly) fill this void, the freshwater rotifer Brachionus calyciflorus was selected as model organism for this study. As mentioned in the summary, it has been shown in literature that rotifers of the genus Brachionus are not only very abundant but also play an important role in the dynamics of pelagic biological communities. The main "practical" reasons which warrant the selection of this species are its small size, its short generation time and the availability of resting eggs of this species. Indeed, the recent developments in the controlled production under laboratory conditions, of B. calyciflorus resting eggs - often called cysts - have greatly increased the potential use of this test organism for routine toxicity assessment. The use of cysts as biological starting material for obtaining test organisms completely eliminates one of the main bottle-necks in routine aquatic toxicology, i.e. the need for the continuous culture and maintenance of test organisms in sufficient numbers. This concept, which has been gaining ground in recent years, has led the development of toxicity screening tests (24 h LC_{50}) with the freshwater rotifer B. calyciflorus and its estuarine counterpart B. plicatilis. Similar screening tests with the crustaceans Streptocephalus proboscideus and Artemia salina (for freshwater and marine toxicity testing, respectively) have also been proposed.

Considering these important facts and building on the experience gained during the development of the acute toxicity test, the potential use of B. calyciflorus for the development of sublethal toxicity tests was examined in the present study. The main objectives of this research were to : (a) determine the sensitivity of various sublethal criteria in comparison to the "conventional" mortality endpoint, (b) examine the potential use of B. calyciflorus for the development of a (short) chronic toxicity test, (c) determine the degree of correlation between the different test endpoints and evaluate the "ecological" relevance of the developed test methods.

Although the development and use of aquatic toxicity tests with fish, using behaviourial responses as test endpoints, has increased during the last few years, very little is known about the influence of pollutants on the behaviour of invertebrates. In studies with fish, however, it has been shown that behaviourial modifications not only provide an index of sublethal toxicity, but may also reflect the potential for subsequent mortality, possible impairment of food intake and for reproduction. Although causal links between these different test criteria are hard to establish, the present study has demonstrated the potential use of the swimming activity of B. calyciflorus as a rapid and sensitive indicator of toxic stress. It was found that for the 4 chemicals (see summary) tested the results of the swimming activity assay (3 h EC_{50}'s) were very similar to those obtained in the acute toxicity tests (24 h LC_{50}'s). In addition, the lowest concentrations at which significant reductions in the swimming activity of the rotifers were noted, were usually of the same order of magnitude as those at which increased mortality and reduced reproduction was noted in the life cycle tests. The major drawback associated with the swimming assay is that it is rather labour intensive. One test requires 3 to 5 man hours to complete, a large part of which is "observation time", i.e. observing the rotifer's swimming activity with the aid of a dissection microscope. However, because of the demonstrated potential use and the associated advantages (i.e. sensitivity and short exposure) of this type of assay, we believe that a behavioural test with rotifers could be a powerful new tool for rapid toxicity assessments. Furthermore, it is suggested that the adaptation of existing computer-assisted tracking systems for toxicity testing purposes might be an attractive line of research to develop. This type of work would not only increase the fundamental knowledge of toxicant induced behavioural changes, but could also lead to the development of an automated, less labour-intensive toxicity test procedure.

This study has also shown that the feeding behaviour of the rotifers was a rapid and sensitive indicator of toxic stress. The results obtained in 5 hour feeding tests, in which the filtration and ingestion rates were measured, were very similar to those found in the 24 hour acute toxicity tests (the difference being less than a factor 2). The proposed test protocol, however, should be considered as preliminary. Indeed, for routine applications of this type of toxicity testing with B. calyciflorus more research is needed to improve the practicability of the test. Test variables such as the type of exposure vessel, the exposure time and the algal counting method can certainly be improved. This should lead to an increased standardization and reproducibility of this method. Furthermore, the substitution of the algae by an inert food could also enhance the potential use of this method for routine toxicity

assessments. Additional, more fundamental work on the effects of toxicants on rotifer feeding behaviour during long-term exposures, gut passage times and toxicant-induced changes in the assimilation rates and energy budgets should also be encouraged. Indeed, this type of research would help to fully assess the ecological relevance of the "feeding" of rotifers as an ecotoxicological test criterion.

