Meiofauna in a chemosynthetic groundwater ecosystem: Movile Cave, Romania

Dissertation

zur Erlangung des akademischen Grades eines
Doktors der Naturwissenschaften
der Fakultät für Biologie
Universität Bielefeld

vorgelegt von

Daniel Muschiol

Bielefeld, Mai 2009









"I must now introduce you to
a class of animals peculiarly microscopic,
since without our marvel-showing instrument,
they are wholly beyond the sphere of human cognizance"
Philip Henry Gosse, 1859

Table of contents

1. Summary

Discovered in 1986, Movile Cave is a peculiar groundwater ecosystem sustaining abundant and diverse invertebrate communities. Isolated from the surface since preglacial times, the cave lacks input of allochthonous photoautotrophically based food. Instead, all life in Movile Cave entirely depends on *in situ* chemoautotrophic production. Chemoautotrophic microbial mats floating on a cave pond of thermomineral, highly sulphidic and methanic water are inhabited by dense populations of partly endemic meiofauna from a wide range of taxonomic groups. Yet, the mats' meiofauna comprises no more than about 20 species, suggesting an extraordinary simple food web in comparison to other habitats. Along with the unique properties of this ecosystem (extreme isolation, *in situ* chemoautotrophic production alone, constant abiotic factors), Movile Cave thus offers the opportunity to characterize and eventually understand an ecosystem as a whole.

The main objective of our studies on the floating microbial mats of Movile Cave was to identify their key species and major trophic links, thereby getting a first general idea of the dynamics and functioning of this self-contained ecosystem.

Since chances of quantitative *in-situ* sampling are negligible in Movile Cave, we experimentally simulated the cave's unique physicochemical conditions in order to develop a model system allowing for laboratory investigation of the mat community. By manipulating the atmosphere in enclosures set atop thermomineral water, we created hypoxic conditions that permitted the development of floating microbial mats. Both in the laboratory and the cave itself, these cultivated mats were quickly colonized by Movile's highly adapted invertebrates. In terms of total abundance and biomass, both native and cultivated mats were clearly dominated by five bacterivorous nematode species and a predacious copepod species. Throughout a year-long investigation period, we monitored meiofaunal community structure and observed strong fluctuations in the relative importance of individual nematode species. These fluctuations also characterized samples of native mat material and may reflect a succession of decreasing bacterial food availability within the mats. However, previous reports on the occurrence of nematodes surviving and reproducing under complete anoxia could not be confirmed.

In a series of electron microscopic studies, we investigated possible associations with symbiotic bacteria because previous studies had proposed a microbial nature of characteristic inclusions visible within specimens of Movile's nematode *Chronogaster*. However, the inclusions possessed a homogenous ultrastructure, providing no evidence of symbionts associated with nematodes. Likewise, an involvement in a sulfide detoxification system is doubtful since the inclusions evinced no marked sulphur concentrations.

In order to gather basic, so far missing ecological information, life-cycle studies were conducted with the two most abundant nematode species from Movile. Under excess food conditions, both *Poikilolaimus* sp. and *Panagrolaimus* sp. emerged as comparatively fast-growing species, with population doubling times of 4.21 and 2.24 days, respectively.

Panagrolaimus produced less progeny than *Poikilolaimus* during its life but exhibited faster maximum population growth due to its earlier maturation. As indicated by subsequent studies under varying food regimes and the fact that competitive exclusion does not occur in Movile, the nematodes apparently evince different food density preferences.

An improved method for the estimation of life-cycle parameters in nematodes was developed and subsequently applied to *Caenorhabditis elegans*; it may ultimately prove to be more convenient than traditional cultivation techniques.

Food web studies aimed to investigate if nematodes, which doubtless consume a considerable amount of chemoautotrophically produced biomass in Movile Cave, in turn serve as a food base for higher trophic levels. With daily consumption rates exceeding its own body weight by a factor of 2.5, the abundant copepod *Eucyclops subterraneus scythicus* emerged as a voracious predator of nematodes, a finding that is also supported by preliminary investigations of nitrogen stable isotope ratios ($\delta^{15}N$). Calculations of nematode biomass production and copepod feeding rates suggested that *Eucyclops* might top-down control nematode populations in Movile Cave. As indicated by predator-prey experiments with a common surface-dwelling copepod, *Diacyclops bicuspidatus*, the revealed trophic linkage between nematodes and copepods might be of considerable importance in many other aquatic habitats, providing an important benthic-pelagic coupling between microbial carbon, endobenthic nematodes, epibenthic copepods, and pelagic fish.

First experiments employing Amplified Ribosomal DNA Restriction Analysis (ARDRA) suggested that the bacterial diversity of Movile's floating microbial mats is probably higher than expected. The investigation of how nematode grazing affects the microbial community of the mats appears a promising field for further investigations.

In summary, our investigations provided a first sketch of the main energetic pathways in the food-web of Movile. The emerging picture shows an extraordinary simple food web, with chemosynthetic mats sustaining countless bacterial-feeding nematodes, which are, in turn, heavily predated upon by copepods.

2. Introduction

2.1. Meiofauna – The microscopic motile fauna of aquatic habitats

The term meiofauna denotes microscopically small, motile aquatic invertebrates living mostly in and on marine and limnic soft substrates. The formal size boundaries of meiofauna are operationally defined, based on the standardized mesh width of sieves with 500 µm as upper and 42 µm as lower limits: all fauna that pass through the coarse sieve but are retained by the finer sieve are considered meiofauna (Fenchel 1978). Being important as both producers and consumers, meiofauna represents a key component in the transfer of energy from microbenthos (bacteria, algae, protozoa) to higher trophic levels (e.g., crustaceans, insect larvae, fish fry). In the last decades meiofaunal research has been dominated by studies of the marine environment so that our understanding of meiofaunal biology and ecology in the sea is more advanced in many areas than in fresh waters (Giere 2009). However, comprising most of the animal species present in lakes and rivers (Särkkä 1995, Robertson *et al.* 2000, Traunspurger *et al.* 2006) meiofauna are increasingly recognized as diverse and abundant constituents of freshwater ecosystems. There is also growing evidence that meiofauna fulfils important roles in the trophic and functional dynamics of freshwater ecosystems (Schmid & Schmid-Araya 2002, Hakenkamp et al. 2002).

Freshwater meiofauna involves a wide range of taxonomic groups, among which nematodes are one of the most important. These cylinder-shaped, mostly 0.5−2 mm long transparent worms are widely distributed throughout all types of substrates in aquatic and terrestrial ecosystems. Benthic freshwater habitats are regularly dominated by a high number and variety of described and undescribed nematode species. Palaearctic lake sediments typically contain more than 50 nematode species (Traunspurger 2002, Michiels & Traunspurger 2005) which, in total, may exceed 10^7 individuals m^{-2} sediment (Traunspurger et al. 2006, Muschiol & Traunspurger 2009). However, our knowledge of the ecology and global distribution of the roughly 11,000 valid nematode species (Andrássy 1992) is very limited, and 90% of the estimated 10^5 nematode species worldwide are still undescribed (Lambshead 1993). By today, there exist only a handful of autecological studies on nematodes and for the vast majority of species information like generation time, reproductive rate, food preference or role in food webs are completely lacking.

2.2. Movile Cave – A unique chemosynthetic groundwater ecosystem

On the continental earth, 97% of all unfrozen freshwater is subsurface, whereas lakes and rivers represent less than 2% (Gilbert & Deharveng 2002). Yet, our ecological knowledge of groundwater ecosystems lags far behind that of lakes and rivers (Boulton *et al.* 2003). Conditions in aquifers can be relatively harsh for life, with low concentrations of nutrients and carbon, limited dissolved oxygen, an absence of light, and restricted free space (Hancock *et al.* 2005). Thus, subterranean ecosystems have long been considered as extreme environments, inhabited only by a few specialized species. This paradigm is now being

revised as many studies have shown that this environment harbours diverse animal communities (Gilbert & Deharveng 2002). For example, Botosaneanu (1986) listed a known (minimal) species diversity of 322 aquatic subterranean nematode species. Since subterranean ecosystems are protected against surface environmental changes—in contrast to most surface ecosystems, which are short-lived (rivers, wetlands)—they may persist relatively unchanged for millions of years.

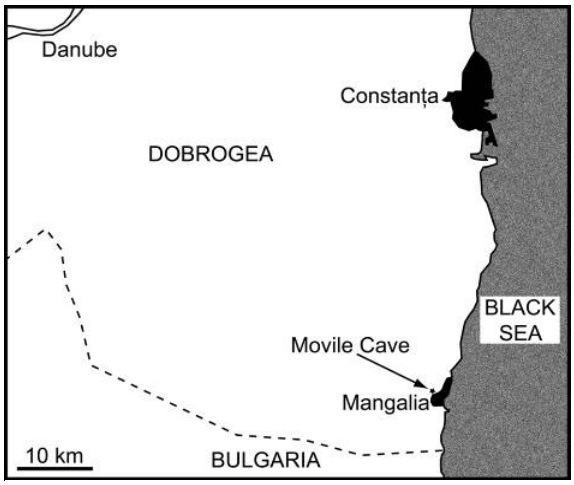

Fig. 1: Romania, Southern Dobrogea, and the location of Movile Cave.

In 1986, a shaft dug for geological investigations in the proximity of Mangalia, Romania (Fig. 1), intersected a natural cave passage at a depth of 18 m (Constantinescu 1989, Lascu 1989). The narrow cave passage (Movile Cave) is situated in the surroundings of Obanul Mare, a large sinkhole located 3 km west of the Black Sea coast, and represents a window into a vast network of fissures and cave passages of phreatic origin. Isolated from the surface since preglacial times (Sarbu et al. 1996), Movile Cave consists of a 200-m long dry level and a 40-m long submerged level. The lower submerged level is flooded by thermomineral groundwater ascending along natural faults from a captive aquifer at a depth of ~200 m. The completely anoxic water has a temperature of 21°C and contains high amounts of NH_4^+, CH_4, and H_2S (Sarbu & Kane 1995). At a depth of 25 m below earth's surface, the water forms a small lake which permits access to the lower submerged level of Movile Cave (Fig. 2). Via this lake, divers may attain several air pockets ('air bells'). In contrast to the almost normoxic atmosphere in the upper cave passages, these air bells are poor in oxygen (7–10%), rich in carbon dioxide (2–3.5%), and contain 1–2% methane (Sarbu & Kane 1995, Sarbu et al. 1996). On their water surface, i.e., at the redox interface between the hypoxic atmosphere and the completely anoxic waters, thick (1–3 mm) microbial mats exist. The mats have a brownish colour and are kept afloat by gas bubbles containing high CH_4 concentrations (up to 13.5%). The mats depend on the chemoautotrophic production of methanotrophic and sulphur-oxidizing bacteria which have been identified as primary producers in the mats (Rohwerder *et al.* 2003, Hutchens *et al.* 2004). At the same time, allochthonous photoautotrophically based food seems to be completely absent in the cave (Sarbu & Kane 1995). Similar to deep-sea hydrothermal vent communities, the cave's ecosystem is thus supported solely by *in situ* chemoautotrophic production. It is this situation that distinguishes the cave from the majority of the world's cave streams and groundwater ecosystems, both of which principally depend on energy derived from surface detritus (Hancock *et al.* 2005). Movile's microbial primary productivity acts as the food base for dense meiofauna populations associated with the mats, comprising about 20 species including Amphipoda, Annelida, Copepoda, Gastropoda, Heteroptera, Isopoda, Nematoda, Ostracoda, Rotifera, and Turbellaria (Sarbu & Kane 1995, Riess et al. 1999).

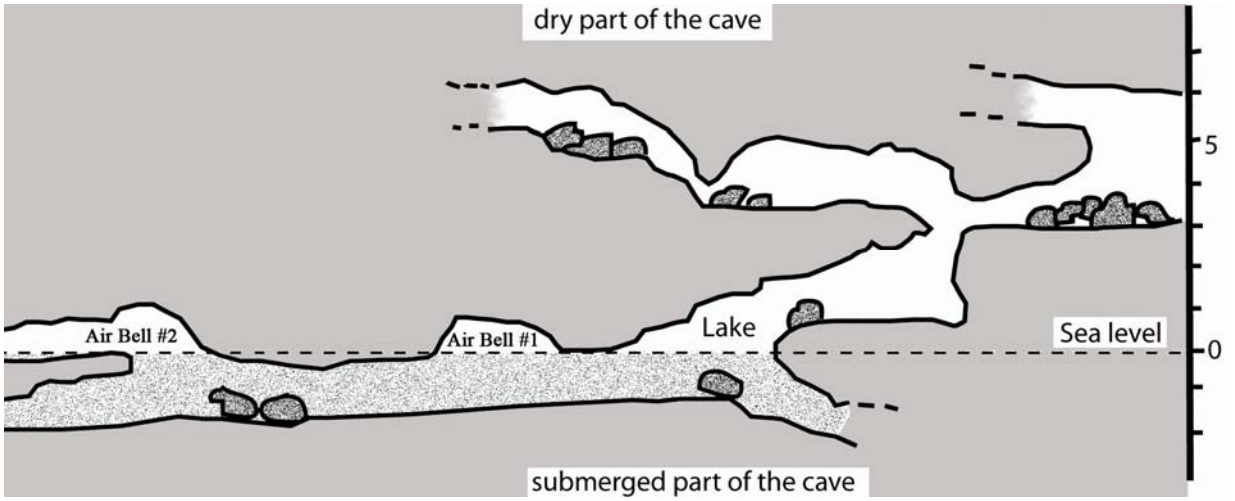

Fig. 2: Cross-sectional map of Movile Cave, Romania. Note that fluctuating water levels repeatedly establish a connection between the Lake Room and Air Bell #1. Redrawn from Sarbu et al. (1994).

