THE ROLE OF CYANOBACTERIAL TOXINS AS GRAZING

INHIBITORS IN THE FRESH WATER CLADOCERAN

DAPHNIA MAGNA STRAUS

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DECLARATION

This thesis embodies the results of scientific experimental investigations carried out by L. P. Jayatissa at the Institute of Aquaculture, University of Stirling, during the period of November 1991 to September 1994. The thesis has been composed independently by L. P. Jayatissa and no part of this work has been submitted for any other degree.

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Abstract

Life history parameters of the cladoceran *Daphnia magna* were compared under several combinations of defined media and green algal diets in order to select a standardized and therefore repeatable culture system for *D. magna*. It was found that none of the defined media tested were nutritionally sufficient to replace natural media. However, Elendt M_4 medium when used in conjunction with *Chlorella minutissima* was deemed the best combination representing a defined medium for *Daphnia* culture. It was noted that bacterial contaminants in algal diets can compensate for some of the nutritional deficiencies present in defined culture media.

The effects of short term (24 h) and long term (4 - 5 d) exposure to toxic *Microcystis aeruginosa* on the feeding rate of *D. magna* were investigated by comparing 24 h feeding rates on *Chlorella vulgaris*, non-toxic *M. aeruginosa* (strain CYA 43) and toxic *M. aeruginosa* (strain PCC 7820). Toxic *M. aeruginosa* suppressed the feeding rate of *D. magna* not only on *M. aeruginosa* itself but also on other algae present. However the cyanobacterial toxin, microcystin-LR, was not the main causative factor for the feeding inhibition. It was hypothesised that factors present on the surface of *M. aeruginosa* cells caused a rapid suppression of feeding rate. Inhibition of feeding was further suppressed by the poor quality of *M. aeruginosa* as a food source for *D. magna*.

Microcystin-LR was the primary cause of the death of daphnids which were exposed to toxic *M. aeruginosa* cells, confirming that microcystin-LR is toxic to *D. magna*. However, at concentrations which occur in natural waters, dissolved purified microcystin-LR had no measurable effect on the feeding rate or life history of *D.*

magna. The toxicity of intact cells of *M. aeruginosa* was three orders of magnitude greater than that of purified microcystin-LR. A difference in bioavailability of microcystin-LR or a cumulative effect of an undescribed toxicant were thought to be the reason for toxicity differences.

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CHAPTER 1

GENERAL INTRODUCTION

1.1 Introduction

Cyanobacteria are widely recognized as a group of phytoplankton which predominate over other phytoplankton groups in eutrophic fresh waters, particularly during summer (Codd & Bell, 1985; de Bernardi & Giussani, 1990; Carmichael, 1994). As a result, heavy growth of cyanobacteria or cyanobacterial blooms are a more frequent phenomenon in eutrophic fresh waters. Since early in the last century, cyanobacteria and their blooms have been the subject of many studies for academic, economic and environmental reasons (Fay, 1983). However in recent times there has been a upsurge of interest in cyanobacteria due mainly to the fact that they have been recognized as a toxigenic phytoplankton group.

Survey reports over the last few years indicate that about 50% of all summer cyanobacterial blooms tested were toxic (Sivonen *et al.*, 1990a; Lawton & Codd, 1991; Carmichael, 1992). Toxic cyanobacterial blooms have been responsible worldwide for intermittent but repeated cases of sickness and death in livestock, pets and wildlife as a result of consumption of water containing toxic cyanobacteria or cyanobacterial toxins (Codd & Bell, 1985; Sivonen *et al.*, 1990a; Lawton & Codd, 1991; Carmichael, 1992). The first scholary report on field poisoning by cyanobacteria and their blooms was published by Francis in 1878 (cited in Carmichael, 1994). Published literature on toxic cyanobacteria even goes back to 1800s, but most of the early literature are in circulation under names other than that of cyanobacteria (Carmichael, 1981).

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1.2 Taxonomic clarification of cyanobacteria

A certain group of prokaryotic organisms containing chlorophyll-a and phycobilins (*i.e.* a group of water soluble plant pigments) has been considered as a special group of algae since early in the last century. According to the international code of botanical nomenclature, it has been named variously as Mixophyceae, Phycochromophyceae, Cyanophyceae and Schizophyceae etc. (Fay, 1983; Castenholz & Waterbury, 1989). These names have been used at the liberty of the authors, but the name Cyanophyceae (Gr. n. *kyanos*, greenish blue; *Phykos*, algae; hence blue-green algae) has survived the longest and is still widely used by phycologists.

The term "cyanobacteria" was first proposed by Stanier (1974) based on the prokaryotic and bacterial culture properties of these phototrophs. The traditional characters employed for the identification of blue green algal genera and species were those determined from specimens collected from the field. However, studies on axenic cultures revealed that many of those characters used to discriminate not only different species but even different genera were not expressed in cultures or varied with culture conditions (Castenholz & Waterbury, 1989). Manipulation of culture conditions directly influenced the morphology of blue green algae (Ecker *et al.*, 1981). As a remedy to these problems Stanier *et al.*, (1978) proposed that cyanobacteria be named under the rules of International Code of Nomenclature of Bacteria (ICNB). Although many objections arose from the botanical community against using the ICNB classification and nomenclature for blue green algae, it is now applied in parallel with the previous nomenclature system (Castenholz & Waterbury, 1989). The name

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cyanobacteria is now widely used by bacteriologists as well as many botanists.

1.3 Cyanobacterial blooms

Algal blooms or heavy growth of algae in waters are not a recent discovery; according to Hallegraeff (1993) the first written reference to algal blooms appears in the Bible. In general, algal blooms are a result of eutrophication which is a natural phenomenon caused by enrichment in waters with nutrients. Although the appearance of blooms is a natural phenomenon, scientists believe that some human activities (*e.g.* man induced eutrophication) may be involved in increasing the frequency and intensity of algal blooms worldwide (Skulberg *et al.* 1984; Maclean, 1993).

A series of factors appears to be involved in the ecological success of this group in eutrophic waters. As King (1970) suggested, one reason for the success of cyanobacteria in water at elevated pH levels is their great efficiency in utilizing CO_2 at such pH levels. This fact has been confirmed by Shapiro (1973) in experiments with mesocosms. Castenhloz & Waterbury (1989) report that the temperature optimum for growth of most cyanobacteria is higher by at least several degrees than for most eukaryotic algae. This tendency may also play an important role in the summer dominance of cyanobacteria in temperate latitudes. Moreover, it is likely that the intensity and frequency of cyanobacterial blooms will increase as a result of global warming.

It is recognized that cyanobacteria grow well and form blooms by dominating the

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other algae when the following four conditions coincide; neutral or alkaline water, mild or quiet wind, water temperatures of between 15 and 30°C and moderate to high levels of nutrients, particularly phosphorus and nitrogen (Skulberg, 1984; Carmichael, 1994). The ratio of total nitrogen to total phosphorus is one of the main factors which determines the composition of cyanobacterial blooms (Codd & Bell, 1985), since phosphorus loading may make nitrogen the limiting nutrient and enhance the growth of nitrogen-fixing species.

It is now recognized that algal and cyanobacterial blooms in natural waters are frequent and extensive, and furthermore are becoming increasingly unpredictable and difficult to control (Skulberg *et al.*, 1984).

1.4 Toxic cyanobacterial blooms.

The first indications that algal blooms could be harmful resulted with a marine bloom which developed in Papua New Guinea in 1972 (Hallergraeff & Maclean, 1989). Since then public attention has been attracted not only by marine blooms but also by freshwater blooms. Reports of toxic fresh water phytoplankton blooms are mainly due to cyanobacteria (Codd & Bell, 1985; Carmichael, 1988).

While cyanobacterial blooms are found world wide, it was generally thought that toxic blooms were somewhat restricted in their distribution (Skulberg *et al.* 1984); however, this view has recently changed. Toxic blooms have been found in inland and coastal waters throughout the world including Argentina, Australia, Bangladesh, Bermuda,

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Brazil, Canada, Chile, China, Europe (16 countries), India, Israel, Japan, Newzealand, South Africa, Thailand and United States (22 states), (Codd & Bell, 1985; Skulberg *et al.*, 1984; 1993; Carmichael *et al.*, 1990). This distribution implies that toxic cyanobacterial blooms can be found wherever the climate supports heavy summer blooms or a permanent standing crop.

A toxic bloom can occur uniformly throughout the water body or in a mosaic pattern with one part of the bloom being toxic and a nearby part being non-toxic (Carmichael & Gorham, 1982). Alteration between toxic and non-toxic blooms have also been observed (Ekmann-Ekebom *et al.*, 1992). Spatial and temporal variations in bloom toxicity as well as in the distribution of toxic cyanobacteria have been recorded, even within the same water body (Codd & Bell, 1985; Skulberg *et al.*, 1984; Vakeria *et al.*, 1985; Ekman-Ekebom *et al.*, 1992; Watanabe *et al.*, 1992). Variations in environmental factors and age of the bloom may be the main factors determining such variability in toxicity (van Der Westhuizen & Eloff, 1983; 1985; van Der Westhuizen *et al.*, 1986; Sivonen, 1990a; Utkilen & Gjolme, 1992), but changes in environmental factors cannot convert a non-toxic strain to a toxic strain or vice versa (Eloff & van Der Westhuizen, 1982, Sivonen, 1990a), implying that the ability of a cyanobacterium to produce toxin is a genetically-determined character (Skulberg *et al.*, 1993).

1.5 Toxic cyanobacteria and their toxins.

From a taxonomic point of view, cyanobacteria are a small group comprising some 150 genera and 2000 species (Fott, 1971 cited in Skulberg *et al.* 1993). They are

distributed over a vast array of habitats throughout the world ranging from hot springs (up to 74°C) to arctic regions (Rippka, 1988a) and from aquatic and semi-aquatic habitats to terrestrial habitats. However, most of the cyanobacteria inhabit fresh and brackishwater ecosystems where they provide a principal source of matter and energy in food webs (Skulberg *et al.*, 1984).

Published literature on toxic cyanobacterial species goes back to the 1800s, but most papers and articles have appeared since 1940 (Carmichael, 1981). Early publications primarily cover poisoning cases in the field and suspected cyanobacterial species. During the last 20 - 30 years, many of the problems of culturing cyanobacteria in the laboratory have been overcome (Rippka, 1988b) and as a result, toxic cyanobacteria and their toxins have become more available for study.

The present known toxic cyanobacteria constitute some 45 species (Table 1.1) and more species are likely to be discovered as investigations continue. Although cyanobacteria are found in a wide variety of habitats, known toxic species are mostly planktonic in fresh or coastal waters (Carmichael, 1986), and belong to the dominant group of species in eutrophic fresh waters (Codd & Bell, 1985).

Cyanobacterial toxins (cyanotoxins) discovered to date are classified into three main categories as cytotoxins, neurotoxins and hepatotoxins (Carmichael, 1992). Cytotoxins are not lethally toxic to animals but do show a wide spectrum of bioactivity against mammalian cell lines and microorganisms *e.g.* bacteria, algae, fungi. Neurotoxins and hepatotoxins are the most important cyanotoxins from an environmental and public

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Table 1.1 Cyanobacterial species that have been found to have toxin producing strains and their toxins; (** not identified). (Hawkins *et al.*, 1985; Carmichael, 1992; Carmichael *et al.*, 1992; Sivonen, 1990a; Sivonen *et al.*, 1992; Skulberg *et al.*, 1993)

SPECIES	TOXIN TYPE	TOXIN
Anabaena circinalis Rabenh.	Neurotoxin	Anatoxin-a
Anabaena flos-aquae (Lyngb.) Breb.	Hepatotxin	Microcystin
	Neurotoxin	Anatoxin-a
		Anatoxin-a(s)
Anabaena hassallii (Kutz.) Witttr.	**	**
Anabaena lemmermannii P. Richt	Neurotoxin	**
Anabaena spiroids Kle.	Neurotoxin	Anatoxin-a
Anabaena variabilis Kutz.	**	**
Anabaenopsis milleri Woron.	**	**
Aphanizomenon flos-aquae (L.) Ralfs	Neurotoxin	Anatoxin-a
		Saxitoxin
		Neosaxitoxin
Coelosphaerium kutzingianum Nag.	**	**
Coelosphaerium Nagelisp.	Hepatotoxin	**
Cylindrospermum Kutzing sp.	**	**
Cylindrospermopsis raciborskii	Hepatotoxin	**
Fischerella epiphytica Ghose	**	**
Gloeotrichia echinulata	Hepatotoxin	**
Gomphosphaeria lacustris Chod.	**	**
Gomphosphaeria nageliana (Unger) Lemm	Neurotoxin	**
Haphalosiphon fontalis (Ag.) Born.	Cytotoxin	Haphalindole
Hormothamnion enteromorphoides Grun.	Cytotoxin	**
Lyngbya majuscula Harvey	**	**
Microcoleus sp.	**	**
Microcystis aeruginosa Kutz.	Hepatotoxin	Microcystin
Microcystis cf botrys Teil.	**	**
Microcystis viridis (A. Br.) Lemm.	Hepatotoxin	Microcystin
Microcystis wessenbergii Kom.	Hepatotoxin	Microcystin
Nodularia spumigena Mertens	Hepatotoxin	Nodularin
Nostoc linckia (Roth) Born.	**	**
Nostoc paludosum Kutz.	**	**
Nostoc revulare Kutz.	**	**
Nostoc zetterstedii Areschoug	**	**
Nostoc Vaucher sp.	Hepatotoxin	Microcystin
Oscillatoria acutisima Kuff.	Cytotoxin	Acutiphycin
Oscillatoria agardhii Gom.	Hepatotoxin	Microcystin
Oscillatoria rubesence	Neurotoxin	Homoanatoxin-a
Oscillatoria nigro-viridis Thwaites	**	**
Oscillatoria Vaucher sp.	Neurotoxin	anatoxin-a
Oscillatoria formosa Bory	**	**
Pseudoanabaena catenata Lauterb	Neurotoxin	**
Schyzothrix calcicola (Ag.) Gom.	**	**
Scytonema pseudohopmanni Bharadw.	Cytotoxin	Scytophycin
Scytonema hopmanni	Cytotoxin	Cyanobacterin
Spirulina sp.	**	**
Symploca sp.	**	**
Synechococcus Nageli sp.	**	**
Synechocystis Sauvageau sp.	**	**
Tolypothrix byssoidea (Hass.) Kirchn.	**	**
Trichodesmium erythraeum Ehrb.	**	**

health standpoint as they can be lethally toxic to animals.

Five chemically-defined neurotoxins are now known to be produced by several species as shown as shown in Table 1. Many species can produce more than one toxin. Their chemical structure and average LC_{50} values are shown in Fig. 1.1. Anatoxin-a, an alkaloid neurotoxin, was the first chemically defined cyanotoxin (Huber, 1972; and Devlin *et al.*, 1977, cited by Carmichael, 1992). Subsequently, a further two neurotoxins, anatoxin-a(s), a natural organophospate (Mahmood & Carmichael, 1987) and homoanatoxin-a, a methylene homologue of anatoxin-a (Skulberg *et al.*, 1992) were found. Two primary toxins of paralytic shellfish poisoning, saxitoxin and neosaxitoxin have also been found in the freshwater cyanobacterium *Aphanizomenon flos-aquae* (Mahmood & Carmichael, 1986).

Bishop *et al.* (1959, cited in Carmichael, 1992) isolated a hepatotoxin from *Microcystis aeuruginosa* strain NRC-1 and were the first to report that hepatotoxins were peptides. The toxin was later named as microcystin. Botes *et al.* (1982a; 1982b; 1982c and 1985) and Santikarn *et al.* (1983) concluded that the structure of microcystin was a monocyclic peptide containing D- and L- amino acids. Although the various extracts of toxic cyanobacteria had similar toxic properties, there were substantially different amino acid combinations in different extracts. Subsequently it has been realised that there are different microcystins with different L-amino acid combinations. To date at least 53 related cyclic peptides have been identified in the cyanobacterial hepatotoxin family (Carmichael, 1992; 1994). Those consisting of seven amino acids are called microcystins; those containing five amino acids are called

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HEPATOTOXINS

Microcystin-LR
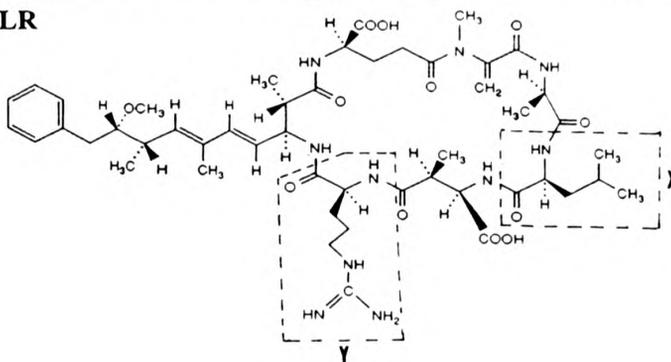
X & Y are variable amino acids in other microcystins
LD_{50} = 60 - 70 µg Kg^{-1} Bd.Wt.

Nodularin
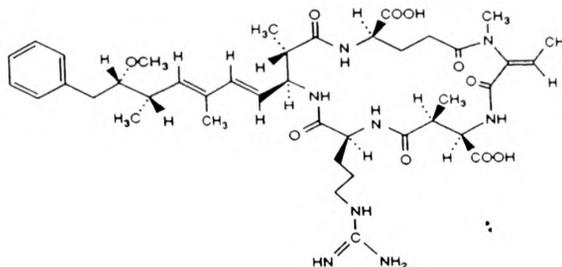
LD_{50} = 60 - 70 µg Kg^{-1} Bd.Wt.

NEUROTOXINS
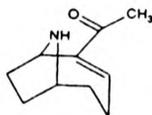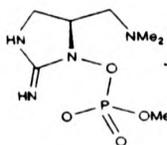
Anatoxin-a   **Anatoxin-a(s)**   **Saxitoxin (R=H) and Neosaxitoxin (R=OH)**
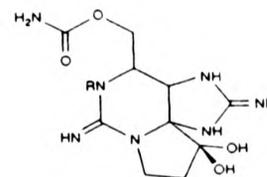
LD_{50} =200 µg Kg^{-1} Bd.Wt.;   =20 µg Kg^{-1} Bd.Wt.;   =20 µg Kg^{-1} Bd.Wt.

Figure 1.1 Chemical structures and approximate LD_{50} (ip., mice) values of most important cyanobacterial toxins (Source: Carmichael *et al.*, 1990; Carmichael, 1994).

nodularin. Most (*i.e.* in excess of 40) are microcystin variants and their analogues (Edwards *et al.*, 1993). Some non-toxic isomers and derivatives of microcystins (*i.e.* non-toxic up to 1 mg kg^{-1} Body wt) have also been found (Carmichael, 1992; Stotts *et al.*, 1993). Although the names of two hepatotoxins, microcystin and nodularin were derived from the generic names of cyanobacteria in which those toxins were originally found, these terms are not restricted to those particular genera; species which belong to other genera are now also known as hepatotoxin-producing species (see Table 1).

Knowledge of the synthesis, nature, effects on biota and fate of cyanobacterial toxins is an important prerequisite to protect not only the public but also the environment as a whole. Moreover, cyanobacteria constitute a major source of natural toxins (Carmichael, 1992) and the study of these toxins is very important in the search for new drugs and other compounds usable in agriculture and other industries (Tu, 1988). For example, the cyanobacterial toxin anatoxin-a(s), is probably the only natural organophosphate yet discovered (Carmichael, 1994); it is more soluble in water and more biodegradable than synthetic options and may provide an important model from which to develop suitable alternatives to synthetic organophosphate insecticides (Carmichael, *ibid*).

1.6 Effects of cyanobacterial toxins on animals

1.6.1 Acute and chronic effects on Vertebrates

The effects of cyanobacterial toxins on animals have mainly been studied using higher

animals. According to these studies, neurotoxins produced by cyanobacteria affect the nervous system in various ways. As explained by Carmichael (1994), four neurotoxins have been studied in detail and all lead to death by causing paralysis of respiratory muscles, which in turn leads to suffocation.

Anatoxin-a mimics the normal neurotransmitter in animals, acetylcholine. Under normal situation, acetylcholine molecules bind to receptors on muscle cells, thereby inducing muscle cells to contract. The enzyme acetylcholinesterase then degrades acetylcholine, freeing receptor sites and allowing muscle cells to return to their resting state. But when anatoxin-a is present, it also binds to acetylcholine receptors, triggering contraction. However, the anatoxin-a cannot be degraded by acetylcholinesterase and as a result it continues to act on muscle cells which eventually become exhausted and stop contracting.

Anatoxin-a(s) acts more indirectly. It allows acetylcholine to bind its receptors and induce contractions as usual, but it blocks acetylcholinesterase from degrading acetylcholine. As a result muscles are over-stimulate and once again become too fatigued to operate.

Both saxitoxin and neosaxitoxin act on animals in a similar way. These two neurotoxins stop nerve impulse propagation in muscle cells by blocking sodium channels thus preventing sodium ions from passing into the neurons. Under normal conditions, sodium ions flow into the neurons in order to relay impulses to muscle cells. When nerve cells are inactivated by saxitoxin or neosaxitoxin, muscle cells

receive no stimulation and become paralysed.

The activity of hepetotoxins produced by cyanobacteria on higher animals is also described by Lawton *et al.* (1994) and Carmichael (1994). Hepatotoxins affect the liver by entering into hepatocysts, the functional cells of the liver, and inhibiting protein phosphatase PP1 and PP2A in a specific and irreversible manner. Protein phosphatases work in concert with other enzyme, protein kinases, to regulate the number of phosphate groups on proteins. The kinases add phosphate groups and the phosphatases remove them.

Such phosphorylation and dephosphorylation reactions influence the structure and function of components of the cytoskeletal system in cells *e.g.* microfilaments and intermediate filaments. It seems, therefore, that microcystins and nodularins disrupt the structural systems of cells. Such disruptions collapse the cytoskeletal system and induce shrinkage on cells.

In the liver the shrinking of hepatocytes creates passages which cause blood to spill into the surrounding liver tissue. The bleeding can leads animals to death by intrahepatic haemorrhagic shock (within hours) or hepatic insufficiency (within days) (Carmichael, 1992; 1994).

Scientists have wondered why microcystins and nodularins act most powerfully on the liver. As indicated by Carmichael (1994), the answer probably is that they are moved to hepatocytes by the unique transport system responsible for carrying bile salts into

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the cells.

Protein phosphatases and protein kinases also play a major role in regulating cell division. It is thus suspected that sublethal levels of hepatotoxins are a contributing factor in the development of cancers (Carmichael & Falconer, 1993; Carmichael, 1994) in animals and humans. Some studies have already shown that microcystins and nodularins can stimulate tumour development in cultured cells (Carmichael, 1994).

As Skulberg *et al.* (1984) and Carmichael (1994) have explained, cyanobacterial blooms would probably not harm terrestrial animals directly if the cells or colonies were distributed evenly throughout the euphotic zone since the cell concentration would not be high enough to allow consumption of a fatal dose. However cyanobacteria migrate vertically to obtain the optimum light for photosynthesis and in the process they often float to the surface. Current and winds can then push them towards the land resulting in poison-filled thick scums accumulated on or near the shoreline. Animals can readily consume a fatal dose when they drink water containing such concentrated scums.

Although humans are as susceptible to cyanobacterial toxins as other mammals, so far no deaths have been recorded. This is probably because people are repelled by the idea of ingesting water containing an algal bloom, thus preventing consumption of a fatal dose. Furthermore, there are no known food vectors such as shellfish which concentrate cyanobacterial toxins in the human food chain (Carmichael, 1992). However there are clinical reports of illnesses such as gastroenteritis, hepatoenteritis

and contact dermatitis *etc.* due to consumption or contact with cyanobacterial toxins (Moore, 1981; Carmichael & Falconer, 1993). Some clinical evidence also suggests that long-term exposure to sublethal levels of cyanobacterial toxins can cause disfunction of liver, stomach and intestine in human populations (Skulberg *et al.,* 1984; Carmichael, 1981). It is likely that the above health risks will increase because the size and duration of cyanobacterial blooms is likely to increase through anthropogenic eutrophication. Recently a toxic cyanobacterial bloom was observed in the Darling River, Australia, which extended for 1000 km along the river (Humpage, *et al.,* 1993).

1.6.2 Acute and Chronic effects of cyanotoxins on invertebrates

The biological mechanisms of cyanobacterial poisoning in invertebrates has not been studied. However it is reasonable to assume that cyanotoxins also act in invertebrates in the same way as in vertebrates because the same target points in the metabolism (*e.g.* acetylcholine, acetylcholinesterase, protein phosphatase) are present in both invertebrates and microorganisms(Waterman, 1961; Lockwood, 1968; Cohen *et al.,* 1989). Furthermore, some studies have clearly demonstrated that toxic cyanobacteria as well as the purified toxins are lethal to some invertebrates *e.g. Daphnia* (Lampert, 1981a; 1981b; 1982; Nizan *et al.,* 1986), *Diaptomus* (Demott *et al.,* 1991), *Artemia* (Kiviranta *et al.* 1991), mosquito (*Aedes aegypti*) larvae (Kiviranta *et al.,* 1993). However the chronic effects of toxic cyanobacteria or their toxins have been largely neglected in previous studies.

Most studies on the effects of toxic cyanobacteria on zooplankton have concentrated mainly on feeding inhibition (Lampert, 1981a, 1981b; Nizan *et al.*, 1986; Demott *et al.*, 1991; Henning *et al.*, 1991).

1.7 Effects of toxic cyanobacteria on zooplankton feeding

1.7.1 Suitability of cyanobacteria as a food for zooplankton

The suitability of cyanobacteria as food for fresh water zooplankton has been widely investigated but results are contrasting and inconclusive. There is evidence from field observations (de Bernardi & Giussani, 1978; de Bernardi *et al.*, 1982) and laboratory experiments (de Bernardi *et al.*, 1981; Hanazato & Yasuno, 1984) that some cyanobacteria provide high quality food for some zooplankton species. Despite such reports, cyanobacteria have also long been reported as an inadequate or unsuitable food for zooplankton (Arnold, 1971; Lampert 1981a; Holm & Shapiro, 1984; Hanazato & Yasuno, 1987; Fulton, 1988a; 1988b). This ambiguity is due not only to differences among cyanobacteria species and strains (Arnold, 1971; Nizan *et al.*, 1986; Henning *et al.*, 1990) but also to differences among zooplankton species (Lampert, 1982; 1987; Hanazato & Yasuno, 1987).

In general, rotifers and copepods are less sensitive to cyanobacteria than cladocerans (Fulton & Pearl, 1987; Lampert, 1987). Selective feeders such as copepods are able to avoid ingestion of unfavourable foods by discriminating particles by size and taste (Demott, 1985; Fulton & Pearl, 1987; Fulton, 1988; Lampert, 1987; Demott & Moxter,

1991). Cladocerans, and daphnids in particular, are unable to do this and can only prevent large particles from entering the filtering chamber by narrowing the carapace gape (Gliwicz & Sielder, 1980). However some small cladocerans, particularly *Bosmina,* can probably discriminate between cyanobacteria and other particles (Demott, 1982; Lampert, 1987).

Three characteristics of cyanobacteria are known to affect their suitability as foods for zooplankton: mechanical properties, nutritional composition, and toxicity (Lampert, 1987; de Berdardi & Giussani, 1990; Kohl & Lampert, 1991). Most colonial and filamentous cyanobacteria mechanically interfere with the filtering process of zooplankton and reduce not only their own collectability but also the uptake of other food particles. Mechanical interference reduces the growth and reproduction of zooplankton, particularly of large bodied cladocerans as a result of the reduced food intake and higher respiratory costs associated with increased handling and rejection of food items (Porter & McDonough, 1984; Gliwicz & Lampert, 1990). However small-bodied cladocerans, copepods and rotifers are known to be less affected than large bodied cladocerans by colonial or filamentous species (Edmondson & Litt, 1982; Fulton & Pearl, 1987; Fulton, 1988a; Gliwicz, 1990a; Gliwicz & Lampert, 1990; Hawkins & Lampert, 1989; Hanazato, 1991), probably because of the selective ability of copepods, and because of the mechanical exclusion of large cyanobacteria by smaller Cladocera. Hence such groups of zooplankton can coexist with cyanobacterial blooms (Porter & McDonough, 1984; Kohl & Lampert, 1991; Hanazato, 1991).

Despite the fact that colonial and filamentous cyanobacteria are difficult to handle,

there is some evidence that they can be ingested by cladocerans, at least to a slight degree, when size and concentration of colonies or filaments is below a critical level (Lampert, 1981a; Holm *et al.*, 1983; Fulton, 1988a; 1988b; Dawidowicz, 1990; Gliwicz, 1990a; 1990b; Rothhaupt, 1991).

It has been suggested that cyanobacteria can pass through the gut of zooplankton unharmed (Nizan *et al.*, 1986) although there is no published evidence for this. If cyanobacteria is an inadequate food when ingested, poor assimilation and/or poor nutrition may be the prime reason for the inadequacy (Arnold, 1971; Holm *et al*, 1983; Holm & Shapiro, 1984; Hawkins & Lampert, 1989). However it is not clear whether poor assimilation efficiency is a major factor with regard to the unsuitability of cyanobacteria as a food for zooplankton because assimilation efficiencies reported in the literature are highly variable (Lampert, 1987).

1.7.2 Interactions between toxic cyanobacteria and zooplankton

An important question regarding cyanotoxins is: what is their function ?. Some suggest that they may function as anti-grazing compounds (Lampert, 1981a; 1982; Demott *et al*, 1991) as many phytotoxins do in vascular plants (Harbone, 1988). As reported above, more than 40 species of cyanobacteria have already been recorded as toxigenic and toxic effects are mainly reported in field poisoning cases of mammals (Codd & Bell, 1985; Sivonen *et al.*, 1990a; Lawton & Codd, 1991; Carmichael, 1992). Moreover, the toxicities of most species to warm blooded animals are well established (Carmichael *et al.*, 1990). However, mass mortalities of zooplankton are not evident

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in the field and toxic effects on zooplankton have only been observed in the laboratory.

Ecologists have concluded that toxic cyanobacteria have a negative effect on zooplankton largely as a result of observations of inverse relationships in the field between cyanobacterial blooms and particular groups of zooplankton (Edmandson & Litt, 1982; Nizan *et al.*, 1986; Fulton & Pearl, 1987; Gliwicz, 1990b) but it is not clear whether this relationship is due to the toxicity of cyanobacterial blooms. Nevertheless, negative effects of toxic cyanobacteria have been confirmed by laboratory experiments (Lampert, 1981a; 1981b; 1982; Nizan *et al.*, 1986; Hanazato & Yasuno, 1987; Demott *et al.*, 1991; Henning *et al.*, 1991; Rothhaupt, 1991).

In laboratory experiments a particular strain of cyanobacterium is considered to be toxic if zooplankton die more rapidly in the presence of that cyanobacterial strain than in the absence of food. In some cases zooplankton do not die but show serious inhibition of feeding (Lampert, 1987). However, there are a few factors to be taken into account when extrapolating results from laboratory experiments to the natural environment. Feeding rates on laboratory cultures of cyanobacteria might not be comparable to those measured in the field because most of the toxigenic species of cyanobacteria which occur in the field as colonies or filaments become single cells or small colonies in laboratory cultures. Reductions in feeding rates in the field are then due not only to toxicity but also to mechanical inhibition. Furthermore, the survival of zooplankton in the presence of toxic or non-toxic cyanobacteria may be better in the field than they would be in the laboratory because in natural waters cyanobacteria

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coexist with other species of algae and bacteria which might compensate for essential nutrients and mask toxic effects (Hanazato & Yasuno, 1984).

1.8 *Microcystis aeruginosa* as a test organism

M. aeruginosa is one of the most commonly used cyanobactera in toxicity studies. Sivonen (1990b) reported that more than 65% of all the literature published on toxic cyanobacteria deals with *M. aeruginosa*. There are several reasons for the popularity and importance of this species.

(1) *M. aeruginosa* is one of the phytoplankton species which typically dominates eutrophic fresh waters.

(2) *M. aeruginosa* is the most widely distributed toxigenic cyanobacterial species in the world including tropical and temperate regions (Skulberg *et al.*, 1993).

(3) Microcystin, the toxin produce by *M. aeruginosa*, has also been recorded from several other species of cyanobacteria (Table.1)

(4) To date, in excess of 40 microcystin variants and analogues have been identified (Edwards *et al.*, 1993).

(5) Availability of toxic *M. aeruginosa* is rather high. More than 50% of *M. aeruginosa* blooms tested were toxic (Sivonen *et al.*, 1990a; Lawton & Codd, 1991; Carmichael, 1992).

(6) The species is easy to culture in the laboratory (see Chapter 2).

(7) *M. aeruginosa* is one of the easiest toxic cyanobacteria to use in grazing experiments with zooplankton because it becomes a single cell or a few-cell colony suspension in laboratory cultures. Hence, there are unlikely to be any mechanical

constraints to grazers.