Two short-chronic toxicity tests with B. calyciflorus were proposed. The 3-day static population growth test, in which the population growth of a small cohort of rotifers under toxic stress is assessed, has a very simple test design and requires little expertise to perform. The second test is based on life table experiments in which the effects of the toxicants on the age-specific survival and reproduction are examined. From this data the demographic parameters : intrinsic rate of natural increase, net-reproduction, life expectancy at hatching and generation time can be calculated and used as test criteria. None of the test criteria was the most sensitive for all chemicals tested. Nevertheless, the intrinsic rate of natural increase was suggested as test criterion for the life table toxicity studies, because of its ecological importance and the fact that it integrates survival and reproduction. A life table test performed at 25°C typically takes 10 days to complete. However, we have shown that when using the intrinsic rate of natural increase as test parameter the test duration can be reduced to only 4 days, without loss of information on the toxicity of the chemical. Considering the increasing need for short chronic toxicity tests, the two short chronic bioassays developed in this study appear to be attractive new tools for routine toxicity evaluations. Further assessment of the intra- and interlaboratory reproducibility of the results obtained with these methods is needed. Additionally, more information on the sensitivity of B. calyciflorus (as compared to other test species) is required. Finally, it is suggested that, in analogy to the feeding tests, attempts should be made to substitute the live rotifer food (algae) with a suitable inert food. Indeed, the use of rotifer cysts in combination with an inert food would completely eliminate the need for culturing both test organisms and their food, thus greatly enhancing the cost-efficiency and routine application of these tests.

In literature, unrealistic high food levels used in chronic toxicity tests with invertebrates have been suggested as one of the obstacles which hamper the field validation of toxicity test results obtained in laboratory systems. The extent to which food availability can change chronic bioassay results has, however, received little or no attention. This issue was addressed in the present study. From the life table toxicity experiments with B.

calyciflorus performed at different food levels it can be concluded that food availability does affect the results of chronic toxicity tests (more food = "less toxic") although the differences observed were rather small. Considering the relatively large inter-laboratory variability of even the most standardized toxicity tests, the difficulties in extrapolating single species laboratory test results to real world situations and the large "safety factors" incorporated in hazard assessment schemes, this study has shown that the issue of food availability in chronic bioassays should not be a major concern for routine toxicity evaluations.

One of the potential weaknesses of the currently used chronic toxicity assays with invertebrates is the fact that the test organisms are not exposed to the toxicant during important parts of their life cycle (oogenesis and embryogenesis). Additionally, in all regulatory chronic toxicity tests with invertebrates only the number of living young are scored and no attempt is made to investigate if the F_1 generation shows disturbed development, growth or reproduction. Both of these "hypothesized" weaknesses have never been examined. In this context, the life history characteristics of four generations of rotifers continuously exposed to the same toxicant concentrations were examined. For the two chemicals tested (copper and pentachlorophenol) this multi-generation approach did not provide more information on the toxicity of the chemicals than the "conventional" single generation bioassays. The two "potential weaknesses" hypotheses associated with the currently used chronic bioassays, mentioned above, can thus be rejected, at least for B. calyciflorus exposed to copper and pentachlorophenol. Additionally, it should be mentioned that multi-generation experiments are very labour intensive and certainly not suited for routine applications.

The flow-through bioassay system developed for performing long-term toxicity experiments with large populations of rotifers proved to be a useful and reliable tool. Indeed, variation among the test replicates was acceptable and no major technical problems were encountered. Although this test design should not be used for routine applications (too labour intensive), it was, however, extremely important for the validation of the results obtained with the other test methods developed in this study. In the framework of single species laboratory toxicity testing, the highest level of biological organization which can be tested is the population level. Significant reductions in the carrying capacity of rotifer populations were observed at approximately the same toxicant concentrations which adversely affected the organisms in the single generation life table tests. It can thus be concluded that within the

constraints of laboratory toxicity testing, the currently used life table approach provides ecologically relevant information which is predictive of events occurring at the population level, at least for B. calyciflorus exposed to copper, pentachlorophenol, 3,4-dichloroaniline and lindane.