This isolated meiofauna community contains a high proportion of endemic species (Sarbu et al. 1996) and persists under low oxygen tensions and permanent exposition to high concentrations of poisonous hydrogen suphide. Since its discovery in 1986, taxonomists assembled a rather comprehensive overview of Movile's species inventory (Sarbu *et al.* 1996), however, our understanding of many ecological aspects (e.g., temporal dynamics, food web) remains quite preliminary (see Popa & Sarbu 1991).

3. Aims and scope

According to Krebs (1994), ecology can be defined as "the scientific study of the interactions that determine the distribution and abundance of organisms". Ecological research is thus interested in *where* organisms are found, *how many* occur there, and *why*. While the answer to the first two questions often lies in adequate sampling effort, the *why* is much more difficult to answer. In the majority of habitats, attempts to uncover the main drivers of observed community patterns are a challenging (and often frustrating) task because of the immense variety of direct and indirect biotic and abiotic factors they depend on. Especially trophic interactions can be very complex and we know that (aquatic) food webs are more reticulate than previously assumed, particularly if micro- and meiofaunal-sized members are considered (Schmid & Schmid-Araya 2002). The situation is complicated by the fact that basal information on the biology of most meiofaunal organisms (e.g., food preferences, developmental time, reproduction rates) is completely missing.

In the face of these difficulties, the investigation of small isolated ecosystems (e.g., Galápagos Islands) may facilitate attempts to uncover generalizable patterns and processes. Such straightforward systems with relatively few species and consequently simple food webs offer the opportunity to reveal connections that in other habitats are hidden by the sheer mass of interactions.

For investigations that aim to characterize and eventually understand an ecosystem as a whole, Movile Cave appears particularly suited since (1) the extreme isolation makes

immigration and emigration processes negligible, (2) allochthonous energy sources are missing since the system depends on *in situ* chemoautotrophic production alone, (3) abiotic factors like temperature, light regime, and water chemistry are constant, and (4) the number of interacting species is relatively low.

The intention of our studies on Movile Cave was to get a first general idea of the functioning and dynamics of this self-contained ecosystem, with emphasis its key species and major trophic links. Specifically, we aimed

(1) to reassess previous studies which suggested that nematodes in Movile are able to survive and even reproduce under complete anoxia (section 4.1),

(2) to elucidate the nature (sulphur compounds, symbionts?) and function (sulfide detoxification?) of curious internal inclusions characteristic for nematodes from Movile (section 4.2),

(3) to identify the –in terms of abundance and biomass– key species among the various meiofaunal taxa reported from Movile (section 4.3),

(4) to gather basic biological information on these species (food preference, generation time, population growth rates) (section 4.4),

(5) and to finally develop a first sketch of the main energetic pathways in the food-web of Movile (section 4.6).

4. Major outcomes

The following results have been presented in form of five manuscripts, appended in section 11. However, some supplementary, so far unpublished results have been included in sections 4.2, 4.4, and 4.6.

4.1. Role of oxygen

The floating microbial mats of Movile Cave occur exclusively under the significantly modified atmospheric conditions of the air bells. Apparently, the high oxygen content of Lake

Fig. 3: Cultivation of artificial mats. (A) Experimental tubes in the field laboratory in Mangalia, Romania. (B) Floating rafts containing experimental tubes in the Lake Room of Movile Cave. (C) Sieve used for harvesting cultivated mats. The sieve was used to shut the open lower end of the tubes before they were removed from the frames.

Room (Fig. 2) hampers the development of such mats, presumably via grazing through aerobic heterotrophs (flagellates, crustaceans).

In an attempt to simulate the atmospheric composition of Movile's air bells, Riess et al. (1999) permanently installed Plexiglas enclosures in the Lake Room of Movile Cave. Set afloat on the surface of the hydrothermal cave lake, these enclosures in fact permitted the development of microbial mats and their associated meiofauna. Surprisingly, dense nematode populations were observed not only within hypoxic enclosures but also within enclosures that had been completely anoxic for several months. Subsequent cultivation experiments "showed that the nematodes could be kept alive and active for more than 1 yr without any supply of oxygen in a clearly sulphidic environment" (Riess et al. 1999, p. 161). These observations challenge our current physiological understanding. It is believed that all free-living and parasitic metazoans need oxygen at least for their reproductive stages (Barrett 1991). While there is a consensus that many metazoans are able to survive for extended periods in sulphidic environments and, thus, are well-adapted to hypoxic conditions, the permanent existence of truly anaerobic metazoans under anoxic conditions has never been experimentally proven. Yet, there are reliable reports of meiofauna from other habitats with indubitably permanent anoxia (Giere 2009). Considering the debate about animals under complete anoxia, it was a central aim of our investigation to reassess the anoxic cultivation experiments in Movile Cave (**Manuscript I**).

Advantageously, an artificial artesian well in a field laboratory in Mangalia taps the same aquifer that supplies Movile Cave allowing for permanent laboratory access to sulphidic thermomineral water. Thus, we established sealed enclosures on top of anoxic thermomineral water both in the cave lake and in the laboratory (Fig. 3). During the year-long investigation period, we regularly inspected these enclosures for developing microbial mats and associated meiofauna. However, under truly anoxic conditions, floating microbial mats were never observed, neither in the laboratory nor in the Lake Room. Only a delicate iridescent film was noticed on the water surface and, except for some decaying remains, viable nematodes were not found in the anoxic cultivation tubes. Likewise, anoxic sediment samples from the Lake Room and the air bells contained only decomposing invertebrate remains. Short-term anoxic incubation of native microbial mats collected in Air Bell #2 resulted in a complete immobilization and increased mortality of the associated nematode fauna.

In summary, the results of our study clearly indicate that Movile's metazoan fauna not only depend on atmospheric oxygen for trophic reasons (growth of microbial mats only at the oxic/anoxic interface), but also fail to survive under anoxia. Riess et al. (1999) may have misinterpreted an initial growth of a mat after an accidental entrapment of air or oxic water when they started their cultivation experiments. They also may have underestimated the oxygen permeability of the materials which they had used for their enclosures. Obviously, the nematodes from Movile Cave are able to tolerate severe hypoxia, but, according to our experiments, complete anoxia is fatal.

4.2. Structural adaptations to the thermomineral, chemoautotrophic situation in the cave

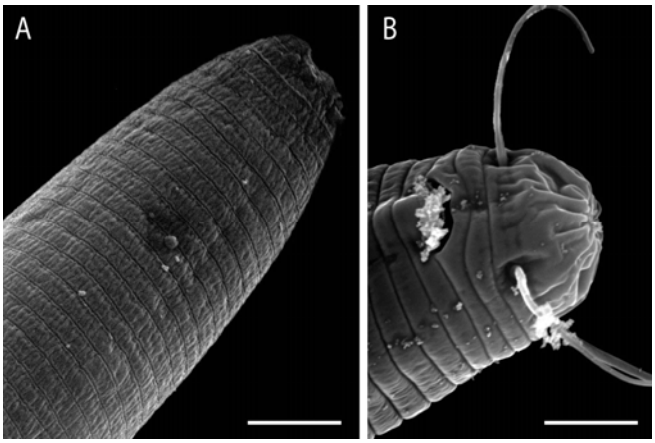

Fig. 4: Exemplary SEM pictures of nematodes from Movile.
A *Panagrolaimus* B *Chronogaster*; Scale Bars 3 µm

The highly adapted nematode community of Movile Cave persists under permanent exposition to hydrogen suphide. It is well documented that sulfide at nanomolar to millimolar concentrations adversely affects cytochrome *c* oxidase, various other enzymes, oxygen transport proteins, cellular structures, and consequently the physiological functions of organisms (review in Bagarinao 1992). Among the various adaptations aquatic organisms have evolved against sulfide toxicity, associations with sulfide-oxidizing bacteria are probably the most fascinating. Several sulfide-tolerant invertebrates, e.g., priapulids (Oeschger & Schmaljohann 1988), vent polychaetes (Fisher 1990), and nematodes (Hentschel *et al.* 1999) form associations with various types of epibacteria; these bacteria contribute not necessarily to the nutrition of the worms but may detoxify sulfide to some degree. Other benthic invertebrates, e.g., nematodes (Ott *et al.* 1982) various molluscs, vestimentiferans, pogonophorans and annelids (Bagarinao 1992) have entered into obligate symbiotic associations with sulfide-oxidizing bacteria. However, in freshwater meiofauna, such associations are still unknown.

In order to uncover possible associations with epibacteria, specimens of Movile's five nematode species were fixed in 4% formaldehyde, dehydrated in an increasing ethanol series, critical point dried, sputtered with gold and observed with a Hitachi S-450 scanning electron microscope (SEM) under 20 kV. However, in none of the five investigated nematode species we found evidence for epibacteria on the cuticle (Fig. 4).

For the nematode *Chronogaster troglodytes*, which seems to be endemic to Movile Cave, characteristic internal "crystalloids" have been reported (Poinar & Sarbu 1994). Also in our samples from Movile Cave, *Chronogaster* frequently contained such inclusions (Fig. 5). The composition and function of these structures is enigmatic. At least, comparable structures in the freshwater nematode *Tobrilus gracilis* have been related to a detoxification system for sulfide ions (Nuß 1984). Since the crystalloids in *Chronogaster* apparently do not represent

Fig. 5: Inclusions within the body of *Chronogaster* sp. from Movile (Differential Interference Contrast, 1000-fold magnification)

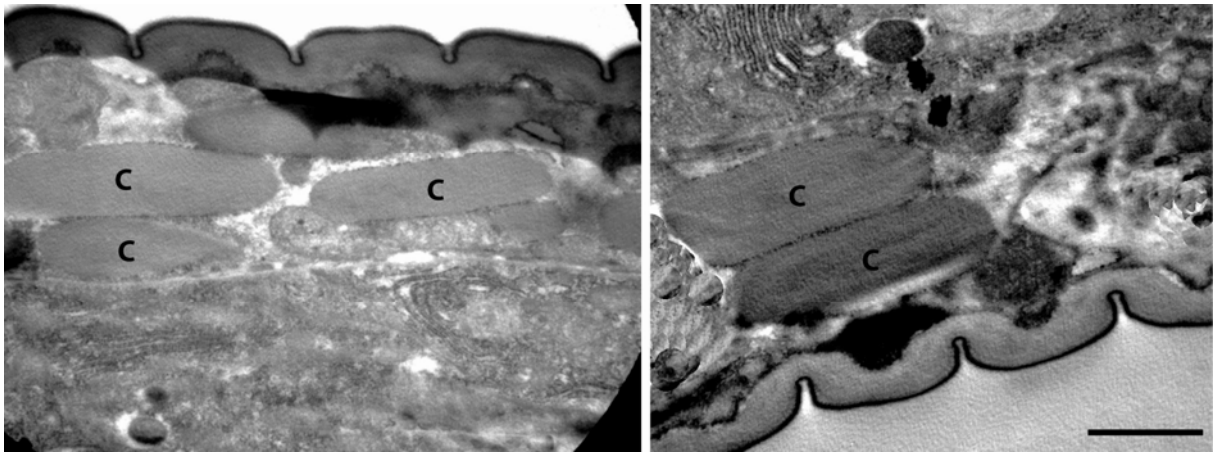

Fig. 6: Exemplary TEM pictures of *Chronogaster* from Movile. C structures which can be homologized to crystalloids in Fig. 5. Scale Bar 1 µm

inorganic crystals, Poinar and Sarbu (1994) speculated on a possible microbial nature of these structures and recommended ultrastructural studies.

For transmission electron microscope (TEM) observation, specimens of *Chronogaster* were fixed in Trump's fixative (McDowell 1978) buffered in sodium cacodylate. Specimens were washed in cacodylate, postfixed in 1% osmium tetroxide, dehydrated in an acetone series (up to 70%) and embedded in Spurr's resin. Ultrathin sections, mounted on copper grids and contrasted in aqueous uranyl acetate and lead citrate, were examined in a transmission electron microscope (Zeiss EM 902 A). However, the ultrastructural studies on *Chronogaster* provided no evidence of a microbial nature of the observed structures. Inclusions which could be homologized in form and location to lightmicroscopically cognizable crystalloids showed a homogenous ultrastructure (Fig. 6). Therefore, we used uncontrasted ultrathin sections to perform element analysis by Electron Energy Loss Spectrography (EELS and ESI) to reveal a possible mechanism of sulfide detoxification. Sulphur could be detected throughout the whole body wall (cuticle, epidermis, inclusions therein). However, a marked concentration to a certain structure was not found. At the moment, we can thus only speculate on the function of the observed crystalloids in *Chronogaster*. Future studies could investigate the influence of environmental factors (mineral and sulfide concentrations). However, such incubation experiments (see Thiermann *et al.* 2000) require large numbers of individuals. Since any attempts to cultivate *Chronogaster* failed, the nature and function of the crystalloids remains ambiguous at present.

4.3. Meiofauna diversity and population dynamics

Our knowledge of the functioning of Movile's ecosystem (population dynamics, biomass turnover, trophic interactions) is by and large preliminary (Popa & Sarbu 1991, Sarbu et al. 1995). Reports of meiofauna population densities are either missing or rough estimates only (e.g., Sarbu & Popa 1992). Previous studies in the cave were hampered by the difficult access to this isolated subterranean habitat and by the fragility of its ecosystem which restricts

sampling and prohibits major manipulations. Accordingly, chances of quantitative *in-situ* sampling are negligible.