1.9 *Daphnia magna* as a test organism

D. magna is a common freshwater cladoceran, inhabiting temperate ponds and lakes throughout Europe and North America (Hrbacek, 1987). Its biology has been studied and reviewed extensively (Green, 1954; 1956; Hebert, 1978; Peters & de Bernardi, 1987).

1.9.1 The life cycle of *Daphnia*

D. magna has a simple iteroparous life cycle (Sibly, 1989). Under favourable and constant conditions, *e.g.* low population density, high food, long day, populations reproduce by ameiotic parthenogenesis and may consist exclusively of asexually producing females and their clonal descendants (Hebert & Ward, 1972; Carvalho & Crisp, 1987). The offspring produced (neonates) are released as miniature versions of the adults (Green, 1954). They grow to maturation via a series of moults, and the time period between two such moults is termed an instar. Growth in length takes place immediately after moulting while the new carapace is still soft and flexible, but growth in mass is continuous.

Maturation is usually reached in the fourth to seventh instar, depending on food level and temperature (Lei & Armitage, 1980; Stephenson & Watts, 1984; Taylor, 1985). Parthenogenetic eggs are deposited from the ovaries into the brood pouch, which is

the space between the upper side of the body and the dorsal part of the carapace, and develop into free swimming neonates, the timing of which is controlled by temperature (Zaffagnini, 1987), before being released into the surrounding medium. Immediately after the brood is released, the mother moults, grows in length and deposits another brood into the pouch.

Under unfavourable conditions such as high population density or low food, sexual reproduction can be induced (Zaffagnini, 1987) and therefore genetic variability can be generated. Male daphnids are produced by parthenogenesis, and females produce haploid eggs. Broods can be single sexed or, where both sex occur, one sex is usually predominant. The females are then fertilised by the males and two ephippial (fertilised) eggs are produced. These are extruded into the brood chamber and are shed with the next moult. Ephippial eggs are generally produced in wild populations prior to winter or drought and are capable of withstanding freezing, desiccation and digestion. They hatch after an indefinite period when favourable conditions return (Doma, 1979).

Since culture conditions in the laboratory are generally maintained as favourable, only parthenogenetic reproduction occurs. Hence genetic variability is minimised and can only occur through spontaneous mutation.

1.9.2 The use of *Daphnia* in aquatic toxicological studies.

Toxicity tests in aquatic toxicology are generally performed with several fresh water

and marine species including algae, invertebrates and fish (Maltby & Calow, 1989; Calow, 1993). *D. magna* is probably one of the oldest and most widely used test organism in aquatic toxicology. According to Anderson (1980), *D. magna* was first used in bioassays by Naumann in 1933. Nowadays *D. magna* is a popular choice of test organism for chronic and acute tests in aquatic toxicology because of its ecological importance and also for practical and economical reasons:

(1) *D. magna* is widely distributed in the holarctic and plays and important role in freshwater trophic chains. It is one of the most efficient filter feeders and converters of algal energy and is also an important food for vertebrate and invertebrate predators in the water. Therefore, any effect of a pollutant on daphnids may have implications for the function of natural ecosystems.

(2) The short life span and reproductive cycle allows chronic tests on more than one generation to be performed.

(3) Their parthenogenetic mode of reproduction provide offspring which are genetically identical to their parent.

(4) Toxicity tests are relatively simple and cheap to run in comparison to most other bioassay animals.

(5) The maintenance of laboratory cultures is relatively easy and inexpensive.

(6) *D. magna*, being the largest of the genus, facilitates easy observation and handling.

(7) *Daphnia* is relatively sensitive to toxic compounds compared to other aquatic organisms (Buikema *et al.*, 1980; Adema & vink, 1981; Baudo, 1987; Wong, 1987) and so screening the sensitivity of daphnids to toxicants may allow the vulnerability of the trophic chain to be predicted to some degree.

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1.10 Interactions between toxic *Microcystis aeruginosa* and *Daphnia*.

As already noted *Daphnia* is one of the oldest and most widely used aquatic invertebrate in aquatic toxicology. Moreover, inverse relationships have been observed between cyanobacterial dominance and abundance of large bodied cladocerans in water bodies (George & Edwards, 1974; Jones *et al.* 1979; Edmondson & Litt, 1982: Infante & Riehl, 1984; Nizan *et al.*, 1986; Hanazato, 1991). Several studies have been carried out on the interaction between toxic *M. aeruginosa* and daphnids.

Grazing studies have demonstrated that the feeding rates of daphnids on cyanobacteria can be suppressed by toxin producing species (Lampert, 1981a; 1981b; Nizan *et al.*, 1986; Demott *et al.*, 1991; Henning *et al.*, 1991). Lampert (1981a) reported that *Daphnia* can recover from feeding inhibition very quickly when returned to a standard green algal food and suggested that this rapid response of *Daphnia* is an algal defence mechanism against grazing. However, Jungmann *et al.* (1991) have suggested that *Daphnia* toxicity and the inhibition of feeding rate are caused by different compounds in *M. aeruginosa* cells. Moreover, different cladoceran species exhibit different sensitivities in terms of feeding inhibition to the same toxic strain of *M. aeruginosa* (Lampert, 1982). On the other hand strains of *M. aeruginosa* differ in their ability to inhibit the feeding rate of *D. magna*, irrespective of their toxicity, implying that there is no correlation between the mouse toxicity of *M. aeruginosa* and its effect on feeding inhibition of *Daphnia* (Nizan *et al.*, 1986; Henning *et al.*, 1991). All these results indicate that the underlying mechanisms of feeding inhibition of *Daphnia* and the toxicity of *M. aeruginosa* remain unclear and necessitate a further study under standard conditions and defined toxicity regimes.

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1.11 Aims of the thesis

The research reported in this thesis is an attempt to understand the underlying mechanisms of feeding inhibition and the other toxic effects of the cyanobacterium *M. aeruginosa* on *D. magna*. Variability of results with regard to the interaction between *M. aeruginosa* and *Daphnia* in preliminary experiments necessitated the use of standard conditions, including defined media in experiments to increase the reproducibility of results. Therefore the aims of the thesis were to:

(1) select a suitable food and defined culture medium for *Daphnia* for use in the cyanobacterial exposure experiments.

(2) investigate the feeding behaviour of *D. magna* on toxic *M. aeruginosa* with

      (a) different cell concentrations

      (b) different exposure periods

      (c) different proportions of co-existing food.

(3) investigate the acute and chronic toxicity of purified microcystin-LR and *M. aeruginosa* cells on *D. magna*.

CHAPTER 2

GENERAL MATERIALS AND METHODS

The materials and methods described below have been used throughout the study. Experimental protocols specific to particular sections are given in relevant chapters.

2.1 Culture of *Microcystis aeruginosa*

Two strains of *M. aeruginosa*, a toxic strain PCC 7820 (LC_{50} = 30 - 40 mg dry wt kg body wt^{-1} (mouse bioassay); (Codd & Carmichael, 1982) and non-toxic strain CYA 43 (defined as non-toxic by interperitoneal mouse bioassay at a dose of 1000 µg dry wt kg body wt^{-1}; Beveridge *et al.*, 1993), were obtained from the Department of Biological Sciences, University of Dundee and used in this study. In addition to the toxicity difference, care was taken to select particles of roughly diameter and shape, in order to avoid differences in mechanical processing characteristics to be confounded with toxicity differences. Both strains of *M. aeruginosa* grew as double cell colonies (henceforth referred to as cells) with near equivalent particle size (Plate, 2.1).

Both strains were maintained in a two-tier culture system *i.e.* in small batch cultures and large-scale, semi-continuous batch cultures. Both steps of the culture were performed in a standard culture medium, nitrate supplemented BG 11 (Stanier *et al.* 1971; Table 2.1) at 20±2 °C under continuous true light supplied by 20W fluorescent tubes. Culture medium has been made in nanopure water (*i.e.* the water passed through four cartridges and three filters in BARNSTEAD nanopure ultrapure water system, Fisons, in order to remove all bacteria and organic and inorganic impurities; conductivity < 0.05 µs cm^{-1}). Although both cultures were not axenic, all procedures were carried out under sterile conditions to avoid contamination and to ensure that the
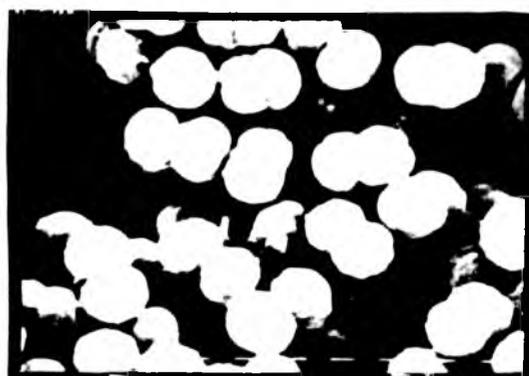
(a) *C. vulgaris* CCAP 211/12   (b) *M.aeruginosa* CYA 43
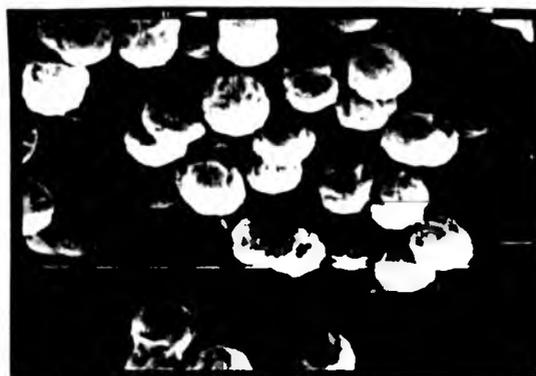
(c)*M. aeruginosa* PCC 7820

Plate 2.1 Scanning electron micrograph (x 5000) of (a) *C. vulgaris* strain CCAP 211/12 showing also mother cell walls (w), (b) non-toxic *M. aeruginosa* strain CYA 43 and (c) toxic *M. aeruginosa* strain PCC 7820 (Each scale bar = 1 μm).

Table 2.1 Blue-Green II (BG II) culture medium for *M. aeruginosa* and *C. minutissima*.

Following nutrients are prepared as 1000-fold stock solutions, which are stored in a fridge (at 4-8 °C). The medium is prepared by addition of 10 ml from stock solution 1 and 1 ml from all the other stock solutions into nanopure water to a final volume of 1 l.

Chemical	g/l
1. $NaNO_3$	150
2. $MgSO_4.7H_2O$	75
3. K_2HPO_4	40
4. $CaCl_2. 2H_2O$	36
5. Na_2CO_3	20
6. $FeSO_4. 7H_2O$	6
7. Citric acid	6
8. H_3BO_3	2.68
9. $MnCl_2. 4H_2O$	1.81
10. EDTA	1
11. $Na_2MoO_4. 2H_2O$	0.39
12. $ZnSO_4. 7H_2O$	0.22
13. $CuSO_4. 5H_2O$	0.08
14. $Co(NO_3)_2. 6H_2O$	0.05

Table 2.2 Proteose-peptone agar medium for *Chlorella vulgaris* culture (solutions made in nanopure distilled water).

Chemical	g per 100 ml water
Proteose peptone	0.1
KNO_3	0.02
K_2HPO_4	0.002
$MgSO_4. 7H_2O$	0.002
Agar	1.0

degree of bacterial contamination was minimal.

Each month, a number of 250 ml conical flasks containing 100 ml sterile BG 11 medium was inoculated with each strain of *M. aeruginosa* and maintained in an illuminated orbital incubator (Gallenkamp), shaken at 100 rpm. After two weeks the purity of the cultures was checked by withdrawing 1 ml of each suspension and viewing the sample under high power microscope. Suitable cultures (*i.e.* in which bacteria content was minimal) were used as inocula to start semi-continuous batch cultures.

Four-litre glass vessels containing 2.5 l of growth medium were used for semi-continuous batch cultures. Each culture vessel was connected to an 11 l plastic (Nalgene) storage bottle containing 10 l growth medium and it has been used as a reservoir for medium replacement. Culture vessel, storage bottle and connecting tubings were autoclaved at 121 ^{0}C and 124.1 kPa for 20 min prior to use. To start the culture, 2.5 l culture medium was inoculated with approximately 100 ml of small batch-culture inocula of either toxic or non-toxic strains described above and the culture system was completed by connecting culture vessel and storage bottle under sterile conditions. The culture medium was aerated with filtered air at a rate of 2 - 4 l min^{-1}. and the storage bottle covered with black polythene to prevent photodegradation of chemicals in the medium.

Both strains of *M. aeruginosa* grew mainly as suspensions of double cell colonies. Cells were harvested during the late log phase of growth (21 - 28 d). Samples of 500

ml were withdrawn from each culture and replaced with the same volume of fresh media from the storage bottle, ensuring that the cultures remained in the same growth phase throughout the experimental period. If any culture appeared abnormal (*e.g.* cloudy or foam producing) it was checked for purity and discarded if contaminated.

2.2 Culture of *Chlorella minutissima* Fott & Novakova

C. minutissima was found as a contaminant in a laboratory culture of cyanobacteria and subsequently isolated. The identification was confirmed by Dr. F. Santos, Coimbra University, Portugal. *C. minutissima* was also cultured in the same culture system following the same procedure, medium and environmental condition as those used in the culture of *M. aeruginosa*. This species was also non-axenic and grew as a unicellular suspension with occasional small colonies. Cells were harvested during the late log phase of growth (10-15 d).

2.3 Culture of *Chlorella vulgaris* Beijerick

An axenic culture of *C. vulgaris* (Strain CCAP 211/12) which was also grew as single cells (Plate 2.1) was obtained from the Freshwater Biological Association's CCAP (Culture Collection for Algae and Protozoa) unit (Ambleside, UK.) and maintained axenically in a three stage culture system *i.e.* in agar slopes, small batch cultures and large-scale semi-continuous batch cultures, according to the following procedure.

2.3.1 Inoculation and maintenance of slopes

Cultures were maintained on proteose peptone agar slopes (Table 2.2). All chemicals except agar, were dissolved in nanopure water and the solution then heated to 95°C. Agar was then added gradually to the solution while stirring. When all agar was dissolved and the mixture was homogenous, 10 ml quantities were poured into 28 ml screw top glass bottles which was then autoclaved at 121 °C and 124.1 kPa. While the agar mixture was still warm, bottles were tilted and allowed to settle. The agar slopes were left for 3 d to ensure that no bacterial contamination was present; if any contamination was discovered the slopes were discarded.

A number of slopes were inoculated from a slope culture which had already been grown, using a wire loop under sterile conditions. The slopes were incubated under continuous light at 20 ± 2 °C and after cultures had become established on agar they were kept at room temperature for up to six months for use as inocula in the next stage of culture. New slopes were prepared at least once every six months.

2.3.2 Batch cultures

The second stage of culture was the establishment of small batch cultures in a standard algal growth medium, Woods Hole MBL (Stein 1973; Table 2.3). Once every three weeks 250 ml conical flasks containing 100 ml of previously autoclaved medium were inoculated with *C. vulgaris* colonies grown on agar slopes. The inoculated cultures were then placed in an orbital incubator, shaken at 100 rpm (Gallencamp).

Table 2.3 Woods Hole MBL (pH = 7.2) culture medium for *Chlorella vulgaris*. The macro and micro nutrients are prepared as 1000-fold stock solutions, which are stored at 4°C. Vitamin stocks are membrane filtered and stored frozen. The medium is prepared by consecutive addition of a, b, c, and d to nanopure water.

a. Macronutrients (use 1 ml per each litre of medium)

Chemical	g/l
$NaNo_3$	85.01
$MgSO_4.7H_2O$	36.97
$CaCl_2. 2H_2O$	36.76
$Na_2SiO_3.9H_2O$	28.42
Na_2CO_3	12.60
K_2HPO_4	8.71

b. Micronutrients (use 1 ml per each litre of medium)

Chemical	g/l
$Na_2.EDTA$	4.36
$FeCl_3.6H_2O$	3.15
$MnCl_2. 4H_2O$	0.18
$ZnSO_4. 7H_2$	0.022
$CoCl_2.6H_2O$	0.01
$CuSO_4. 5H_2O$	0.01
$Na_2MoO_4. 2H_2O$	0.006

c. Vitamins

Thiamine.HCl	0.1mg/l
Biotin	0.5µg/l
Cyanocobalamin	0.5µg/l

d. Tris (use 2 ml per each litre of medium)

Tris (hydroxymethyl)-aminomethane	50g/200ml
Adjust pH to 7.2 with HCl	

After 2-3 weeks a 1 ml sample was withdrawn from each culture and checked for purity under a high power microscope (Olympus BH-2). Pure cultures were used as inocula for the next stage of culture.

Large scale, semicontinuous batch cultures were also maintained in Woods Hole MBL medium, following the same procedure and conditions as those used for *C. minutissima* (see 2.2 above).

2.4 Preparation of algal / cyanobacterial food for daphnids.

In order to obtain *Microcystis* or *Chlorella* cells for use in experiments or as daphnid food, samples withdrawn from a culture were spun down in a bench top centrifuge (MSC Minor "s") at 3000 rpm for 5 min. The supernatant was then decanted and pellets of cyanobacterial or algal cells resuspended in ASTM water. The cell concentration of the suspension was determined at X 100 dilution by electronic particle counter (Coulter Multisizer, Coulter Electronics Ltd.) fitted with a 30 µm orifice tube. The final suspension was used in experiments or kept at 4 °C for up to 3 d for use as a food for daphnids. Cell biovolume was also estimated using the electronic particle counter.

2.5 Culture maintenance of *Daphnia magna*

A single clone of *D. magna*, (designated F, Baird *et al.*, 1990), originally obtained from the University of Sheffield, has been maintained in the laboratory in Stirling for

several years. For the present study animals were maintained in a three-tier culture system:

- brood stock cultures

- bulk cultures

- semi-sterile individual cultures

All cultures were maintained in a constant temperature room (20 ± 2 ^{0}C) under a 14:10 light: dark regime. This constant temperature and photoperiod regime together with careful observations prevent sexual reproduction occurring (Stross & Hill, 1965)

The culture medium routinely used for both brood stock cultures and bulk cultures was ASTM hard water (ASTM; 1980) with an organic additive. ASTM hard water consists of four inorganic salts dissolved in nano-pure water (Table 2.4). Fifty millilitres each of KCl, $MgSO_4.7H_2O$ and $NaHCO_3$ and 500 ml of $CaSO_4.2H_2O$ were added to nanopure water to a final volume of 10 l in a plastic carboy. The medium was left standing overnight to aerate and reach the required temperature (20 °C) before use.

This synthetic medium was enriched with an organic additive - a water soluble extract of the seaweed *Ascophyllum nodosum* (Glenside Organics Ltd. Throsk, Stirlingshire. UK; Table 2.5) as recommended by Baird *et al.* (1989). The seaweed extract comes in a concentrated form; a stock solution was prepared by diluting the concentrated extract 100 fold using nanopure water and then filtered to remove any large particles. The stock solution as well as concentrated extract were stored at 4°C in the dark to avoid photodegradation and microbial growth. The stock solution of the extract was

Table 2.4 Quantities of Analar grade chemicals required to prepare ASTM hard water (artificial fresh water) and the resulting water qualities (ASTM, 1980)

Salts	Amount required (mg/l)
$MgSO_4.7H_2O$	245.5
$NaHCO_3$	192.0
$CaSO_4.2H_2O$	120.0
KCL	8.0
pH	7.6 - 8.0
Hardness (as mg/l of $CaCO_3$)	160 - 180

Table 2.5 Average analysis of Marinure (seaweed extract) in dry powder form (data supplied by Glenside Organics Ltd.)

Dry matter	92-95%
Organic matter	50-55%
Inorganic matter	40-45%
Chlorine	3.0%
Sulphur	2.7%
Potassium	2.5%
Nitrogen	1.4%
Calcium	1.2%
Magnesium	0.8%
Phosphorus	0.05%
Iodine	1800.0 ppm
Iron	1500.0 ppm
Boron	110.0 ppm
Zinc	100.0 ppm
Manganese	13.0 ppm
Aluminium	5.0 ppm
Nickel	5.0 ppm
Copper	3.0 ppm
Cobalt	1.6 ppm
Vanadium	0.7 ppm

Cytokinins and other natural growth stimulants 130 - 260 ppm

added to ASTM hard water only when the media was renewed and at a concentration of 4%.

2.5.1 Brood stock cultures

Brood stock cultures were initiated using three neonates (<24 h) per 500 ml screw top glass jars containing 450 ml of the culture medium described above. Each group was fed daily with *C. vulgaris* at a rate of 1×10^7 μm^3 cells ml^{-1} (equivalent to 6×10^5 cells ml^{-1}; see Table 4.1 for cell diameter values) neonate culture medium and medium being renewed every other day. (In this study, biovolume has been used to express ration level and feeding rate in order to facilitate comparison among foods which may differ in particle size and chemical composition)

Cultures were established in order to provide neonates for experiments and for establishing other cultures. However, first and second brood offspring were not used because they usually consist of individuals which are more variable in size and quality (Waddell, 1993). Third and subsequent brood offspring were checked for the presence of males and if none was present they were used in experiments or to establish new cultures.

2.5.2 Bulk cultures.

Bulk cultures were set up in order to produce sufficient numbers of adult animals for experiments. Twenty neonates (<24 h) were stocked per 3 l transparent plastic tank,

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containing 2.5 l of the medium and fed with *C. vulgaris* at the same concentration. Media was renewed once before the first brood and then routinely after each brood was released. Following their third brood, daphnids (14 d) were used in experiments.

2.5.3 Semi-sterile individual cultures

Individual cultures were established only for experiments. Although the environmental conditions were the same as those used in the other cultures, media used in individual cultures varied in different experiments (see Chapter 3). However, the basic procedure given below, was similar for all experiments unless otherwise stated.

Individual cultures were initiated by transferring neonates (<24 h) or single gravid females (14 d) from brood stock cultures or bulk cultures respectively, into 150 ml media in 190 ml screw top glass jars. Animals were fed every day with *Chlorella* or *Microcystis* (except for animals which were starved, as determined by the experiment) at a rate of 1×10^7 μm^3 cells ml^{-1}. Media and jars were renewed every other day and any neonates were removed within 24 h of hatching. Individual glass pipettes (0.7mm diameter) were used to transfer parent animals to fresh media. Pipettes were washed with salt solution (NaCl, 50 g/l) after use and again by nanopure water before being used again. Glass jars were also acid and detergent washed prior to use. Standard screw caps of glass jars were replaced by aluminium caps which were made from thick aluminium catering foil. Cleaned glass jars with the aluminium caps were sterilized in a hot-box oven (Gallenkamp) at 180 ºC.

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2.6 Extraction and analysis of microcystin-LR

In order to get purified toxins for experiments and to know the toxin concentration of cells, microcystin-LR was extracted from *M. aeruginosa* cells and purified. For both purposes, harvested cells of *M. aeruginosa* were freeze-dried and stored at -20 °C, until extracted. Lyophilized cells were extracted in 5% acetic acid (0.5 g / 50 ml, W/V) for 20 min with continuous stirring. After centrifugation at 1500 X g for 10 min, the supernatant was removed and the pellets used for re-extraction. Supernatant from the two extractions was pooled and passed through a Whatmann GF/C filter to remove particulates. The filtrate was then applied to a preconditioned C_{18} environmental sep-pack cartridges (Millipore) using a peristaltic pump. The cartridge was washed with 20% aqueous methanol (20 ml) and microcystins eluted with 100% methanol (20 ml). The methanol was removed *in vacuo* at 45 °C and the residue stored at -20 °C until analysis.

When water (*i.e.* culture media) samples to be analyzed for microcystin were filtered through a GF/C filter and acidified by addition of trifluoro acetic acid (TFA) to a final concentration of 0.1%. Addition of TFA resulted in severe precipitation which necessitated a second filtration. The filtrate was then applied to a C_{18} environmental sep-pack column and extracted as above.

All toxin samples were redissolved in HPLC grade methanol and analyzed by photodiode array HPLC as explained in Lawton *et al.* (1994). The analysis was carried out using waters instrumentation consisting of a model 600E solvent delivery system,

a model 991 photodiode-array PDA detector at 200 - 300 nm with 3 nm resolution. A Waters μBondapak C_{18} column (300 x 3.9 mm i.d.) (Millipore) was used. The column temperature was maintained at 40°C using a Waters temperature control module. Injections of 25 μl samples from redissolved toxin samples were made in triplicate.

The limit of detection for pure microcystin with the described HPLC system giving a consistently reproducible peak area was found to be 5 ng on the column in a 25 μl injection (Lawton, 1994). Detection limit of cell bound microcystin was less than 10 ng l^{-1} mg dry wt cells (Lawton, L. A., Pers. comm). All reagents used were of analytical reagent or HPLC grade.

The toxin extraction and HPLC analysis were performed with the collaboration of Prof. Geoffrey A. Codd and his research staff, at the Department of Biological Sciences, University of Dundee.

2.7 Scanning Electron Microscopy (SEM).

Scanning Electron Microscopy was used to show that shape and relative size of each cell type used in feeding experiments (see Chapter 4) were of near equivalent particle size (Plate 2.1). Log phase culture of the three cell types (*C. vulgaris*, non-toxic *M. aeruginosa* and toxic *M. aeruginosa*) were used to prepare scanning electron micrographs. SARTOLON, 0.45 mm polymide filters were used to collect cells for processing for SEM. Prior to collection of cells on the filters, 5.ml of 1% gelatine in

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1% chromo alum solution was passed through the filters in order to enhance adhering ability. suspensions (5 ml) of each cell type were then passed through the filter and the resulting cell concentrates left in 1% and 3% glutaraldehyde for 1h and overnight respectively for fixation. Cell concentrates were post-fixed in 1% OsO_4 and dehydrated through an acetone series. Samples were then critical point dried (BioRad critical point drier), mounted on aluminium stubs and coated with gold in a sputter coater (Edwards-150b) and examined under scanning electron microscope (Phillips 500).

CHAPTER 3

SUITABILITY OF DIFFERENT MEDIA AND ALGAL DIETS FOR *DAPHNIA* CULTURE

3.1 Introduction

Daphnids have been extensively used in many areas of study related to aquatic environments because they are widely distributed, common, sensitive, easily sampled animals which, more importantly, play a major role in the food web of freshwaters (Peters & de Bernardi, 1987). A preliminary stage in conducting laboratory experiments with *Daphnia* is to develop and maintain cultures in the laboratory, and in particular, to ensure a culture medium with a balanced ionic composition and a nutritionally adequate food source (Peters, 1987). During the last few decades, various culture systems have been tested and employed to culture *Daphnia* in the laboratory. According to Peters (1987) these systems can be divided into two general groups: detrital and autotrophic systems.

In detrital systems and some autotrophic systems *e.g.* green water culture, both food and culture medium are complex and undefined. The use of such systems is now less common, mainly because of higher variations of performance among daphnids in these cultures (Peters, 1987).

3.1.1 Foods for *Daphnia* culture.

Daphnia laboratory cultures are generally designed to supply healthy neonates of constant quality in sufficient numbers for experiments (Baird *et al.* 1989). Therefore many researchers have preferred culture systems with simple food sources, because the quantity and quality of food in the system is easy to control; moreover, all the

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biotic elements in the culture system are then known. These are probably the most common systems now being used to culture *Daphnia*. In general, a natural diet, unicellular green algae, has been found to be a very successful diet for *Daphnia* culture because it gives healthy neonates of constant quality in sufficient numbers (Baird *et al.*, 1989; Naylor *et al.*, 1992). Such diets usually consist of one or a few species of algae (Lampert, 1976; Baird *et al.*, 1989; Lundstedt & Brett, 1991). Although more effort is required in the maintenance of algal culture under axenic conditions, better performances in *Daphnia* culture have been achieved through the use of monoaxenic algal diets (Baird *et al.*, 1989).

3.1.2 Media for *Daphnia* culture

Most laboratories have used natural media to culture *Daphnia e.g.* filtered lake water (Lampert, 1981; 1982; Fulton & Paerl, 1987; Gliwicz, 1990a; Demott *et al.*, 1991; Jungmann *et al.*, 1991), aged or dechlorinated tap water (Arnold, 1971; Nizan *et al.*,1986; Penaloza *et al.*, 1990), aged well water (Klapes, 1990; Dave *et al.*, 1991), or biologically-conditioned water (Taub & Dollar, 1968). Natural media may not be suitable for some laboratory experiments since its composition varies among laboratories and time. *Daphnia* can be extremely sensitive to changes in surrounding water (Peters, 1987). Moreover these natural media may create additional problems in toxicological studies; metals and a number of chemicals in natural waters can be complexed, at least in part, by chelating agents *e.g.* humic acid, which may change their availability and toxicity (Alloway & Ayres, 1993). Therefore the standardization of culture media was also an important goal in this study in order to achieve

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reproducible and less variable results.

Aquatic toxicologists expect two main utilities from a standard culture medium: first, it should be able to supply healthy neonates of constant quality in sufficient numbers to carry out acute and chronic toxicity tests and second, the same medium should be suitable to run toxicity tests: *i.e.* there should be no unknown carrier compounds (Baird *et al.* 1989). The solution is to use a fully defined medium as this is the only way to guarantee a defined and reproducible composition of the medium. Currently this is difficult to achieve as no optimal ionic composition for *Daphnia* culture has been established (Peters, 1987). Nevertheless, several defined media have been proposed (*e.g.* ISO, 1982; OECD, 1983; Keating, 1985; ASTM, 1988). Keating's (1985) medium, which is called "MS" and appears to be a more complete medium, has been found to be regularly contaminated with bacteria resulting in low oxygen tension in the medium (Barber, 1990). These problems may arise from the presence of large quantities of an organic buffer glycylglycine which is an ideal food source for bacteria (Elendt & Bias, 1990). All other media are made up of only a few salts and their composition is inadequate to meet the animals requirements (Barber, 1990; Elendt & Bias, 1990).

The above facts indicate that there is still no adequately defined medium for *Daphnia* culture. As a result Baird *et al.* (1989) introduced a partially defined medium which was ASTM water (ASTM, 1988) enriched by an extract of the seaweed *Ascophyllum nodosum*. This medium (hereafter ASTM+SE) has been used in conjunction with a uni-algal diet by several laboratories to culture *Daphnia* as well as to run toxicity tests

(Soares, 1989; Dave *et al.*,1991; Sims *et al.*, 1993; Waddell, 1993). Although the seaweed extract is not a fully defined additive, it comes from a single brown algal species. Therefore in terms of nutritional composition, this culture system can assumed to be a combination of a fully defined medium (*i.e.* ASTM) and two algal diets (*i.e.* *Ascophyllum nodosum* and the live algal diet). However the medium does not provide standard conditions for toxicity testing purposes because of the unknown organic complexes present in the medium.

Elendt & Bias (1990) have proposed another fully defined medium, M_4, for *Daphnia* culture as a modified combination of micro-nutrients from Keating's "MS" medium and macro-nutrients from ISO medium. They have used this medium with a uni-algal diet to culture *Daphnia* successfully for about three years. Although Elendt & Bias (1990) recommended M_4 medium for *Daphnia culture*, there was no other published evidence on the success of Elendt M_4 medium (hereafter M_4) when the present study began.

3.1.3 Performance of culture systems.

It was necessary to test the M_4 medium for *Daphnia* culture before use in toxicity testing. Two media were used as controls: ASTM+SE as the upper limit control and ASTM hard water only (hereafter ASTM) as the lower limit control. The use of two controls may give a better assessment than using only one control since ASTM is the simplest defined medium reported for *Daphnia* culture and ASTM+SE has given good performance in *Daphnia* culture in previous studies (Soares, 1989; Barber, 1990).

Moreover in preliminary experiments, the two control media have shown better and poorer performances respectively, in *Daphnia* culture. It is worth pointing out, however, that Elendt M_4 medium contains a remarkably high concentration of Na_2EDTA (Table 3.1). It is well known that EDTA has an ability to alter the bioavailability of metals and some nutrients by complexation (Elendt & Bias, 1990). Therefore, when this medium is used to run toxicity tests, higher concentrations of Na_2EDTA may provide a higher capacity for complexation with toxins and toxicant which may lead to an alteration in their availability and toxicity. But, if the concentration of Na_2EDTA is reduced, the availability of some nutrients, particularly of trace elements, might be increased. An extensive supply of trace nutrients can cause toxic effects to *Daphnia*. By taking all these factors into account another Elendt medium called M_7 which contains less amounts of both Na_2EDTA and trace nutrients has been proposed (Anonymous, 1991). In the present study, Elendt M_7 (hereafter M_7) medium was also tested as a culture medium.