SAMENVATTING

Het proefschrift beschrijft de bevindingen omtrent het gebruik van de zoetwaterrotifeer Brachionus calyciflorus als testorganisme voor subletale toxiciteitstesten. Rotiferen van het genus Brachionus in het algemeen, en van de soort B. calyciflorus in het bijzonder, zijn uitstekende biologische testmodellen vermits ze zowel een aantal fundamenteel biologische als practische voordelen bieden in vergelijking met andere testorganismen.
De voornaamste voordelen van deze proefdieren zijn:

- ze zijn klein zodat toxiciteitstesten weinig laboratoriumruimte innemen,
- ze bezitten een (zeer) korte generatietijd zodat reproduktietesten met meerdere generaties kunnen uitgevoerd worden,
- ze zijn zeer eenvoudig te kweken in het laboratorium,
- de rusteieren (cysten) van deze soort zijn (commercieel) verkrijgbaar,
- de cysten kunnen op een eenvoudige manier ontloken worden zodat testorganismen continu beschikbaar zijn en de kweek van deze rotiferen volledig overbodig wordt.

De keuze van B. calyciflorus is tevens ecologisch verantwoord omdat deze soort niet alleen een wereldwijde verspreiding heeft, maar ook omdat deze organismen in grote aantallen voorkomen in zoetwaterbiotopen en een belangrijke rol spelen in de dynamiek van pelagische gemeenschappen.

In dit proefschrift werden de gevolgen onderzocht van toxische stress op diverse aspecten van de ecologie van B. calyciflorus met de bedoeling subletale toxiciteitstesten met deze soort te ontwikkelen.

In hoofdstuk 1 worden de morfologie, de systematiek en diverse aspecten van de biologie en ecologie van B. calyciflorus kort besproken.

In hoofdstuk 2 worden aan de hand van een korte literatuurstudie de rol van rotiferen in zoetwatergemeenschappen geschetst en wordt het gebruik van deze organismen

in de aquatische toxicologie beschreven. Verder worden de methoden en de concepten geïntroduceerd die van belang zijn voor de volgende hoofdstukken van dit proefschrift. Deze aspecten worden geïllustreerd aan de hand van een voorbeeld ; namelijk de invloed van koper op het zwemgedrag, de voedselopname en de demografische parameters werd onderzocht en de relaties tussen deze verschillende testcriteria werden geëvalueerd.

In hoofdstuk 3 wordt de invloed van 4 in de ecotoxicologie veel gebruikte chemicaliën (koper (Cu), pentachloorphenol (PCP), 3,4-dichlooraniline (DCA) and lindaan) op het zwemgedrag van B. calyciflorus onderzocht. Een toxiciteitstest wordt beschreven waarin de zwemactiviteit van een rotifeer wordt bepaald. Voor 3 chemische stoffen werd een duidelijke concentratie-respons gevonden, waarbij de zwemactiviteit afnam bij toenemende concentraties. Voor rotiferen blootgesteld aan Cu daalden de EC_{50}'s van 220 µg/l na een blootstellingsperiode van 5 minuten tot 68, 38, en 14 µg/l na respectievelijk, 30, 60 en 300 minuten. Een gelijkaardig verloop van de concentratie-response curve werd waargenomen met PCP en DCA. De zwemactiviteit van rotiferen blootgesteld aan lindaan daarentegen, vertoonde een ander patroon : de EC_{50}'s namen geleidelijk toe in functie van de blootstellingsduur. De EC_{50}'s bepaald na 5, 60 en 300 minuten waren respectievelijk 13.7, 16.4 and 18.5 mg/l lindaan. De waarden van de 3 uur EC_{50}'s bekomen met de in deze studie ontwikkelde "zwemactiviteit" toxicititeitstest waren vergelijkbaar met de 24 uur LC_{50}'s bepaald met acute mortaliteitstesten.

In hoofdstuk 4 wordt de invloed van de 4 chemische stoffen op de voedselopname van B. calyciflorus bestudeerd. In een reeks preliminaire experimenten werden de standaard testcondities bepaald voor een eenvoudige "voedselopname" toxiciteitstest. In deze biotest werd de voedselopnamesnelheid gemeten van een kleine populatie rotiferen blootgesteld aan de toxische stof gedurende 5 uren. De berekende 5 uur EC_{50}'s waren 0.032, 1.85, 41.2 and 8.5 mg/l voor respectievelijk Cu, PCP, DCA en lindaan. Gezien het ecologisch belang van dit testcriterium en de snelheid en eenvoud waarmee het kan gemeten worden, kan besloten worden dat een "voedselopname" toxiciteitstest met rotiferen een waardevolle nieuwe benaderingswijze blijkt te zijn voor de snelle evalutie van de toxiciteit van xenobiotische stoffen.