A promising approach to overcome these difficulties is the artificial cultivation of microbial mats. Riess et al. (1999) already demonstrated that a cultivation of mats in hypoxic enclosures set atop the water surface of the cave lake is principally possible. Hence, we asked ourselves if it is also possible to cultivate microbial mats and associated meiofauna under controlled laboratory conditions since this would offer new possibilities to investigate the functioning of Movile's ecosystem. The establishment of such a laboratory model would considerably facilitate investigations because quantitative samples could be obtained much easier than from the cave itself (no caving and diving equipment required) and conservation reasons would no longer constrain manipulations.

In our experimental set-up (Fig. 3), hypoxic enclosures were thus installed both in a laboratory discharge system and in the cave itself (**Manuscript I**). Each enclosure was inoculated with a piece of natural mat (~ 50 mm^2) collected from Air Bell #2. Our aim was to monitor species inventory and population dynamics of meiofauna during one year. The obtained data should be compared to samples of native microbial mats from Movile's air bells in order to examine the usability of the model system. The obtained quantitative estimates of population densities should be used to identify key taxa in the meiofaunal food web and thus develop hypotheses concerning the most important energetic pathways.

Among all metazoans encountered in samples of native microbial mat, 94.7% were nematodes, followed by specimens of the cyclopoid copepod *Eucyclops subterraneus scythicus* (3.2%), its nauplii (0.3%) and ostracods (1.1%). The remaining taxa comprised harpacticoid copepods, gammarids, isopods, acarids, rotifers, and gastropods.

In the hypoxic enclosures, developing microbial mats could be observed within weeks after the start of the experiment. Both in the laboratory and the cave itself, these cultivated mats were quickly colonized by Movile's highly adapted invertebrates, among which nematodes were the most important, both in terms of total abundance and biomass. Once the water surface in the enclosures was covered by a microbial film, nematode populations grew quickly, reaching maximum densities of 1932 nematodes 10 cm^{-2} in the laboratory and 1308 nematodes 10 cm^{-2} in the Lake Room.

In addition to estimates of total abundances, we also investigated nematode species composition: During the one-year investigation period, a total of 806 nematodes from natural mats and 4451 nematodes from cultivated mats were prepared on permanent slides and identified to species level. As a matter of fact, cultivated mats in the laboratory and in the Lake Room possessed the same nematode species inventory as the natural mats in Movile Cave, comprising five species belonging to the genera *Chronogaster*, *Panagrolaimus*, *Poikilolaimus*, *Udonchus*, and *Monhystrella*. Both in native and cultivated microbial mats, strong fluctuations in the relative importance of individual species were observed over time (see Figs. 5 and 6 in Manuscript I). The cultivation experiment revealed a specific pattern of successive population maxima of the different nematode species: *Panagrolaimus* was

abundant only at the very beginning of the one-year period while *Chronogaster* and *Udonchus* became abundant in the second half of the one-year period. *Poikilolaimus*, being frequent in the majority of samples, reached its highest relative abundance after 3 months. In contrast, *Monhystrella* was either absent from samples or reached only very low relative abundances.

In summary, our experiments demonstrated that it is possible to cultivate microbial mats that are, at least macroscopically, identical to the native mats from Air Bell #2 and serve as suitable habitats for the highly adapted and partly endemic stygofauna. Thus, the laboratory mats can provide a useful model for the investigation of the cave's floating mats. Our investigation revealed that among the various meiofaunal taxa within the mats, nematodes are by far the most important, followed by the copepod *Eucyclops subterraneus scythicus*. In the following section, we describe efforts to uncover the underlying mechanisms causing the observed fluctuations in the importance of individual nematode species in native and cultivated microbial mats.

4.4. Autecology of nematodes

According to the morphological characteristics of their buccal cavities (see Traunspurger 1997), all nematode species in Movile Cave are bacterial grazers. Based on their high abundance and biomass, it is clear that as consumers of chemoautotrophic bacteria and as a potential food base for higher trophic levels they play a major role in the cave's food web. However, the literature contains very little information on these nematodes and several key questions remain unanswered. For example, as the nematodes coexist in a quite homogeneous, unchanging environment and compete for the same food resources, mechanisms to reduce interspecific competition and thus allow coexistence should be present. The paucity of food, which is characteristic for the majority of groundwater ecosystems, accounts for the slow metabolism, long life, slow growth, and few young characteristic of most stygobite (i.e., obligatory hypogean) species (Gilbert & Deharveng 2002); however, it is not known whether this is also true for the nematodes of the highly productive Movile Cave.

In **Manuscript II**, we conducted a life-cycle experiment with the two most frequent species of Movile's microbial mats, *Panagrolaimus* sp. and *Poikilolaimus* sp., in order to assess the general biological features of the two species and to create a basis for further ecological experiments. Since preliminary experiments revealed that traditional cultivation techniques (both solid and liquid media) are unsatisfying for a precise estimation of life-cycle parameters in nematodes, we developed an improved method ('hanging drop') which involves the use of semi-fluid culture medium. In our life-cycle experiments, we provided food in excess (10^9 cells *Escherichia coli* ml^{-1}), as determined in preliminary experiments. The experiment started with newborn juveniles and was conducted until the last animal died (day 72).

During juvenile development, both species showed a strict linear increase in mean body length (Fig. 3 in Manuscript II), whereas body weight increased exponentially (Fig. 6 in Manuscript II): Juvenile *Poikilolaimus* and *Panagrolaimus* doubled their body weights every

73 h and 36 h, respectively. At the beginning of the reproductive period, *Panagrolaimus* had a body length of 1688 µm and a biomass of 1.53 µg, whereas *Poikilolaimus* was a lot stubbier by then (1133 µm/1.92 µg). Life tables and fecundity schedules for the two species were created (Table 3 in Manuscript II). In addition, important demographic parameters (total life span, generation time, population doubling time, intrinsic rate of increase, net reproductive rate, total fertility rate) and a regression relating body weight and length (to simplify future calculations of body mass) were calculated.

In summary, *Panagrolaimus* started reproduction at an average age of 9.5 days and produced 64 juveniles, whereas *Poikilolaimus* needed twice as long to start reproduction (19.5 days) but produced 108 juveniles. However, due to its faster maturation, *Panagrolaimus* exhibited a much higher population growth rate (population doubling times: 2.24 vs. 4.21 days).

Since population growth rates integrate the entire age schedules of survival and fertility into a single measure, they are a measure of fitness (Charlesworth 1994). Thus, our results imply that *Panagrolaimus* would inevitable outcompete *Poikilolaimus* under constantly high food supply. Nevertheless, the two nematode species (and three more) obviously do coexist in Movile's microbial mats, at least since 20 years (Poinar & Sarbu 1994, Riess et al. 1999). Which factors have permitted the long-term coexistence of these nematodes in the mats? Movile Cave's microbial mats are unaffected by seasonal or diurnal variations in temperature, illumination, or humidity and receive a continuous supply of methane and sulphide through the cave's well-buffered and constantly tempered hydrothermal water. This seemingly stable equilibrium situation without trophic or spatial separation does not accord with the principle of competitive exclusion (Hardin 1960). But is the micro-ecological situation, particularly the trophic supply of the cave nematodes really stable and unchanging? Consequently, we conducted further experiments with Movile's nematodes in which we varied food densities. The results of these studies suggested that the nematodes indeed evince different food density preferences. For example, at intermediate food densities around 10^8 cells ml^{-1}, *Poikilolaimus* is a superior competitor in comparison to *Panagrolaimus* (Schroeder, Muschiol & Traunspurger, in preparation). Under severe food limitation, all nematode species in samples of native microbial mat successively became extinct until only *Chronogaster* remained (see Manuscript I). Consequently, regular fluctuations of food availability within the floating microbial mats may in fact account for the observed long-term coexistence of different bacterivorous nematode species in the mats.

Several lines of evidence, compiled in Manuscript I, let the following scenario appear reasonable: Ascending gas bubbles keep Movile's microbial mats afloat on the water surface as long as the mat texture is light and fluffy. By aging, the mats become compacted and heavier, and after some time (months to years) they exceed the critical point of stability and finally sink with all their inhabitants into the hostile anoxic depth just to allow the start of a new growth cycle of mats at the surface. These young mats provide plenty of microbial food to nematodes, thereby favouring fast-growing *r*-selected species (*Panagrolaimus*,

Poikilolaimus). Nematode populations develop until microbial food becomes a limiting factor. Simultaneously, the accumulation of dead, less-degradable organic material increasingly acts as a diffusion barrier for oxygen and sulphide, so that microbial production declines. The growing intra- and interspecific competition favours more *K*-selected species (*Chronogaster*, *Udonchus*), which gradually displace the *r*-selected species. Hence, a stable equilibrium, i.e., competitive exclusion, is not obtained. This cyclicity of mat development would explain both phenomena, the extreme variability in nematode community structure in the native mats of Air Bell #2 (Fig. 5 in Manuscript I) and the succession in community structure in the cultivation experiments (Fig. 6 in Manuscript I).

4.5. Excursus: Life-cycle studies on Caenorhabditis elegans

While comparing our results to existing literature data, we realized that our understanding of the dynamics of natural nematode populations suffers from the very restricted knowledge of life-cycle parameters in nematodes. Generally, only a handful of nematode species have ever been investigated in life-cycle studies (see Discussion in Manuscript II). However, as demonstrated above, such data are essential for a meaningful interpretation of observed community structures. The scarcity of life-cycle studies in nematodes may be partly due to methodological shortcomings: Current cultivation techniques of these minute organisms are unsatisfying in many aspects (Moens & Vincx 1998). During our life-cycle studies on Movile's nematodes, we developed an improved method ('hanging drop') that overcomes some problems associated with traditional cultivation techniques. Since we felt that this method has considerable potential in life-cycle studies of nematodes (and other meiofauna), we decided to test the method on the predominant model organism in biological research, *Caenorhabditis elegans*. In Manuscript III, the life cycles of wild-type *C. elegans* strains N2 (Bristol, UK) and MY6 (Münster, Germany) were compared at 20°C with 5×10^9 *E. coli* ml^{-1} as food source.

Discussed in the light of earlier studies on *C. elegans*, our investigation demonstrated certain advantages of the hanging drop method (e.g., convenience, negligible juvenile mortality, speed). We supplemented existing data on *C. elegans* with complete high-resolution life tables and fecundity schedules and accurately assessed life-cycle parameters commonly used in various research fields. Partly in contradiction to earlier studies, we found no evidence that adaptation to the laboratory altered the life history traits of *C. elegans* strain N2 in comparison to the recently isolated strain MY6. A possible laboratory evolution has been discussed in the literature since N2, the canonical "wild-type" *C. elegans* strain used in laboratories throughout the world, has been maintained in the laboratory for about 60 years.

In conclusion, the method might help to reduce the inter-laboratory variability of life-history estimates and may ultimately prove to be more convenient than the current methods used to investigate nematode life-cycles.

4.6. Food web

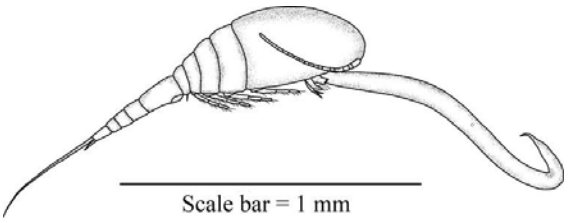

Fig. 1: *Eucyclops* from Movile Cave feeding on adult *Poikilolaimus*. Note the remarkable prey size relative to the predator.

Our studies on the meiofaunal diversity and population dynamics emphasized the enormous importance of nematodes in Movile's microbial mats. It is clear that nematodes consume a considerable amount of chemoautotrophically produced biomass – but do they represent an energetic dead end or do they, in turn, serve as food for invertebrate predators? Although a preliminary study on the cave's food web was carried out by Popa and Sarbu (1991), to date there is no evidence whether any of the taxa (e.g., planarians, amphipods, copepods, ostracods) reported from Movile Cave's microbial mats exploit the huge nematode standing-stock as a food resource to any significant extent. Based on our studies of meiofaunal diversity and population dynamics, only cyclopoid copepods are sufficiently abundant within the mats to potentially affect nematode populations. In preliminary experiments, we observed that Movile's most numerous copepod, *Eucyclops subterraneus scythicus,* indeed readily fed on nematodes (Fig. 7). But further experiments in our home laboratory were hampered by the fact that a laboratory cultivation of this species proved to be impossible. Since meaningful predator-prey experiments required a previously established experimental set-up, we decided to begin our investigations with a copepod species that is easier to gain and handle. These preconditions were met by *Diacyclops bicuspidatus*, a very common Holarctic cyclopoid copepod. With the nematode *Panagrolaimus* sp. offered as prey, we developed and conducted a functional-response experiment (**Manuscript IV**). In fact, *D. bicuspidatus* consumed up to 45.1 nematodes in 2 h, that is, 43.5% of the copepod's body mass. The ability of *D. bicuspidatus* to overwhelm and consume adult nematodes of considerable size, often longer than its own body length, was quite remarkable. Additionally, the efficiency of capturing, overwhelming, and devouring the nematodes suggested that this type of feeding is not uncommon and might be important in many aquatic habitats: Due to their high abundance and wide distribution, copepods play a pivotal role in aquatic food webs, both as primary and secondary consumers as well as a major source of food for many larger invertebrates and vertebrates (Williamson & Reid 2001).