Variability in performance of a specified clone of *D. magna* under controlled environmental conditions seems to arise not only from the use of different media but also from the use of different foods (Elendt & Bias, 1990; Sims *et al.*, 1993). Varying the algal species can potentially alter the quality of food available to *Daphnia* (Naylor *et al.*, 1992). In this study, two green algal species, *Chlorella vulgaris* and *C. minutissima*, were tested for *Daphnia* culture. The quality of algal foods will vary depending also on growth phase or age (Baird *et al.*, 1989; Ahlgren *et al.*, 1992; Mitchell *et al.*, 1992). Moreover senescent *Chlorella* cells have been suspected as producing an antibiotic named chlorellin (Pratt *et al.*, 1945; Ryther, 1954). Therefore

effects of the age of algal culture on *Daphnia* culture were also examined in this study.

Bacterial contamination can also alter the quality of algal foods (Baird *et al.*, 1989). Therefore an experiment was performed to compare performances of *Daphnia* fed on axenic and non axenic cultures of the same strain of *C. vulgaris*.

3.1.4 Aims of the study

The aim of the experiments discussed in this section was to select the best food-media combination for *Daphnia* culture, in order to maximize,

(1) the survival and growth and reproductive performance of *D. magna*, and

(2) the reproducibility of experimental conditions.

To meet this challenge, effects of the following factors on *Daphnia* culture were ascertained.

(1) four different culture media: ASTM+SE, ASTM, M_4 and M_7

(2) two different green algal foods: *C. vulgaris* and *C. minutissima*.

(3) the age of algal cultures used as foods: log phase and stationary phase cultures

(4) bacterial contaminations: axenic and non axenic algal suspensions.

3.2 Materials and Methods

3.2.1 Culture media

The daphnid culture media tested in this study were ASTM+SE, ASTM, M_4 and M_7. The preparation of ASTM and ASTM+SE has already been described (see Chapter 2). The other two media, M_4 and M_7 share a similar chemical composition, but differ in the concentrations of some of the chemicals. The preparation of final media and stock solutions of both media are given in Table 3.1. The concentrations of most of the stock solutions given in the original publication (Elendt & Bias, 1990) have been changed in Table 3.1, in order to obtain a consistent withdrawal volume from each solution to be added to nanopure water to prepare M_4 and M_7. Of course, the actual media concentrations remain consistent with the published recipe.

All culture media were prepared by dissolving analytical grade chemicals into nanopure water, and subsequently left overnight to aerate and reach an appropriate temperature ($20 \pm 1^\circ C$).

3.2.2 Foods

Two green algal (Chlorophyceae) species were used: *C. minutissima* and *C. vulgaris*. Both were cultured in BG 11 and Woods Hole MBL media respectively as described in Chapter 2. Separate cultures were set up in order to harvest cells in the log and stationary phases of growth. Under the temperature and light regimes used, cultures

Table 3.1 The chemical composition and preparation of Elendt M_4 and Elendt M_7 media.

Chemical formula	Quantity required for stock solution mg l^{-1}	Volume required from stock solution to 10 l of;	
		M_4	M_7
Macronutrients			
$CaCl_2.2H_2O$	29380	100	100
$MgSO_4.7H_2O$	12330	100	100
$NaHCO_3$	6480	100	100
$Na_2SiO_3.5H_2O$	10000	10	10
KCl	14500	4	4
$NaNO_3$	685	4	4
KH_2PO_4	357.5	4	4
K_2HPO_4	460	4	4
Micronutrients			
H_3BO_3	7148.7	4	1
$MnCl_2.H_2O$	901.2	4	1
LiCl	765	4	1
RbCl	177.5	4	1
$SrCl_2.6H_2O$	380	4	1
NaBr	40	4	1
$Na_2MoO_4.2H_2O$	157.5	4	1
$CuCl_2.2H_2O$	42	4	1
$ZnCl_2$	32.5	4	4
$CoCl_2.6H_2O$	25	4	4
KI	8.1	4	4
Na_2SeO_3	5.5	4	4
NH_4VO_3	1.4	4	4
$Na_2EDTA.2H_2O$[a]	5000	-	-
$FeSO_4.7H_2O$[a]	1991	-	-
Fe-EDTA		10	2.5
Vitamins[b]			
Thiamine-HCl	750		
Cyanocobalamine	10	1	1
Biotin	7.5		

(a) Both solutions should be prepared singly, poured together and autoclaved immediately in order to produce the Fe-EDTA solution.

(b) Vitamin stock should be immediately filtered through a 0.2μm filter and stored frozen in individual portions.

of both species reached the stationary phase within 15-18 days. This could be determined readily as the onset of rapid colour change from pale green to dark grass green. Therefore cultures were harvested by withdrawing 500 ml, at age 10-15 days for cells in the log phase of growth and 28-35 days for cells in the stationary phase of growth. At the same time cultures were maintained by replenishing with equal volumes of fresh media ensuring cultures remained in the same phase of growth throughout the experimental period. Cells were harvested once every 4 days from cultures in the log phase of growth and every 8 days from cultures in the stationary phase of growth.

The strain of *C. vulgaris* used in this study was normally maintained as an axenic culture; in order to obtain non-axenic cultures, small batch cultures (see Chapter 2) were kept open to the atmosphere in different locations for 24 h and then again incubated under sterile conditions. After 3-4 days of incubation, cultures were checked under a high power microscope (x 400: Olympus BH2) for contaminants and those suspected to be contaminated by bacteria were selected. In order to confirm bacterial contamination, three agar (Tryptone Soya Agar, Oxoid) plates were inoculated by selected algal cultures and incubated at 20 ± 1 °C for 48 hour. A culture which contaminated by bacteria only was used as an inoculum to start semi-continuous batch cultures under sterile conditions. Cells were harvested at the log phase of growth as described for axenic *C. vulgaris* cultures (Chapter 2.)

3.2.3 Chemical analysis of foods

When harvesting cells for the first time from a culture, three 50 ml samples were taken and concentrated by centrifugation (see Chapter 2). Each concentrated sample was resuspended in 5 ml of nanopure water and cell number and mean cell diameter determined using an electronic particle counter (see Chapter 2). Samples were then freeze dried and weighed using a Mettler (Model MT5) microbalance (sensitivity, ± 1 µg). The relative proportions of carbon, hydrogen and nitrogen in the freeze dried cells were determined by Perkin-Elmer C H N S/O analyzer (Model PE 2400, Series II).

3.2.4 Experimental protocol

Four life-history experiments were carried out under different combinations of media and foods in order to test four media, two green algal foods, effects of the age of algal cultures and effects of bacterial contamination of algal cultures, for *Daphnia* culture in this study.

In the first experiment, four culture media: ASTM+SE, ASTM, M_4 and M_7 in conjunction with *C. vulgaris* were tested in order to select the best medium. *C. vulgaris* at its log phase of growth was selected as the only food for this experiment because it has been successfully used as the sole food for *Daphnia* culture by several workers (McMahon & Rigler, 1963; Baird *et. al.*, 1989; Soares, 1989; Barber, 1990; Sims *et. al.*, 1993; Waddell, 1993).

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Following the results of the first experiment, two media ASTM+SE and M_4 were used as culture media in the second experiment to test the two algal foods *C. minutissima* and *C. vulgaris* during the log phase of growth.

In the third experiment, life history performance of daphnids in ASTM+SE was tested under log phase and stationary phase cultures of each *Chlorella* species in order to compare the effects of the algal-culture age on daphnid performance.

The final experiment was to compare effects of axenic and non-axenic cultures of algae on *Daphnia* culture. The result of the first experiment demonstrated that the survival of neonates in all the defined media tested in conjunction with axenic *C. vulgaris*, were poor whilst all the neonates in ASTM+SE were healthy. But Elendt media in conjunction with non-axenic algal diet, have been used successfully for *Daphnia* culture over three year period by Elendt & Bias (1990). Moreover ASTM+SE contains some organic compounds which can be suitable substrate for bacterial growth. Therefore M_4 was used as the culture medium in order to reveal the effects of non-axenic algal food on *Daphnia* culture. Generally the conversion of a non-axenic culture to an axenic culture is a difficult and time consuming process (Court *et. al.*, 1981; Parker, 1982). Therefore an axenic *C. vulgaris* culture was used to obtain a non-axenic culture as already described.

The following nine food-medium combinations were tested.

(a) *C. vulgaris* (log phase) and ASTM+SE

(b) *C. vulgaris* (log phase) and ASTM

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(c) *C. vulgaris* (log phase) and M_4

(d) *C. vulgaris* (log phase) and M_7

(e) *C. minutissima* (log phase) and ASTM+SE

(f) *C. minutissima* (log phase) and M_4

(g) *C. vulgaris* (stationary phase) and ASTM+SE

(h) *C. minutissima* (stationary phase) and ASTM+SE

(i) *C. vulgaris* (non-axenic, log phase) and M_4

These combinations were tested in three steps. In the first instance, (a), (b), (c) and (d) combinations were tested. Then (e), (f), (g) and (h) were tested once again with (a) and (c). Finally axenic and non-axenic *C. vulgaris* in conjunction with M_4 (*i.e.* (a) and (i)) were tested. Whenever the same experiment was repeated, results were pooled. However all combinations were tested as follows.

Neonates were placed individually in 150 ml of each culture medium in screw top glass jars and were fed every day with each food to a concentration of 1×10^7 μm^3 cells ml^{-1}. There were seven replicates under each combination and all were maintained as described in Chapter 2 for individual culture of *D. magna*. Cultures were checked every day for moults, neonates, aborted eggs and the health of the parent animal and observations recorded. Life history experiments in all combinations were conducted up to 30 d. Body lengths of animals were measured directly at the end of the experiment (*i.e.* after 30 d or whenever they died) using an Olympus dissecting microscope (x40) fitted with an eyepiece micrometer. When animals released their clutches, living, dead or immobile neonates were counted separately.

In the evaluation of the life history performance, single variables *e.g.* body length, brood size, may not be good measures to indicate the authentic performance. For example, an animal which produces smaller broods can make an equivalent contribution to population growth as to an animal which produces larger broods if the former reproduces before the latter. Therefore life history performance can only be properly evaluated using an integrated measure. In this study, population growth capacity (r_c) of daphnids was used as an integrated measure to evaluate the life history performance. It was calculated from the following equation (Southwood, 1978),

$$r_c = \log_e R_o/T_c$$

where, R_o is sum of the live neonates released until day 30 and

$$T_c = \Sigma \text{ ages at clutch release/number of clutches.}$$

3.2.5 Statistical analysis.

Population growth capacity, body length data and values of the mean brood size in terms of live offspring, measured in the experiment which was to test two algal diet in conjunction with two culture media were analyzed using two-way analysis of variance (ANOVA) with Tukey multiple range test (Zar, 1984). One-way analysis of variance with Tukey multiple range test was used to analyze same parameters measured in the rest of the experiments. Carbon, hydrogen and nitrogen percentages of algal foods were transformed firstly to proportions (p), and then to arcsin values (P') by P'=arcsin$\sqrt{P}$ (Zar, 1984). The resultant data were also analyzed using one-way analysis of variance with Tukey multiple range test.

3.3 Results

3.3.1 Chemical analysis of algae

Three comparisons of chemical compositions were made: between log phase cultures of *C. minutissima* and *C. vulgaris*, between log phase cultures and stationary phase cultures of each species and between axenic cultures and non-axenic cultures of *C. vulgaris*. Variations in terms of percentage carbon, hydrogen and nitrogen in these cultures are given in Fig. 3.1a, 3.1b and 3.1c respectively.

Percentage of both carbon and hydrogen in different foods varied in a similar way. In terms of inter-specific differences, *C. vulgaris* was higher than *C. minutissima* in percentage carbon as well as in percentage hydrogen during the log phase of growth. Carbon and hydrogen content in log phase cells of both species of *Chlorella* were lower than in stationary phase cells. Again, the percentages of both elements in axenic, log phase *C. vulgaris* were higher than that of non axenic *C. vulgaris* as well as *C. minutissima*. Except one food, *C. minutissima* at the stationary phase of growth, all the other foods tested were significantly different from one another, in terms of both their percentage carbon ($F = 70.4$; $df = 4, 10$; $P < 0.05$) and percentage hydrogen ($F = 40.42$; $df = 4, 10$; $P < 0.05$).

The proportion of nitrogen present in *C. vulgaris* cells was much lower than in cells of *C. minutissima*. The percentage nitrogen in *C. vulgaris* cells during the log phase of growth was higher than that of the stationary phase of growth, by contrast, there was
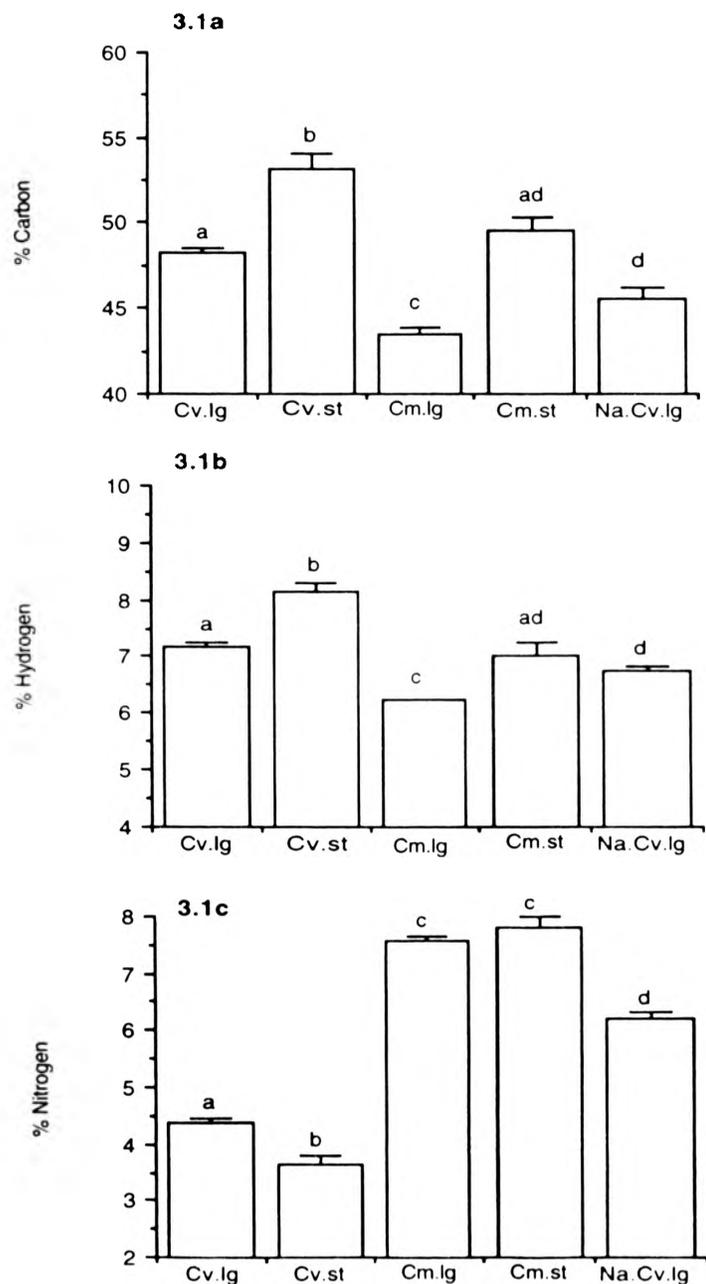
Figure 3.1 Percentage dry weights of (a) C, (b) H, and (c) N in different foods tested for *Daphnia* culture (lg, log phase; st, stationary phase; Na, non-axenic; Cv, *Chlorella vulgaris*; Cm, *C. minutissima* (Note that data presented are means ± standard deviations; statistical analysis was performed on transformed data; different superscripts indicate significantly different groupings).

no significant difference in percentage nitrogen content of log phase and stationary phase cultures of *C. minutissima*. Non-axenic cultures of *C. vulgaris* were higher than axenic cultures in terms of nitrogen content. Except for log phase and stationary phase cultures of *C. minutissima*, all the other foods were significantly different from one another in their nitrogen content ($F = 307.58$; df = 4, 10; $P < 0.05$).

3.3.2 Performance of *D. magna* in different media

The population growth capacity, body length and mean brood size of *D. magna* cultured in four different media in conjunction with the same food, log phase *C. vulgaris*, is given in Fig. 3.2 (a), (b) and (c) respectively. ASTM showed the lowest population growth of *D. magna* whilst ASTM+SE gave the highest and among the least variable population growth. Both M_4 and M_7 showed intermediate and not significantly different population growth but M_4 was superior to M_7 as there was no significant difference in population growth capacity between ASTM+SE and M_4 ($F = 86.8$; df = 3,24; $P > 0.001$).

Culture media had a significant effect on the growth of *D. magna* ($F=31.0$; df=3, 19; $P < 0.05$). Variations in body lengths of daphnids reared in four media showed the same pattern as for population growth capacity. Daphnids held in ASTM+SE and ASTM showed the highest and lowest growth respectively whilst those in M_4 and M_7 showed intermediate performance. However body lengths of daphnids cultured in M_4 and M_7 were not significantly different.
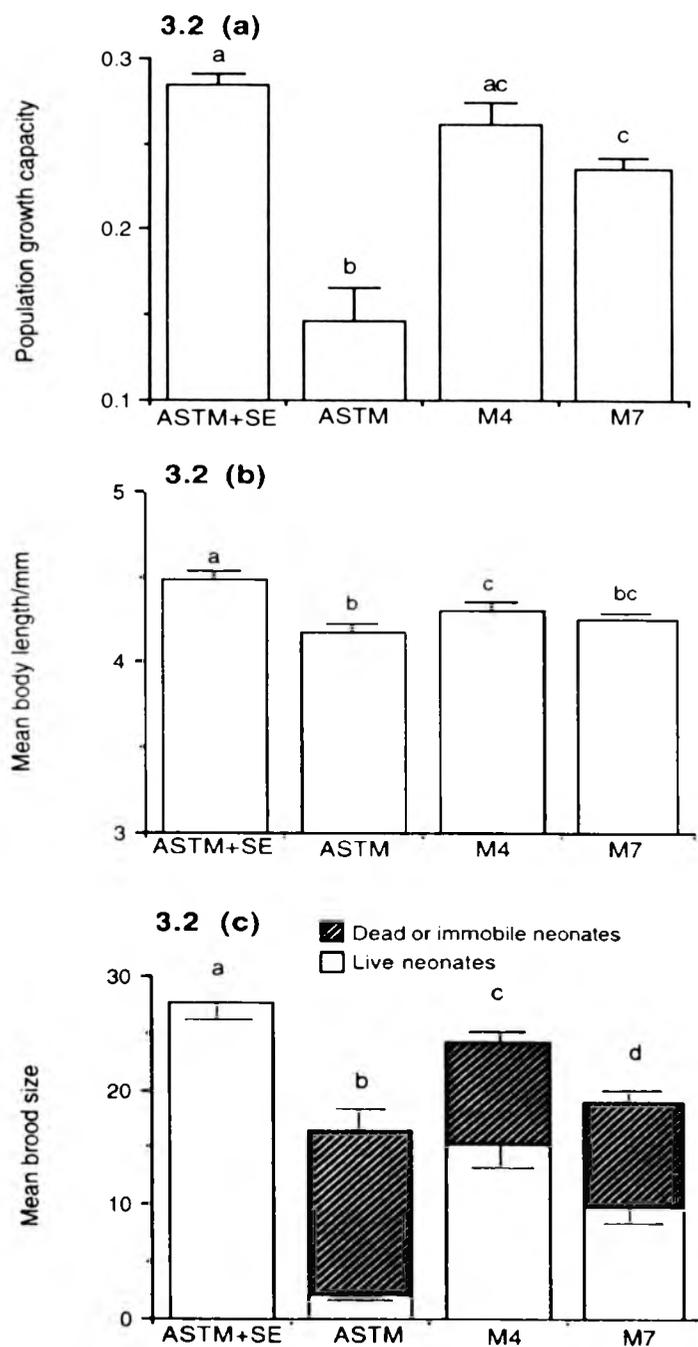
Figure 3.2 (a) Population growth capacity, (b) individual growth and (c) mean brood size of *D. magna* cultured in different media over 30 d period with the same food *C. vulgaris* (data presented are means ± standard deviations; different superscripts indicate significantly different groupings).

Different culture media also had a highly significant effect on the reproduction of daphnids as the mean brood size of animals cultured in four media in terms of live offspring differed significantly from one another (F = 516.7; df = 3, 24; P < 0.05). Again, the medium ASTM+SE gave the highest fecundity in which all the neonates were healthy. However, more than one third of neonates produced by daphnids in the other three media were dead or irrecoverably immobilized. This defect was more severe in ASTM as more than 90% of neonates were dead or immobile. Again, variations in total fecundity as well as in numbers of live offspring among the four media followed the same pattern as with body length or population growth capacity.

3.3.3 Performance of *Daphnia* fed on two different green algal species

The performance of daphnids cultured in ASTM+SE and M_4 in conjunction with two *Chlorella* species is summarised in Fig. 3.3 (a), (b) and (c). Population growth of animals in combination of M_4 with *C. minutissima* was more variable and lower than that in combination of ASTM+SE with each algae and in M_4 with *C. minutissima*. However the result of two-way analysis of variance showed that medium, diet and interaction between medium and diet had not significant effect on the population growth (Table 3.2).

Body lengths of daphnids reared in ASTM+SE in conjunction with either of the two algae were greater than those of daphnids reared in M_4 in conjunction with either alga (Fig.3.3(b)). Moreover, when the culture media was the same the body lengths between *C. vulgaris*-fed animals and *C. minutissima*-fed animals were almost same. Two way

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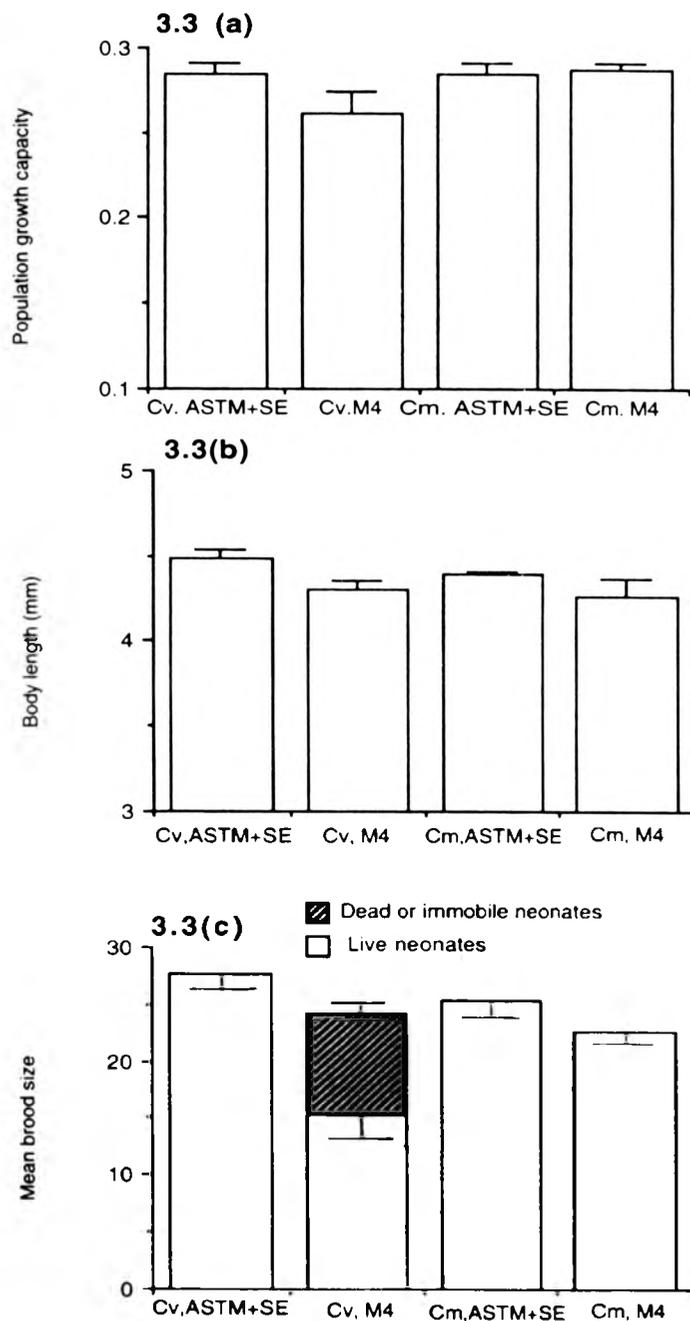
Figure 3.3 (a) Population growth capacity, (b) individual growth and (c) mean brood size of *D. magna* cultured in ASTM+SE and M_4 over 30 d period under two different diets (Cv, *C. vulgaris;* Cm, *C. minutissima;* data presented are means ± standard deviations).

Table 3.2 Two-way analysis of variance for the population growth capacity in the experiment to test two algal diets(* significant at P<0.05; n.s., not significant).

Source of variation	d.f.	Sum of squares	F value
Treatments	2	0.0018	
Medium	1	0.0007	7.73 (n.s.)
Diet	1	0.0012	12.78 (n.s.)
Medium↔Diet	1	0.0012	12.78 (n.s.)
Residual	24	0.0022	
Total	27	0.0052	

Table 3.3 Two-way analysis of variance for the body length in the experiment to test two algal diets(* significant at P<0.05; n.s., not significant).

Source of variation	d.f.	Sum of squares	F value
Treatments	2	0.1754	
Medium	1	0.1259	23.74 *
Diet	1	0.0295	5.57 (n.s.)
Medium↔Diet	1	0.0075	1.41 (n.s.)
Residual	21	0.1114	
Total	24	0.2944	

Table 3.4 Two-way analysis of variance for the mean brood size in the experiment to test two algal diets(* significant at P<0.05; n.s., not significant).

Source of variation	d.f.	Sum of squares	F value
Treatments	2	428	
Medium	1	384	182 *
Diet	1	43	20.4 *
Medium↔Diet	1	171	81 *
Residual	24	50	
Total	27	649	

analysis of variance also revealed that diet and the interaction between medium and diet had not a significant effect on the body length, only medium had a significant effect on the body length (Table 3.3).

However, medium, diet and the interaction between diet and medium had a significant effect on mean brood size of *D. magna* (Table. 3.4). Poor survival of neonates was observed only in the combination of M_4 and *C. vulgaris*; almost one third of neonates in this combination were dead or immobile. However the mean brood size of all the combinations were considerably higher *i.e.* greater than 22.

3.3.4 Effect of the age of algal cultures on daphnid performance.

Performance of *D. magna* cultured in ASTM+SE in conjunction either with log phase or stationary phase cells of *C. minutissima* and *C. vulgaris* are shown in Fig. 3.4(a), (b) and (c). Population growth capacity of daphnids in all combinations were high and significantly not different from one another (F = 2.6; df = 3, 24; P > 0.05) although that of animals fed on stationary phase *C. vulgaris* was slightly lower and more variable.

Mean body lengths of daphnids fed on each of the four algal diets were significantly different from one another (F= 171.88; df= 3, 21; P<0.05). The highest and lowest body lengths were observed in animals fed on log phase and stationary phase cells of *C. vulgaris* respectively. Daphnids fed on log phase and stationary phase cells of *C. minutissima* showed intermediate body lengths. The difference between log phase cells and stationary phase cells of *C. minutissima* in terms of growth of daphnids was less striking than that of *C. vulgaris* (Fig. 3.4(b)).

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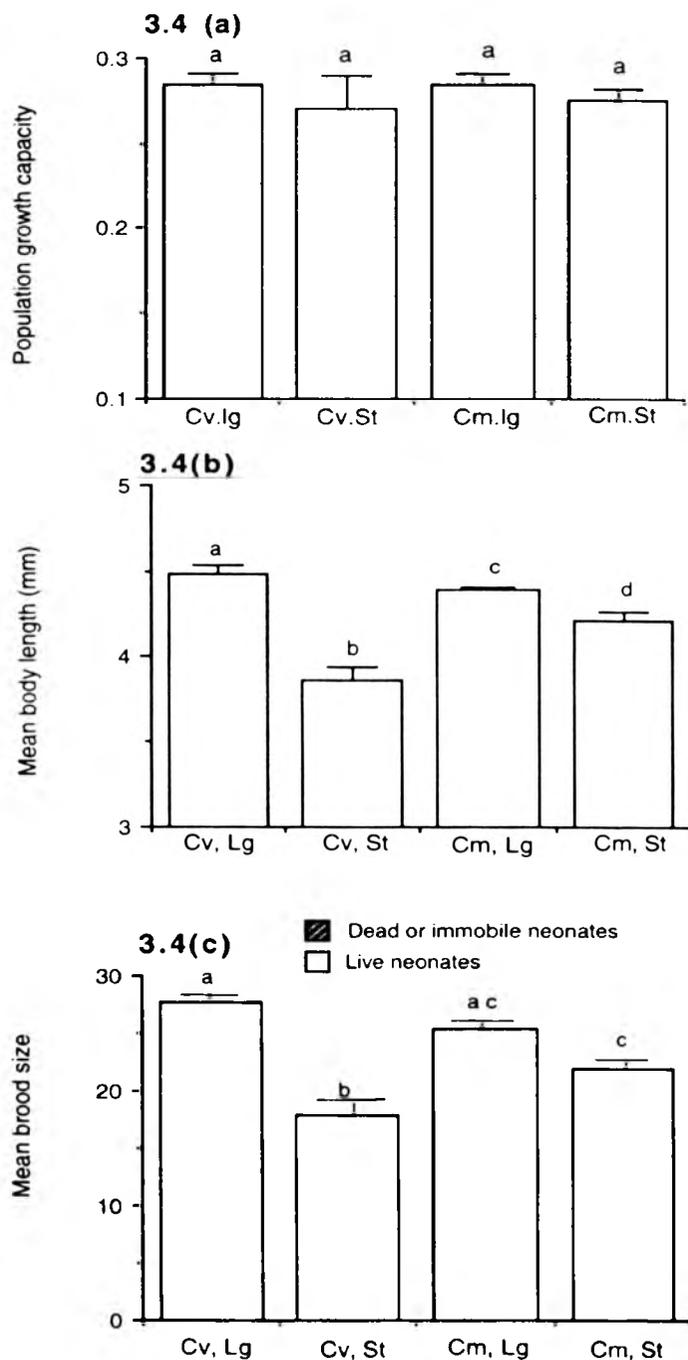
Figure 3.4 (a) Population growth capacity, (b) growth and (c) mean brood size of *D. magna* cultured in ASTM+SE over 30 d period under different diets (Cv, *C. vulgaris;* Cm, *C. minutissima;* Lg, log phase; st, stationary phase; data presented are means ± standard deviations; different superscripts indicate significantly different groupings).

All the neonates produced by daphnids fed on each of the four diets were healthy, but there were significant differences among mean brood size (F = 20.25; df = 3, 24; P < 0.05). The brood size of daphnids fed on log phase and stationary cells of *C. vulgaris* were significantly different whilst those of *C. minutissima* were not. However, the mean brood size of daphnids fed on log phase cells of each species was higher than that of stationary phase cells.