In hoofdstuk 5 wordt de ontwikkeling en het potentieel gebruik van een 4 dagen semi-statische toxiciteitstest (4 dagen LevensTabel test) en een 3 dagen statische test (3

dagen PopulatieGroei test) behandeld. De toxiciteit van de 4 hoger vermelde chemische stoffen werd bepaald met deze methoden. Met de 4 dagen L.T. test werden de volgende NOEC's bekomen (testcriterium r_m : momentane aanwasmaat) : 0.025, 0.4, 5 en 20 mg/l voor respectievelijk Cu, PCP, DCA en lindaan. Gelijkwaardige NOEC's (respectievelijk 0.005, 0.8, 20 en 10 mg/l) werden gevonden met de 3 dagen P.G. test. De resultaten bekomen met deze toxiciteitstesten bleken goed reproduceerbaar te zijn. Voor beide biotesten was de gevoeligheid van B. calyciflorus voor Cu en PCP vergelijkbaar met deze van chronische Daphnia magna testen. Voor de twee andere chemische stoffen daarentegen bleek B. calyciflorus weinig gevoelig te zijn.
De conclusie van dit deel van het onderzoek is dat deze 2 "short-chronic" testen, betrouwbare en aantrekkelijke testmethodes zijn voor routine bepalingen van de toxiciteit van xenobiotische stoffen. De belangrijkste troeven van deze testen zijn : (a) ze kunnen beëindigd worden binnen 1 werkweek, (b) doordat testorganismen verkregen worden door het ontluiken van rusteieren is het continu kweken van organismen overbodig.

In hoofdstuk 6 wordt de invloed van de temperatuur en het voedselaanbod op de demografische parameters van de testorganismen bestudeerd. Hiervoor werden levenstabel-experimenten uitgevoerd bij 3 temperaturen en 3 voedselconcentraties. De momentane aanwasmaat (r_m) van de organismen was positief gecorreleerd met de temperatuur, wat vooral een gevolg was van de hogere individuele ontwikkelingssnelheid en van de snellere reproductieve aanzet. Hogere waarden van de r_m werden ook vastgesteld bij hogere voedselconcentraties wat een gevolg is van de hogere netto-reproductie. Uit de resultaten van deze experimenten werden praktische testcondities afgeleid voor het uitvoeren van toxiciteitstesten met B. calyciflorus, steunend op levenstabeltechnieken. In dit hoofdstuk werd ook nagegaan in welke mate verschillende voedselconcentraties de resultaten van chronische toxiciteitstesten beinvloeden. Hiertoe werden levenstabelexperimenten uitgevoerd met rotiferen, gevoed bij lage, matige en hoge voedselconcentraties, terwijl de proeforganismen tevens blootgesteld waren aan koper of pentachloorphenol. Voor beide chemische stoffen namen de LOEC's (met de demografische parameters als testcriteria) toe in functie van stijgende voedselconcentraties. De verhouding tussen de LOEC's verkregen in de testen met een hoog en laag voedselaanbod was 4 voor beide chemicaliën. De implicaties van deze observaties voor routine toxiciteitstesten worden besproken.

In hoofdstuk 7 worden (in een reeks multigeneratie-toxiciteitstesten) de effecten van Cu en PCP op de demografische parameters van 4 opeenvolgende rotiferen generaties bestudeerd. In een tweede reeks experimenten wordt de invloed van de leeftijd van het moederdier op de gevoeligheid van haar nakomelingen (voor Cu and PCP) besproken.
Voor beide chemicaliën waren de LOEC's (met r_m als testcriterium), bepaald voor de eerste (F_1) en tweede (F_2) generatie organismen, gelijk aan de LOEC's bekomen in het moedergeneratie-experiment (P). Binnen de gebruikte concentratiereeks kon echter geen LOEC bepaald worden in de toxiciteitstesten met de derde generatie rotiferen.
In de tweede reeks experimenten waren de LOEC's, bekomen in de moedergeneratie-testen (P) voor beide chemische stoffen identiek aan de LOEC's afgeleid uit de testen met nakomelingen van "jonge" moeders (F_{11}). De nakomelingen geproduceerd door "oude" moeders (F_{14}) waren echter minder gevoelig aan toxische stress en voor beide chemicaliën kon geen LOEC bepaald worden. In vergelijking met de conventionele toxiciteitstesten (één generatie), beschreven in hoofdstukken 5 en 6, blijken multigeneratietesten geen bijkomende informatie op te leveren over de toxiciteit van Cu en PCP. Op basis van de verkregen resultaten werden verschillende aspecten van chronische toxiciteitstesten besproken.