Surprisingly, a thorough review of the relevant literature revealed that possible predator-prey interactions have been widely disregarded and no more than four reports of copepod predation on nematodes existed (Marcotte 1977, Rocha & Bjornberg 1988, Lehman & Reid 1992, Reversat et al. 1992). We discussed our findings in the light of earlier studies (limitations of gut content analysis) and concluded that nematophagous nutrition is potentially quite common among freshwater copepods. A trophic linkage between nematodes and copepods might provide an important benthic-pelagic coupling between microbial carbon, endobenthic nematodes, epibenthic copepods, and pelagic fish predators.

With the experience collected in our experiments with *Diacyclops*, we conducted a functional response experiment in the field laboratory in Romania, with access to cave water und recently collected *Eucyclops subterraneus scythicus* (**Manuscript V**). As potential prey, we offered the two most numerous nematode species occurring in Movile's microbial mats, *Panagrolaimus* sp. and *Poikilolaimus* sp. As indicated by previous observations, the copepods readily fed on the two offered nematode species. Within 20 min, the maximum number of consumed nematodes was 25.8 *Panagrolaimus* sp. or 37.5 *Poikilolaimus* sp., corresponding to 28.7% and 31.1% of the predator's average body weight. At different prey densities, the copepod's feeding rates followed Holling's (1959b) type II functional responses. Within 24 h, the predator's mean consumption corresponded to 264.4% of its own body weight. Using these copepod feeding rates and previously estimated population growth rates of the mat's nematodes (Manuscript II), we calculated that *Eucyclops* might top-down control Movile's natural nematode populations. This study was the first attempt to quantify a predator-prey relationship between coexisting copepods and nematodes at the population level.

In subsequent experiments we aimed to answer the question if the trophic linkage between nematodes and copepods can be further supported by biochemical data. In recent years, the examination of stable isotope ratios ($\delta^{15}N$ and $\delta^{13}C$) has emerged as a useful tool for the reconnaissance of trophic connections in food webs (Fry 2006). Somewhat simplified, animal carbon isotopes usually reflect and index the time-integrated average diet ("You are what you eat"), while $\delta^{15}N$ assays measure trophic levels and provide a "trophometer" for the investigation of food webs in field conditions. Per trophic level, the $\delta^{15}N$ value has been found to increase by 2.2 to 3.4 ‰ in the consumer versus its diet (Vander Zanden & Rasmussen 2001, McCutchan et al. 2003).

For measurements, fragments of microbial mat, nematodes, copepods, and their nauplii were collected from Movile Cave and freeze dried. In nematodes, of which ~5000 specimens had to be collected to attain the required biomass for analysis, no separation concerning species was conducted. Sample preparation was performed in an elemental analyser (Euro EA, Hekatech, Wegberg, Germany), followed by measurement in a continuous-flow isotope ratio mass spectrometer (IsoPrime, GV Instruments, Manchester, UK). Samples were measured against IAEA-N1 and IAEA-NO_3 (International Atomic Energy Agency, Vienna, Austria). Values are reported relative to VPDBee.

Concerning nitrogen isotope ratios, we found that copepods ($\delta^{15}N$:-6.66) were elevated by 2.54 ‰ relative to nematodes ($\delta^{15}N$:-9.20). This increase lies within the range expected for one trophic level (see above). The mat fragments, evinced a $\delta^{15}N$ value of -9.36, which is only slightly lower than in nematodes. This finding is consistent with the fact that the microbial mats represent a mixture of both producers (chemoautotrophic bacteria) and consumers (heterotrophic bacteria, filamentous fungi). The nauplii (i.e., early larval stages of copepods) evinced a $\delta^{15}N$ value of -8.45, which is intermediate between nematodes and adult copepods. This result might well indicate a niche shift from microbivorous to predacious nutrition during ontogenesis. In summary, these findings fit well in the emerging picture of Movile's food

web. However, as a word of caution, we should mention that these findings are quite preliminary because (1) the huge number of required specimens, especially in minute organisms like nauplii and nematodes (~5000!) did not permit replicate measurements and (2) we found considerable variance in $\delta^{13}C$ values. For unknown reasons, measured $\delta^{13}C$ values of copepods and nauplii (-38.91 and -37.29, respectively) were by more than 8 ‰ increased relative to nematodes and mat (-46.00 and -46.87, respectively). These findings hint on a carbon source other than the chemoautotrophic mats. Maybe, the copepods utilize dissolved hydrogen carbonate from the surrounding limestone to build up their carapace, but this remains mere speculation at the moment.

In summary, our investigations suggest that Movile's bacterivorous nematodes face extensive predation through predacious copepods. But how do nematodes impact, in turn, on their bacterial food source? All nematode species in Movile Cave are bacterial grazers. Due to their high abundance (up to 9.8×10^6 nematodes m^{-2}: Riess *et al.* 1999) and productivity (up to 213 mg dry mass m^{-2} daily: Manuscript V), it is clear that nematodes consume a considerable amount of chemoautotrophically produced biomass. But can nematodes significantly affect bacterial abundance and diversity? At least, the observed succession in nematode community structure in the cultivation experiments (Manuscript I) suggested that nematodes are (temporarily) food limited. In freshwater sediments, nematode grazing has been reported to significantly affect bacterial activity and abundance (Traunspurger *et al.* 1997b). To date, very few is known about the microbial identity of Movile's mats (Rohwerder *et al.* 2003, Hutchens *et al.* 2004) and basic questions on, e.g., the rough number of coexisting bacterial species in Movile Cave remain unanswered.

Within a bachelor's thesis conducted at Bielefeld University (Bandeili 2006), a first attempt was conducted to characterize the floating microbial mats' bacterial diversity. In collaboration with Prof. Dr. K. Niehaus (Bielefeld University), 16S rDNA genes were isolated from Movile's mats and investigated employing Amplified Ribosomal DNA Restriction Analysis (ARDRA; see Wegener 2000 for methodological details). We hypothesized that bacterial diversity is low due to the extreme conditions in Movile Cave.

Our results, however, suggested that the microbial diversity of the mats is rather high: 32 randomly chosen sequences comprised 25 species. Only one bacterial sequence, "*biogas-DE-b63*" (Moletta *et al.* 2007) was relatively abundant in the mats (21.9% of all sequences). Of course, these findings are preliminary due to the low number of investigated sequences; however, they convincingly demonstrate the potential of ARDRA in the investigation of Movile's microbial diversity and how it may be affected by nematode grazing.

5. Conclusions

The present thesis provides a comprehensive characterization of the meiofaunal community inhabiting floating microbial mats in Movile Cave, Romania. It throws light on the diversity, population dynamics, autecology, and trophic connections of the key species inhabiting Movile's peculiar self-contained groundwater ecosystem (Fig. 8).

More specifically, the presented results clearly indicate that Movile's dominating meiofaunal component, bacterivorous nematodes, not only depend on atmospheric oxygen for trophic reasons, but also fail to survive under anoxia. Attempts to uncover the nature and function of enigmatic "crystalloids" encountered in the nematode *Chronogaster* were in vain; however, we could vitiate speculations that these structures represent microbial symbionts or localized concentrations of sulphur compounds.

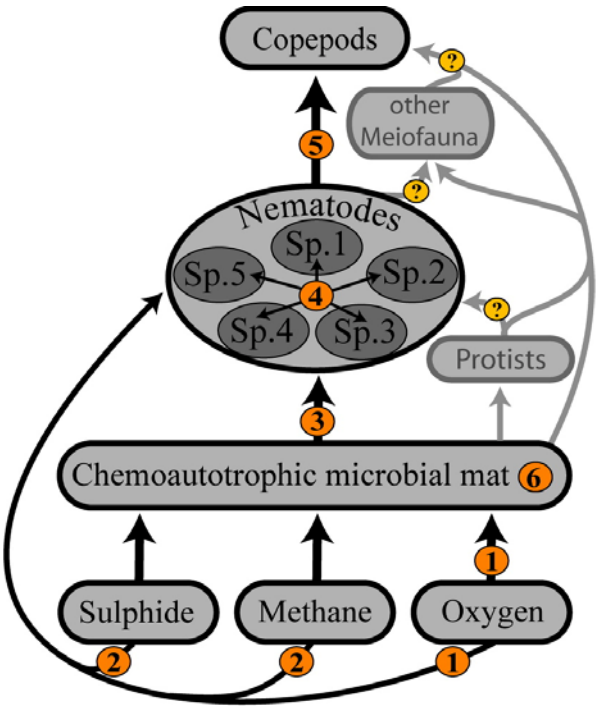

Fig. 8: Topics addressed in studies on Movile Cave.
(1) Oxygen dependence of mats and nematodes?
(2) Symbiotic bacteria? Sulphide detoxification?
(3) Food resources of nematodes? Niche separation?
(4) Autecology? Biomass production? Competition?
(5) Copepod predation on nematodes?
(6) Microbial diversity of mats?

In the laboratory, we successfully simulated the hypoxic cave habitat, allowing for artificial cultivation of microbial mats. This model system offers new possibilities to investigate the functioning of Movile's ecosystem, as demonstrated by the revealed succession in nematode community structure. We presented a new concept that explains why competitive exclusion does not reduce the nematode community to a single species (no stable equilibrium due to cyclicity of mat development; different food density preferences among nematode species).

An improved method to perform life-cycle studies in nematodes was developed and successfully applied to *Caenorhabditis elegans*. The estimation of life-cycle parameters in the two most frequent nematodes in Movile's microbial mats, *Panagrolaimus* sp. and *Poikilolaimus* sp., revealed that these two species, in comparison to the majority of stygobites, possess short life-cycles and high reproduction rates.

Predator-prey experiments, together with calculations of nematode biomass production, suggested that Movile's nematodes are extensively preyed upon by the copepod *Eucyclops subterraneus scythicus* and may (temporarily) face top-down control.

The emerging picture is that the food web of Movile's floating mats is an extraordinary simple one, consisting of only few key species and major trophic links (Fig. 8). It is this simple assemblage structure that offers the opportunity to quantify an entire food web, thereby understanding connections that in other habitats are hidden by a mass of details. For

instance, the revealed trophic linkage between nematodes and copepods may ultimately prove to be of considerable importance in a wide range of aquatic ecosystems.
Future studies in Movile might elucidate the role of less abundant taxa (e.g., Annelida, Heteroptera, Rotifera, and several crustaceans), leading to a more comprehensive understanding of this unique ecosystem. The inclusion of stable isotope / fatty acid analysis may help to identify und quantify trophic links in Movile's food web. Furthermore, the application of molecular methods could scrutinize the microbiological identity of native and cultivated microbial mats and how they are affected by meiofaunal grazing.

6. Zusammenfassung

Die 1986 entdeckte Movile-Höhle stellt ein eigentümliches Grundwasserökosystem dar, das eine individuen- und artenreiche Gemeinschaft von Wirbellosen beherbergt. Jedoch erreicht keinerlei photoautotroph produzierte Nahrung die Höhle, die seit mindestens der letzten Eiszeit vollständig von der Erdoberfläche isoliert ist. Stattdessen beruht alles Leben in der Höhle allein auf *in situ* chemoautotropher Produktion. Auf einem thermomineralem, stark methan- und sulfidhaltigem See schwimmen chemoautotrophe mikrobielle Matten, die von dichten, teilweise endemischen Meiofaunapopulationen der verschiedensten taxonomischen Gruppen besiedelt sind. Diese Meiofaunagemeinschaft umfasst jedoch nur etwa 20 Arten, so dass das Nahrungsnetz der Matten außergewöhnlich einfach aufgebaut zu sein scheint. Aufgrund der übrigen Besonderheiten dieses Ökosystems (extreme Isolation, alleinige Abhängigkeit von chemoautotropher Produktion, konstante abiotische Parameter), bietet die Movile-Höhle deshalb die Gelegenheit, ein Ökosystem gänzlich zu charakterisieren und letztendlich umfassend zu verstehen.

Das Hauptanliegen unserer Studien in Movile war die Identifikation von Schlüsselarten sowie das Aufdecken der wichtigsten trophischen Verbindungen, um so einen ersten Einblick in die Struktur und Dynamik dieses abgeschlossenen Habitats zu erhalten.

Da es leider kaum Gelegenheiten gibt, quantitative Probennahmen in Movile durchzuführen, simulierten wir experimentell die einzigartigen physikalisch-chemischen Bedingungen der Höhle um ein Modellsystem zu etablieren, welches die Untersuchung der Artengemeinschaft auch im Labor ermöglicht. Durch die Manipulation der Atmosphäre in Experimentalgefäßen schufen wir hypoxische Bedingungen, die die Entwicklung von schwimmenden mikrobiellen Matten an der Oberfläche von thermomineralem Wasser ermöglichten. Diese kultivierten Matten wurden sowohl in der Höhle selbst als auch im Laborsystem bald von den hochadaptieren Höhlenorganismen kolonisiert. Gemessen an Individuenzahl und Biomasse wurden sowohl kultivierte als auch natürliche Matten deutlich von fünf Arten bakterienfressender Nematoden sowie einem räuberischen Copepoden dominiert. Die Entwicklung der Nematodengemeinschaft wurde über ein Jahr verfolgt, wobei sich zeigte, dass die relative Zusammensetzung auf Artniveau starken Fluktuationen unterlag. Ähnliche Fluktuationen wurden auch in natürlich gewachsenen Matten beobachtet und

könnten eine sukzessive Abnahme bakterieller Nahrung in den Matten widerspiegeln. Frühere Berichte, dass die Nematoden auch unter vollständiger Anoxie gedeihen, konnten jedoch nicht bestätigt werden.