3.3.5 Effect of axenic and non-axenic cultures.

The performance of *D. magna* reared in M_4 and fed on of axenic and non-axenic cultures of *C. vulgaris* is shown in Fig. 3.5 (a), (b), and (c). The population growth capacity of animals fed on axenic cultures of *C. vulgaris* was significantly lower than that of animals fed on non-axenic *C. vulgaris* (F = 6.8; df = 1, 12; P < 0.05). There was no significant difference in body length of animals fed on each diet (F = 3.7; df = 1, 9; P > 0.05). However mean brood size of daphnids in terms of live offspring was significantly different from that of animals fed on non-axenic *C. vulgaris* (F = 46.39; df = 1, 12; P < 0.05). Moreover, more than one third of neonates produced by animals fed on axenic *C. vulgaris* were dead or immobile whilst all neonates produced by animals fed on non-axenic cultures were healthy. The presence and absence of bacteria in non-axenic and axenic cultures respectively were confirmed by inoculated agar plates (Plate 3.1)
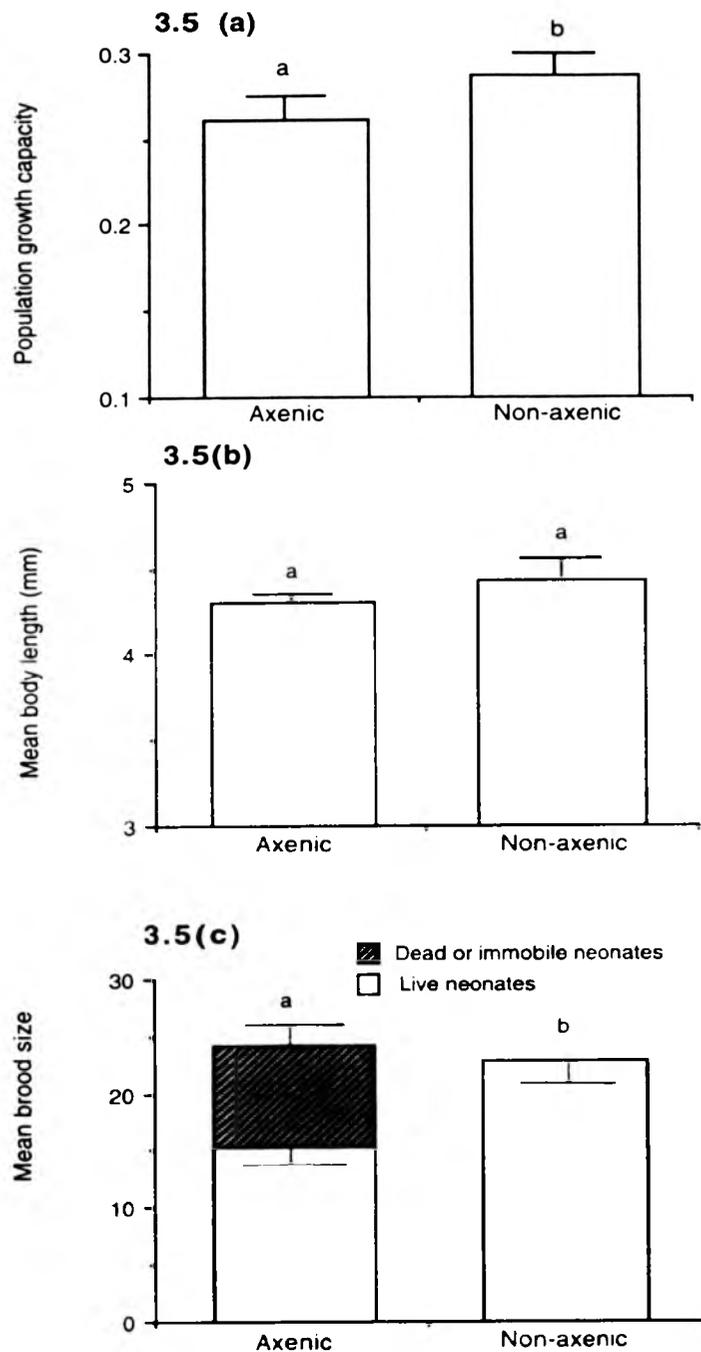
Figure 3.5 Performance of *D. magna* reared in M_4 and fed on axenic and non-axenic cultures of *C. vulgaris* over 30 d period, (a) population growth capacity, (b) growth and (c) body length (data presented are means ± standard deviations; different superscripts indicate significantly different groupings).

Table 3.5 Percentages of 30 day survival of *Daphnia magna* under different food-media combinations.

Food-Medium combination	% Survival
C. vulgaris (log phase) and ASTM+SE	100
C. vulgaris (log phase) and ASTM	47.5
C. vulgaris (log phase) and M_4	59.3
C. vulgaris (log phase) and M_7	71.1
C. minutissima (log phase) and ASTM+SE	100
C. minutissima (log phase) and M_4	100
C. vulgaris (stationary phase) and ASTM+SE	47.5
C. minutissima (stationary phase) and ASTM+SE	100
C. vulgaris (non-axenic, log phase) and M_4	100
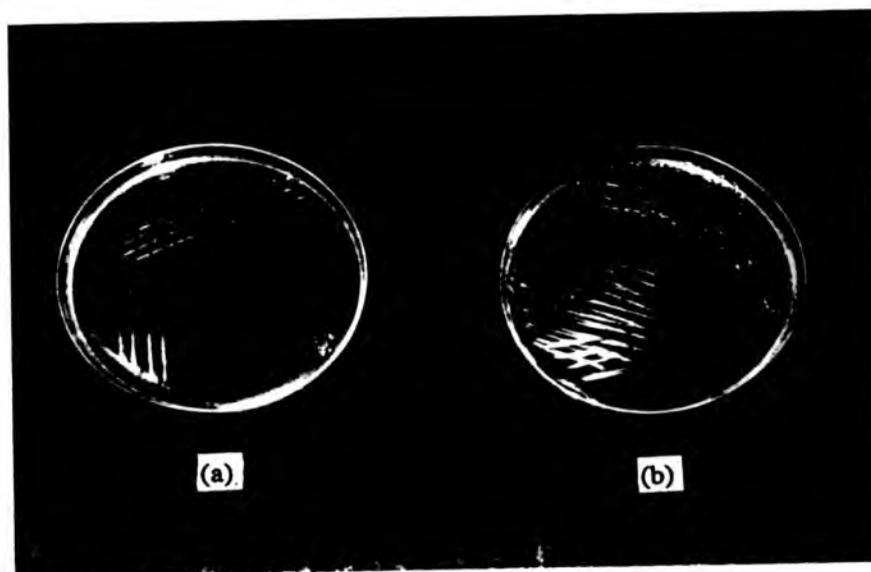
Plate 3.1 Bacterial growth on agar (TSA) plates after 48 h of incubation. (a) inoculated by axenic and (b) non-axenic cultures of *C. vulgaris*.

3.3.6 Survival of *Daphnia magna*

The percentages of parent daphnids surviving for 30 d in different food and media combinations are given in Table 3.2. ASTM with *C. vulgaris* and ASTM+SE with stationary phase cells of *C. vulgaris* showed the lowest survival. M_4 and M_7 also gave poor survival when *C.vulgaris* has used as food. Animals held in these conditions showed algal clogging in their antennae and the ends of the tail spines during the days immediately prior to death. The remaining combinations gave the maximum survival during the experimental period.

3.4 Discussion

This study aimed to ascertain variations in terms of the performance of daphnids maintained in four different culture media (ASTM+SE, ASTM, M_4, and M_7) two algal foods (*C. vulgaris* and *C. minutissima*), the log phase and stationary phase cells and using axenic and non-axenic algal diets. Except in the experiment in which stationary phase cells were tested for their suitability as food, only log phase cultures of each algae were used in the experiments because log phase cultures are generally superior to stationary phase cultures in *Daphnia* culture (Ahlgren *et al.*, 1992; Mitchell *et al.* 1992).

Of the four culture media tested, ASTM-only gave the poorest performance in terms of reproduction, population growth and survival of *D. magna*; growth of individuals was also reduced. These results indicate that the chemical composition of ASTM is

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unsuitable for daphnid culture in combination with a mono-axenic diet. Baird *et al.* (1989) and Barber (1990) also reported similar problems in the use of ASTM with a unialgal diet in the culture of *Daphnia*. ASTM+SE gave the best performance in all *D. magna* life history trials studied, supporting the hypothesis of Baird *et. al.* (1989) that the extract of the seaweed *Ascophylum nodosum* can compensate nutritional deficiencies in ASTM for *Daphnia* culture.

Both M_4 and M_7 showed intermediate performance in terms of population growth, individual growth, reproduction and survival of *D. magna*. However, an additional problem in the use of these media was in terms of neonate survival. When *C. vulgaris* was used as the only food, about 38% and 50% of newborn neonates produced by *Daphnia* in M_4 and M_7 respectively, were dead or immobile. In other aspects, the performance of *D. magna* in M_4 was similar to that in ASTM+SE. For this reason, ASTM+SE and M_4 were used in an experiment to test the suitability of two algal diets *C. minutissima* and *C. vulgaris*.

In terms of daphnid performance, there was no significant differences between *C. minutissima* and *C. vulgaris* when ASTM+SE was used as the medium. But when either of the two algae was used with M_4, *C. minutissima* appeared the more suitable food. Although *C. vulgaris* has been recorded as a good quality food for *Daphnia* culture (McMahon & Rigler, 1963; Baird *et al.*, 1989; Barber, 1990; Sims *et al.*, 1993) a poor survival of adults in addition to neonates resulted when used in conjunction with M_4 medium. It is not clear how the survival of neonates was affected when *C. vulgaris* was offered as food. There may be no anti-nutritional factors in *C. vulgaris*

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cells because it resulted in the best performance of *D. magna* when ASTM+SE was used as the culture medium. It is probable that some nutritional deficiency in *C. vulgaris* is the reason for the poor survival of neonates in Elendt media.

There was little difference in daphnid performance between animals fed on log phase cells and stationary phase cells of *C. minutissima*: *D. magna* showed good performance even with stationary phase cells. But the growth and mean brood size of daphnids fed on stationary phase cells of *C. vulgaris*, were poorer than that of animals fed on log phase cells. The nitrogen contents of log phase and stationary phase cells of *C. minutissima* were not significantly different, whilst those of *C. vulgaris* were significantly different, reflecting differences in daphnid performance with these diets. Ryther (1954) also found that the performance of *D. magna* was impaired when feeding on senescent (*i.e.* stationary phase) cells of *C. vulgaris*. He attributed this result to the accumulation of a suspected antibiotic, chlorellin, in senescent cells which was never confirmed.

However the results of the experiment which was to test the age of algal cultures on *D. magna*, show that the population growth of *D. magna* was not seriously affected by the diet of stationary phase *C. vulgaris* although mean brood size of daphnids was low. The reason is that daphnids fed on stationary phase cells of *C. vulgaris* reproduced earlier than the animals fed on other diets. The growth rate and survival of animals fed on stationary phase *C. vulgaris* were also low and probably it might stress animals to reproduce earlier in order to keep the survival potential higher. Bradley *et al.* (1991) and Guisande & Gliwicz (1992) also reported a rather similar

response in daphnids reproduction: when the quality or quantity of food was low, daphnids were able to respond by decreasing clutch size and increasing egg size in order to increase the survival of neonates. However this result emphasize that the importance of using an integrated measure of life-history response *e.g.* population growth capacity, to evaluate performance instead of single life history variables *e.g.* growth rate, brood size, as already explained.

The reason for the observed differences between the two algal species and their two growth phases in terms of nutritional composition as well as daphnid performance, might also be due to different levels of bacterial contaminants in the cultures. If there are bacteria in an algal culture, their biomass could increase during the stationary phase of algal growth. In such situations the food quality of algal cells can be reduced, but may be compensated by increased bacterial numbers. Although the *C. minutissima* cultures used in this study were non-axenic, the contribution of bacteria was initially considered unimportant since bacteria have never been identified as a primary resource for *Daphnia* (Lampert, 1987) except in wastewater treatment ponds (Uhlmann, 1961) and the biomass of bacteria even in non-axenic algal cultures is negligible when cultures are in their log phase of growth (Fulton & Paerl, 1987). Moreover, microscopical observations of the culture revealed that the biomass of bacteria was negligible (*ca.* < 1% of the algal biomass).

However, consideration of all results led to the development of the hypothesis that the success of *C. minutisima* as a diet for *Daphnia* in M_4 medium may be due, at least in part, to the presence of bacteria. To test this hypothesis, axenic and non-axenic

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cultures of the same algal strain were compared as food sources for *Daphnia* in conjunction with M$_4$. Life-history parameters of *D. magna* in M$_4$ fed on log phase cultures of axenic and non axenic *C. vulgaris* revealed that bacterial contaminants in non-axenic cultures can apparently compensate for the nutritional deficiency in M$_4$ medium which caused the poor survival of neonates. This deficiency seemed to be due to some compound (*e.g.* vitamin, hormone, amino acid, fatty acid) rather than an element because the same culture medium was used to culture both axenic and non axenic *C. vulgaris*. Moreover the deficiency was compensated by bacteria which were very low in biomass (*ca.* < 1% of the algal biomass) in log phase cultures of *C. minutissima,* indicating that a trace amount of deficient factor is enough to improve the survival of neonates. Lampert (1987) and Baird *et al.* (1989) also reported that better utilization of algal foods can be observed when cultures are contaminated by bacteria.

This study emphasized the importance of both culture medium and the food for optimum performance of *Daphnia* in culture. Although food is the primary source of energy, *Daphnia* can also absorb essential elements directly from the medium. It is possible that this may be the cause of the unpredictable performance of daphnid cultures under different food-medium combinations (Taub & Dollar, 1968; Elendt & Bias, 1990; Dave *et al.*, 1991; Naylor *et al.*, 1992; Sims *et al.*, 1992).

The main purpose of this study was to find a combination of a algal diet and a defined medium for *Daphnia* culture which would be suitable to conduct experiments described in forthcoming chapters. M$_4$ in conjunction with either non-axenic *C.*

vulgaris or *C. minutissima* appears to be the best for the purpose. It became apparent, however, that the culture of non-axenic *C. vulgaris* and its associated bacterial flora prepared for this study was an unstable system. By contrast, the bacterial flora in *C. minutissima* appeared more stable and did not cause the culture to collapse, even during the late stationary phase of growth. Furthermore, the nutritional composition of *C. minutissima* (particularly in terms of nitrogen content) was superior to non axenic *C. vulgaris*. Therefore *C. minutissima* in conjunction with M_4 has been used in this study for chronic experiments. However, axenic *C. vulgaris* was used in feeding experiments because the particle size of *C. minutissima* was rather small and determination of ingested cell numbers proved difficult due to the presence of small aggregations.

CHAPTER 4

GRAZING INHIBITION OF *DAPHNIA MAGNA* BY TOXIC *MICROCYSTIS AERUGINOSA*

4.1 Introduction

Cyanobacterial blooms (in general and *Microcystis* blooms in particular) are increasing in size and duration due to the increase of man-induced eutrophication (Carmichael, 1994). As toxic cyanobacterial blooms become more common in freshwaters, the interest in the knowledge of the role of cyanobacteria in aquatic food chains has also increased. The flow of energy from phytoplankton to zooplankton, particularly to filter-feeding species, is a major link in the food chains of freshwaters (Mitchell *et al.*, 1992). Since *Daphnia*, the most commonly studied filter feeding zooplankton, occupies a central position in fresh water food webs, its feeding and nutrition are important to both phytoplankton and predator populations. It is also worth noting that filter-feeding zooplankton are also important from an ecotoxicological standpoint because they filter large volumes of water, which increase the contact of contaminant present in the water.

4.1.1 Filter feeding in *Daphnia*

The feeding apparatus of *Daphnia*, consisting of highly specialized thoracic appendages for the collection of the food particles, is probably the most developed feeding apparatus within the Branchiopoda (Lampert, 1987). Five trunk limbs *i.e* thoracic appendages, together with the carapace form a suction and pressure pump. Only the third and fourth pairs of appendages carry large filter-like screens which are supposed to retain the particles from the water as they pass through the filtering apparatus.

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In the process of filter feeding, water is pumped between the two valves of the carapace and through filter screens, by rhythmic movements of thoracic appendages (Fig. 4.1). Particles retained by the screens are transferred to the food groove and then towards the mouth for ingestion. If the ingestion rate is lower than the collection rate, particularly when the food concentration is high, excess food may be accumulated in the food groove. Excess food or unsuitable particles can be rejected by a raking movement of the post abdominal claw. *Daphnia* can control the food uptake by regulating the post abdominal rejection rate (hereinafter rejection rate) and thoracic appendage beat rate (hereinafter beat rate) which control the collection of food in the food groove (McMahon & Rigler, 1963).

4.1.2 Particle selection by *Daphnia*

Daphnia's natural food spectrum includes bacteria, cyanobacteria, algae, protozoa and detritus (Lampert, 1987). Generally, *Daphnia* can utilize all these foods although dietary importance is likely to be dependent upon the physical and chemical characteristics of individual food items. McMahon & Rigler (1965) showed that *D. magna* can filter particles within the size range 0.9 - 18 000 μm^3 (equivalent to 0.96 - 20.2 μm spherical diameter) equally efficiently, but obviously the optimum particle size will vary with body size (Knoechel & Holtby, 1986).

Unlike copepods which can discriminate between particles on the basis of size and taste (Demott, 1985) *Daphnia* feed rather non-selectively (Demott, 1986; Kerfoot & Kirk, 1991), *i.e.* they are unable to discriminate particles by size and taste when
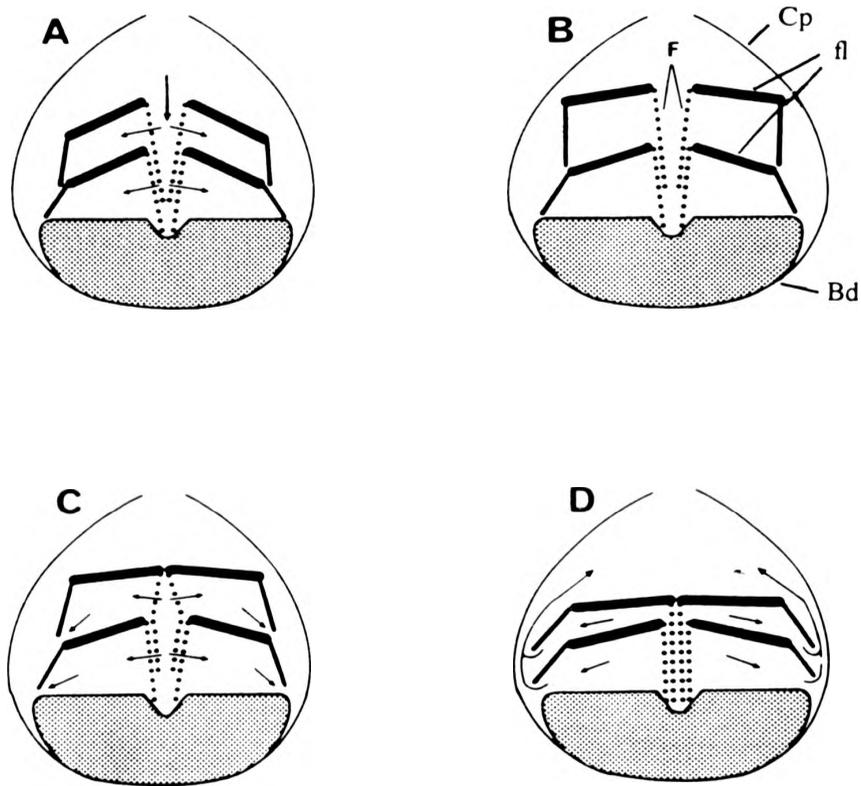
Figure 4 1 A simplified and schematic section through the filtering apparatus of *Daphnia*. A - C represent the four phases of a filtering cycle, and the arrows indicate the flow of water. A & B Limbs move forward, water is sucked into the filter chamber and into the widening interlimb spaces. C & D: Downward stroke, water is pressed through the filtering screens and escapes from the narrowing interlimb spaces. Cp = carapace; fl = filtering limbs, Bd = body; f = filter meshes. (based on Lampert, 1987)

presented in a mixture. However, some reports suggest that *Daphnia* can restrict passage of large particles into the filter chamber by narrowing of the carapace gape (Gliwicz & Sieldar, 1980), the response being based on mechanical stimuli. Furthermore, Lampert (1982) has suggested that *Daphnia* can also reject particles on a chemosensory basis. In this instance the entire contents of the food groove, which might also include high quality food particles, would be rejected non-selectively.

4.1.3 Measurement of feeding rate in *Daphnia*

The feeding rate (synonym = ingestion rate) is a measure of the amount of food that passes through the mouth and enters the gut and is defined as the number of cells consumed by an animal over a set time period (Rigler, 1961). In this study, *Daphnia* feeding rate is expressed as the biovolume consumed per individual per hour in order to facilitate comparison among foods which may differ in particle size.

There are three main methods of measuring the feeding rate of zooplankton; radio-labelling, chlorophyll-a (Chl-a) determination and direct cell counting. Radiolabelling is the most commonly used method for estimating feeding rate in cladocerans (Arnold, 1971; Lampert, 1981; 1982; Hanazato & Yasuno, 1984; Demott *et al.*, 1991). The food organism is labelled either with phosphorus (P^{32}) or carbon (C^{14}) and the filter feeder is allowed to graze on the food for a period less than the gut passage time. The amount of radioactive food ingested by the animal is then determined by scintillation counting techniques and the feeding rate is calculated. These methods determine a true ingestion rate. However, Lampert (1977) found losses of C^{14} due to respiration during

the feeding of *D. pulex* on radiolabelled algae, ranging from 10 to 35% of the assimilated carbon. Care must be taken when using this method therefore, and appropriate corrections must be applied if measurements are to be accurate. Radio-labelling is useful in dealing with short exposure times of a few minutes, with single animals and dilute food concentrations (Peters, 1984). But, perhaps these values may over- or underestimate feeding rate since they do not adequately address the problem of long-term variations in feeding rates *e.g.* diel rhythms of feeding (Lampert, 1987).

The chlorophyll-a determination method has also been used by some authors (Nizan *et al.* 1986). In this method, chlorophyll-a in feeding suspensions is extracted before and after a suitable exposure period, with acetone or ethanol. The colour of the extraction is then determined by spectrophotometer and the respective cell numbers or biovolumes are determined from a previously prepared calibration curve. The change in chlorophyll-a concentration in feeding suspension is considered as a measure of food ingested. In some instances daphnid faeces may contain chlorophyll-a and then an estimation closer to assimilation rate rather than ingestion rate is yielded. Chlorophyll-a concentration in algae or cyanobacteria can vary with environmental factors such as light, and for these reasons care must be taken when using this method.

Cell-count methods are based on the observed change in the number of cells counted before and after a suitable exposure period (usually hours), the change in food concentration being assumed to be a measure of the amount of food eaten (Peters, 1984). Since undigested cells are defecated during long incubation period and may be

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counted, a rate closer to assimilation than ingestion is derived. Two basic methods of cell counting are currently in use - the haemocytometer and electronic particle counter. Waddell (1993) reported that the electronic particle counter is precise and easier than the haemocytometer in estimating cell numbers in feeding suspensions.

4.1.4 Feeding by *Daphnia* on *M. aeruginosa*

Estimation of feeding rate by daphnids on toxic and non-toxic *M. aeruginosa* is important when considering the role of daphnids as control agents of algal population dynamics and energy flow. However, there remains some ambiguity with regard to the utilization of cyanobacteria by *Daphnia* and other cladocerans (Lampert, 1987; de Bernardi & Giussani, 1990; Gliwicz, 1990b). The consensus is that cyanobacteria are an unsuitable food for *Daphnia* (Arnold, 1971; Lampert, 1987) due a combination of several factors: many cyanobacteria species can have inhibitory effects on grazing by zooplankton and are unsuitable foods simply because of their filamentous or colonial morphology (Lampert, 1987; Gliwicz, 1990a; 1990b) *e.g. Anabaena, Aphanizomenon* and *Oscillatoria* species, rendering them invulnerable to grazing, and these mechanical constraints will receive no further consideration here. However some strains of *M. aeruginosa* are potentially capable of being grazed by *Daphnia*, at least when the colony size is below a critical level (Lampert, 1981a; 1981b), and some reports indicate that *M. aeruginosa* can be a good quality food for *Daphnia* (de Bernardi *et al.*, 1981) and other cladocerans (Hanazato & Yasuno, 1984).

Many studies, however, have demonstrated that grazing by *Daphnia* on cyanobacteria

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can be suppressed by toxin-producing species (Lampert, 1981a; 1981b; 1982; Nizan *et al.*, 1986; Demott *et al.*, 1991; Henning *et al.*, 1991). Experiments by Nizan *et al.* (1986) and Lampert (1981a) showed that *Daphnia* can recover from feeding inhibition immediately on transfer to their usual food. As a result, Lampert (1981a; 1982) claimed that the response supported the idea that inhibition of filtering rate is a defence mechanism in the presence of toxic cyanobacteria. However Demott *et al.*,(1991) showed that the reduced food uptake did not recover immediately on transfer to their standard (*i.e.* green algal) food suspensions. Moreover, he showed that daphnids are 10 - 20 times less sensitive to purified microcystin than the copepod, *Diaptomus birgei*.

If microcystin is an evolutionary adaptation against grazing pressure, it seems logical that microcystin would be more toxic to non-selective feeders than to selective feeders because selective feeders may be able to discriminate particles with even a slight toxicity. Hence, it is questionable whether the toxicity of *M. aeruginosa* is an evolutionary adaptation against grazing pressure. Furthermore, it raises another question: "Does the same compound in *M. aeruginosa* cause both filtering inhibition and toxicity to daphnids?" (Jungman *et al.*, 1991). It has been observed that there is no correlation between the mouse toxicity of different strains of *M. aeruginosa* and their effects on feeding inhibition among *Daphnia* (Nizan *et al.*, 1986; Henning *et al.*, 1991). Whilst there is no controversy regarding the fact that some strains of *M. aeruginosa* are toxic to *Daphnia* and that most strains inhibit daphnid feeding (Lampert, 1981a; 1981b; 1982; Nizan *et al.*, 1986; Demott *et al.*, 1991) the underlying mechanisms remain unclear.

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One problem inherent in all of the above cited studies on the toxic effects of microcystin-LR is that they are carried out in different aqueous media, using animals either sampled directly from the field, or produced under unspecified culture condition. Recently Baird (1993) has emphasised the value of standard toxicity testing procedures, such as the OECD *D. magna* 48 h acute and 21 d chronic toxicity tests (OECD, 1984), in ecological studies. Such approaches offer ecotoxicologists the opportunity to carry out studies of toxic effects which can be easily replicated between laboratories. To ascertain the effects of cyanobacterial toxins on *D. magna*, this study used standardised culture conditions and exposure conditions, using artificial media, which allowed for easier replication of experiments.

4.1.5 Aims of the study

The aim of this component of the thesis was firstly to test the hypothesis that microcystin-LR is an antigrazing compound. To do this, 24 h feeding rates of *D. magna* on toxic *M. aeruginosa* were compared with two controls, (*i.e.* feeding rates on non-toxic *M. aeruginosa* and standard food *C. vulgaris*) under the following conditions;

(a) with different cell concentration (*i.e.* functional response)

(b) with different free microcystin concentrations

(c) with different proportions of co-existing non-toxic food concentrations

Secondly, the variations of

(d) feeding rate,

(e) filtering rate (*i.e.* thoracic appendage beat rate; henceforth beat rate), and

(f) post abdominal rejection rate (henceforth rejection rate)

were studied with the increasing exposure time (4 - 5 d), in order to determine the long term feeding response.

Finally (g) recovery from feeding inhibition and

(h) apparent digestibility of foods

were also studied.

4.2 Materials and methods

4.2.1 Some physico-chemical characteristics of *Chlorella* and *Microcystis*

When harvesting *C. vulgaris* and toxic and non-toxic *M. aeruginosa* for feeding experiments, three 50 ml samples were also taken from each culture and concentrated by centrifugation (5 min at 3000 rpm). Each concentrated sample was examined in order to determine cell numbers and cell sizes using the Coulter multisizer (see Chapter 2). Samples were then freeze-dried and weighed using a Mettler Model MT5 microbalance (sensitivity ± 1 µg). The carbon content of freeze-dried cells was determined by Perkin-Elmer C H N S/O Analyzer (Model PE 2400, Series II).

Lampert (1987b) and Nizan *et al.* (1986) reported that *M. aeruginosa* cells in the stationary phase were more toxic than those in the log phase of culture. Hence it is important to define the exact time when cultures reach the stationary phase to harvest algae/cyanobacteria for the purpose of grazing experiments. For most of the green algal species, including *C. vulgaris*, this could be determined readily by the onset of

a rapid colour change from blue-green to dark grass green (Arnold, 1971). But according to laboratory experience in this study, there is no such spectacular colour change for *M. aeruginosa* with the onset of the stationary growth phase; rather the change is gradual. Moreover, the duration of the log phase depends on the initial cell density of the culture. Therefore periodic measurements of some culture characteristics were a prerequisite in deciding the onset of the stationary phase.

A 3 l culture of toxic *M. aeruginosa* was maintained under sterile conditions (see Chapter 2) for 60 d without the addition of fresh medium. Three 50 ml samples were withdrawn once each week, concentrated by centrifugation to a volume of 5 ml and cell/particle size and numbers quantified by Coulter Multisizer. All samples were then freeze-dried and weighed. Freeze-dried samples were then analyzed by photodiode array HPLC (Waters Associates) in order to determine the concentrations of the cyanotoxin microcystin-LR (see Chapter 2).

4.2.2 Feeding experiments

Adult daphnids, after release of their third brood (14 d old; body length = 3.7 ± 0.1 mm) and which were taken from bulk cultures (see Chapter 2) were used for all the following experiments. Whenever toxic *M. aeruginosa* was used for experiments, microcystin-LR concentrations in cells were analyzed by the HPLC method given in Chapter 2.

4.2.2.1 Functional response

The use of low food concentration for feeding experiments may result in food depletions which may lead to lower feeding rates of animals particularly during the latter part of the 24 h exposure period. On the other hand, the use of excessively high food concentrations may cause deleterious effects on animals such as clogged filtering apparatus or oxygen depletion of the medium. Therefore the functional responses of *D. magna* feeding on *C. vulgaris*, toxic and non-toxic *M. aeruginosa* were studied in order to determine the optimum food level to use in other feeding experiments.

Six food levels ranging from 2.5 to 48.0 x 10^6 μm^3 ml^{-1} were used to determine the functional response. Each food regime was prepared by adding the appropriate amount of cells of each species/strain to 1.5 l of M_4 medium and mixing thoroughly. For each of the six food regimes, eight 190 ml screw-top glass vessels were each filled with 180 ml of cell suspension. From the remaining cell suspension, two 1 ml samples were withdrawn by micropipette and cell numbers enumerated by Coulter multisizer (see Chapter 2).

One of each set of 180 ml cell suspensions was used as an acclimation chamber, and four adult daphnids were assigned to each chamber and left to feed for 1 h. Another four of each set of cell suspensions were used as grazing chambers and the experiment began when acclimatized daphnids were transferred individually to the experimental grazing chambers containing the same food type and concentration as in the acclimation chambers. The remaining three suspensions of each food served as

controls. The position of the grazing chambers was randomized and grazing was allowed to proceed for 24 h at 20 ± 1 °C under a 14L:10D photoperiod.

At the end of the feeding period, animals were removed from the vessels and each suspension was vigorously shaken to re-suspend any settled cells. Two 1 ml samples from each vessel were enumerated by Coulter multisizer as previously described. Body lengths of the animals removed were directly measured using an Olympus dissecting microscope. The ingestion rate I_r (ingested biovolume μm^3 per daphnid per hour) was calculated as follows:

$$ I_r = \{(C_o - C_t)\, v\} / t $$

where C_o is the initial cell concentration ($\mu m^3\ ml^{-1}$), C_t is the final cell concentration ($\mu m^3\ ml^{-1}$), t is the exposure time (h), and v is the total volume of the feeding suspension (ml). If there was any increase in cell numbers in controls during the exposure period, the mean value of the initial and final cell numbers was used as C_o.

4.2.2.2 Feeding rate in the presence of free microcystin-LR

Feeding rate of adult (14 d) daphnids on *C. vulgaris* was measured in the presence of dissolved purified microcystin-LR. The concentration of microcystin-LR used in this experiment was 1000 $\mu g\ l^{-1}$ which was well below the LC_{50} value for adult *D. magna* (see Chapter 5).

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The feeding experiment was carried out using only a single concentration (1×10^7 μm³ ml⁻¹) of *C. vulgaris*. The procedure followed was identical to that of the functional response studies except that both strains of *M. aeruginosa* were not used as the main aim of the experiment was to reveal whether there is any effect of free microcystin-LR on the grazing loss of other algal foods by daphnids. Feeding rates of daphnids were determined in two treatments: in the controls, without added microcystin-LR, and in the treatments, in which microcystin-LR was added. A stock solution of microcystin-LR with a concentration of 40 000 μg l⁻¹ (see Chapter 2) was used. After measuring out eight 180 ml samples from the *C. vulgaris* suspension with toxin added, two 20 ml samples were withdrawn from the remaining suspension and analyzed to confirm the concentration of microcystin-LR (see Chapter 2).

4.2.2.3 Feeding rates on mixtures of *Chlorella* and *Microcystis*

Feeding rates of adult (14 d) daphnids on different mixtures of *C. vulgaris* and both strains of *M. aeruginosa* were determined in order to discover if there was any effect of toxic *M. aeruginosa* (*i.e.* cell-bound toxin) on the ability of daphnids to graze other algal foods. Two series of mixtures: *C. vulgaris* plus toxic *M. aeruginosa* and *C. vulgaris* plus non-toxic *M. aeruginosa* were used. In each series of experiments, there were five treatments: 100% *C. vulgaris*, 75% *C. vulgaris* + 25% *M. aeruginosa*, 50% *C. vulgaris* + 50% *M. aeruginosa*, 25% *C. vulgaris* + 75% *M. aeruginosa* and 100% *M. aeruginosa* based on percentages of a constant biovolume concentration of 1×10^7 μm³ ml⁻¹. Feeding rates of *Daphnia* in these treatments were measured simultaneously. The same procedure as in the functional response studies was followed, except that

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only one food concentration was used.

4.2.3 Feeding behaviour with increasing exposure time

4.2.3.1 Long term feeding rates

Long term feeding rates were determined using only a single concentration (*i.e.* 1×10^7 μm^3 ml^{-1}) of each species/strain. The procedures followed were identical to those in the functional response studies, except that feeding rates were measured daily for 4-5 d using the same animals. After each 24 h exposure period, animals were transferred to fresh suspensions with the same concentration of each species/strain. As daphnids were expected to release their fourth brood on the third day of the experiment, every 8 h on the third day two 1 ml samples were withdrawn from each jar and algal cell numbers enumerated as in the functional response studies. If animals had released a brood in the first or second 8 h period the feeding suspensions were discarded and adults animals were transferred to fresh suspensions at the beginning of the next 8 h period. The fresh samples also had controls.