In hoofdstuk 8 wordt de invloed van de 4 gebruikte chemicaliën op de populatiedynamica van grote rotiferenpopulaties onderzocht. Een doorvloeisysteem voor het uitvoeren van dergelijke toxiciteitstesten wordt beschreven. De testen werden gestart met kleine, exponentieel groeiende rotiferenpopulaties en bestudeerd gedurende 28 dagen. De meeste testpopulaties vertoonden een typische sigmoide groeicurve gevolgd door oscillaties rond de evenwichtsdensiteit : de draagkracht (K) van de populatie. De gemiddelde K-waarde in de controlepopulaties bedroeg 70 rotiferen per ml. De draagkracht van de populaties blootgesteld aan 0.01 mg/l Cu en deze blootgesteld aan 0.4 mg/l PCP was significant lager dan deze in de controlepopulaties (reducties van respectievelijk 50 en 40 %). De populaties behandeld met 10 mg/l DCA waren volledig uitgestorven na 18 dagen terwijl 2.5 mg/l DCA geen effect had op de draagkracht van de testpopulaties. Significante reducties van K werden genoteerd in beide lindaanbehandelingen (10 en 20 mg/l). De NOEC's bekomen met deze lange duur experimenten waren vergelijkbaar met deze bekomen in de "short chronic" testen beschreven in hoofdstuk 5.

Eén van de hoofdobjectieven van dit onderzoek was het nagaan van de mogelijke correlatie tussen de resultaten verkregen met de verschillende testmethoden, met

verschillende blootstellingstijden en met verschillende testcriteria. Voor alle chemische stoffen waren de waargenomen verschillen tussen de NOEC's verkregen met de eenvoudige toxiciteitstest, namelijk de 3 dagen populatie groei test (P.G.) en de NOEC's bekomen met de complexe, 28 dagen doorvloeitesten, relatief klein. De verhouding van deze beide NOEC's was 4, 1 en 4 voor respectievelijk Cu, PCP en lindaan. Voor DCA kon geen NOEC afgeleid worden. Er kan dus geconcludeerd worden dat (zeer) weinig bijkomende informatie over de toxiciteit van de chemische stoffen bekomen wordt door het verlengen van de blootstellingstijd en/of de toenemende complexiciteit van de testopstelling. Er dient echter vermeld te worden dat deze conclusie gebaseerd is op slechts 4 chemische stoffen. De relatief eenvoudige 3 dagen P.G. en de 4 dagen L.T. testen blijken een goede "predictie" te geven van de concentraties van chemicalieën die nadelige effecten hebben op populaties.

Veelbelovende resultaten werden ook bekomen met de 2 (zeer) korte toxiciteitstesten, waarin de zwemactiviteit en de voedselopname van de rotiferen werd nagegaan. De verhouding tussen de 3 uur EC_{50}'s, verkregen in de zwemactiviteit testen en de 24 uur LC_{50}'s, bekomen in de acute mortaliteitstesten, varieerde tussen 0.38 en 1.2 (voor de 4 gebruikte chemische stoffen. Voor dezelfde vergelijking voor de 5 uur voeselopnametest (EC_{50}) en de 24 uur mortaliteitstest (LC_{50}) varieerden de ratios tussen 2.3 en 0.58. Hieruit kan besloten worden dat deze (zeer) korte bioassays, waarin gebruik wordt gemaakt van gedrags- en fysiologische parameters als testcriteria, een goede indicatie geven van de concentraties die acute mortaliteit veroorzaken (na een blootstelling van 24 uur). Dergelijke korte toxiciteitstesten zijn dus uiterst geschikt voor snelle toxiciteitsevaluaties van xenobiotische stoffen.

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