Aufgrund früherer Studien, die nahelegten, dass es sich bei eigenartigen Einschlüssen in dem Höhlennematoden *Chronogaster* um Bakterien handeln könnte, führten wir eine Reihe von elektronenmikroskopischen Untersuchungen durch, die darauf abzielten, mögliche Assoziationen mit symbiotischen Bakterien nachzuweisen. Die Einschlüsse wiesen allerdings eine homogene Ultrastruktur auf und lieferten damit keinerlei Hinweise auf Symbionten. Auch konnten keine erhöhten Schwefelkonzentrationen in den Einschlüssen gemessen werden, so dass sie vermutlich auch nicht Teil eines Systems zur Sulfiddetoxifikation sind.

Um basale, bisher fehlende ökologische Informationen zu sammeln, wurden mit den beiden häufigsten Nematodenarten der Höhle Untersuchungen zum Lebenszyklus durchgeführt. Sowohl *Poikilolaimus* sp. als auch *Panagrolaimus* sp. erwiesen sich als vergleichsweise schnell wachsende Arten, die ihre Populationsgrößen alle 4,21 bzw. 2,24 Tage verdoppeln können, wenn Nahrung im Überfluss vorhanden ist. *Panagrolaimus* produziert zwar während seines Lebens insgesamt weniger Nachkommen als *Poikilolaimus*, weist aber aufgrund seiner kürzeren Entwicklungszeit ein höheres maximales Populationswachstum auf. Anschließende Studien unter variablen Nahrungsbedingungen sowie die Tatsache, dass in Movile kein kompetitiver Ausschluss von Nematodenarten stattfindet, legen nahe, dass die Nematoden unterschiedliche Ansprüche bezüglich der bevorzugten Nahrungsdichte aufweisen.

Eine verbesserte Methode zur Untersuchung von Parametern des Lebenszyklus von Nematoden wurde entwickelt und anschließend auf *Caenorhabditis elegans* angewendet; möglicherweise wird sie sich im Vergleich zu traditionellen Kultivierungstechniken als vorteilhaft erweisen.

Studien zum Nahrungsnetz zielten darauf ab, zu untersuchen, ob die Nematoden, die zweifellos einen erheblichen Anteil der chemoautotroph produzierten Biomasse konsumieren, auch höheren trophischen Ebenen als Nahrungsgrundlage dienen. Mit täglichen Konsumptionsraten, die das 2,5-fache seines eigenen Körpergewichtes übersteigen konnten, erwies sich der häufige Copepode *Eucyclops subterraneus scythicus* als gefräßiger Räuber von Nematoden − ein Ergebnis, dass auch von vorläufigen Untersuchungen zu Stabilen-Isotopen-Verhältnissen ($\delta^{15}N$) gestützt wird. Berechnungen der Biomassenproduktion durch Nematoden sowie der Fraßraten des Copepoden legen nahe, dass *Eucyclops* in Movile eine top-down Kontrolle auf die Nematodenpopulationen ausüben könnte. Weitere Räuber-Beute-Experimente mit dem an der Erdoberfläche weitverbreiteten Copepoden *Diacyclops bicuspidatus* deuten an, dass die beobachtete trophische Verbindung zwischen Nematoden und Copepoden auch in zahlreichen anderen aquatischen Habitaten von Bedeutung ist: Sie könnte eine wichtige benthisch-pelagische Kopplung zwischen mikrobiellem Kohlenstoff, endobenthischen Nematoden, epibenthischen Copepoden und pelagischen Fischen darstellen.

Erste Untersuchungen anhand von Amplified Ribosomal DNA Restriction Analysis (ARDRA) zeigten, dass die bakterielle Diversität der schwimmenden mikrobiellen Matten in Movile möglicherweise höher als erwartet ausfällt. Weiterreichende Untersuchungen zum Einfluss des Nematodenfraßes auf die mikrobiellen Matten würden vermutlich ein fruchtbares Forschungsgebiet darstellen.

Zusammenfassend lässt sich festhalten, dass unsere Untersuchungen eine erste Skizze der wichtigsten energetischen Wege im Nahrungsnetz von Movile entwerfen. Das entstehende Bild zeigt ein außergewöhnlich einfach aufgebautes Nahrungsnetz, in dem chemoautotrophe mikrobielle Matten die Nahrungsgrundlage bakterienfressender Nematoden sind, die wiederum stark von Copepoden bejagt werden.

7. Author's contributions

The present thesis comprises five publications with a varying set of authors. Four of the manuscripts have already been published in international, peer-reviewed scientific journals. All corresponding authors gave permission for using the publications in this thesis. Daniel Muschiol is the main author of each manuscript. He has done all steps from sample collection and preparation, setting up experiments, microscopic observation and documentation, digital drawing and figure preparation, data and literature collection, statistical analyses, to the writing of the manuscripts. The co-authors participated in conceptualisation, planning the investigations, data collection, and discussing the results. A detailed listing of the author's contributions is given below. Prof. Dr. Walter Traunspurger was supervisor of the project "Meiofauna in chemoautotrophen Bakterienmatten – Diversität, Ökologie und Dynamik in einem isolierten Höhlensystem" funded by the German Research Foundation (DFG).

Manuscript I. Muschiol, D., Giere, O. & Traunspurger, W. **Thiobiotic meiofauna in a chemosynthetic subterranean freshwater ecosystem (Movile Cave, Romania)**. *submitted*.
DM is the main author and drafted the manuscript. Assisted by M. Baciu and A. Hillebrand, Bucharest, Romania, he collected samples and set up the experiments. WT and W. Sudhaus, Berlin, conducted nematode species determination. OG and WT participated in the discussion of results and revised the manuscript.

Manuscript II. Muschiol, D. & Traunspurger, W., 2007: **Life cycle and calculation of the intrinsic rate of natural increase of two bacterivorous nematodes, *Panagrolaimus* sp. and *Poikilolaimus* sp. from chemoautotrophic Movile Cave, Romania**. – *Nematology* **9:** 271-284.
DM is the main author and drafted the manuscript. WT conceived of the study, participated in the discussion of results and revised the manuscript. M. Bökamp, Bielefeld, carried out large parts of the experimental work. Species determination was confirmed by W. Sudhaus. M. Baciu and A. Hillebrand helped to obtain the samples.

Manuscript III. Muschiol, D., Schroeder, F. & Traunspurger, W., 2009: **Life cycle and population growth rate of *Caenorhabditis elegans* studied by a new method**. – *BMC Ecology* **9:** 14.
DM and FS contributed equally to the conception, design and accomplishment of the study, DM conducted statistical analysis and drafted the manuscript. WT conceived of the study, and participated in its design and coordination and revised the manuscript.

Manuscript IV. Muschiol, D., Marković, M., Threis, I. & Traunspurger, W., 2008a: **Predator-prey relationship between the cyclopoid copepod *Diacyclops bicuspidatus* and a free-living bacterivorous nematode**. – *Nematology* **10:** 55-62.
DM coordinated experimental work, conducted raw data processing, figure preparation and drafted the manuscript. MM and IT carried out large parts of the experimental work. WT conceived of the study and revised the manuscript. Copepod species determination was confirmed by T. Glatzel, Oldenburg.

Manuscript V. Muschiol, D., Marković, M., Threis, I. & Traunspurger, W., 2008b: **Predatory copepods can control nematode populations: A functional-response experiment with *Eucyclops subterraneus* and bacterivorous nematodes**. – *Fundamental and Applied Limnology* **172:** 317-324.
DM coordinated experimental work, conducted raw data processing, figure preparation and drafted the manuscript. MM and IT carried out large parts of the experimental work. WT conceived of the study and revised the manuscript. Copepod species determination was confirmed by T. Glatzel.

8. Acknowledgements

Prof. Walter Traunspurger is acknowledged for his supervision of all the studies and investigations done for this thesis. Special thanks go to Prof. Olav Giere for introducing me to the fascinating world of Movile's groundwater meiofauna. I would also like to thank all members of the workgroup "Tierökologie" at Bielefeld University who provided an excellent and inspiring academic environment and became good friends over the years.

Many thanks go to my parents, my sister and brothers as well as Andrea Brinkmann and my daughter Jana who formed the non-scientific pole of my life during the last years and before.

9. Declaration / Erklärung

Hereby, I certify that the present thesis was prepared independently and without aid or sources other than those explicitly quoted.

Bielefeld, 27.05.2009

Daniel Muschiol

10. Curriculum Vitae

Name	Daniel Muschiol
Day of birth	16.10.1977
Place of birth	Paderborn, Germany
Nationality	German

1997	Abitur (overall grade 2.1) at Gymnasium Schloß Neuhaus
1997 – 1998	Civilian Service (Hermann-Schmidt-Schule für Geistig Behinderte, Paderborn)
1998 – 2004	Studies of biology at Bielefeld University Diploma in biology ('with distinction'; overall grade: 1.0) Subject of diploma thesis: *Meiobenthos of the caldera lakes of an active volcano of the Galápagos Islands*.
2004 – present	Doctoral studies at Bielefeld University

11. Appendix: Manuscripts I to V

In the following, the five manuscripts of this thesis are appended. The particular layout and pagination of the scientific journal in which the respective manuscript was published has been retained. A continuous pagination has additionally been added.

Manuscripts II and **IV** are reprinted from *Nematology* (http://www.brill.nl/nemy) with kind permission from Koninklijke Brill NV, Leiden, The Netherlands.

Manuscript III was published in the open access journal *BMC Ecology* (http://www.biomedcentral.com/bmcecol).

Manuscript V is reprinted from *Fundamental and Applied Limnology* (http://www.schweizerbart.de/j/archiv-hydrobiologie) with permission from E.Schweizer-bart'sche Verlagsbuchhandlung OHG (Naegele u. Obermiller). The publisher interdicts extraction and distribution of the article from this thesis.

11.1. Manuscript I

Muschiol, D., Giere, O. & Traunspurger, W.: **Thiobiotic meiofauna in a chemosynthetic subterranean freshwater ecosystem (Movile Cave, Romania)**. (*submitted*)

Thiobiotic meiofauna in a chemosynthetic subterranean freshwater ecosystem (Movile Cave, Romania)

DANIEL MUSCHIOL[1,2], **OLAV GIERE**[3], and **WALTER TRAUNSPURGER**[2]

Abstract

Discovered in 1986, Movile Cave is a peculiar groundwater ecosystem sustaining abundant and diverse invertebrate communities. Isolated from the surface since preglacial times, the cave lacks input of allochthonous photoautotrophically based food. Instead, metazoan life in Movile Cave entirely depends on in situ chemoautotrophic production. Chemoautotrophic microbial mats floating on a cave pond of thermomineral, highly sulphidic and methanic water are inhabited by dense populations of five bacterivorous nematode species and a predacious copepod species. We experimentally simulated the unique physicochemical conditions of Movile Cave in order to develop a model system allowing laboratory investigation of the mat community. By manipulating the atmosphere in enclosures set atop the thermomineral water, we created hypoxic conditions that permitted the development of floating microbial mats. Both in the laboratory and the cave itself, these cultivated mats were quickly colonized by Movile's highly adapted invertebrates. The nematode community structure was monitored for one year and strong fluctuations in the relative importance of individual species were observed. These fluctuations also characterized samples of native mat material and may reflect a succession of decreasing food conditions caused by the aging of the mats. However, previous reports on the occurrence of nematodes surviving and reproducing under complete anoxia could not be confirmed. Under truly anoxic conditions, neither mats nor nematodes developed. Successful simulation of the hypoxic cave habitat under laboratory conditions offers new possibilities to investigate the functioning of Movile's ecosystem. The possibility that Movile Cave is not as isolated from the surface as previously assumed is discussed regarding the first report of a presumably epigean gnat in the cave.

[1]Corresponding author (daniel.muschiol@uni-bielefeld.de)
[2]Universität Bielefeld, Tierökologie, Morgenbreede 45, D-33615 Bielefeld, Germany
[3]Biozentrum Grindel, Zoologisches Institut und Zoologisches Museum, Universität Hamburg, Martin-Luther-King-Platz 3, D-20146 Hamburg, Germany

Introduction

The question whether a "thiobios," a specialized meiofaunal community inhabiting the sulphidic, often anoxic zones of (marine) sands, exists and how it should be defined has been debated since its first description by Fenchel and Riedl (1970). Today, there is a consensus that many metazoans are able to survive for extended periods in sulphidic environments and, thus, are well-adapted to hypoxic conditions. Although the permanent existence of truly anaerobic metazoans under anoxic conditions has never been experimentally proven, there are reliable reports of nematodes and other meiofauna from sites with indubitably permanent anoxia (Giere 2009). These observations challenge our current physiological understanding. It is believed that all free-living and parasitic metazoans need oxygen at least for their reproductive stages (Barrett 1991).

In 1999, Riess et al. described a community of highly adapted freshwater nematodes occurring in floating microbial mats in the peculiar, thermomineral ecosystem of Movile Cave (Romania). The nematodes were reported to permanently live and reproduce under completely anoxic conditions. Moreover, cultivation experiments "showed that the nematodes could be kept alive and active for more than 1 yr without any supply of oxygen in a clearly sulphidic environment" (Riess et al. 1999, p. 161). Plexiglas enclosures set afloat on the surface of the cave lake allowed development of hypoxic and anoxic conditions under which the nematode populations could be studied for months. Considering the debate about animals under complete anoxia, it was a central aim of our present investigation to reassess those anoxic cultivation experiments. We established anoxic and hypoxic enclosures on top of anoxic thermomineral water both in the laboratory and in the cave lake in order to cultivate floating microbial mats. The development of microbial mats and the nematode populations inhabiting them were monitored for one year. Life-cycle (Muschiol and Traunspurger 2007) and predator-prey (Muschiol et al. 2008b) experiments were carried out and compared with observations under natural cave conditions.