4.2.3.2 Beat rate and rejection rate

As already described, *Daphnia* can control food intake by regulating the thoracic appendage beat rate and the rejection rate of foods by the post abdominal claw. Therefore an experiment was performed to record beat rate and rejection rate of daphnids after increasing exposure to toxic and non-toxic *M aeruginosa*. Fifteen adult

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daphnids (14 d) were assigned individually to 180 ml suspensions of each strain of *M. aeruginosa* with the concentrations of 1×10^7 μm^3 ml^{-1} in order to expose them to these foods prior to observation of beat rate and rejection rate. Simultaneously, the same number of animals was exposed to 180 ml of food-free medium and *C. vulgaris* suspensions. Animals which were to be exposed for more than 24 h were transferred to fresh media after each 24 h exposure. Three animals from each treatment were used to record beat rate, and rejection rate after 24 h, 48 h, 72 h and 96 h exposure.

It was necessary to physically immobilise *Daphnia* by tethering animals in order to record these parameters. Individual daphnids were attached dorsally (*i.e.* just below the antennal sockets on the dorsal midline) to the centre of a glass slide with petroleum jelly (vaseline). The glass slide was then introduced into 180 ml of the same food suspension/medium in a 250 ml beaker and inclined against the wall of the beaker so that the animal was on the upper surface of the slide (Fig. 4.2). With practice, it proved possible to reduce the time for this procedure to 20 seconds per animal.

The beat rate, and rejection rate of each animal was measured by recording with a Panasonic CCD video camera (F 15) coupled to an Olympus trinocular dissecting microscope (SZ 30) at x40 magnification. Each *Daphnia* was filmed for 5 min; a digital video timer was superimposed on the tape in order to keep track of the time during filming. The recorded sequences were replayed at a rate sufficient to observe individual movements of the thoracic appendages and the numbers of thoracic appendage beats and post abdominal rejections observed during one minute intervals recorded.

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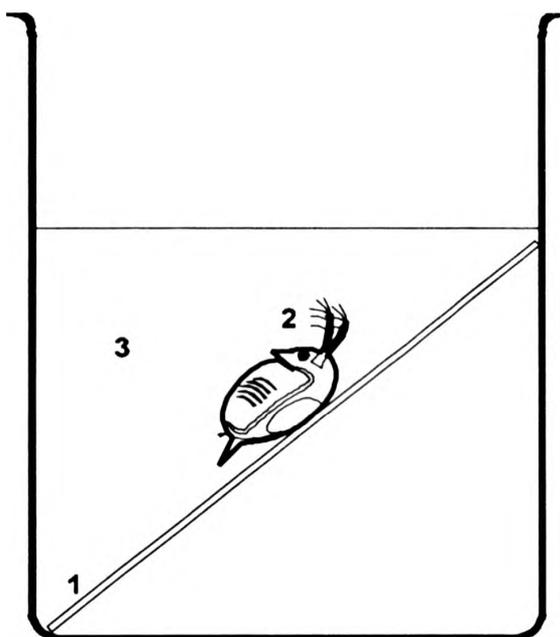
Figure 4.2 Schematic representation of the setup for direct observation and vedio recording of thoracic appendage beat rate and post abdominal rejection rate of *D. magna* (1 = glass slide; 2 = *Daphnia*; 3 = culture medium).

4.2.4 The recovery from feeding inhibition

Preliminary experiments showed a significant inhibition of feeding rates of *Daphnia* on toxic as well as non-toxic *M. aeruginosa*. Hence, recovery from inhibition was studied by measuring daily feeding rates of *Daphnia* which were pre-exposed individually to different treatments as follows:

- four daphnids in toxic *M. aeruginosa* for 24 h;

- four daphnids in toxic *M. aeruginosa* for 72 h;

- four daphnids in non-toxic *M. aeruginosa* for 24 h;

- four daphnids in non-toxic *M. aeruginosa* for 72 h;

- four daphnids in food-free medium (M_4) for 24 h;

- four daphnids in food-free medium (M_4) for 72 h;

- four daphnids in *C. vulgaris* for 72 h;

Food concentration in all pre-exposure suspensions, except that of food free media, was 1×10^7 μm^3 ml^{-1}. In order to achieve a single temporal end point for all pre-exposures, animals exposed for 72 h periods were assigned to their suspensions/media 48 h prior to the other animals; moreover, after each 24 h of exposure, animals in this treatment were also transferred to fresh suspensions of the same food.

After the pre-exposure period, all animals were transferred to fresh suspensions of *C. vulgaris* with the same food concentration and daily feeding rate measured for up to 4-5 d. The procedure followed was identical to that in long-term feeding response studies.

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4.2.5 Gut defecation rate and digestibility of foods

It is difficult to quantitatively measure the rate of defecation as preliminary observations showed that the sizes of faecal masses ejected from the gut vary depending on food type. Hence the frequency of gut defecation of daphnids which were exposed to each food was recorded and considered as the defecation rate. Gut defecation frequency depends on the gut retention time of foods which is probably affected by feeding rate. Prior to measuring defecation rate, three daphnids were exposed to each food for 24 h. They were then each attached to glass slides and introduced into a suspension of the same food following the procedure used to measure beat rate (see above). Each animal was watched for two periods of 5 min under an Olympus dissecting microscope and the number of defecations counted.

After recording the defecation rate, animals were further observed under the dissecting microscope. A fine glass pipette (1 mm in diameter) was directed towards the anus of the daphnids and when it defecated the faecal mass was immediately withdrawn into the pipette and the contents observed under the high power microscope (Olympus BH-2) in order to assess the digestibility of each food.

4.2.6 Statistical analysis

Comparisons of feeding rates on log phase and stationary phase cultures of each food were carried out using directional t-test (Zar, 1984). Two-way analysis of variance (ANOVA) and Tukey HSD multiple range test were used to compare daphnid feeding

rates, beat rates, and rejection rates under increasing exposure to three different diets and feeding rates on different mixtures of *C. vulgaris* and *M. aeruginosa*.

Since the functional response data followed a curvilinear mode, comparison between treatments was carried out by linearising the data using a reciprocal transformation (*i.e* 1/x). Responses were then compared by analysis of covariance to test the hypothesis that the slope of the response lines were different *i.e.* the null hypothesis was that b1 = b2 = b3 at a probability level of p = 0.05.

4.3 Results

4.3.1 Cell characteristics of *C. vulgaris* and *M. aeruginosa*

Key characteristics of *C. vulgaris* and the two strains of *M. aeruginosa* cultured in this study are given in Table 4.1. The spherical diameter of *C. vulgaris* was 3.14 ± 0.47 µm whilst non-toxic and toxic *M. aeruginosa* cells were 3.47 ± 0.45 and 3.33 ± 0.47 µm respectively (Mean ± S.d.; n = 20). Carbon content was also similar for all three species/strains. The data given Table 4.1 facilitate conversion between biovolume, carbon weight, dry weight and cell numbers - often a source of confusion in zooplankton feeding studies.

Fig. 4.3 (a)-(e) illustrate the variation in cell density, cell diameter, cell dry weight and cell toxin content (expressed as total mass per cell and concentration per unit cell volume) within a culture of toxic *M. aeruginosa* strain PCC 7820 over a 60 d period.

Table 4.1 Some characteristics of *C. vulgaris* cells and *M. aeruginosa* doublets (here referred to cells) used for feeding experiments.

Species / Strain	Spherical diameter of cells		Mean Dry Wt. of a cell (pg)	Carbon content (%)	Microcystin-LR content of cells (μg mm^{-3})
	Mean (μm)	Range (μm)			
C. vulgaris	3.1	1.6-4.6	9.0 ± 0.08	48.23 ± 0.25	not analyzed
Toxic *M.aeruginosa*	3.3	2.4-5.4	7.61 ± 0.19	45.67 ± 0.04	1.5
Non-toxic *M.aeruginosa*	3.5	2.2-5.0	9.57 ± 0.25	46.99 ± 0.29	0.0
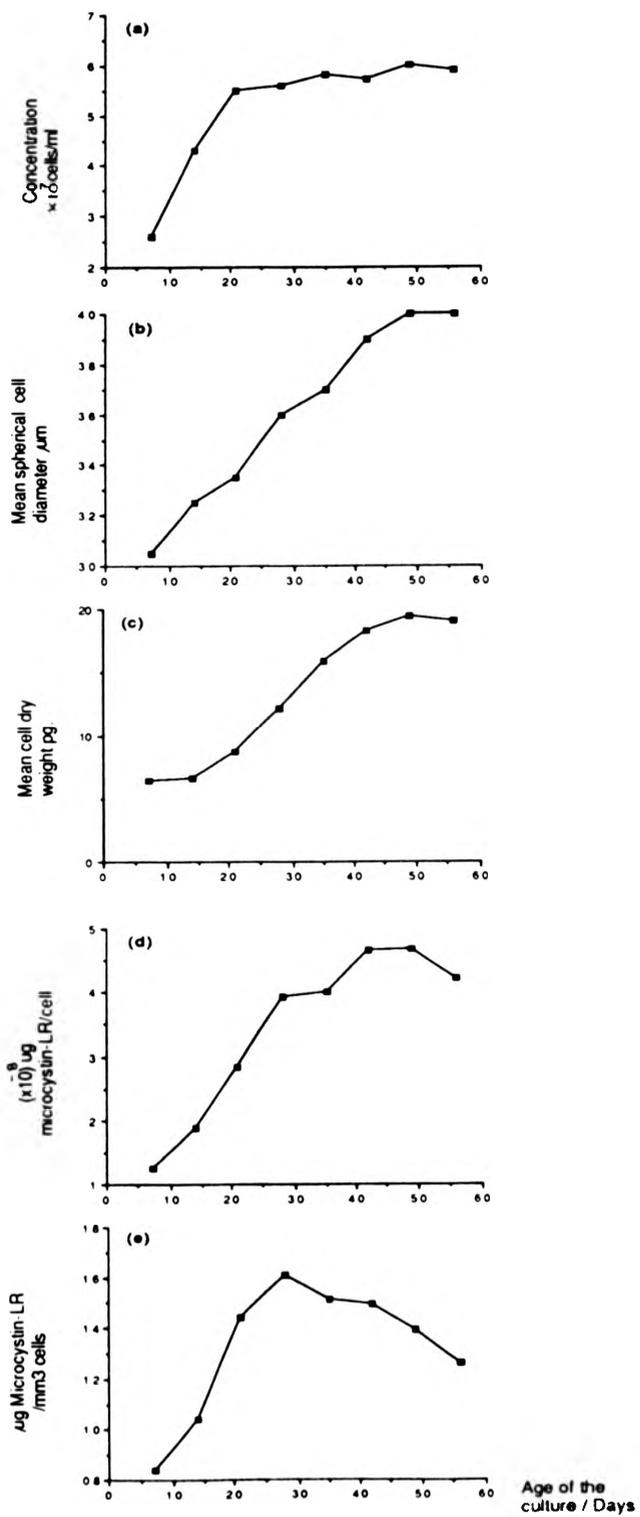
Figure 4.3 Variations of some cell characteristics of a toxic *M. aeruginosa* culture.

The general pattern is of a rapid increase in cell size and density during the early growth phase of the culture, after which cell density stabilises while cell diameter and dry weight continue to increase. However, when toxicity is expressed in terms of microcystin-LR content per cell, it also appears to increase until the late growth phase of the culture. By contrast, when toxicin concentration is expressed in terms of microcystin-LR concentration per unit cell volume, it seems to decrease during the late growth phase of the culture.

4.3.2 Feeding experiments

4.3.2.1 The functional response.

The relationship between food concentration and feeding rate (often described as the functional feeding response) of 14 d old *D. magna* feeding on each of the three cell types over 24 h period is given in Fig. 4.4. For all cell types, the functional response was fitted to a simple first order saturation curve (type II of Holling, 1966). It was apparent that although a similar shape of feeding response was obtained for all three cell types, the maximum feeding rate was substantially lower for the two *M. aeruginosa* strains relative to *C. vulgaris*. In addition, it was also seen that the feeding rate of animals presented with cells of the toxic *M. aeruginosa* strain (toxin content measured as 1.20 µg mm^{-3} microcystin-LR) was reduced in comparison with those fed on the non-toxic strain, although this difference was less striking than between *C. vulgaris* and *M. aeruginosa*. Covariance analysis revealed that feeding rate values on each diet were significantly different from one another (Table 4.2).

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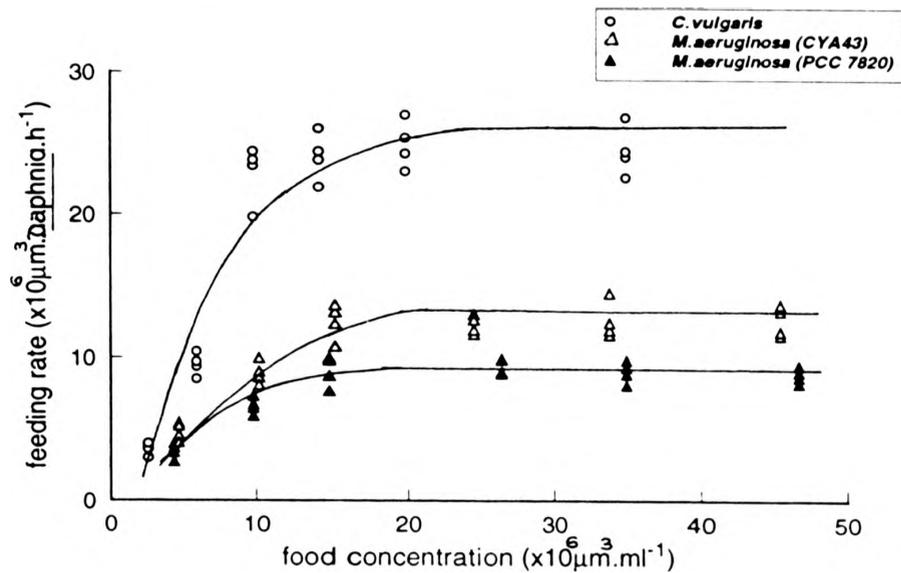
Figure 4.4. Functional response of *D. magna* feeding on *C. vulgaris*, a non-toxic strain (CYA 43) and a toxic strain (PCC 7820) of the cyanobacterium *M. aeruginosa*. The microcystin-LR content of the toxic strain was 1.2μg mm^{-3}. (Curves fitted by eye).
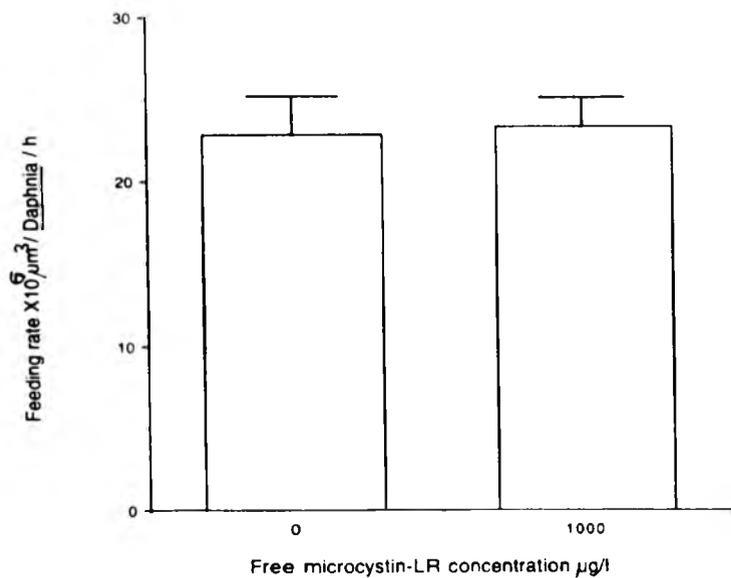
Figure 4.5 Feeding rate of *D. magna* on *C. vulgaris* after 24 h exposure to dissolved purified microcystin-LR (data presented are means ± standard deviations).

Table 4.2 Results of the analysis of covariance performed on the functional response data of adult feeding on different diets. The asterisk indicate significant difference between diets at a probability level of p = 0.05.

	C. vulgaris	Non-toxic M. aeruginosa
Non-toxic M. aeruginosa	F = 73.8; 1, 14 df; p < 0.05	
Toxic M. aeruginosa	F = 70.44; 1, 14 df; p < 0.05	F = 4.97; 1, 14 df; p < 0.05

Table 4.3 Two-way analysis of variance for the feeding rate on mixtures of C. vulgaris and M. aeruginosa (* significant at P<0.05; n.s., not significant).

Source of variation	d.f.	Sum of squares	F value
Treatments	5	1214	
M.aeruginosa strain	1	55	33.3 *
Percentage	4	1158	173 *
Percentage↔Strain	4	20	3.0 (n.s.)
Residual	30	1.7	
Total	39	1284	

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4.3.2.2 Feeding rate in the presence of free microcystin-LR

Fig. 4.5 illustrates feeding rates of adult *D. magna* on *C. vulgaris* in the presence of dissolved purified microcystin-LR in the medium. It revealed that *Daphnia* exhibited no acute feeding response to the purified toxin at a concentration of 1000 µg l^{-1}, some 25 times higher than that might occur in natural waters, as there was no significant difference between feeding rates in the presence and absence of free toxin (t= - 0.28; df = 6, P > 0.05).

4.3.2.3 Feeding rate on mixtures of *C. vulgaris* and *M. aeruginosa*

Observations on *D. magna* feeding on mixtures of *C. vulgaris* and toxic or non-toxic *M. aeruginosa* with a constant total food concentration (10^7 µm^3 ml^{-1}) are represented in Fig. 4.6 (a)-(b). Feeding rates on both mixtures decreased gradually with the increase in percentage of *M. aeruginosa* biovolume. However, feeding rates on mixtures with 25% of toxic and non-toxic *M. aeruginosa* were 15.97 and 18.3 µm^3 *Daphnia*$^{-1}$ h^{-1} whilst those exposed to pure (*i.e.* 100%) suspensions of toxic and non-toxic *M. aeruginosa* and *C. vulgaris* were 8.15, 13.3 and 25.2 µm^3 *Daphnia*$^{-1}$ h^{-1} respectively indicating that the addition of even small amounts of toxic or non-toxic *M. aeruginosa* cells was sufficient to inhibit feeding substantially. Two-way analysis of variance showed that *M. aeruginosa* strain as well as the percentage of *M. aeruginosa* had a significant effect on feeding rate but the interaction between *M. aeruginosa* strain and the percentage had not a significant effect on the feeding rate (Table 4.3).
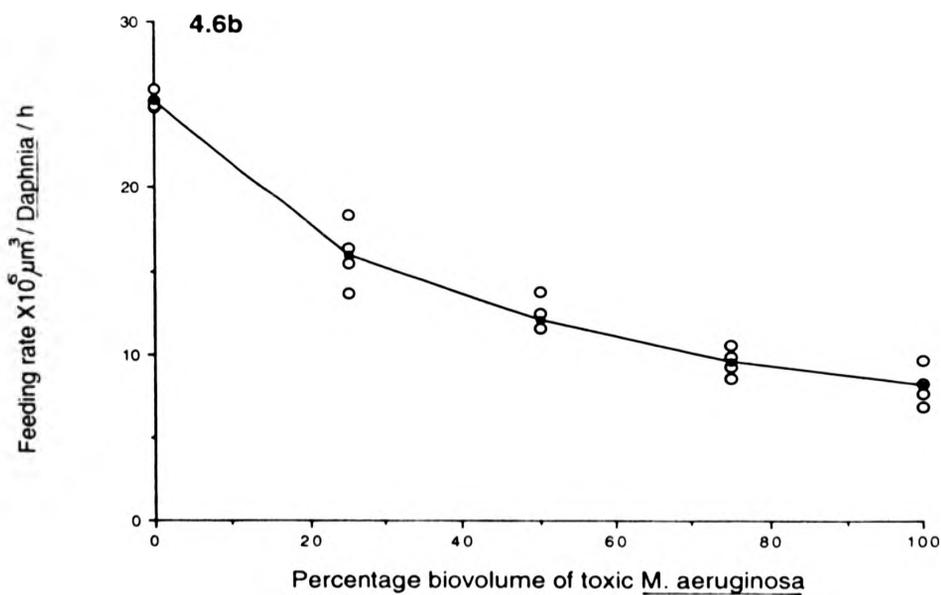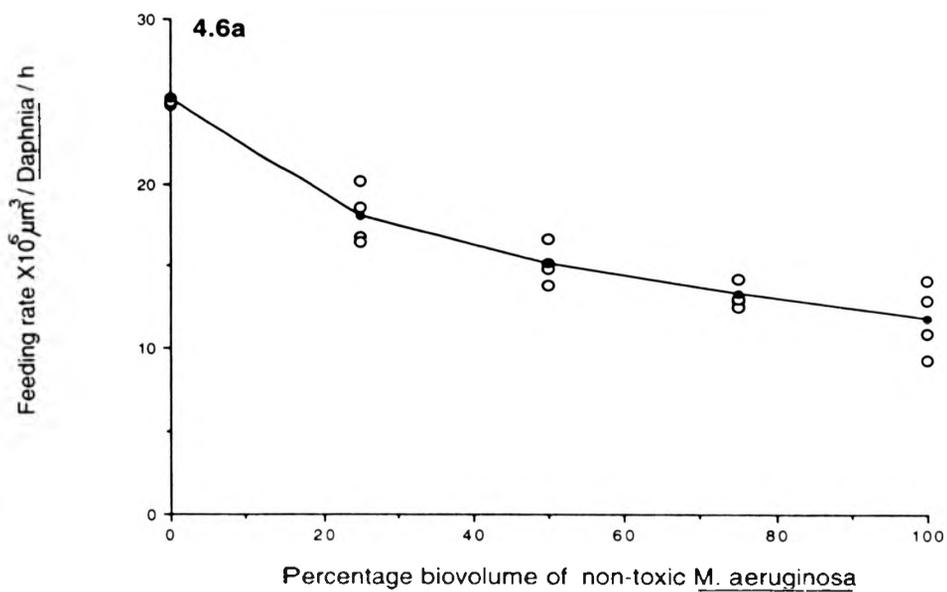
Figure 4.6 Feeding rate of *D. magna* at a constant food density of 10^7 µm^3 ml^{-1} on mixtures of *C. vulgaris* and (a) non-toxic *M. aeruginosa* and (b) toxic *M. aeruginosa* over 24 h (line indicate joined mean values).

4.3.3 Variations of feeding behaviour with the increasing exposure time

4.3.3.1 Long term feeding response

Further observations of *D. magna* feeding on the three cell types over a more prolonged period, 120 h, at a single food level (10^7 μm^3 ml^{-1}) are shown in Fig. 4.7. It can be seen that feeding rate on *C. vulgaris* remained at constantly high levels. But again the relatively lower initial (*i.e.* first 24 h) feeding rates of animals fed on *M. aeruginosa* declined substantially over the five day period, indicating that feeding inhibition of *D. magna* on toxic and non-toxic *M. aeruginosa* occurs in two ways; initial inhibition and further inhibition over time. However, daphnids fed on toxic *M. aeruginosa* (the toxin content measured was similar to that used in functional response studies) died between 96 h and 120 h of the start of the experiment whilst those fed on non-toxic *M. aeruginosa* seemed to stabilise at a lower level of feeding rate. The two-way analysis of variance revealed that the exposure time and diet had a significant effect on the feeding rate but there was not a significant interaction between time and the diet (Table 4.4)

4.3.3.2 Beat rate

Variation of beat rate of adult *D. magna* fed on each of the three cell types over a protracted period of 120 h are given in Fig. 4.8. Starved animals showed the highest beat rate which remained constant for 72 h and then slightly reduced, but was still higher than that of the other treatments. The beat rate of animals in the control

Table 4.4 Two-way analysis of variance for feeding rate in the long term feeding response studies. (* significant at P<0.05; n.s., not significant).

Source of variation	d.f.	Sum of squares	F value
Treatments	5	2953	
Time	3	100	11 *
Diet	2	2876	496 *
Time↔Diet	6	34	1.9 (n.s.)
Residual	35	101	
Total	46	3089	

Table 4.5 Two-way analysis of variance for the thoracic appendage beat rate (* significant at P<0.05; n.s., not significant).

Source of variation	d.f.	Sum of squares	F value
Treatments	6	207173	
Time	3	26483	13 *
Diet	3	181680	95 *
Time↔Diet	9	16375	2.8 (n.s.)
Residual	37	23499	
Total	52	247047	

Table 4.6 Two-way analysis of variance for the post abdominal rejection rate (* significant at P<0.05; n.s., not significant).

Source of variation	d.f.	Sum of squares	F value
Treatments	6	2687	
Time	3	321	2.0 *
Diet	3	798	18 *
Time↔Diet	9	784	2 (n.s.)
Residual	36	1537	
Total	51	5008	
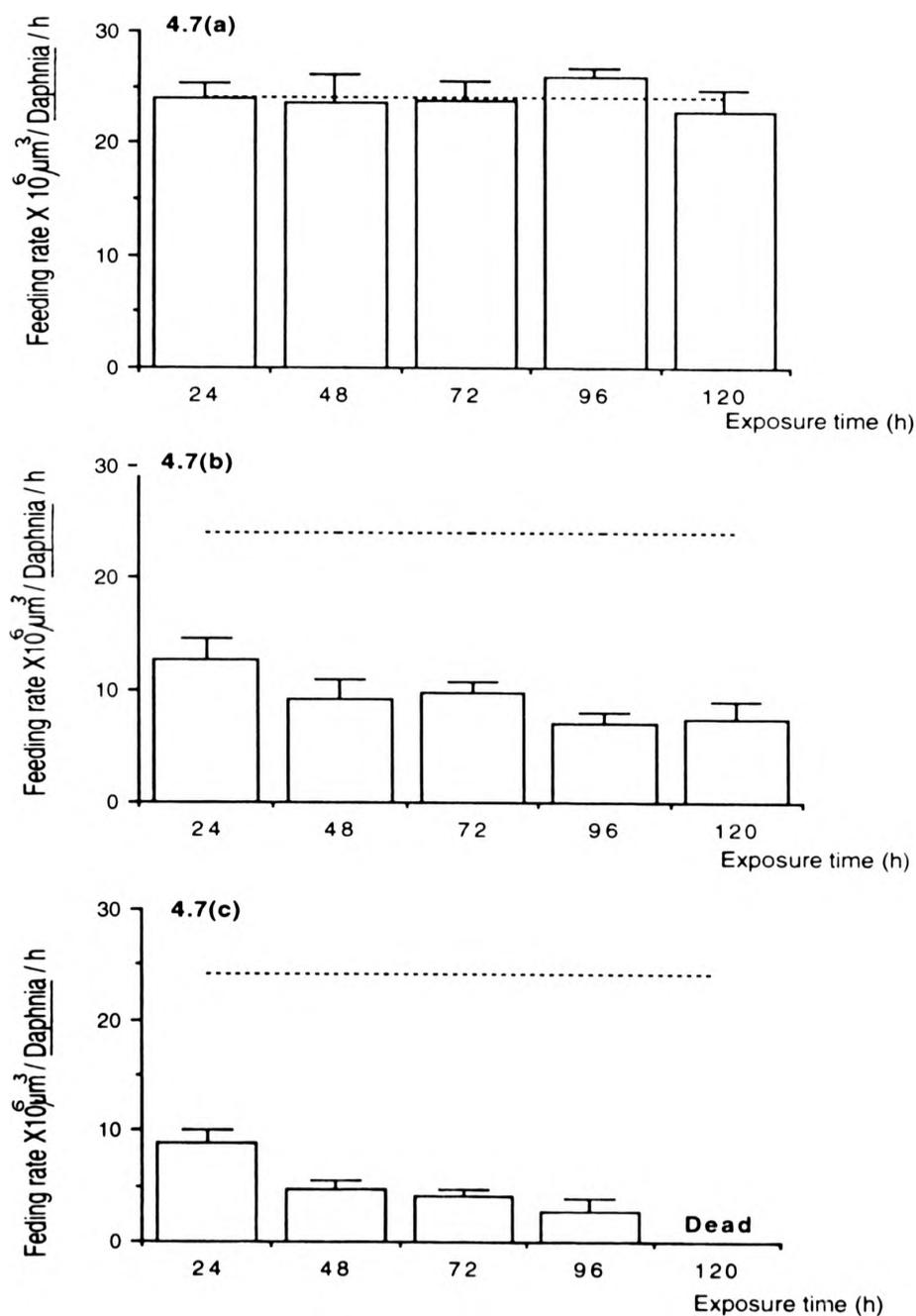
Figure 4.7 Variations of feeding rate of *D. magna* on (a) *C. vulgaris* (b) non-toxic *M. aeruginosa* and (c) toxic *M. aeruginosa* under continuous (4 - 5 d) exposure (data presented are means ± standard deviations; dotted line indicate the average of control feeding rate)

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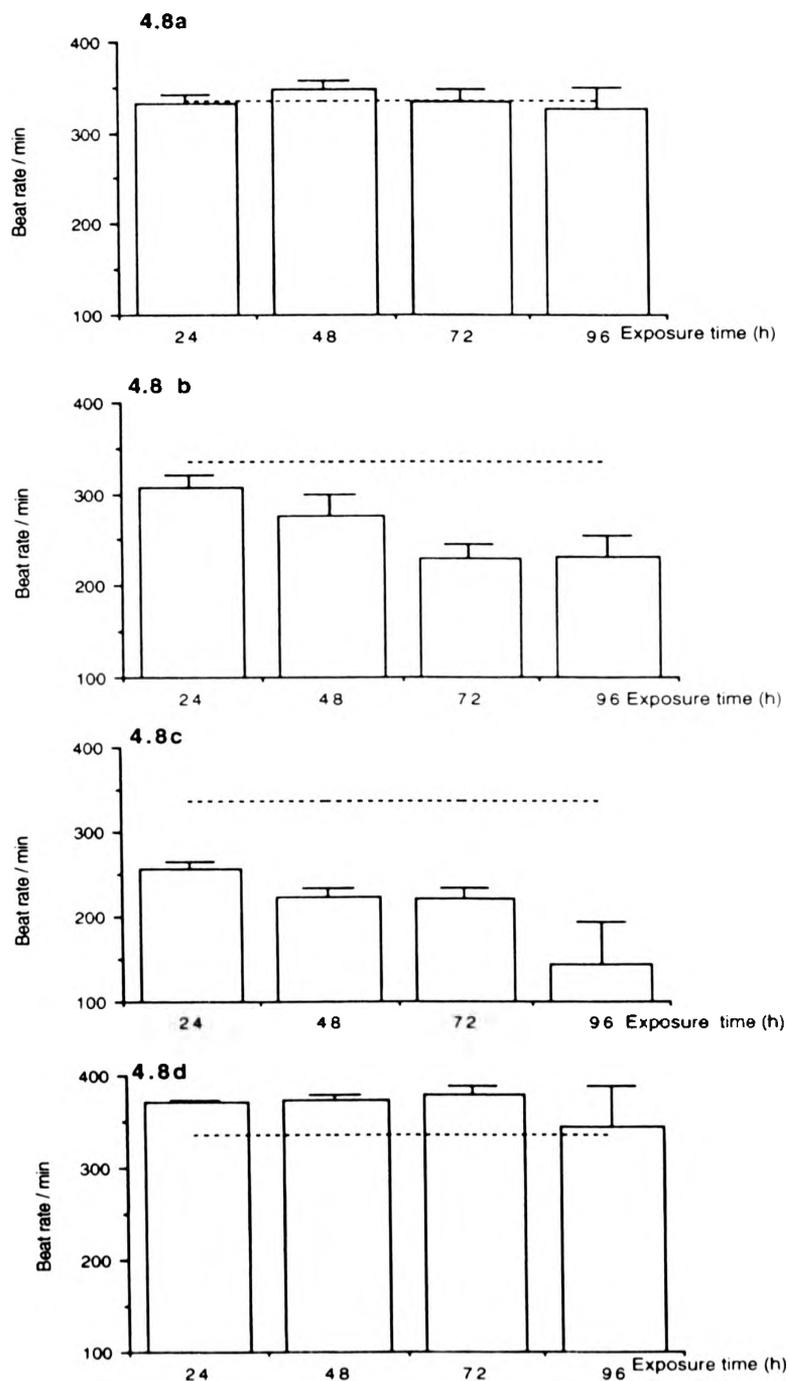
Figure 4.8 Thoracic appendage beat rate of *D. magna* after increasing exposure to (a) *C. vulgaris* (b) non-toxic *M. aeruginosa* (c) toxic *M. aeruginosa* and (d) food free medium (data presented are means ± standard deviations; dotted line indicate the average of control beat rate).

treatment (*i.e.* continuous exposure to *C. vulgaris*) showed the second highest beat rate and it remained constant over the 5 d period. The third highest and the lowest beat rate were apparent among daphnids exposed to non-toxic and toxic *M. aeruginosa* respectively. The initial (*i.e.* after the first 24 h exposure) beat rates reduced further as a result of continuous exposure to the same food, in a similar pattern to the feeding inhibition which was observed in long term feeding responses. The result of two-way analysis of variance was also similar to that of long term feeding response. The exposure time and the diet had a significant effect on the beat rate but there was not a significant interaction between exposure time and diet (Table 4.5). The toxin content of toxic *M. aeruginosa* used in this study was determined to be 1.32 μg microcystin-LR mm^{-3}.

4.3.3.2 Rejection rate

The variation in rejection rate of adult *D. magna* exposed to a constant food level (10^7 μm^3 ml^{-1}) of each of the three cell types over 96 h are shown in Fig. 4.9(a)-(d). Daphnids exposed to food-free media and *C. vulgaris* showed lower and rather similar rejection rates which remained at approximately the same level over the 96 h period. However, the rejection rates of animals exposed to toxic and non-toxic *M. aeruginosa* were almost twice as high as those of starved animals and those fed on *C. vulgaris;* moreover, these rejection rates also remained at a higher level with little or no variation over a 96 h period. Time as well as the interaction between time and diet had no significant effect on rejection rate; only diet had a significant effect on rejection rate (Table 4.6).
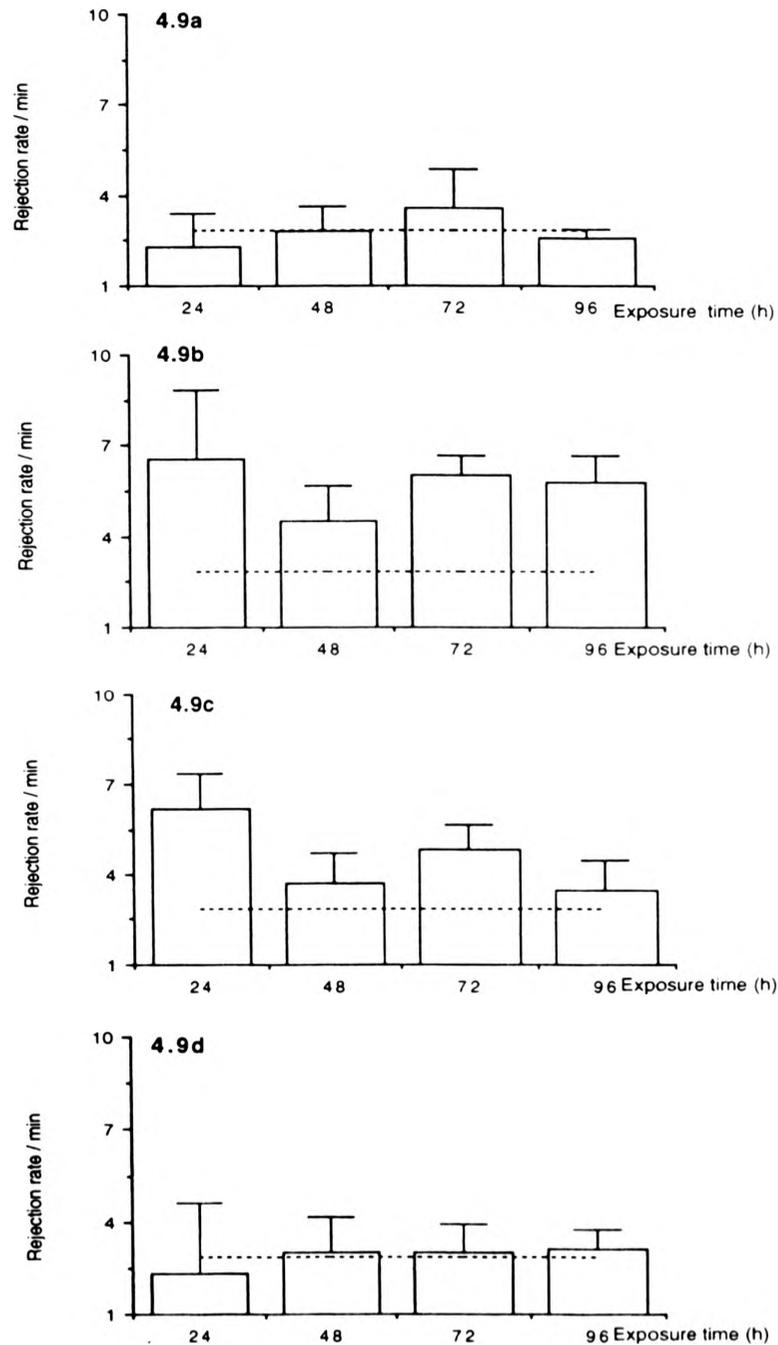
Figure 4.9 Post abdominal rejection rate of *D. magna* after increasing exposure to (a) *C. vulgaris* (b) non-toxic *M. aeruginosa* (c) toxic *M. aeruginosa* and (d) food free medium (data presented are means ± standard deviations; dotted line indicate the average of control rejection rate).

4.3.4 Recovery of feeding inhibition

Feeding inhibition in *D. magna* as a result of pre-exposure to food-free medium and toxic and non-toxic *M. aeruginosa* and recovery on transfer to *C. vulgaris* are shown in Fig. 4.10 (a)-(d). Feeding rates of *D. magna* on *C. vulgaris* were inhibited by 17%, 26% and 46% with respect to the control value as a result of 24 h pre-exposure to food-free medium, non-toxic and toxic *M aeruginosa* respectively. However, with respect to the first two, the inhibition effect disappeared within a day but took two days for complete recovery when pre-exposure was to toxic *M. aeruginosa*. Animals pre-exposed to the same treatments for 72 h, showed 52.6%, 46.9% and 48.9% suppression of feeding respectively. It seems that the degree of feeding inhibition in all treatments was somewhat similar although the recovery periods were not. Daphnids which were pre-exposed to non-toxic *M. aeruginosa* and food-free medium recovered normal feeding rates within 2 and 3 days respectively whilst those of animals pre-exposed to toxic *M. aeruginosa* took 5 d to recover to a level which was still depressed relative to the control.

4.3.5 Defecation rate and digestibility of foods

Plate 4.1(a)-(c) show faecal material produced by adult *D. magna* after 24 h exposure to each food type. It was apparent that *D. magna* can digest all three cell types but that there were pronounced differences in the digestibilities among the three cell types. Gut contents of animals fed on toxic *M. aeruginosa* appeared to be completely digested (Plate 4.1c) whilst those taken from animals fed on non-toxic *M. aeruginosa*

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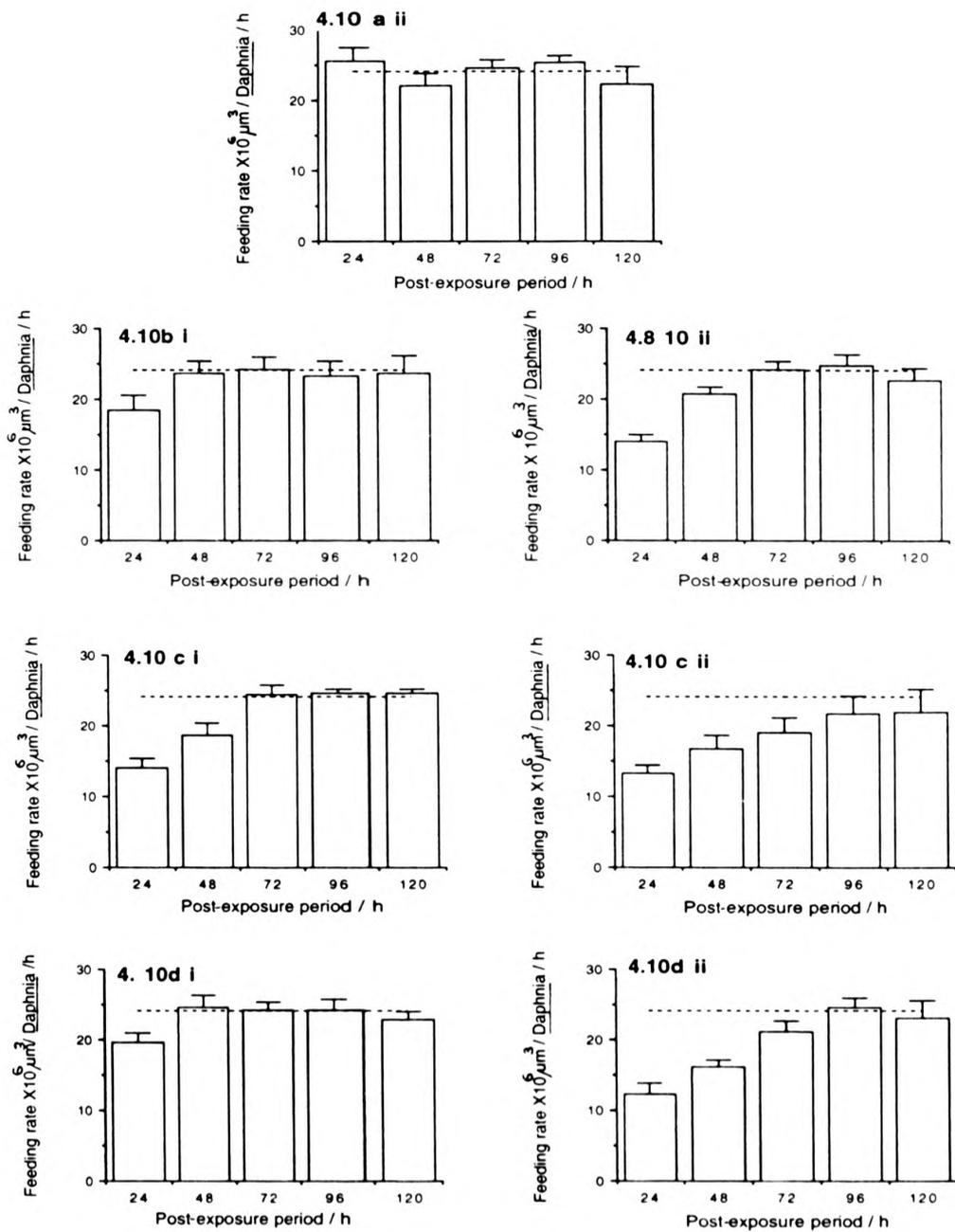
Figure 4.10 Post-exposure feeding rates of adult *D. magna* on *C. vulgaris*. Animals were pre-exposed to (a) *C. vulgaris*, (b) non-toxic *M. aeruginosa* (c) toxic *M. aeruginosa* and (d) food free medium for (i) 24 h and (ii) 72 h (data presented are means ± standard deviations; dotted line indicate the average of control feeding rate).
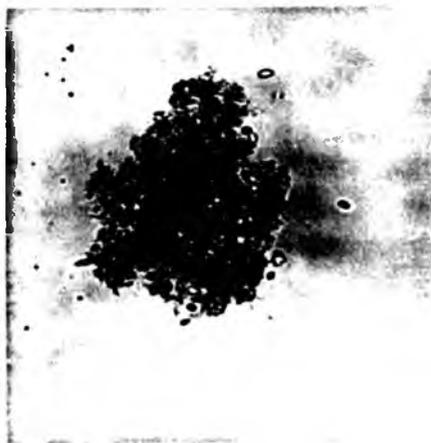
(a)

(b)
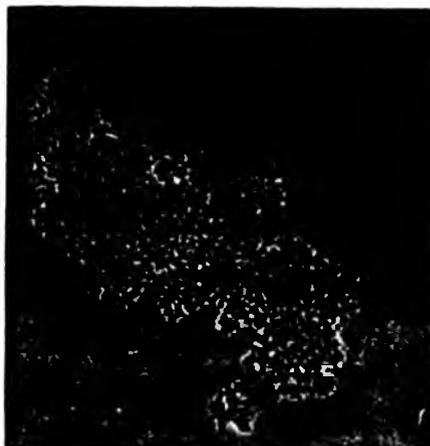
(c)

Plate 4.2 Faeces of *D. magna* fed on (a) *C. vulgaris* strain CCAP 211/12 (b) non-toxic *M. aeruginosa* strain CYA 43 and (c) toxic *M. aeruginosa* strain PCC 7820. Note that all the cells are digested in (c) but that there are some undigested cells in (a) and (b), (x600).

(Plate 4.1b) and *C. vulgaris* (Plate 4.1a) contained undigested or partially digested cells. The defecation rates of animals fed on toxic and non-toxic *M. aeruginosa* and *C. vulgaris* were 0.075, 0.1 and 0.46 min^{-1} respectively.

4.4 Discussion

The dependence of ingestion rate of *D. magna* on food concentrations of the green alga *Ankistrodesmus falcatus* and toxic *M. aeruginosa* was studied by Henning *et al.* (1991) who described the functional relationship between ingestion and food concentration as a simple type II response (Holling, 1966), characterised by a curvilinear increase in ingestion rate with increasing food concentration. The present results also showed a similar pattern, although the minimum food concentration which gave the highest feeding rates were not comparable. The discrepancies may be due to differences in methods and materials used (*i.e.* exposure time, species and strains of algal/cyanobacterial foods, *etc.*). However, the results from this study are in agreement with the maximum feeding rate values of *D. magna* on green algae reported by Henning *et al.* (1991), although again the suppression of feeding rate on toxic *M. aeruginosa* relative to the typical feeding rate is not comparable. According to results of this study, feeding rates on non-toxic and toxic *Microcystis* were reduced by 50% and 60% respectively, relative to the feeding rate on *C. vulgaris*.

It is obvious that there should be strain-specific differences in feeding rates of *D. magna* on *M. aeruginosa* (Henning *et al.*, 1991; Nizan *et al.*, 1986). However other factors must also be important, since there are deviations even with the same strain.

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Nizan *et al.* (1986) found that the feeding rate of *D. magna* on toxic *M. aeruginosa*, (strain PCC 7820) was approximately 15% of the normal feeding rate. However, the percentage value obtained in this study was more than double that for the same strain of *M. aeruginosa*. Methodological differences are the most probable explanation for this discrepancy. Indirect quantification of cyanobacteria and algae based on chlorophyll-a determination is unlikely to be an accurate method of determining ingestion rate, particularly in comparisons between different algal/cyanobacterial food types. However, such discrepancies can only be fully accounted for examining all the factors which affect feeding rate. Apart from the genetic differences between organisms, there are environmental factors (*e.g.* temperature, light intensity, photoperiod), differences in food characteristics (*e.g.* concentration, particle size, age of cells, purity of the culture, toxicity), and some factors specific to the grazer (*e.g.* body size, health) which might affect feeding rate among daphnids (Lampert, 1987a). This study compared only the effect of toxicity of *M. aeruginosa* on feeding rates of *D. magna* using defined toxicities, culture media and environmental factors.

The sizes of the particles of the three algal/cyanobacterial strains used were almost identical and appropriate for collection and ingestion by *Daphnia* since they were unlikely to interfere mechanically with the filtering apparatus. The pattern of variations in culture characteristics in toxic *M. aeruginosa* emphasizes that the age of the culture can be an important source of discrepancies in the results from zooplankton feeding studies.

In addition to the study of short term (*i.e* 24 h) feeding responses, the long term (*i.e.*

4 - 5 d) feeding responses which have been largely neglected in previous studies were determined in this study. When animals were exposed to *M.aeruginosa* for long periods, feeding rates on both strains (*i.e.* toxic and non toxic) continued to decline. There was no substantial difference in the pattern of decrease between strains, although, the feeding rate of daphnids on toxic *M. aeruginosa* declined continuously until death on day 5 and 6 of exposure, while daphnids exposed to non-toxic *M. aeruginosa* established a very poor rate of ingestion after 72 h of exposure. The results of both the short-term functional response study and the long term feeding response study indicate that feeding inhibition of *D. magna* on toxic and non-toxic *M. aeruginosa* can be separated in two phases: an rapid initial inhibition (*i.e.* observed during the first 24 h) and further slower inhibition (*i.e.* observed during the protracted exposure period of 120 h).

The initial and further inhibition of feeding of *D. magna* on *M. aeruginosa* and their pattern of variation observed in the feeding experiments, were reproduced by an another method; the direct measurement of beat rate, which has been used in several studies to characterize feeding rate (McMahon & Rigler, 1963; Burns, 1968; Jones *et.al.*, 1991; Forsyth *et. al.*, 1992) and the results demonstrated that the reductions in beat rate can be used as a direct measure of feeding inhibition. The beat rates of daphnids exposed to non-toxic and toxic *M. aeruginosa* for 24 h were 307±14 and 257±6.8 min^{-1} respectively whilst that of animals exposed to *C. vulgaris* for the same period was 332±10.8 min^{-1} (mean ± SD, n=3 for all treatments). These results are in agreement with the initial inhibition of feeding rate observed in feeding experiments, except that the difference between *C. vulgaris* and non-toxic *M. aeruginosa* was less

striking than between non-toxic *M. aeruginosa* and toxic *M. aeruginosa*. Such minor discrepancies can happen because the calculated feeding rate is an average value for the entire 24 h period whilst beat rate represents the situation at the end of the 24 h period. Beat rates of daphnids held in non-toxic and toxic *M. aeruginosa* decreased over the 96 h exposure period in a similar way to the further inhibition observed in long term feeding response studies. These results suggest both an initial and then a further inhibition of daphnid feeding on non-toxic and toxic *M. aeruginosa*, leading to the questions: is microcystin-LR toxicity responsible for both forms of inhibition, and if not, what could be the alternative mechanism behind inhibition?.

It is reasonable to expect that microcystin-LR toxicity plays a role in the initial rapid inhibition response rather than in the slow inhibition response because pronounced differences between toxic and non-toxic *M. aeruginosa* can be seen in the former but not in the latter. In terms of biovolume, the toxicity of toxic *M. aeruginosa* was high (*i.e.* 1.20 µg microcystin mm^{-3}) when used in the feeding experiment. The suppression of the feeding rate on toxic *M. aeruginosa* was also high relative to the feeding rate on *C. vulgaris*. But these factors alone are not enough to prove that microcystin-LR is an antigrazing compound, because no correlation was found between the degree of feeding inhibition of *D. magna* on different toxic strains of *M. aeruginosa* and toxicity (Nizan *et al.*, 1986, Henning *et al.*, 1991). Therefore, three possibilities should be tested in order to validate the above hypothesis. These are that the toxicity is (i) the only causative agent, (ii) only one of a number of causative agents and (iii) is not a causative agent, for the feeding inhibition of *D. magna*.

Clearly, the microcystin toxicity is not the sole causative agent for feeding inhibition, because, the feeding rate on non-toxic *M. aeruginosa* was also suppressed. No toxin (*i.e.* microcystin-LR) was detected in the non-toxic *M.aeruginosa* samples analyzed. The suppression of feeding rate on toxic *M. aeruginosa* was higher than under exposure to non-toxic *M. aeruginosa*, but the difference was not great (*ca.* 10% of control). Therefore it seems doubtful that microcystin-LR toxicity is the primary causative agent of feeding inhibition. If the toxin (*i.e.* microcystin-LR) does not affect feeding, there must be other factor(s) *e.g.* cell surface properties or other toxins responsible for feeding inhibition.

Jungmann *et al.* (1991) observed that the inhibition of ingestion rate of *D. pulicaria* exposed to toxic *M. aeruginosa* was reduced by 30% when cells were freeze thawed, and by 100% when cells were treated with lipophilic solvent. This observation does not clearly prove whether the reduction of feeding inhibition is due to the removal of chemical/s from cells or changes in surface properties of cells. The fact that there might be some strain specific variations between the two strains of *M. aeruginosa* other than in the presence of microcystin-LR, cannot be excluded because experiments by Jungmann *et al.* (1991) and Nizan *et al.* (1986) showed some variations in feeding rate also on different non-toxic strains of *M. aeruginosa*.

As further evidence, when animals were exposed to a suspension of toxic *M. aeruginosa* for 48 h without disturbance, readily visible (>1mm) clumps of undigested cells could be seen in the bottom of the vessel. However, this did not happen when daphnids were held in suspensions of non-toxic *M. aeruginosa*, implying a difference

in surface properties between the two strains. These clumps were not the result of defecation, since they always consisted entirely of intact cyanobacterial cells. It is possible that *D. magna* triggers the formation of colonies. When *D. magna* rejects a cell bolus it is ejected from the food groove in the form of a flocculent mass which does not completely disaggregate unless the medium is shaken. As a result of continuous re-collection of those aggregations, larger clumps may be produced. The non-toxic strain did not form such colonies, probably because of different surface properties. Nizan *et al.* (1986), also observed sedimented flocks of *M. aeruginosa*, even after 24 h exposure to *D.magna*, in one strain out of 12 strains used.

The results of this study imply that factors other than microcystin-LR underlie the inhibition of feeding of *D. magna* on toxic *M. aeruginosa*. However, a question remains: what is the main reason for the initial inhibition of the feeding rates of *D. magna* on *M. aeruginosa*?. In an experiment by Nizan *et al.* (1986) on *D. magna* feeding on strains of *M. aeruginosa*, there was no similarity in terms of feeding inhibition among the 12 strains used. This led to the suggestion that different feeding rates may be due to differences in surface tension properties of different strains. In the present study, it is also suspected that some strain-specific differences, probably surface factors, are the cause of the initial (*i.e.* during the first 24 hour) inhibition of feeding rate.

Direct observations of feeding behaviour provided further evidence to support this hypothesis. Post abdominal rejection rates of daphnids after 24 h exposure to toxic or non-toxic *M. aeruginosa* were some three times higher than those of animals exposed

to *C. vulgaris*. The low ingestion rates of both strains of *M. aeruginosa* were not only due to reduced filtering rate, but also to the frequent rejection of captured *M. aeruginosa* cells from the food groove by abdominal claw movement (Hertel 1984, cited by Henning *et al.*, 1991). The higher rejection rates lead to the hypothesis that the inhibition of feeding rate is also due to the rejection of food caused by the "bad taste" of *M. aeruginosa* (Lampert, 1982). Several other workers (Burns, 1968; Porter, 1977; Porter & Orcutt, 1980) have also suggested that particle rejection by *Daphnia* is on a chemosensory basis. However, results of this study do not imply that *Daphnia* can detect microcystin-LR in cells (*i.e.* toxic cells) on a chemosensory basis since they reject both toxic and non-toxic *M.aeruginosa* cells at almost the same rate. Moreover daphnids did not show any response to free microcystin-LR, even at a concentration of 1000 µg l^{-1} which is some 50 times higher than naturally-occuring bloom concentrations (Rodger *et al.* 1994). This situation challenges the hypothesis that microcystin-LR is an antigrazing compound.

Further evidence can be gained from *D. magna* feeding on mixtures of *C. vulgaris* and toxic or non-toxic *M. aeruginosa*: the suppression of daphnid feeding rates on mixtures was non-linearly proportional to the percentage of *M. aeruginosa* cells in the food. These results are in agreement with Lampert (1981a) who reported that very small quantities of *M. aeruginosa* present in standard food caused a significant suppression of the filtering rate. Taken together, the results indicate that the cellular factor for the chemosensitivity of *D. magna* is likely to relate to some property of the surface chemistry of *M. aeruginosa* cells. However, there is at present no evidence whether this is a physical factor or chemical factor and, if it is a surface toxin, how

it affects zooplankton. However, it is worthy of further investigation as it apparently plays a major role in zooplankton feeding inhibition.

There may be reasons other than the presence of microcystin-LR or cell surface factors for the slow inhibition response which might be associated with the reasons for initial inhibition. The survival of *D. magna* on non-toxic *M. aeruginosa* is very poor compared with that on normal food (see Chapter 5). Poor assimilation (Lampert, 1987b) or nutritional inadequacy of food may be the reason for poor survival. Although poor digestibility of *M. aeruginosa* has also been suspected in some reports (Nizan *et al.*, 1986) the present study showed *D. magna* can digest all three cell types adequately. However, compared with the faeces of daphnids fed on toxic *M. aeruginosa*, there were fewer and more undigested or partially digested cells in the faeces of daphnids fed on non-toxic *M. aeruginosa* and *C. vulgaris* respectively. But the opposite trend was observed for the gut retention time of each food, with the highest gut retention time recorded for toxic *M. aeruginosa*. It seemed to be that, at lower feeding rates or in low quality foods, the gut retention time of the food becomes longer, so that there would be sufficient time for complete digestion. This implies that the digestibility of all three cell types is similar and unlikely to affect the survival of *D. magna*. Poor assimilation or nutritional inadequacy is the most likely reason for the poor survival of *D. magna* held in non-toxic *M. aeruginosa*. Whatever the reason, daphnids may suffer reduced assimilation rates as a result of low food quality which will reduce feeding to a very low level in order to minimise the energetic costs associated with the collection and digestion of unsuitable particles.

It is also possible to underestimate ingestion rate, particularly with regard to *C. vulgaris* and non-toxic *M. aeruginosa*, due to the fact that defecated undigested cells were available for re-ingestion. However, the number of undigested cells was negligible in comparison to the percentage of ingested cells,. Moreover feeding rate values obtained in this study for *C. vulgaris* and non-toxic *M. aeruginosa* are comparable with values reported by radio-isotope methods (Henning *et al.*, 1991).

Lampert (1981b) and Nizan *et al.* (1986) noted that feeding in *Daphnia* exposed to toxic *M. aeruginosa* recovered rapidly when animals were returned to normal feeding conditions. In the present study *D. magna* took 48 hours for full recovery from feeding inhibition resulting from 24 h pre-exposure to toxic *M. aeruginosa*. It is thus concluded that the observations of Lampert (1981) and Nizan *et. al* (1986) apply only when the pre-exposure period is short. Lampert (1981) suggested that the observed feeding inhibition and fast recovery is a defence mechanism which helps daphnids to survive in patchy distributions of toxic *M. aeruginosa*. However, results from the present study do not support this hypothesis, particularly when toxic and non-toxic *M. aeruginosa* show a patchy distribution, because the feeding inhibition of daphnids which were pre-exposed to non-toxic *M. aeruginosa* for 24 h also took at least 24 h to recover. The feeding rates of *D. magna* which were pre-exposed to food-free medium were also found to be suppressed in this study. During starvation, daphnids reduce metabolic processes to a minimum, reducing energetic costs (Perrin *et al.*, 1992). For this reason, it appears to be that they take a substantial (*i.e.* 1 - 3 d) time to restore metabolic processes to normal levels in the presence of food. Animals exposed to toxic and non-toxic *M. aeruginosa* may also be faced with a similar

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situation due to poor food quality. However daphnids pre-exposed to toxic *M. aeruginosa* take more time to recover from feeding inhibition and this is probably a direct consequence of poisoning (Demott *et al.*, 1991). This is further evident from the recovery pattern of feeding inhibition by animals pre-exposed to the same three treatments for 72 h.

The experiments described in this chapter revealed that a suppression of feeding rate of *D. magna* was recorded not only in the presence of toxic *M. aeruginosa* but also in the presence of non-toxic *M. aeruginosa*. This suggest that microcystin-LR is not the major factor behind the feeding inhibition and surface properties of *M. aeruginosa* cells may be important in feeding inhibition in *D. magna*. This hypothesis is supported by the fact that there is no correlation between the feeding inhibition of *D. magna* on different strains of *M. aeruginosa* and the toxicity of the strain to mice (Nizan *et al.* 1986., Henning *et al.* 1991). Although the suppression of feeding rate on toxic *M. aeruginosa* is higher than that on non-toxic *M. aeruginosa*, the difference is not more than 10% of the normal feeding rate. The present study was unable to confirm or rule out the hypothesis that microcystin-LR is the reason for the difference in suppression of feeding between the two *M. aeruginosa* strains. However, results of this chapter still support the assertion that microcystin-LR is toxic to *D. magna*, since daphnids cannot avoid ingesting some cells in pure suspensions of toxic *M. aeruginosa*, resulting either in lethal (Lampert, 1987b) or sublethal effects, depending on exposure time or number of cells ingested.

CHAPTER 5

CHRONIC AND ACUTE TOXICITY OF *MICROCYSTIS AERUGINOSA*

5.1 Introduction

Two well-documented phenomena associated with the interaction between toxic *M. aeruginosa* and freshwater cladocerans are the suppression of grazing, and increased mortality of the grazers (Lampert, 1981a; 1981b; 1982; Nizan *et. al.* 1986; Demott *et. al.*, 1991; Henning *et. al.*, 1991). These phenomena may account for the observation that herbivorous zooplankton, and large filter-feeding daphnid cladocerans in particular are scarce during cyanobacterial blooms in freshwaters (Edmondson & Litt, 1982; Lampert, 1987a; Gliwicz, 1990; Kohl & Lampert, 1991) in which *M. aeruginosa* is one of the most common constituents (Skulberg *et al.*, 1984; Carmichael, 1990). Nizan *et al.* (1986) also observed that *Daphnia magna* did not occur when *M. aeruginosa* was present in Israeli water bodies. Demott *et al.* (1991) concluded that microcystins (*i.e.* microcystin-LR) are the primary cause of zooplankton mortality when animals consume toxic *M. aeruginosa* cells. However, it is not known whether feeding inhibition is also caused by microcystin toxicity or by some other factor.

The results of the previous chapter showed that microcystin toxicity cannot be the main factor causing feeding inhibition in *D. magna*. Whatever the reason for feeding inhibition, daphnids cannot avoid eating toxic cells resulting in death, particularly when exposed to pure suspensions of toxic *M. aeruginosa* (Lampert, 1987b). Although *M aeruginosa* generally grows as colonies which may be too large to ingest by most zooplankton species, small colonies and fragments of colonies can be ingested (Lampert, 1981; Hanazato & Yasuno, 1984). Demott *et al.* (1991) clearly demonstrated that very low densities (10^4 cells ml^{-1}) of toxic *M. aeruginosa* can strongly inhibit

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feeding by daphnids and cause mortality when ingested, although very high concentrations of dissolved purified microcystin-LR are needed to cause mortality. If it is true that microcystin-LR is the primary cause of death, this leads to the hypothesis that cell-bound microcystin-LR is more bioavailable than free microcystin-LR and thus exerts a greater toxic effect. If this is true it is also reasonable to expect that a crude extract of toxic *M. aeruginosa* cells should have an intermediate toxicity between purified toxins and intact cells because microcystin-LR in crude extracts may be associated with some carriers.

5.1.1 Acute toxicity of purified toxin, cell extract and intact cells of *M. aeruginosa*

Few studies have dealt with the effects of dissolved, purified microcystins on zooplankton. It has been reported that purified microcystin was toxic to *Artemia salina* larvae (Kiviranta *et al.*, 1991) and mosquito (*Aedes aegypti*) larvae (Kiviranta *et al.*, 1993). In perhaps the most authoritative study to date, on the toxicity of purified microcystin to daphnids, Demott *et al.* (1991) have demonstrated that three daphnid species, *D. hyalina, D. pulex* and *D. pulicaria,* show remarkably different sensitivities to purified microcystin-LR. They have attributed these differences to different degrees of feeding inhibition of same species on toxic *M. aeruginosa*. There are no data available on the acute toxicity of purified microcystins to *D. magna* although it is the largest member of the genus *Daphnia* and a standard test organism in ecotoxicology.

In addition to feeding inhibition among zooplankton, studies have clearly demonstrated the acute toxicity of intact cells of toxic *M. aeruginosa* to daphnids (Lampert, 1981a;

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1981b; Fulton & Paerl, 1987; Fulton, 1988; Jungmann *et al.*, 1991) and other cladocerans (Hanazato & Yasuno, 1987), including *D. magna* (Nizan *et al.*, 1986). It has been shown that the *M. aeruginosa* strain used in this study is toxic to *D. magna* (Nizan *et al.*, 1986), *D. parvula* (Fulton, 1988), *D. pulicaria* and *D. pulex* (Demott *et al.*, 1991).

The acute toxicity of crude or partially purified cell extract of *M. aeruginosa* to daphnids has been examined in three previous studies. Stangenberg (1968) showed that crude extracts of *M. aeruginosa* can cause dose-dependent mortalities in *D. longispina*. Panaloza *et al.* (1990) reported that a partially purified peptide obtained from phytoplankton dominated by *Microcystis* sp. was lethally toxic to *D. magna*. Finally, Jungmann *et al.* (1992) found that a crude extract of *M. aeruginosa* was highly toxic to *D. pulicaria*, but this extract contained two variants of microcystins, hence it was not possible to compare directly with the single variant studies involving purified microcystin. In order to study the bioavailability of toxins the crude extract must be prepared without adding any additional chemical and the extract must contain the same microcystin variant which is in the purified toxin.