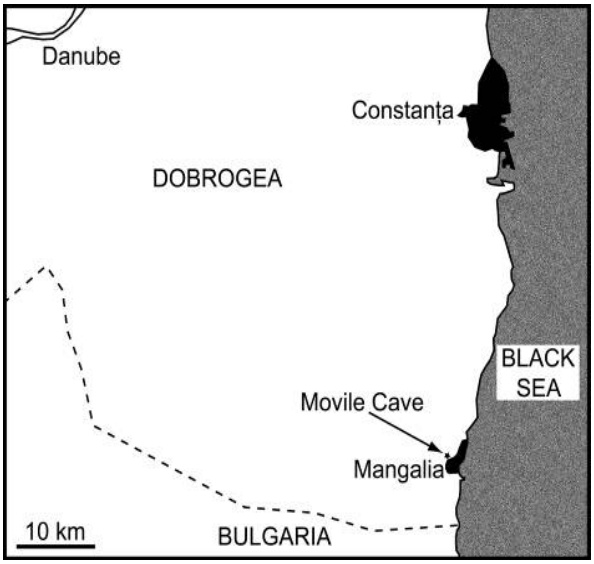

Fig. 1: Romania, Southern Dobrogea, and the location of Movile Cave.

A successful simulation of the cave habitat under controlled laboratory conditions should offer new possibilities to investigate the functioning of this isolated and stable chemosynthetic ecosystem. Key questions were: Which mechanisms prevent competitive exclusion among several coexisting bacterivorous nematode species (e.g., niche differentiation, *r*-selection vs. *K*-selection, interspecific interactions)? What is the advantage of developing only female nematode populations in Movile Cave (Poinar and Sarbu 1994; Riess et al. 1999)

corresponding to numerous other epigean freshwater nematode species (e.g., Michiels and Traunspurger 2005)?

Material and Methods

Habitat—Movile Cave (43°49.611' N, 28°33.684' E) is located in Southern Dobrogea, Romania, close to the coastline of the Black Sea and to the town of Mangalia (Fig. 1). Subterranean karstic voids host a wide thermomineral sulphidic aquifer from which waters ascend to the surface along natural faults and form numerous artesian springs. Movile Cave consists of a 240-m long network of natural passages located 20 m below the surface. Apart from a sealed artificial entrance, Movile Cave seems completely isolated from the surface since preglacial times (Sarbu et al. 1996). A small lake in its deep section permits access to the lower level of the cave, which is partially flooded by thermomineral waters and contains several air bells that can be reached only by divers (Fig. 2).

In contrast to the almost normoxic atmosphere in the upper cave passages, these air bells are poor in oxygen and rich in carbon dioxide and methane (atmospheric O_2: 7–10%, CO_2: 2–3.5%, CH_4: 1–2%) (Sarbu and Kane 1995; Sarbu et al. 1996). This significantly modified atmospheric composition of the cave's air bells and the specific physicochemical conditions at the interface with the thermomineral sulphidic water allow the development of floating microbial mats while in the oxygen-rich atmosphere of the Lake Room the development of such mats on the water surface is hampered. The brownish mats can attain a thickness of 2–3 mm and are kept afloat by gas bubbles containing high CH_4 concentrations (up to 13.5%) (Sarbu and Kane 1995). The completely anoxic hydrothermal water (21°C) contains high amounts of H_2S, CH_4, and NH_4^+ (Sarbu and Kane 1995), potential energy sources for microaerophilic bacteria at the oxic/anoxic interface. Methanotrophic and sulphur-oxidizing bacteria have been identified as primary producers in the mats (Rohwerder et al. 2003; Hutchens et al. 2004). Allochthonous photoautotrophically based food seems to be absent in

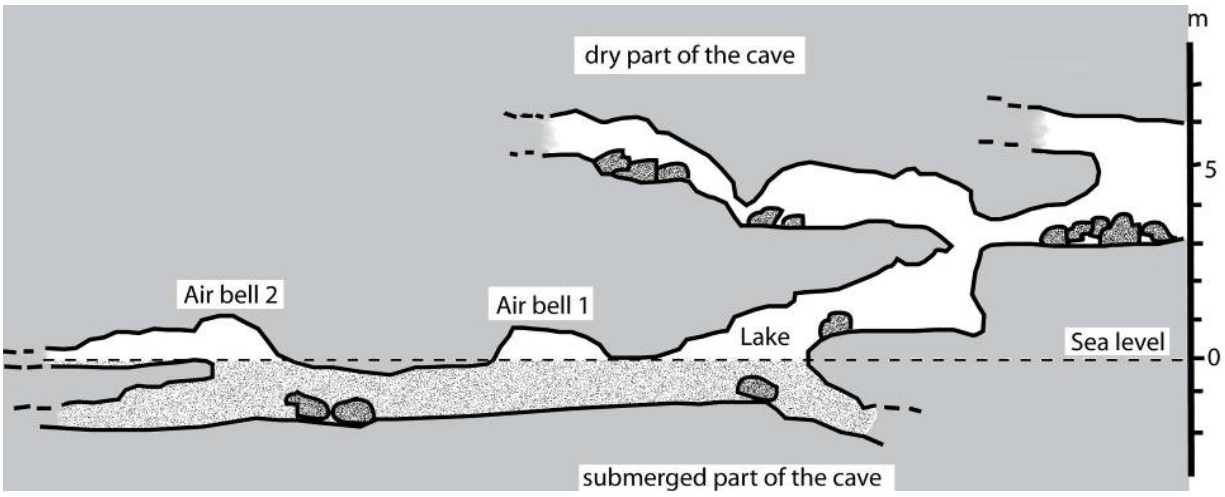

Fig. 2: Cross-sectional map of Movile Cave, Romania. Redrawn from Sarbu et al. (1994). Note that fluctuating water levels can repeatedly establish a connection between the Lake Room and Air Bell #1.

Fig. 3: Cultivation of artificial mats. (A) Experimental tubes in the field laboratory in Mangalia, Romania. (B) Floating rafts containing experimental tubes in the Lake Room of Movile Cave. (C) Sieve used for harvesting cultivated mats. The sieve was used to shut the open lower end of the tubes before they were removed from the frames.

the cave (Sarbu and Kane 1995). Thus, similar to deep-sea hydrothermal vent communities, the cave's ecosystem is supported solely by in situ chemoautotrophic production.

Sampling meiofauna—Samples from four different sources were investigated.

(1) Samples from native microbial mats from the water surface of Air Bell #2 (Fig. 2) taken by scuba diving in May and October 2004 and in January 2005.

(2) Samples from bottom sediment of the cave's lake and Air Bell #1 (n=3) and Air Bell #2 (n=1) (Fig. 2) in May 2004 (~ 1.5 m water depth).

(3) Samples from cultivated microbial mats grown in Movile Cave.

(4) Samples from cultivated microbial mats grown in the laboratory.

Cultivating microbial mats—An artificial artesian well in the field laboratory of the GESS group in Mangalia, Romania (43°49.005' N, 28°35.276' E) taps the same aquifer that supplies Movile Cave allowing for permanent laboratory access to sulphidic thermomineral water. For cultivation experiments, a flow-through basin (452 × 95 cm; ~ 0.6 m^3 h^{-1}) in the field laboratory was filled by this hydrothermal water (total volume: 3.9 m^3, Fig. 3A). Cultivation of microbial mats in experimental tubes on the water surface of both the basin and the native cave lake should be the basis for comparisons of meiofauna development under varying conditions. The delicate texture of the mats allowed for bulk fixation only of the entire mat in each tube. The cultivation tubes (Fig. 4) consisted of a polypropylene tube (length 220 mm; inner diameter 105 mm, cross-sectional area 86.6 cm^2) placed on the water surface so that the tube's upper 120 mm extended above the water surface. The lower end was open to the water underneath. The tubes rested on floating frames (Fig. 3B) to compensate possible fluctuations of the water level. Half of the tubes were closed by a tight lid for development of an anoxic inner atmosphere, whereas in the other half the lid was perforated resulting in hypoxic conditions. As an outlet of emanating methane, a small hose was mounted just below the water surface at each tube (Fig. 4). Each cultivation tube was inoculated with a piece of natural mat (~ 50 mm^2) collected from Air Bell #2. It was positioned on a disc of

polyurethane foam that kept the mat flush to the water surface. 48 of the tubes were installed in the experimental basin in the field-laboratory and 32 in the Lake Room of the cave. At each sampling campaign, four anoxic and four hypoxic tubes were removed for quantitative retrieval of the contents (Fig. 3C), which were washed into a polyethylene bottle and fixed with formaldehyde. In the experimental basin, sampling was carried out after 1, 2, 3, 6, 9, and 12 months, and in the cave after 3, 6, 9, and 12 months.

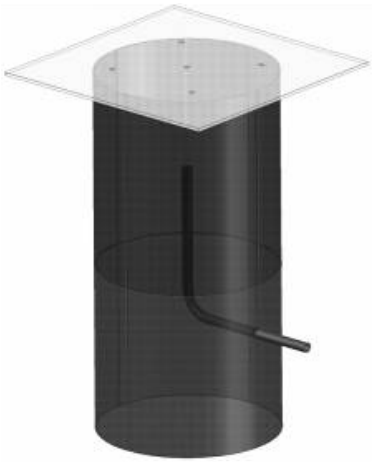

Fig. 4: Tube used for the cultivation of microbial mats in the laboratory and in the Lake Room of Movile Cave. The length of the polypropylene tube is 220 mm, its inner diameter 105 mm. The quadratic lid on top consisted of either glass (anoxic atmosphere inside the tube) or perforated Plexiglas. In anoxic tubes, a polyvinyl chloride hose allowed the bubbling out of surplus methane out-gassing from the hydrothermal water into the tube (see text).

Processing samples—Samples were immediately fixed with formaldehyde solution (4% final concentration), stained with Rose Bengal (300 μg ml^{-1}) prior to extraction of fauna and scanned for meiofauna organisms under a dissection microscope (35-fold magnification). From each sample of native mat material 250 nematodes were hand-picked under the dissection microscope, while from the cultivated mats the first 100 nematodes, if possible, were selected and slide-mounted in glycerol (Seinhorst 1962). This way, in total 806 nematodes from natural mats and 4451 nematodes from cultivated mats were prepared on permanent slides, identified to species level (Zeiss Axioplan 2) and deposited in the nematode collection of W.T. To study the effect of food depletion on the nematode community, we studied sub-samples of unfixed natural mats (room temperature, dark and oxic conditions, evaporation water replaced). After 8 months, the viable nematodes were identified after embedding in glycerol (n=57). For extraction of the fauna from the sediment samples, the formalin-fixed substrate was suspended with Ludox® TM-50 colloidal silica (1.14 g ml^{-1}; mesh size 20 μm: Pfannkuche and Thiel 1988, modified) and centrifuged. Other meiofaunal taxa were identified to the lowest possible taxonomic level.

Water and atmosphere analyses—The water chemistry within the thermomineral Movile Cave was compared to that of the water in the experimental basin (UCL Umwelt Control Labor GmbH, Lünen, Germany). Additionally, a portable analyser WTW 340i (Wissenschaftlich-Technische Werkstätten GmbH & Co. KG, Weilheim, Germany) was used at each sampling to monitor pH, conductivity, oxygen content, and temperature of the water. The concentration of hydrogen sulphide in the water from the cave and from the experimental basin at a depth of approximately 10 cm below the water surface was measured photometrically after immediate fixation with alkaline zinc-acetate solution (Gilboa-Garber 1971, modified). The atmospheric composition in the cave and in the laboratory was analysed with a gas detector (Polytector II G750, Gesellschaft für Gerätebau, Dortmund, Germany).

Results

Water and atmosphere analyses—The sulphide content of the water from the artesian well supplying the laboratory (287 μmol L^{-1}; n=1) was similar to that measured in the water of the cave lake (278 ± 2 μmol L^{-1}; n=3) and Air Bell #2 (299 ± 5 μmol L^{-1}; n=3). Data obtained from our analyses of 34 water-chemistry parameters, both from the artificial well in the laboratory and the cave lake, are given in Table 2. Many values of the laboratory water were considerably higher compared to those of the cave lake (e.g., electrical conductivity: 1927 vs. 1420 μS cm^{-1}; chloride: 440 vs. 250 mg L^{-1}; temperature: 23.9 vs. 21.1° C; Table 3). Only sulphate and temporary hardness were lower than in the cave lake, the remaining 19 values were equal or below the detection limit. The pH values were neutral (around 7.4) in both sites. Atmospheric oxygen content in the laboratory was equal to that of normal air (20.9%), while in the Lake Room the oxygen level was only 17.6%. The carbon dioxide content in the Lake Room was elevated (1.78%) in comparison to the laboratory room (0.09%) and normal air (0.04%). Free hydrogen sulphide in the atmosphere was detected only in the laboratory (1.5 ppm), while free methane was measured neither in the (aerated) laboratory nor in the Lake Room. These measurements are consistent with the findings of earlier studies (e.g., Marin and Nicolescu 1993).

Native microbial mats—Because of the extreme fragility of bacterial mats, quantitative sampling of this substrate proved to be impossible. Hence, we only made one approach to quantify the entire metazoan community structure of the natural mats in Movile Cave. Also this sample (15 January 2005) may possibly be inaccurate. It consisted mainly of nematodes (94.7% of total metazoans), followed by specimens of the cyclopoid copepod *Eucyclops subterraneus scythicus* (3.2%), its nauplii (0.3%) and ostracods (1.1%). Harpacticoid copepods (*Parapseudoleptomesochra italica*), gammarids, isopods, acarids, rotifers, and gastropods were found in densities below 1%. In unfixed samples, high densities of flagellates and−to a lesser extent− ciliates were observed, but no attempt was undertaken to quantify these protists. A sample, collected on 7 May 2004, contained numerous decaying imagines of a species of dark-winged fungus gnat (Sciaridae) but no larvae or pupae, none occurred in the

Table 1: Total nematode abundances 10 cm^{-2} (± S.D.) in hypoxic vs. anoxic cultivation tubes in the laboratory and in the Lake Room.