5.1.2 Chronic toxicity of free and cell bound microcystin-LR

In healthy *Microcystis* blooms, microcystin-LR tends not to occur at detectable levels in the surrounding water, as it is an endotoxin. However, microcystins can be released into the water when cells age or decompose: at this point low levels of microcystins can potentially exert chronic effects on zooplankton. In one study, Rodger *et al.*

(1994) found that the concentration of microcystin-LR in natural water was 16-19 µg l^{-1}. Levels can be rather higher where cyanobacterial scums accumulate and decompose (Lawton, L. A., pers. comm.). However, the chronic effects of dissolved microcystin on zooplankton have been largely neglected in previous studies.

Daphnia maintained in pure suspensions of toxic *M.aeruginosa* die more rapidly than starving animals (Lampert, 1982). Therefore the only way to study the chronic effects of intact cells of toxic *M. aeruginosa* to *Daphnia* is to use mixtures of toxic and non-toxic cells. In fact, in the natural environment *Daphnia* is never exclusively exposed to *M. aeruginosa* or any other cyanobacteria. Especially before the beginning of a bloom, there will be many algae co-existing with *M. aeruginosa* which can serve as food for *Daphnia*. However, very few studies have dealt with the chronic effects of toxic *M. aeruginosa* on *Daphnia*. Lampert (1981a) reported that the growth of *D. pulicaria* was markedly reduced when only about 10% of toxic *M. aeruginosa* was added to the standard food *Scenedesmus* and attributed this to a reduction in food uptake.

5.1.3 Aims of the study

The aims of experiments described in this chapter were;

> (1) to assess the acute and chronic effects of cell-bound microcystins on the survival of *D. magna* by exposure to cell suspensions of toxic *M. aeruginosa* cells in the absence and presence of the green alga *C. minutissima*.

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(2) to study the acute and chronic toxicity of purified microcystin-LR on *D. magna*;

(3) to test the hypothesis that cell-bound microcystin-LR is more bioavailable to cladocerans than free microcystin-LR and thus has a greater potential to exert toxic effects.

5.2 Materials and methods

5.2.1 Experimental animals

Except for chronic toxicity tests with purified microcystin-LR, all the experiments described here were conducted using both neonates (<24 h old, body length = 1.0 ± 0.1 mm) and adult (14 d old, body length = 3.7 ± 0.7 mm) *D. magna* which were taken from broodstock cultures and bulk cultures respectively (see Chapter 2). Chronic toxicity tests were always started with neonates. Elendt M_4 medium was used as the experimental medium for all the experiments, with *C. minutissima* used as food.

5.2.2 Survival of *Daphnia*

The survival of *D. magna* exposed to different diets, different concentrations of toxic *M. aeruginosa* and different mixtures containing *M. aeruginosa* and *C. minutissima* were studied. Daphnids were maintained as individual cultures for a period of 21 days, in order to study survival under the above treatments. The basic procedure followed

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for maintaining animals in chronic tests is as described in Chapter 2.

5.2.2.1 Survival under different diet regimes

In order to ascertain survival on different diets, neonate and adult daphnids were fed three different diets: *C. minutissima* (*i.e.* upper limit control), non-toxic *M. aeruginosa* and toxic *M. aeruginosa* at a concentration of 10^7 μm^3 ml^{-1}. There were seven replicates for each food and each age group of animals. Another seven neonates and seven adults were kept in identical conditions to the control but without the addition of any food (*i.e.* lower limit control). The survivors were recorded every day over the 21 d period.

5.2.2.2 Survival under different concentrations of toxic *M. aeruginosa* alone

The effect of different concentrations of toxic *M. aeruginosa* on the survival of neonates and adult daphnids was tested by exposure to a series of biovolume concentrations of toxic *M. aeruginosa* cells which were 0.25 x 10^7, 0.5 x 10^7, 0.75 x 10^7 and 1.0 x 10^7 μm^3 ml^{-1}. In addition to this concentration series, there were upper limit controls (*C. minutissima*) and lower limit controls (starved) for both neonates and adults, as in the previous experiment. The number of replicates and the procedures followed were also identical to those in the experiment which was to test the survival on different diets, except that non-toxic *M. aeruginosa* was not used.

5.2.2.3 Survival under different proportions of *C. minutissima* and *M. aeruginosa*

Neonates and adult daphnids were exposed to mixtures of *C. minutissima* and toxic *M aeruginosa* in order to ascertain the chronic effect of intact cells of toxic *M. aeruginosa* on the survival of daphnids. In this experiment there were three mixtures in which 25%, 50% and 75% of the total biovolume (*i.e.* 1×10^7 μm^3 ml^{-1}) was composed of toxic *M. aeruginosa*; the remaining biovolume was composed of *C. minutissima*. The survival of animals in these mixtures, as well as in food free-medium and pure suspensions of both foods at the same concentration, were studied simultaneously, following the same procedure as in the above two experiments. The survival of daphnids in mixtures containing non-toxic *M. aeruginosa* and *C. minutissima* was also determined using the same procedure.

5.2.3 Acute toxicity tests with purified toxins.

The effect of purified microcystin-LR on survivorship was tested by separately placing neonates and adults in a concentration series of toxin solution. In order to enhance the solubility of purified toxins, 1 ml of 100% methanol was added to each 5 mg of toxin. The toxin mixture was then dissolved in Elendt M_4 medium in order to prepare a stock solution of 40 000 μg microcystin-LR l^{-1}. Two separate series of concentrations, one for neonates and the other for adults, were prepared by diluting the stock solution. Toxin concentrations used for neonates and adults were 0, 5 000, 10 000, 15 000, 20 000 and 25 000 μg l^{-1} and 0, 10 000, 15 000, 20 000, 25 000, 30 000 and 35 000 μg l^{-1} respectively. Twenty-five ml of the toxin solutions were delivered into 100 ml

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screw top glass jars. Three replicates were established for each concentration, and a control which was 25 ml of M_4 with methanol added in the appropriate concentration. Four adults or 10 neonates were transferred into each jar and the jars left with unscrewed caps. Animals were not fed during the experimental period and observed after 24h and 48h. The test end point was non-recoverable immobilization of daphnids, based on response to gentle prodding with a pipette. Oxygen concentrations in all the solutions were determined at the beginning and end of the experiment using a Strathkelvin oxygen meter (model 781). Two 5 ml samples from the stock solution of microcystin-LR and the highest and the lowest concentration of each series were withdrawn at the beginning of the experiment and analyzed by photodiode array HPLC (see Chapter 2) in order to confirm the nominal concentration of microcystin-LR.

5.2.4 Acute toxicity test with cell extract

An experiment was carried out to measure the acute toxicity of a crude extract of toxic *M. aeruginosa* cells to *D. magna*. Four litres of toxic *M. aeruginosa* culture at late log phase of growth were spun down in a bench top centrifuge (MSC Minor "s") at 3000 rpm for 5 min. The supernatant was then decanted and the cyanobacterial pellet resuspended in 250 ml of M_4 medium. The concentrated cell suspension was then frozen and thawed four times repeatedly, and each time the suspension was stirred for 1 h by placing on a magnetic stirrer. The suspension was then centrifuged at the same rate as above for 10 min and the supernatant filtered through a membrane filter (0.22 μm; Minisart NML, Sartorius). Three 5 ml samples were analyzed by photodiode array HPLC in order to determine the microcystin-LR content (see Chapter

2). In order to determine the LC_{50} values of the filtered extract the same procedure as in acute toxicity tests with purified toxin was followed except that only one concentration series was used for both neonates and adults. The filtrate of the crude extract was used as the stock solution to prepare the series of concentrations (0.0, 1125, 2250, 4500, 7000, 9500, 12000 µg microcystin-LR l^{-1}).

5.2.5 Chronic toxicity tests with purified toxins.

D. magna life table experiments were conducted under three concentrations of microcystin-LR (0, 50 and 1000 µg l^{-1}), with animals fed each of three diets: *C. minutissima*, non-toxic and toxic *M. aeruginosa*. The procedure followed for each series of experiment was identical to that of the 21 day survival experiment with different diets (see 5.2.2) except that there was no starved control and the culture media contained free microcystin-LR. Moreover, each series was started with neonates and culture media was renewed every day. Both survivorship and fecundity of individual animals in the three series of experiment were monitored continuously up to day 21. At the end of the 21 d period the body lengths of all surviving animals were measured under an Olympus dissecting microscope fitted with an eyepiece micrometer. On day 14 of the experiment two 20 ml samples, one immediately after renewal of the media and one taken 24 h later, were withdrawn from each individual culture vessel. All samples were filtered through GF/C glass fibre filter paper and kept at -20°C until analyzed. Samples were then analyzed by photodiode array HPLC (Waters Associates) in order to determine microcystin-LR content (see Chapter 2).

On day 15 of the experiment, after animals had released their third brood, all animals in the control or first series of experiments (*i.e.* in which *C. minutissima* used as the food) were transferred to screw top glass jars containing 180 ml of *C. vulgaris* suspensions which had been prepared by diluting concentrated cell suspension with Elendt M_4 medium to reach a concentration of 1×10^7 μm^3 ml^{-1}. Purified toxin was also added to the relevant treatments. Two 1 ml samples were withdrawn from each jar in order to confirm the initial concentration of cells. Grazing was then allowed to proceed for 24 h with three controls being assigned at random to each treatment. At the end of this period, animals were returned to the normal experimental system containing suspensions of algae. Feeding suspensions were then mixed thoroughly and two 1 ml samples were withdrawn by micropipette from all jars and enumerated by electronic particle counter in order to determine final cell concentrations. Ingestion rates were calculated as in Chapter 4.

5.2.6 Statistical analysis

A proportional mortality response to in microcystin-LR concentrations was calculated using standard probit analysis (Finney, 1971) to estimate 50% lethal concentration (LC_{50}). One way analysis of variance (ANOVA) and Tukey HSD multiple range tests were used to compare body lengths, total fecundities and feeding rates of daphnids after chronic exposure to purified microcystin-LR (Zar, 1984). Results from different diets in the same experiment were analyzed separately.

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5.3 RESULTS

5.3.1 Survival of *Daphnia*

Survival rates of neonates and adult *Daphnia* fed on different diets are shown in Fig. 5.1a and 5.1b respectively. Both neonates and adults fed on toxic *M. aeruginosa* died earlier than starved animals. All animals fed on *C. minutissima* survived over the 21 d period. Survival rates of neonates and adults fed on non-toxic *M. aeruginosa* also remained at 100% during the first 8-9 days of exposure, although thereafter they decreased gradually throughout the remaining period. However at the end of the 21 d period, the number of animals surviving in non-toxic *M. aeruginosa* was higher in the experiment with neonates than in that with adults, but the difference was not very striking.

The survival rates of neonates and adult daphnids held in different concentrations of toxic *M. aeruginosa* cells are shown in Fig. 5.2a and 5.2b respectively. Both neonates and adults in all concentrations died before starving animals. Neonates died 2-3 days before adults.

Figure 5.3a and 5.3b respectively show the survival of neonates and adult daphnids fed on mixtures containing *C. minutissima* and toxic *M. aeruginosa*. Survival rates of both neonates and adults reduced dramatically with increasing proportion of toxic *M. aeruginosa* in the mixtures. Neonates and adults in mixtures containing 75% toxic *M. aeruginosa* died earlier than starved animals and within the same time period as
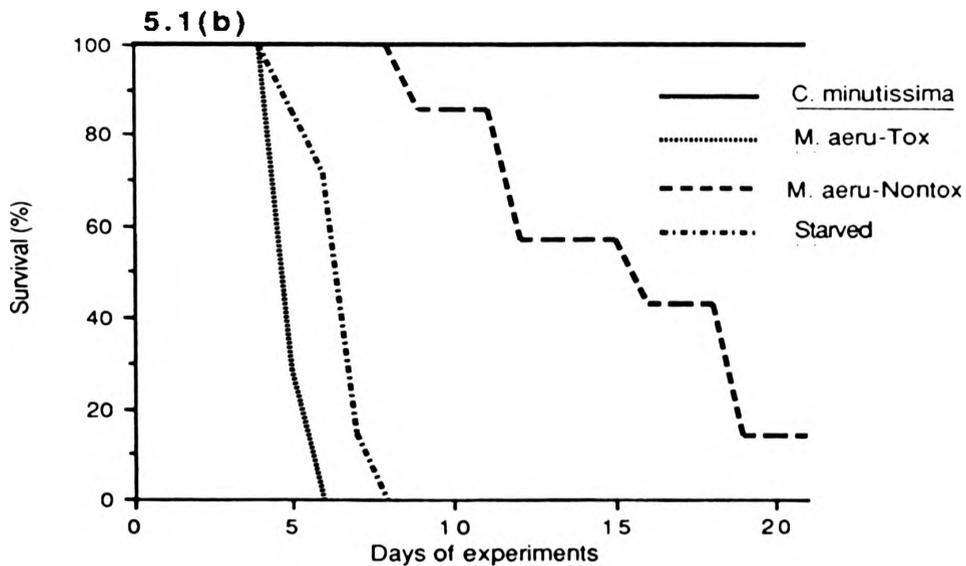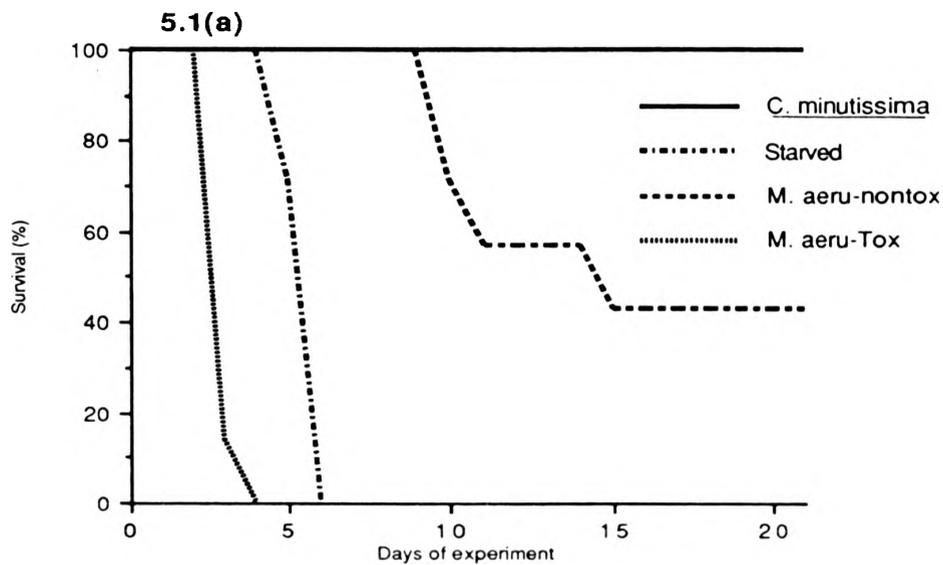
Figure 5.1 Survival of neonates (a) and adult (b) *D. magna* exposed to different diets and food free medium over 21 days (M. aeru = *M. aeruginosa*, Tox = Toxic, Nontox = Non-toxic)

5.2 (a)
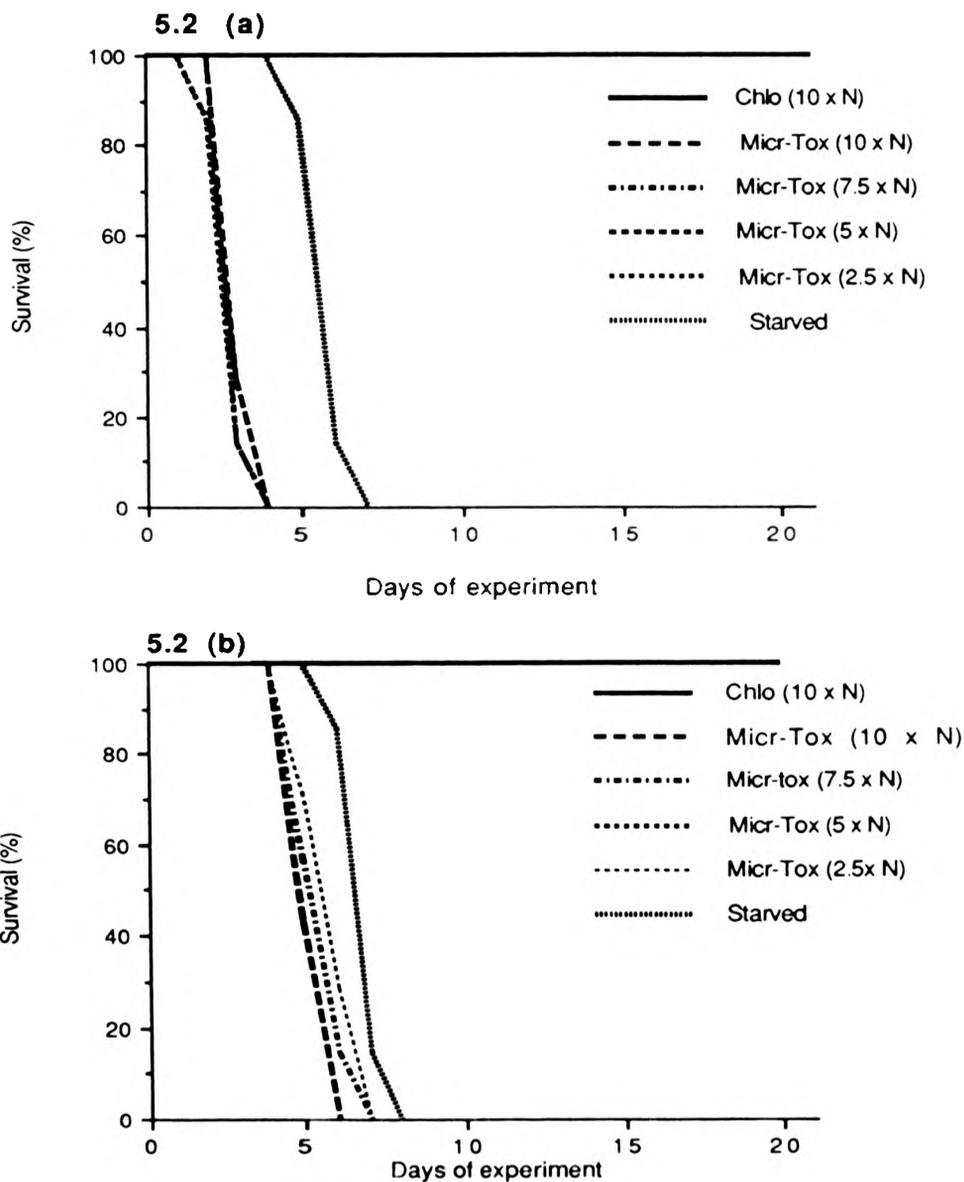
Figure 5.2 Survival of neonates (a) and adult (b) *D. magna* exposed to different densities of toxic *M. aeruginosa* cells (Chlo = *C. minutissima*, Micr-Tox = Toxic *M. aeruginosa*, N = 10^6 μm^3 ml^{-1}, values given in parenthesis are cell densities)

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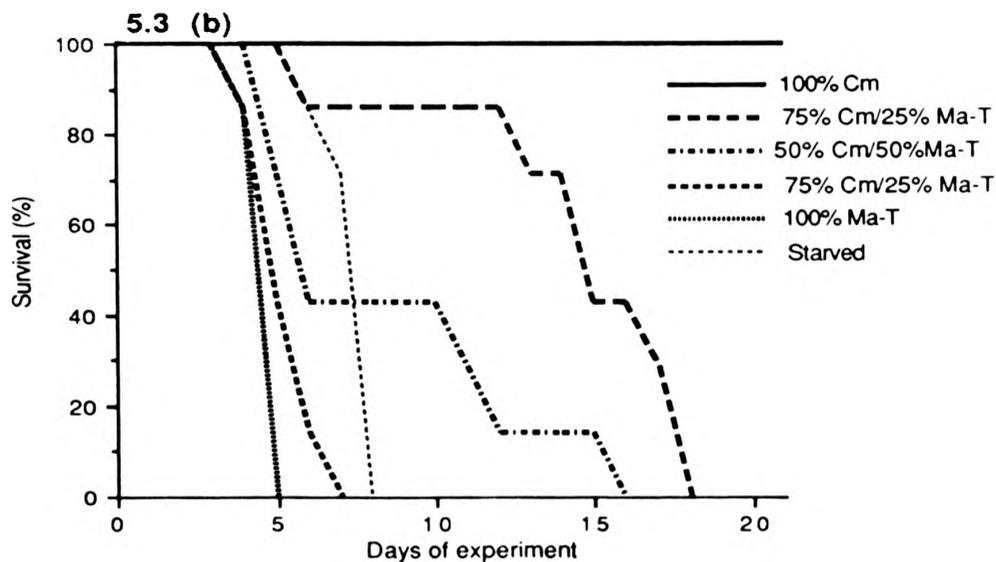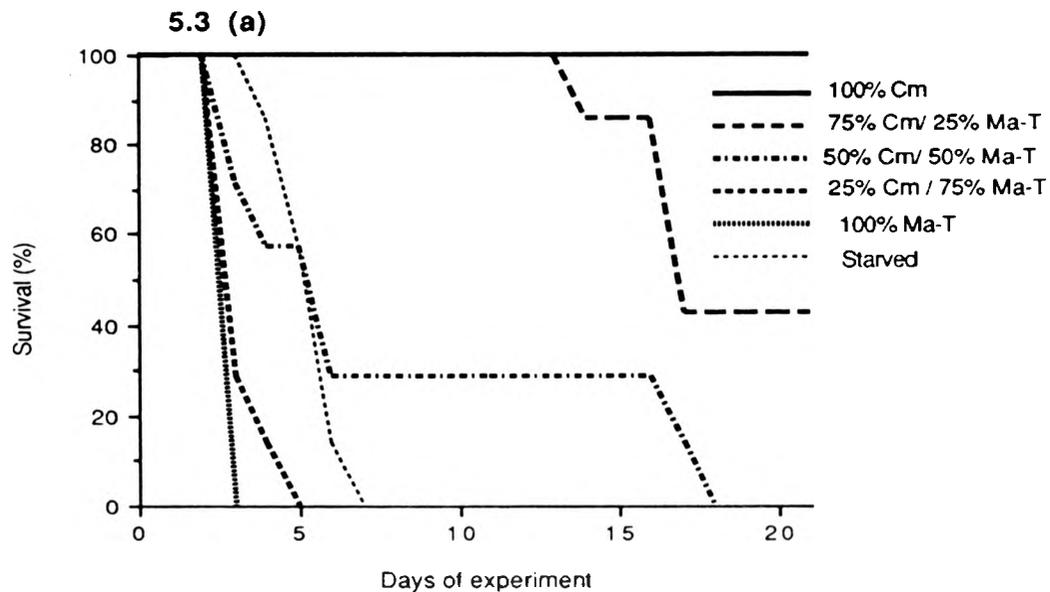
Figure 5.3 Survival of (a) neonates and (b) adult *D. magna* exposed to food mixtures containing toxic *M. aeruginosa* (Ma-T) and *C. minutissima* (Cm), Total food concentration = 10^7 μm^3 ml^{-1}.

animals held in pure suspensions of the same strain. By contrast, the survival of animals held in mixtures containing 50% and 25% toxic *M. aeruginosa* was higher than starved animals, but decreased with increasing exposure time. However, by the end of the experiment only neonates held in the mixture containing 25% toxic *M. aeruginosa* had survived.

Figure 5.4a and 5.4b shows survival over 21 d of neonates and adult daphnids fed on mixtures containing *C. minutissima* and non-toxic *M. aeruginosa*. No effect was observed on the survival of either neonates or adults in mixtures containing 25% and 50% of non-toxic *M. aeruginosa*, although those in the mixtures containing 75% non-toxic *M. aeruginosa* were slightly depressed during the second half of the experimental period. The survival of animals in pure suspensions of non-toxic *M. aeruginosa* was also reduced as already described.

5.3.2 Acute toxicity of purified toxin.

Proportional mortality responses in both neonates and adults observed in acute toxicity tests were used to calculate LC_{50} values. Results are shown in Fig. 5.5 as probability density functions of the 48 h LC_{50}. Kurtosis of each function shows the intensity of the response whilst mid-point of each function indicates the LC_{50} value (15600 µg l^{-1} for neonates and 25300 µg l^{-1} for adults).
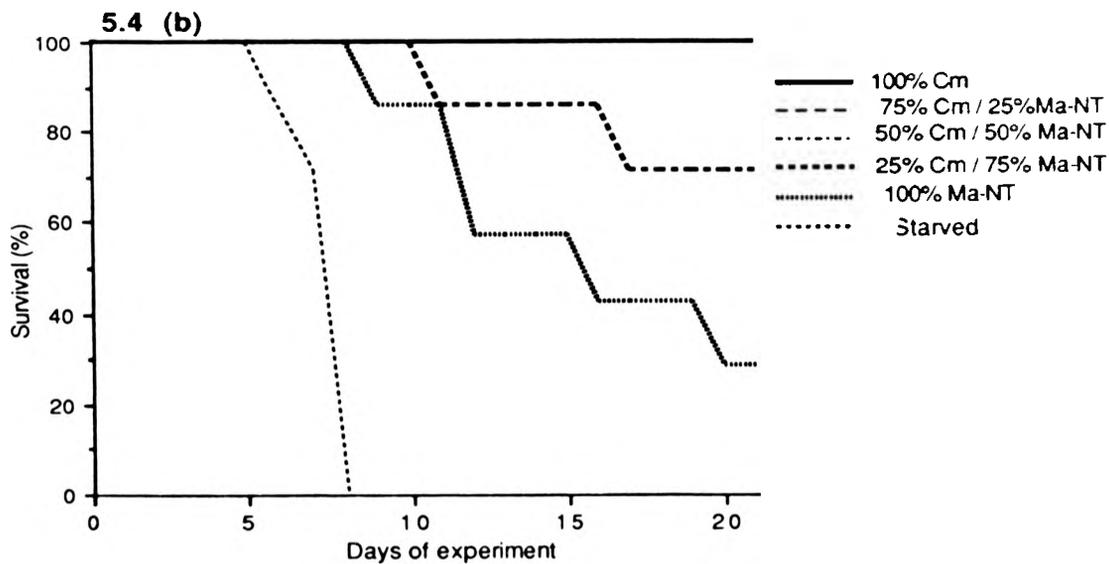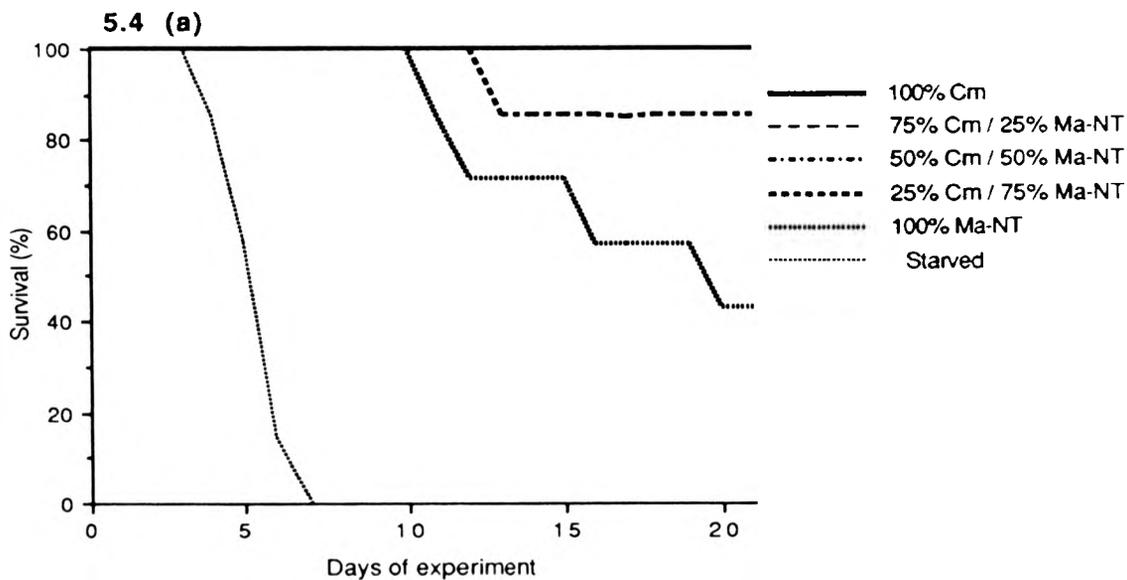
Figure 5.4 Survival of (a) neonates and (b) adult *D. magna* exposed to food mixtures containing non-toxic *M. aeruginosa* (Ma-NT) and *C. minutissima* (Cm), Total food concentration = 10^7 μm^3 ml^{-1}.
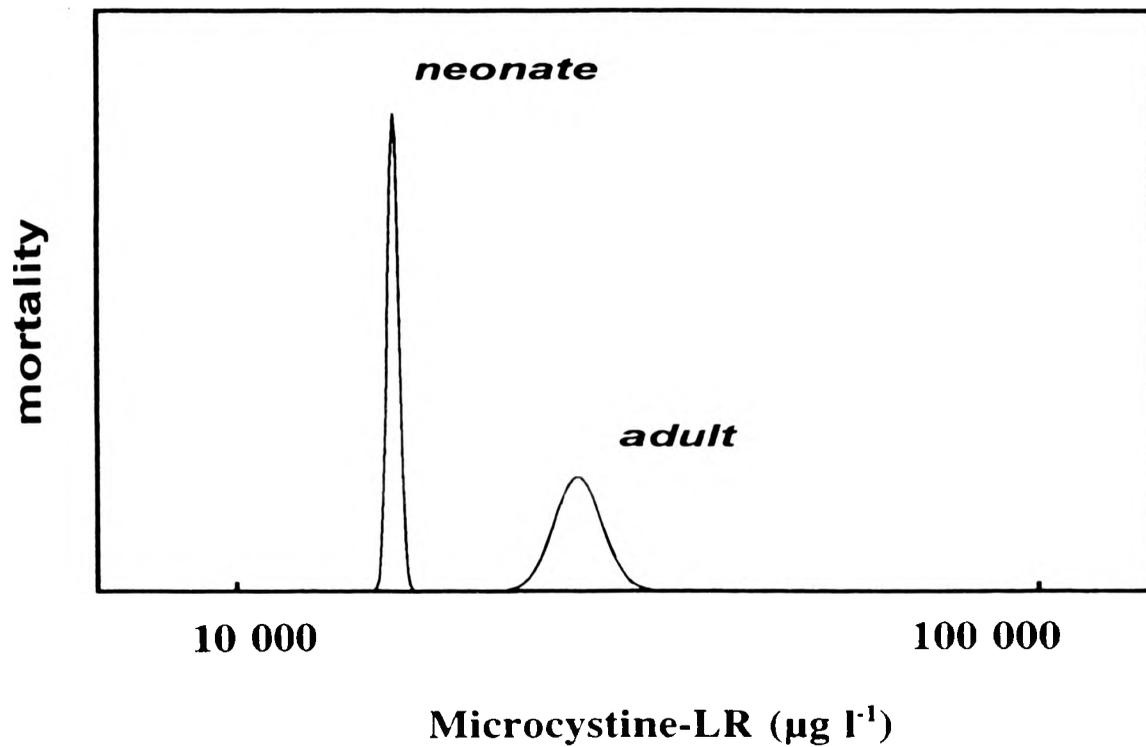
Figure 5.5 Acute toxicity of dissolved, purified microcystin-LR to neonate (age <24h) and adult (age 14d) *Daphnia magna*. Each curve is a probability density function of the 48h EC_{50} response and its 95% confidence limits

5.3.3 Acute toxicity of cell extract.

HPLC analysis of filtered cell extract revealed that it contained only a single microcystin variant, microcystin-LR. The concentration of microcystin-LR in the extract was 12 000 µg l^{-1}.

After 48 h, the mortality patterns of neonates and adult daphnids in the concentration series of cell extract were similar to those held in concentration series of pure toxin but LC_{50} values indicated were lower than those of pure toxin. However these results were unreliable as oxygen concentrations of extracts after 48 h exposure to *Daphnia* were reduced by almost 90%. The same problem arose whenever the experiment was repeated. Examination of extracts revealed that contamination and rapid growth of a bacterium *Aeromonas hydrophila* Chester (identified by the Bacteriology division, Institute of Aquaculture, University of Stirling) had occurred and was the cause of the low oxygen concentrations.