	Laboratory		Lake Room	
Month[a]	Hypoxic	Anoxic	Hypoxic	Anoxic
1	352.6 (± 57.6)	1.4 (± 2.2)		
2	1103.9 (± 508.1)	21.7 (± 14.2)		
3	1931.9 (± 827.5)	5.3 (± 5.9)	280.8 (± 321.1)	0.9 (± 1.1)
6	801.8 (± 465.5)	6.2 (± 5.9)	547.0 (± 530.7)	1.4 (± 2.0)
9	717.4 (± 586.1)	4.0 (± 3.5)	262.8 (± 312.5)	0.1 (± 0.2)
12	517.1 (± 371.3)	0.3 (± 0.6)	1308.1 (± 946.8)	0.9 (± 1.0)

[a]Time in months since the initial incubation of polypropylene tubes with original mat material collected in Air Bell #2. At each sampling, four tubes were harvested.

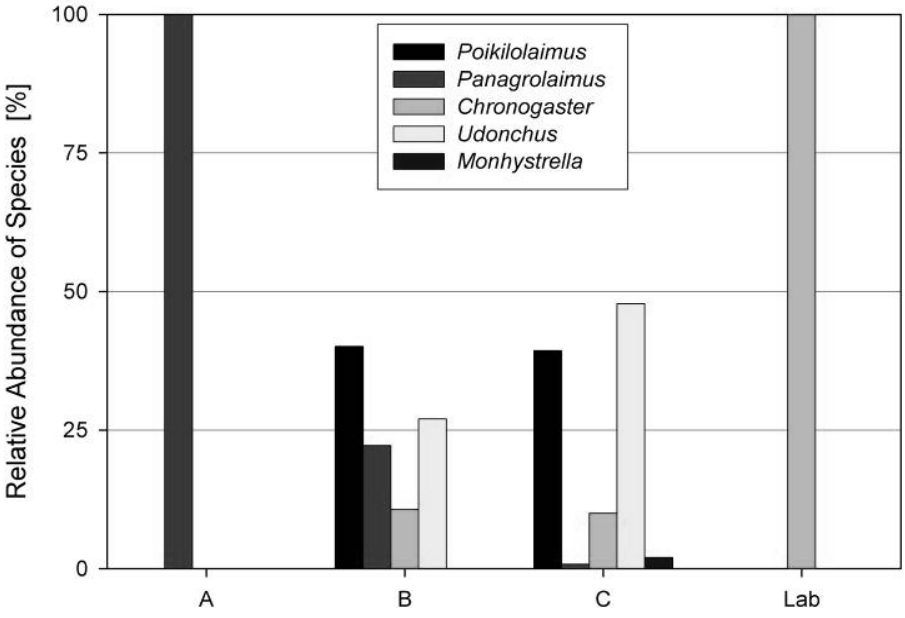

Fig. 5: Relative composition of the nematode community in natural microbial mats from Air Bell #2 in Movile Cave, Romania. Samples A (May 2004; *n*=248), B (October 2004; *n*=252), and C (January 2005; *n*=249) were fixed on the day of sampling. Sample Lab (*n*=57) is a sub-sample of the January 2005 sample that was kept in the laboratory under oxic conditions at room temperature for 8 months prior to fixation.

two subsequent mat samples. This seems to be the first report of sciarids in Movile Cave. In contrast to earlier investigations, none of our samples in 2004 and 2005 revealed oligochaetes or collembolans previously reported to live in high densities in or on the mats (Popa and Sarbu 1991; Sarbu and Popa 1992).

Nematodes were always dominant in native microbial mats. They consisted of five species, *Chronogaster troglodytes* Poinar & Sarbu, 1994, *Monhystrella* sp., *Panagrolaimus* c.f. *thienemanni* Hirschmann, 1952, *Poikilolaimus* sp., and *Udonchus tenuicaudatus* Cobb, 1913. The relative composition of the nematode community inhabiting the mats was highly variable between the sampling dates (Fig. 5). For example, the 248 nematodes identified from the May 2004 sample entirely consisted of *Panagrolaimus* sp., whereas in January 2005 this species comprised not more than 2 out of 249 nematodes (0.8%).

Under laboratory conditions (room temperature, oxic), *Chronogaster troglodytes*, which represented eight months before only 10% of total nematodes, had increased to 100%; all other species were absent from the sample (Fig. 5: sample "Lab"). In our home laboratory, we made extensive attempts to establish laboratory cultures of the five nematode species from Movile Cave. While we succeeded in obtaining cultures of *Panagrolaimus* and *Poikilolaimus* (Muschiol and Traunspurger 2007), cultivation of the three other species proved to be impossible. While *Chronogaster* thrived well in the sample with native bacterial mats for more than one year, the specimens died within hours after transfer to any of the artificial media offered.

In a strongly sulphidic (smell!) sample of native bacterial mats, which had been sealed for three days, nematodes were found completely paralysed. After approximately 1 hour under oxic conditions, a fraction of them recovered again, while a considerable proportion had died (see discussion).

Table 2: Analyses of water samples collected from the cave lake and the artificial well of the field laboratory in Mangalia. n.d.=non-detectable.

Parameter	Laboratory	Cave lake	Detection limit
Chloride (mg L^{-1})[a]	440	250	1
Nitrate (mg L^{-1})[a]	n.d.	n.d.	1
Nitrite (mg L^{-1})[b]	n.d.	n.d.	0.03
Phosphor, total (mg L^{-1})[c]	n.d.	n.d.	0.01
Sulphate (mg L^{-1})[a]	11	18	1
Sulphide (mg L^{-1})[d]	0.94	n.d.	0.1
Ammonium (NH_4, mg L^{-1})[e]	2.8	1.2	0.04
Aluminium (mg L^{-1})[f]	n.d.	n.d.	0.1
Antimony (mg L^{-1})[f]	n.d.	n.d.	0.01
Arsenic (mg L^{-1})[f]	n.d.	n.d.	0.01
Boron (mg L^{-1})[f]	2.1	1.1	0.01
Cadmium (mg L^{-1})[f]	n.d.	n.d.	0.001
Calcium (mg L^{-1})[f]	41	41	1
Cobalt (mg L^{-1})[f]	n.d.	n.d.	0.01
Copper (mg L^{-1})[f]	n.d.	n.d.	0.01
Iron (mg L^{-1})[f]	0.05	n.d.	0.03
Iron 2^+ (mg L^{-1})[g]	0.05	n.d.	0.03
Iron 3^+ (mg L^{-1})[f]	0.05	n.d.	0.03
Lead (mg L^{-1})[f]	n.d.	n.d.	0.01
Magnesium (mg L^{-1})[f]	24	23	1
Manganese (mg L^{-1})[f]	n.d.	0.03	0.01
Molybdenum (mg L^{-1})[f]	n.d.	n.d.	0.01
Nickel (mg L^{-1})[f]	n.d.	n.d.	0.01
Potassium (mg L^{-1})[f]	14	9.0	1
Selenium (mg L^{-1})[h]	n.d.	n.d.	0.01
Silicate (mg L^{-1})[f]	16	15	1
Silicon(mg L^{-1})[f]	8.0	7.0	1
Sodium (mg L^{-1})[f]	290	200	1
Vanadium (mg L^{-1})[f]	n.d.	n.d.	0.01
Zinc (mg L^{-1})[f]	n.d.	n.d.	0.01
Total hardness (mmol L^{-1})[i]	2.0	2.0	0.1
Temporary hardness (mmol L^{-1})[j]	2.8	3.2	0.1
TOC, I (mg L^{-1})[k]	20	20	1
DOC (mg L^{-1})[k]	16	9.5	1

Analyses were conducted on 3 August 2005 by UCL Umwelt Control Labor GmbH, Lünen, Germany. [a]DIN EN ISO 10304 (1/2) [b]DIN EN 26777 [c]DIN EN ISO 6878-7 D11 [d]DIN 38405 D27 [e]DIN EN ISO 11732 [f]DIN EN ISO 11885 [g]DIN 38406 E1 [h]UCL-SOP 92 [i]DIN 38409 H6 [j]DIN 38409 H7 [k]Total/Dissolved Organic Carbon: DIN EN 1484

Experimental tubes under hypoxia—Both in the laboratory (Fig. 3A) and in the Lake Room of Movile Cave (Fig. 3B), microbial mats developed within weeks after the start of the experiment. The initially fluffy whitish mats became increasingly yellowish and compacted towards the end of the 1-year period. Macroscopically, these mats strongly resembled naturally grown mats from Movile Cave.

The cultivated mats turned out to be a favourable habitat for nematodes. Their populations grew quickly reaching maximum densities of 1932 nematodes 10 cm^{-2} in the laboratory and 1308 nematodes 10 cm^{-2} in the Lake Room (Fig. 6; Table 1). In the laboratory populations established faster than in the Lake Room, and after 3 months total nematode abundance in the laboratory was almost 7-fold higher than in the Lake Room (1932 vs. 281 individuals 10 cm^{-2}, Table 1). After 12 months, this proportion was reversed such that nematode abundance in the Lake Room was 2.5-fold higher than in the laboratory.

Cultivated mats in the laboratory and in the Lake Room had the same nematode species inventory as the natural mats in Movile Cave (Fig. 6). However, both total nematode abundance and species composition varied strongly between replicates, as reflected in the large standard error bars in Fig. 6. Nonetheless, a general pattern of successive population maxima became apparent in the cultivated mats: *Panagrolaimus* was abundant only at the very beginning of the 1-year period, constituting after 2 and 3 months 21.3% of total nematodes in the laboratory and 11.9% of those in the Lake Room, respectively. None of the 28 cultivation tubes subsequently sampled after this initial phase showed *Panagrolaimus* in densities higher than 1.5% (Fig. 6). In contrast, *Chronogaster* and *Udonchus* became abundant in the second half of the 1-year period. By then, at least in the laboratory, they developed considerable densities reaching peak values of 39.9% (*Udonchus*, 12 months) and 44.3% (*Chronogaster*, 9 months) of total nematodes. In the Lake Room, the density of *Udonchus* reached 28.9% after 9 months, while *Chronogaster* stayed less abundant (maximum 5.1% after 12 months). *Monhystrella* was detected only once: After 9 months, we found in a single replicate from the Lake Room four non-gravid females, corresponding to 3.8% of nematode species composition. For *Poikilolaimus*, there was no obvious trend, since the species was quite abundant in the majority of samples. Both in the laboratory and in the Lake Room, highest relative abundances occurred after 3 months. At that point, the nematode community of the cultivated microbial mats was essentially a monoculture of *Poikilolaimus* (96.4 and 87.1% of total nematodes, respectively). Particularly in this species, the large variation among replicates is conspicuous: after 12 months, the nematode populations in the four hypoxic replicates from the Lake Room comprised 37.4, 97.0, 98.9, and 100.0% of *Poikilolaimus*, corresponding to 1155, 152, 2448, and 1478 individuals 10 cm^{-2}, respectively. The variation between replicates was, thus, quite high, both on the level of total (152 vs. 3× >1155 ind. 10 cm^{-2}) and relative (37.4 vs. 3× >97.0% of total nematodes) abundances.

In one replicate of the hypoxic cultivation tubes, we discovered two non-gravid females of the nematode species *Diploscapter coronatus* (Cobb, 1893) Cobb, 1913. We interpret the appearance of this terricolous species as the result of contamination (cultivation tubes or sampling equipment, see discussion below).