5.3.4 Chronic toxicity of purified microcystin-LR

The 21 d survival of daphnids exposed to sublethal levels of free microcystin under different diets is shown in Fig. 5.6. The survival rate of animals fed on *C. minutissima* was 100% in both toxin concentrations and in the control, and poor survival was observed in all treatments fed on non-toxic *M. aeruginosa*. Survival was poorest among animals held in the highest (*i.e.* 1000 µg l^{-1}) level of microcystin-LR. Survival at this concentration was reduced by up to 30% over the 21 d period whilst those in
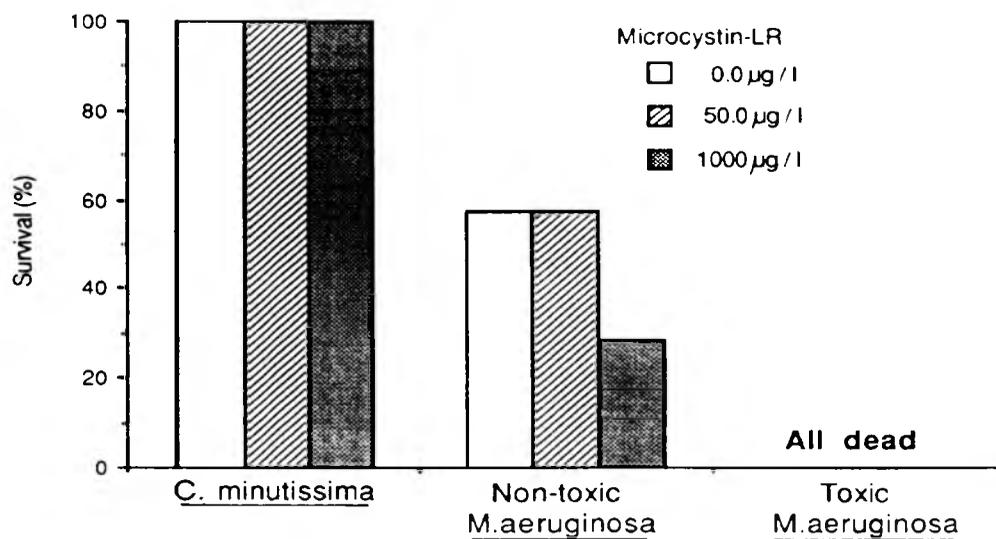
Figure 5.6 Survival of *D. magna* after 21 d exposure to free microcystin-LR with different algal diets (data presented are means ± standard deviations).
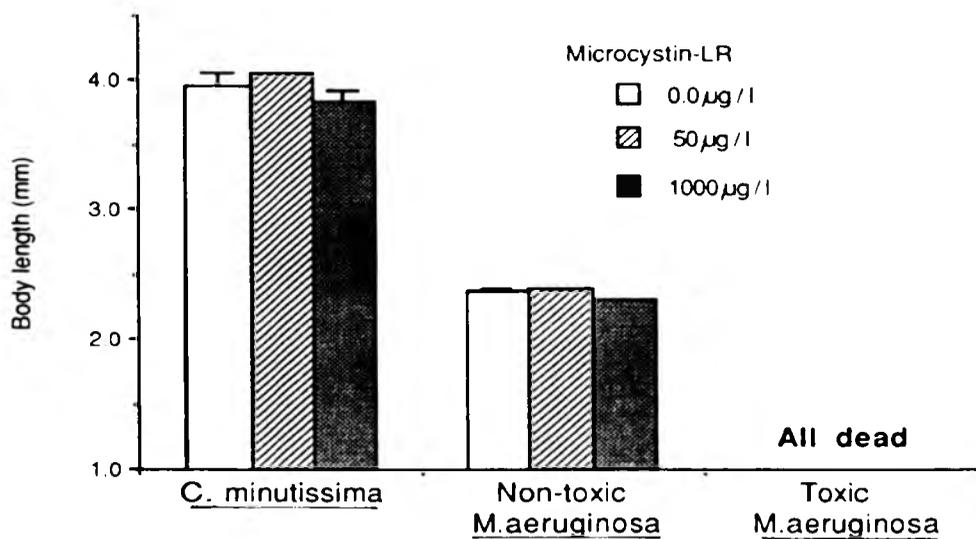
Figure 5.7 Growth of *D. magna* after 21 d exposure to free microcystin-R with different algal diets (data presented are means ± standard deviations).

the control and 50 µg l^{-1} microcystin level were almost 50%. Neonates fed on toxic *M. aeruginosa* died on the third day of exposure, irrespective of dissolved toxin concentrations.

Daphnids fed on *C.minutissima* in all treatments grew faster than those fed on non toxic *M.aeruginosa* (Fig. 5.7). Growth rates of animals at each toxin level in the non-toxic *M.aeruginosa* treatments were not significantly different from the control (F = 6.3, df = 2, P > 0.05). By contrast, growth rates of daphnids fed on *C. minutisima* in the presence of 1000 µg l^{-1} toxin were significantly lower than those fed on the same foods at 50 µg l^{-1} toxin level and the control (F = 13.0, df = 2, P < 0.05).

Daphnids which survived in non-toxic *M. aeruginosa* did not reproduce over the 21 d period, even in the treatment without added toxin (Fig. 5.8). By contrast, daphnids fed on *C. minutissima* reproduced at all toxin levels, and animals exposed to 1000 µg l^{-1} toxin level had significantly lower fecundity, relative to other treatments (F = 21.8, df = 2, P < 0.005)

Feeding rates of adult (14 d) daphnids which were reared in Elendt M$_4$ medium with *C. minutissima* as food and in the presence of dissolved purified microcystin-LR at a concentration of 1000 µg l^{-1} from day 1 to day 14 were significantly lower than those in the other two treatments (F = 21.5, df = 2, P < 0.05) (Fig. 5.9). No significant differences in feeding rates were observed between the control group and that held at 50 µg l^{-1} microcystin even after 14 d of exposure.
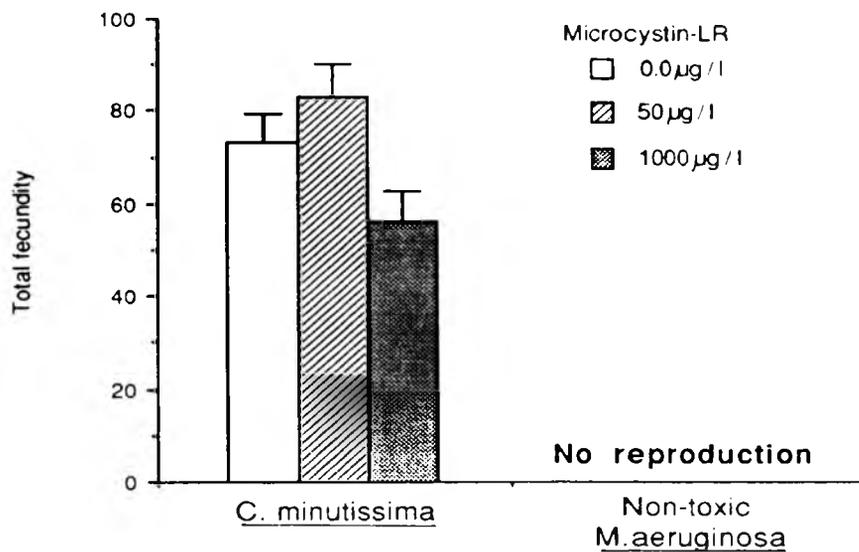
Figure 5.8 Reproduction of *D. magna* after 21 d exposure to free microcystin-R with different algal diets (data presented are means ± standard deviations).
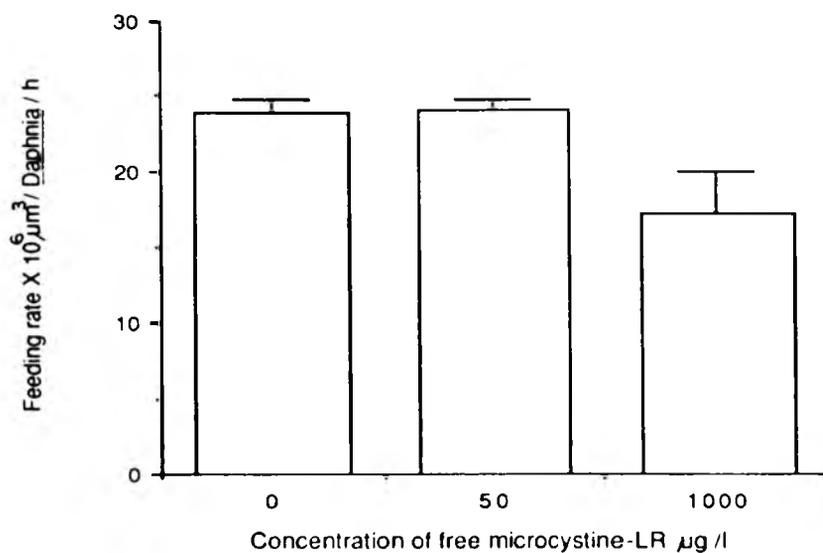
Figure 5.9 Feeding rate of adult (14 d) *D. magna* on *C. vulgaris* after 14 d exposure to free microcystin-LR (data presented are means ± standard deviations).

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The microcystin-LR content of toxic *M aeruginosa* cells used in this experiment was 1.4 - 1.6 µg mm^{-3}. Table 5.1 shows the variation in dissolved microcystin-LR concentrations in Elendt M_4 medium as a result of 24 h exposure to *D. magna* (14 d old).

Table 5.1. Variation in dissolved microcystin-LR concentration in Elendt M_4 medium, as a result of 24 h exposure to *D. magna* (n=7 for all mean values).

Nominal concentration	Mean actual concentration	
	Before exposure	After exposure
µg l^{-1}	µg l^{-1}	µg l^{-1}
50.0	48.57±1.05	43.56±2.61
1000.0	992.28±1.66	906.85±40.13

5.4 DISCUSSION

D. magna died more rapidly in the presence of toxic *M. aeruginosa* cells than in the complete absence of foods, implying that *M. aeruginosa* cells are toxic to daphnids. However during starvation, daphnids reduce their metabolic processes to a minimum (Perrin *et al.*, 1992). An alternative explanation is that starved daphnids may live longer because of reduced metabolic rate compared with animals maintained in the presence of cyanobacteria. However, neonates exposed to toxic cells died three days earlier than starved neonates whilst neonates exposed to different concentrations of toxic *M. aeruginosa* cells died simultaneously. This suggests that toxic particle concentration has no effect on the death rate of *D. magna*. It proves that death of *D. magna* in the presence of toxic *M. aruginosa* cells is not due to starvation at a higher metabolic rate but to poisoning by *M. aeruginosa*. According to Lampert (1981a; 1981b) and Nizan *et al.* (1986) filtrates of toxic *M. aeruginosa* cultures, which were at the same log phase of growth as cultures used in this study, did not cause any effect on *Daphnia*. Therefore, any toxic effect of *M. aeruginosa* on *Daphnia* requires that cells are consumed.

Acute toxicity tests revealed that the LC_{50} values of dissolved purified microcystin-LR were 15790 and 25300 µg l^{-1} for neonates and adults respectively, suggesting that *D. magna* was rather insensitive to purified microcystin-LR. LC_{50} values of the same toxin given by Demott *et al.* (1991) for *D.pulicaria, D. hyalina* and *D. pulex* which are 21400, 11600 and 9600 µg l^{-1} respectively, inferred that *D. magna* was more insensitive to mycrocystin-LR. However, these variations in LC_{50} values may reflect

not only variations in physiological sensitivity among different species but also differences in body mass, as it is evident by different LC_{50} values obtained in this study for neonates and adults of the same species, *D. magna*. When the effect of body size are taken into account, it is doubtful whether *D. magna* is more insensitive to purified microcystin-LR than other *Daphnia* species because *D. magna* is the largest of the genus, the body lengths of the adult used in this study being 3.7±0.1 mm. The results imply that in general daphnids sensitivity to dissolved purified microcystin-LR is low in comparison to other cyanotoxins (Klapes, 1991) and to other organic contaminants (Soares, 1989).

No systematic study of the fate of cyanobacterial toxins in natural waters has yet been done although it is of central interest to public health. Although microcystins are endotoxins, they are present in the water after cyanobacterial blooms have collapsed. As the acute toxicity of purified microcystin-LR is rather low, it may be expected that daphnids can co-exist in sublethal levels of microcystin-LR in natural water. According to the values reported so far (Rodger, *et al.*, 1994), concentrations of microcystins in natural waters might not exceed 50 µg l^{-1}, even following the lysis of toxic blooms. Dissolved purified microcystin-LR, at a concentration of 50 µg l^{-1} had no discernable effect on *D. magna*, even after exposure for 21 d suggesting field concentrations of purified microcystin-LR would have no effect. However, exposure of *D. magna* to microcystin-LR, at a concentration of 1000 µg l^{-1} for 21 d, had conspicuous chronic effects. Body length and total fecundity of daphnids fed on *C. minutisima* were significantly lower at this toxin level compared with the control and exposure to 50 µg l^{-1} microcystin-LR. However, no significant effect on the growth

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of animals fed on non-toxic *M. aeruginosa* was observed over the 21 d period, even at 1000 μg l⁻¹ level of purified microcystin-LR. Probably the growth rate was not high enough to reveal the differences.

In experiments with added purified toxin, the culture medium was renewed every day in order to minimise losses of toxin. Although microcystin-LR is a relatively non-volatile, heat resistant compound (Lawton, L. A., pers. comm.) microbial decomposition can result in loss from the culture media. However, the microbial population in the culture media was negligible and measurement of dissolved microcystin-LR levels in culture media before and after exposure to daphnids showed no significant loss (*i.e.* < 10%) of toxin over a 24 hour period (Table 5.1).

The acute (*i.e.* 24 h) exposure microcystin-LR even at a concentration of 1000 μg l⁻¹ did not inhibit the feeding rate of *D. magna* (Chapter 4). However the feeding rate of *D. magna* was inhibited as a result of chronic (*i.e.* 14 d) exposure to the same concentration of microcystin-LR and it might be associated with the weakened state of animals resulting from continuous exposure to toxin over 14 d. Their body length was also lower than the controls as a result of chronic exposure to toxin.

As found by Lampert (1981a), it was evident that neonates were more sensitive than adults to toxic *M. aeruginosa*, since they died three days earlier than the starved control, while adults died 1-2 days earlier than the starved control. It has been shown from HPLC analysis that the non-toxic *M. aeruginosa* strain CYA 43 did not contain the toxin microcystin in any of its variants. However it is still not a suitable food for

D. magna as the survival of animals held in non-toxic *M. aeruginosa* was intermediate between that of starved animals and those fed on *C. minutisima*. Poor survival of daphnids on non-toxic cyanobacteria of edible size, has also been reported by Arnold (1971), Lampert (1981a), Holm & Shapiro (1984), Fulton (1988a; 1988b) and Henning *et. al.* (1991). It emerges from these studies that not only *M. aeruginosa* in particular but also cyanobacteria in general appear to be inadequate as food, even when they are non-toxic and of an edible size. Reports indicating that *M. aeruginosa* is a good food for *Daphnia* (de Bernardi *et. al.*, 1981) may be because non-axenic cultures of *M. aeruginosa* were used. In the present study, it was observed that *D. magna* could grow and poorly reproduce when fed with late stationary phase cultures of non toxic *M. aeruginosa* (strain CYA 43), in which the bacteria population was rather high. Peters (1987) documented that the rate of reproduction of *Daphnia* increased when fed on algal cultures contaminated with bacteria or protozoa. Lampert (1981a) also concluded that bacteria contaminating a cyanobacterial culture used to feed *Daphnia* stimulated daphnid growth. Wylie and Currie (1991) showed that bacteria represent an important source of carbon for cladocerans in aquatic food webs. They also suggest that when bacterial and algal biomasses are similar, much of the carbon flow to crustaceans is likely to originate from bacteria.

The concentration of toxic *M. aeruginosa* cells in the medium has no apparent effect on the survival of *D. magna*, as daphnids exposed to four different concentrations of *M. aeruginosa* died almost simultaneously and prior to starved animals. This result is in disagreement with those of Fulton & Paerl (1987) and Demott *et al.* (1991) who reported that the survival of daphnids in lower concentrations of toxic *M. aeruginosa*

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cells was unchanged or even improved in comparison to that of starved animals whilst animals in high concentrations of toxic *M. aeruginosa* died before starved animals. The most likely explanation for this discrepancy is that the lowest concentration used in this study was only four times lower than the highest concentration, whereas there was a 100-fold difference between the lowest and highest concentrations in the previous studies mentioned.

The survival pattern of daphnids in mixtures of *C. minutissima* and toxic *M. aeruginosa* showed that daphnids are highly susceptible to even small numbers of toxic cells. Survival decreased with increasing proportions of toxic *M. aeruginosa* cells, again confirming the toxicity of *M. aeruginosa* to *D. magna*. The reduced survival rates of daphnids, in mixtures containing even a small fraction of toxic *M. aeruginosa*, seems to be due to microcystin-LR toxicity rather than suppressed feeding rates or any other mechanisms, because the addition of non-toxic *M. aeruginosa* to the standard food *C. minutissima* had no effect on the survival of daphnids other than in the mixture containing 75% non-toxic *M. aeruginosa*. However, the survival of neonates exposed to mixtures containing toxic *M. aeruginosa* cells was higher than that of adults, although neonates were more sensitive than adults to toxic *M. aeruginosa*.

It is possible that neonates, unlike adults, may have some ability to select food particles. In a recent study on selective feeding of cladocerans, Kerfoot & Kirk (1991) demonstrated that large bodied cladoceran species had no preference for size or flavour of particles but small bodied species tended to be able to discriminate (albeit

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weakly) on this basis. The explanation given is that relative particle size is very important with small bodied daphnids. With cladocerans less than 0.7 mm, the size of the beads used in the study of Kerfoot & Kirk (*ibid*) (*i.e.* 6 and 12 µm) meant that they could probably be processed one at a time, allowing single particle rejection by the mandibles. In larger species, selection or rejection of individual particles is not possible because they are processed into a bolus, and animals either accept or reject the entire bolus rather than individual particles. Perhaps the same concept may be applicable to neonates and adult daphnids of the same species. At the beginning of the survival experiments, the body length of neonates was 0.9 - 1.0 mm and in mixtures containing toxic *M. aeruginosa* and *C. minutissima* they remained somewhat stunted throughout the experimental period. The spherical diameter of toxic *M. aeruginosa* cells (3.3 µm) meant that neonates could probably process only a few cells at a time. It is possible that in some instances the few cells collected might be composed only of *C. minutissima*, particularly in mixtures containing a low proportion of *M. aeruginosa* cells. Adult daphnids may not be able to do this because they would be unlikely to collect a large bolus of cells without toxic *M. aeruginosa* being present, even in mixtures containing only a small fraction of *M. aeruginosa*.

The present study revealed that *D. magna* is rather insensitive to dissolved purified microcystin-LR. Purified toxin showed some measurable effects on the life history of *D. magna* only at a concentration of 1000 µg l^{-1}, but those effects were minor. At a concentration of 50 µg l^{-1}, purified toxin has no observable effect on *D. magna*. However caution must be taken in extrapolating these results to natural conditions because direct bioavailability of microcystin-LR was not investigated in the present

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study. Neonates exposed to toxic *M. aeruginosa* suspensions at a concentration of 2.5 x 10^6 μm^3 ml^{-1}, equivalent to 3.75 μg microcystin-LR l^{-1}, died more or less within two days. Surprisingly, however, the 48 h LC_{50} of purified microcystin-LR for neonates seems to be very high (*i.e.* 15790 μg l^{-1}) implying that the difference in toxin effect concentration could be due to either differences in bioavailability or to the presence of an unknown toxic feature of the cells. It has been reported that there were five more variants of microcystins present in intact cells of the *M. aeruginosa* strain used in this study: microcystin-LY, -LW, -LF, -LM and an analog of microcystin-LR (Lawton *et al.*, in press). But microcystin-LR comprises more than 75% of total microcystins when the cultures are maintained at $20^\circ C$ (Lawton *et al.*, 1994), as in this study. Moreover, the other variants of microcystins also show the same mode of action (*i.e.* inhibition of the enzyme protein phosphatase) and almost similar mouse toxicities (Carmichael, 1992). Therefore the presence of the other microcystin variants is not likely to be the reason for the enormous difference in toxicities between intact cells and the purified toxin.

If a crude extract of toxic *M. aeruginosa* cells gave an intermediate toxicity between the purified toxin and intact cells, it would be consistent with the hypothesis that differences in bioavailability underlie the differences in toxicity reported here. An attempt to study the toxicity of a crude extract failed due to problems of bacterial contamination. Several attempts, including autoclaving the extract and glassware, aimed at avoiding bacterial contamination, failed. It is likely that the source of contamination was from daphnid guts, the same bacterium being observed from dissected guts of healthy daphnids. Bodar (1989) also observed morphologically

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similar bacteria in the gut of *D. magna*. Therefore further studies are required in order to understand the bioavailability of microcystin-LR.

CHAPTER 6

GENERAL DISCUSSION

Since one aim of the present study was to standardize protocols and therefore results are repeatable, several culture media and algal diets were tested in order to select the best combination of defined media and algal diets for *Daphnia* culture. The observations made have a number of implications for the standardization of *Daphnia* culture system in aquatic toxicology. Almost all previous studies aimed at finding a suitable defined medium for *Daphnia* toxicity tests have largely concentrated on the quality and the performance of the medium: the importance of the food in the culture system received little or no attention (ISO, 1982; OECD, 1983; Keating, 1985; ASTM, 1988; Elendt & Bias, 1990). None of the defined media used in conjunction with a mono-axenic algal food has proved to be superior for *Daphnia* culture. The present study emphasized that both the culture medium and the food are important for *Daphnia* culture in aquatic toxicology.

The presence of bacteria in an algal diet can increase the surface area of food particles which in turn increases the bioavailability of contaminants which have a higher sorption potential (Taylor & Baird, 1993). When Elendt M_4 and M_7 media, the best defined media yet to have been developed, were used in conjunction with axenic *C. vulgaris*, the survival of neonates was rather poor. However the same media performed identically in *Daphnia* culture as in the original publication (Elendt & Bias, 1988) when used in conjunction with a non-axenic algal diet. Nevertheless M_4 medium was still acceptable for *Daphnia* reproductive tests, even with axenic *C. vulgaris*, because the total number of surviving neonates reached the level of revised OECD test acceptability criteria *i.e.* mean 21 d fecundity of ≥ 60 neonates (OECD, 1984).

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The present study revealed that bacterial contamination in algal diets can compensate for some of the nutritional deficiencies in defined media and may be one source of variation between laboratories in *Daphnia* reproductive tests. Moreover the present study proved that a defined medium which can replace natural media has not yet been introduced and further studies are needed in order to find a combination of a defined medium and a mono-axenic algal diet which can replace culture system with natural media.

Cyanotoxins have been implicated in the deaths of livestock and wildlife throughout the world (Gorham & Carmichael, 1988) and it has further been suggested that these toxins have also been responsible for ill health in humans (Falconer *et al.*, 1983; Turner *et al.*, 1990). The knowledge of the vectors, victims, production and the ultimate fate of these toxins is the backbone of the ecotoxicology dealing with freshwater cyanobacterial blooms. In the diversity of cyanotoxins, hepatotoxins are the most common cyanotoxins and microcystin is the most abundant hepatotoxin. Out of more than 50 variants of microcystin yet recorded, microcystin-LR is the most common (Carmichael, 1992; Lawton *et al.*, 1994) proving that the main toxin in *M. aeruginosa*, microcystin-LR, is the best to represent the whole group of cyanotoxins.

Effects of short term (24 h) and long term (4 - 5 d) exposure to toxic *M. aeruginosa* on the feeding rate of *D. magna* were investigated by comparing 24 h feeding rates on *C. vulgaris*, non-toxic and toxic *M. aeruginosa* (Chapter 4). The main purpose of these studies was to test the hypothesis that microcystin-LR is an antigrazing compound. This study also investigated the acute and chronic effects of intact cells of

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toxic *M. aeruginosa* and purified microcystin-LR on *D. magna*.

The results from the present study suggest that main causative agent of general poisoning (*i.e.* lethality of animals exposed to toxic *M. aeruginosa*) and the feeding inhibition of *D. magna* appears to be different factors of *M. aeruginosa* (strain PCC 7820) cells. Although *M. aeruginosa* can suppress feeding rates, it did not block the food uptake of *D. magna*, even when held in pure suspensions, in contradiction to the findings of Nizan *et al.* (1986). The ingested cells cause mortality in daphnids and microcystin-LR appears to be the primary cause of death (Demott *et al.*, 1991) since ingested cells of non-toxic *M. aeruginosa* which have been proved to contain no microcystins did not kill daphnids. Survival of daphnids fed on non-toxic *M. aeruginosa* was also low, but was higher than starving animals indicating that poor survival could be due to factors other than the presence of cyanotoxins.

However the present study provided evidence to suggest that microcystin-LR is not the main causative agent for feeding inhibition of *D. magna*. Both thoracic appendage beat rate and measurements of feeding rate proved that the ingestion rate of *D. magna* was suppressed not only by toxic *M. aeruginosa* containing microcystin-LR but also by non-toxic *M. aeruginosa* in which the toxin is absent. Results from the feeding experiment with dissolved purified toxin showed that the presence of microcystin-LR did not inhibit feeding rate even at concentration of 1000 μg l^{-1}. This confirms the finding of Demott *et al.* (1991) on the effect of dissolved purified microcystin-LR on feeding rates of *D. pulicaria* and *D. hyalina*. These results also refute the hypothesis that microcystin-LR is an anti-grazing compound. Lampert (1981a) reported that

Daphnia can recover from feeding inhibition very quickly when transferred to standard food and concluded that the rapid response to the presence and absence of toxic *M. aeruginosa* supported the idea that feeding inhibition is an algal defence mechanism. By contrast, the present study revealed that daphnids previously fed on toxic *M. aeruginosa* takes some time to restore normal feeding rates, and this period increases propotionally with the period of pre-exposure to toxic *M. aeruginosa*. Although daphnids exposed to toxic *M. aeruginosa* died on the 4th and 5th day of exposure, animals pre-exposed to toxic *M. aeruginosa*, even for 72 h, were able to recover and return to normal feeding behaviour when transferred to standard algal food, indicating that there is a delay (*i.e.* about 3 days) prior to being poisoned. Direct observation of feeding behaviour also revealed that the lower feeding rate on both strains of *M. aeruginosa* was not only due to a reduction in filtering rate but also to the increased rejection of cell boluses collected in the food groove by post abdominal claw movements. The two factors, the time lag before poisoning and the inability of daphnids to detect microcystin-LR, permitted toxic *M. aeruginosa* to be grazed. Together, these results refute the hypothesis that microcystin-LR is an antigrazing compound and suggest that factors other than microcystin-LR content are responsible for the feeding inhibition of *D. magna* by cyanobacterial cells.

This conclusion is further supported by the fact that there is no correlation between the feeding inhibition of *D. magna* on different strains of *M. aeruginosa* and toxicity to mice (Nizan *et al.* 1986; Henning *et al.* 1991). Microcystins may be a metabolic by-product of *M. aeruginosa*. Although it is not very effective as an anti-grazing compound, it may confer an advantage on cyanobacteria which contain them, since

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it can reduce the population of their grazers gradually by poisoning and killing them. This phenomenon may lead toxic *M. aeruginosa* to ecological success which is evident by the fact that more than 50% of all *M. aeruginosa* blooms have been found to be toxic to mice (Sivonen *et al.*, 1990a; Lawton *et al.*, 1994).

The present study revealed that feeding inhibition of *D. magna* on toxic and non-toxic *M. aeruginosa* can be separated into two phases: an initial rapid inhibition and further slow inhibition. The results suggest some surface factor of cells, either chemical or physical or both, as a reason for the initial rapid inhibition. Raziuddin *et al.* (1983) reported that *M. aeruginosa* and probably many other cyanobacteria produce a toxin called lipopolysaccharide (LPS) endotoxin as a part of the cell wall which has a low toxicity to laboratory animals. Carmichael (1981) documented that LPS endotoxin is also well known from Gram-negative bacteria and is present in at least some cyanobacteria. Moreover, Carmichael (1988) reported that some cyanobacteria are suspected of causing contact irritation in recreational water supplies, implying the presence of a toxin associated with cell walls.

The mechanisms underlying the slow component of feeding inhibition is likely to be the poor food quality of both strains of *M. aeruginosa*, rather than microcystin-LR toxicity. The present study also proved that non-toxic *M. aeruginosa* is an unsuitable food for *D. magna* resulting in poor survival, poor reproduction and retarded growth, despite its lack of microcystins. Poor assimilation, nutritional inadequacy or a yet undescribed toxin could be the cause of the unsuitability of non-toxic *M. aeruginosa* as a food for *D. magna* in the present study. Traditionally, the term toxicity means

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"the toxicity to mice", not to zooplankton or other invertebrates. Although *M. aeruginosa* strain CYA 43 is non-toxic from the traditional viewpoint, it is possible that it contains some other toxic chemicals which might affect *D. magna*. Whatever the reason for the unsuitability of *M. aeruginosa* as a food, daphnids may reduce feeding rates in order to minimize the energetic cost associated with collection and digestion of unsuitable particles. Toxic *M. aeruginosa* (strain PCC 7820) may also be equally unsuitable, although it is masked by the microcystin toxicity.

The LC_{50} values of purified microcystin-LR for *D. magna* observed in the present study and those reported by Demott *et al.* (1991) for some *Daphnia* species imply that daphnids are rather insensitive to microcystin-LR. Clearly, bioavailability is a key factor behind the toxicity of any toxin. Pure microcystin is a weakly polar and hence a rather hydrophobic chemical (Lawton *et al.*, 1994). Moreover, the wax cuticle of crustaceans may also be a barrier to hydrophobic compounds. Therefore the rate of uptake of the toxin into daphnids body may be slow, without a suitable carrier. An alternative explanation is that the observed low sensitivity of daphnids to purified microcystin-LR may be an evolutionary adaptation because daphnids are generally unselective feeders and cannot avoid eating toxic cells. This assumption is supported by the fact that selective feeders such as copepods which are able to discriminate and avoid ingesting toxic cells are more sensitive to purified microcystin-LR (Demott *et al.*, 1991). If microcystin toxicity is an evolutionary adaptation of *M. aeruginosa* to protect it from being grazed, it would be more toxic to non-selective feeders than selective feeders since the latter are more discriminating.

Cell-bound microcystins are released to the water and become free microcystins following cell lysis. Studies have reported the presence of cyanotoxins not only in natural surface waters (Rodger *et al.*, 1994) but also in treated waters (Himberg *et al.* 1989; Falconer, 1993). The present study revealed that dissolved purified microcystin-LR in concentrations which occur in natural waters appear to have no measurable effect on the life history of *D. magna*. In order to achieve conspicuous chronic effects on daphnids it was necessary to expose animals to concentrations of purified microcystin-LR at least 20 times higher than those likely to occur in the natural environment. However, these result cannot be extrapolated to the natural environment until it is known whether there is any difference in bioavailability between dissolved purified microcystins and free microcystins in natural waters.

The present study revealed that the 48 h LC_{50} of dissolved purified microcystin-LR to *D. magna* was three orders of magnitude greater than the concentration of cell bound microcystin-LR sufficient to kill daphnids more or less within the same period. An either unknown toxic compound or a difference in bioavailability of microcystin-LR could be the cause of this large difference in toxicity between intact cells and pure toxin. Some previous studies of the acute toxicity of microcystins on *Daphnia* provide a clue. Jungmann *et al.* (1992) found that 48 h LC_{50} of a crude extract of toxic *M. aeruginosa* (strain PCC 7806) to *D. pulicaria* was 47.4 ml l^{-1}, equivalent to 1620 μg microcystin l^{-1} (the crude extract contained 22 000 μg microcystin-LR l^{-1} and 12300 μg 3-desmethyl microcystin-LC l^{-1}). The 48 h LC_{50} of purified microcystin-LR reported by Demott *et al.* (1991) for the same species of *Daphnia* is 21400 μg l^{-1}. These results show that the toxicity of the crude extract is intermediate to that of the

pure toxin and intact cells of *M. aeruginosa*, implying that the bioavailability of the toxin is higher in the crude extract than in pure toxin solution, if microcystin is the only reason for the death of *D. pulicaria*. However, the results remain inconclusive as the study by Jungmann *et. al.* (*ibid*) did not use a proper experimental control in order to ascertain whether any other cause (*i.e.* bacterial contaminations and oxygen depletions in the medium, as found in this study) might have been responsible for the death of the daphnids. Moreover, in order to reach a reliable conclusion, the same strain of toxic *M. aeruginosa* must be used to conduct toxicity tests with intact cells and crude extracts and all tests must be conducted under the same standardized conditions. By these criteria, the bioavailability of microcystins remains still to be studied and to do this it is necessary to improve the delivery of purified microcystin using some carrier which does not enhance bacterial growth.

The suppression of feeding rate on toxic *M. aeruginosa* in the present study was higher than that under exposure to non-toxic *M. aeruginosa* although the difference was less than 10% of the control value. It is not clear whether this difference was due to microcystin-LR or some other strain-specific variation. Nizan *et al.* (1986) and Henning *et al.* (1991) reported variations in feeding rates of *Daphnia* even on different non-toxic strains of *M. aeruginosa*. This study, therefore, was unable to rule out microcystin-LR as a contributing factor in feeding inhibition. Further studies are needed and one method would be to measure the feeding rates of *Daphnia* on a series of cultures of the same toxic strain of *M. aeruginosa*, each culture being of different toxicity (*i.e.* microcystin content). If a correlation existed between toxicity and inhibition of feeding it would support the hyphothesis that microcystin is a contributing factor to feeding inhibition.

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