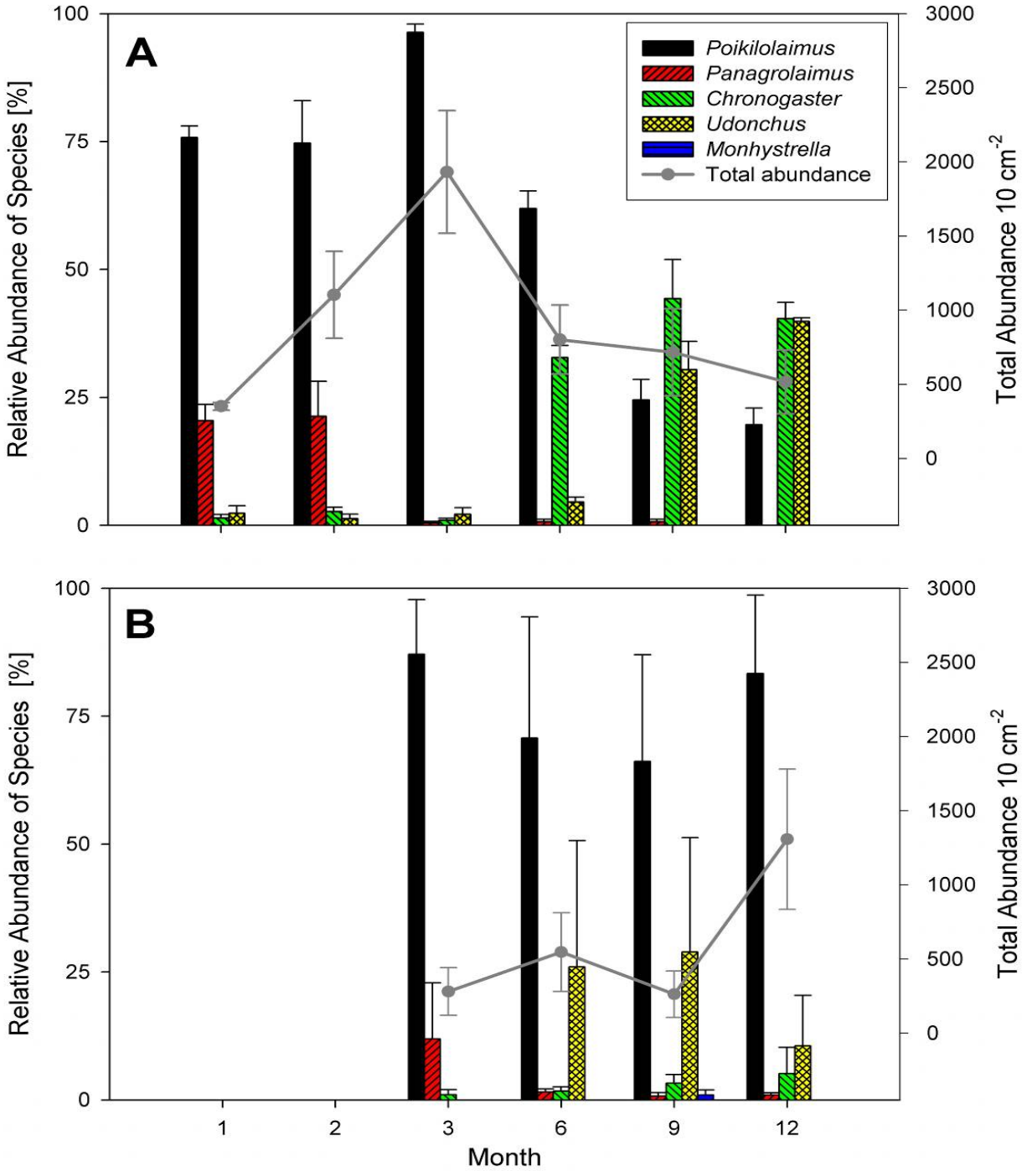

Fig. 6: Relative composition (bar charts) and total abundance (line plots) of the nematode communities in hypoxically cultivated microbial mats in the laboratory (A) and in Movile's Lake Room (B). Numbers on the *x*-axis indicate time in months since the initial incubation of polypropylene tubes with original mat collected in Air Bell #2. At each sampling, four tubes were harvested. S.E. bars. A 6th species, *Diploscapter coronatus*, was represented by only two individuals and was excluded from the graph because its occurrence was probably the result of contamination (see text).

Table 3: Atmospheric composition and physicochemical para-meters of the atmosphere and water in the laboratory and the Lake Room of Movile Cave.

	Parameter	Laboratory	Lake Room
Atmosphere	CO [ppm]	0.0	0.0
	H_2S [ppm]	1.5	0.0
	CO_2 [%]	0.09	1.78
	O_2 [%]	20.9	17.6
	CH_4 [%]	0.0	0.0
Water	T [°C]	23.9 [±0.4]	21.1 [±0.1]
	pH	7.42 [±0.09]	7.33 [±0.06]
	μS cm^{-1}	1927 [± 32]	1420 [±10]

Atmosphere was analysed on 27 July 2005 with a Polytector II G750 device (Gesellschaft für Gerätebau). Water parameters represent the average [± S.D.] of 3–11 individual measurements conducted in 2004 and 2005.

Among the thousands of nematode specimens in native and cultivated microbial mats we never observed a male specimen. Apparently, under the conditions of Movile Cave none of the five nematode species develops males.

Experimental tubes under anoxia—Microbial mats were never found developed within the anoxic cultivation tubes, neither in the laboratory nor in the Lake Room. Only a delicate iridescent film was noticed on the water surface and, in the experimental tubes in the laboratory, a greenish-blackish slime on the submersed tube walls. Except for some decaying remains, viable nematodes were not found in the anoxic cultivation tubes (Table 1), their thriving is apparently coupled to an appropriate substratum (sediments, bacterial mats). Also in the anoxic sediment samples from the Lake Room, from Air Bells #1 and #2, only decomposing nematodes, exoskeletons of acarids and ostracods, and undefined limbs of larger arthropods were found.

Discussion

Unlike the vast majority of freshwater cave ecosystems, Movile Cave harbours rich microbial mats with an intense chemosynthetic primary production. They support, in turn, fairly diverse aquatic and terrestrial animal communities. Thus far, 48 species of cave-adapted terrestrial and aquatic invertebrates have been described from Movile Cave, of which 33 are endemic. Associated with the chemoautotrophic mats are at least 20 aquatic invertebrates, including Amphipoda, Annelida, Copepoda, Gastropoda, Heteroptera, Isopoda, Nematoda, Ostracoda, Rotifera, and Turbellaria (Sarbu and Kane 1995; Riess et al. 1999). Five nematode species belonging to the genera *Chronogaster*, *Monhystrella*, *Panagrolaimus*, *Poikilolaimus*, and *Udonchus* (Riess et al. 1999; Muschiol and Traunspurger 2007) have been reported so far. At least one of them, *Chronogaster troglodytes* Poinar & Sarbu, 1994, seems to be endemic to

Movile Cave (Poinar and Sarbu 1994). With peak densities of 1932 ± 828 nematodes 10 cm^{-2} (Table 1) our studies confirmed the enormous importance of nematodes in the ecosystem of Movile Cave. Here, they are roughly as abundant as nematodes in other benthic freshwater habitats (Traunspurger et al. 2006; Muschiol and Traunspurger 2008). Riess et al. (1999) reported an even higher maximum density of 9800 nematodes 10 cm^{-2} in cultivated microbial mats from Movile Cave. The exclusively bacterivorous nematode species in Movile Cave (Traunspurger 1997) doubtlessly consume a large proportion of the mat's microbial primary production. Life-cycle and functional-response experiments (Muschiol and Traunspurger 2007; Muschiol et al. 2008a; Muschiol et al. 2008b) indicated that a significant amount of this chemoautotrophically fixed microbial carbon is transferred to higher trophic levels via predatory copepods that feed on Movile's nematodes. Hence, nematodes not only dominate the metazoan fauna of the mats, they play a major role in the cave's food web as consumers of chemoautotrophic bacteria and as a food base for predators (Muschiol et al. 2008b).

Previous studies in Movile Cave were hampered by the difficult access to this isolated subterranean habitat and by the fragility of its ecosystem which restricts sampling and prohibits major manipulations. For technical and conservation reasons chances of quantitative in-situ sampling are negligible and population densities are rough estimates only. With the experimental set-up described here, microbial mats can now be cultivated under controlled laboratory conditions. This offers new possibilities to investigate the functioning of Movile Cave's ecosystem, since, by and large, the physicochemical properties of the tapped water in the laboratory correspond to conditions in the cave (Table 2). The cave water appears to be a diluted version of the tapped aquifer. Especially the sensible sulphide and methane balance seems comparable. Both types of water allowed the development of floating microbial mats that were, at least macroscopically, identical to the native mats from Air Bell #2 and served as suitable habitats for a highly adapted and often endemic stygofauna. Thus, the laboratory mats can provide a useful model for the investigation of the cave's floating mats. The application of molecular methods in future studies may scrutinize the microbiological identity of the mats.

Are there anoxic nematodes in Movile Cave?—The study of Riess et al. (1999) suggested that the nematodes of Movile Cave are able to survive, grow, and reproduce under entirely anoxic conditions. The unique thermomineral ecosystem of Movile Cave with apparently stable conditions during several million years (Lascu 1989) may favour long-term adaption of metazoans to anoxia. Nonetheless, the results of our study clearly indicate that Movile's metazoan fauna not only depend on atmospheric oxygen for trophic reasons (growth of bacterial mats only at the oxic/anoxic interface), but also fail to survive under anoxia. Riess et al. (1999) may have misinterpreted an initial growth of a mat after an accidental entrapment of air or oxic water when they started their "cage experiments". They may also have underestimated the oxygen permeability of the materials which they had used for their cultivation experiments. Anyway, they reported (Riess et al. 1999, p. 161) "that the nematodes could be kept alive and active for more than 1 yr without any supply of oxygen in

a clearly sulphidic environment." However, for nematodes, there are several important metabolic pathways that are obligatory aerobic (e.g., collagen synthesis: Powell et al. 1980). Obviously, the nematodes from Movile Cave are able to tolerate severe hypoxia, but, according to our experiments, complete anoxia is fatal. In addition, if there were oxygen-independent metazoans in Movile Cave, they should also have colonized the nutrient-rich bottom sediments beneath the anoxic water column. Summarizing, the reasons for the conflicting evidence between our results and those of Riess et al. (1999) must remain unclear.

Absence of males among the nematodes of Movile Cave—In contrast to marine environments, where sexual reproduction predominates, unisexual populations are more common in freshwater systems. Many freshwater nematodes, but also tardigrades, ostracods, and rotifers, are known to reproduce unisexually (Michiels and Traunspurger 2005, and references therein). This trend is especially pronounced in populations of bacterivorous nematodes. Here, females dominate most natural populations and a balanced sex-ratio is the exception rather than the rule (Traunspurger 1991). For example, in the genus *Chronogaster*, males have been described for only five of the ~ 30 known species (Poinar and Sarbu 1994). In Movile Cave we never observed the occurrence of males, neither in natural populations nor in laboratory cultures. It remains unclear whether the ancestors of the Movile nematodes disposed of their males after colonization of the cave or, alternatively, whether already their surface ancestors were unisexual. In Movile Cave the ecological or mutation-clearing advantages of sex (Jokela et al. 1997) that would maintain sexual reproduction in other habitats are obviously missing. Yet, the factors shaping nematode sex-ratios are still poorly understood (Triantaphyllou 1973; Huettel 2004) and remain an interesting field for further investigations.

Movile Cave - a completely isolated subterranean ecosystem?—The chemosynthetic ecosystem of Movile Cave with its isolated fauna containing at least 33 endemic species (Sarbu et al. 1996) ranks among the most fascinating freshwater habitats. Is this isolation based on rigid physical barriers or rather on ecological conditions which may allow for short-lived access, but prevent longer-term establishment? Oxygen from the epigean atmosphere is certainly penetrating through the permeable carbonate layers with its numerous cracks and voids. We witnessed a temporary colonization of Air Bell #2 by a surface-dwelling sciarid fly species, but could not find any developmental stages. Either the limited resources of the cave or competition/predation by better adapted indigenous species led to an early extinction of the invaders. During prehistoric times a repeated contact of Movile Cave with the surface has been documented (Stiuca and Ilinca 1995). Those species which managed to establish permanent populations in Movile Cave became well adapted to their subsurface environment during their long evolutionary history. By their ecological and evolutionary fitness they have possibly outcompeted several invasions from surface organisms. This scenario would also explain the ephemeral appearance of two non-gravid females of the nematode species *Diploscapter coronatus* in our experimental tubes, where they were apparently not able to establish stable populations.

Fluctuations in nematode species composition: A succession cycle?—The same nematode community which we encountered, has been reported in earlier studies since at least 20 years (Poinar and Sarbu 1994; Riess et al. 1999) (with the previously listed *Protorhabditis* sp. now correctly identified as *Poikilolaimus* sp., Prof. W. Sudhaus, Berlin). Which factors have permitted the long-term coexistence of these nematodes in the mats? Movile Cave's microbial mats are unaffected by seasonal or diurnal variations in temperature, illumination, or humidity and receive a continuous supply of methane and sulphide through the cave's well-buffered and constantly tempered hydrothermal water. This seemingly stable equilibrium situation without trophic or spatial separation does not accord with the principle of competitive exclusion (Hardin 1960). Predation has been proposed as a factor permitting coexistence among competitors (e.g., Shurin and Allen 2001). Among the potential predators in Movile Cave, the copepod *Eucyclops subterraneus scythicus* is abundant enough to significantly influence the vast number of nematodes within the mats. However, the copepod is a non-selective predator feeding on each of the nematode species, regardless of size, or body shape.

Is the micro-ecological situation, particularly the trophic supply of the cave nematodes really stable and unchanging? Ascending gas bubbles keep Movile's microbial mats afloat on the water surface as long as the mat texture is light and fluffy. By aging, the mats become compacted and heavier, and after some time (months to years) they exceed the critical point of stability and finally sink with all their inhabitants into the hostile anoxic depth just to allow the start of a new growth cycle of mats at the surface. This periodicity would explain observations that young, 1st stage mats can cover natural surfaces which had not been disturbed for years. These young mats provide plenty of microbial food to nematodes, thereby favouring fast-growing (Muschiol and Traunspurger 2007) *r*-selected species (*Panagrolaimus*, *Poikilolaimus*, see Fig. 6). Nematode populations develop until microbial food becomes a limiting factor. Simultaneously, the accumulation of dead, less-degradable organic material increasingly acts as a diffusion barrier for oxygen and sulphide, so that microbial production declines. The growing intra- and interspecific competition favours more *K*-selected species (*Chronogaster*, *Udonchus*, see Fig. 6), which gradually displace the *r*-selected species. Hence, a stable equilibrium, i.e., competitive exclusion, is not obtained. This cyclicity of mat development would explain both phenomena, the extreme variability in nematode community structure (Fig. 5) in the native mats of Air Bell #2 and the succession in community structure in the cultivation experiments (Fig. 6).

Conclusions

Among the diverse and abundant invertebrate populations inhabiting the groundwater ecosystem of Movile Cave, nematodes are the most numerous. Associated with floating chemoautotrophic microbial mats, five bacterivorous nematode species outnumber all other metazoan groups in abundance and total biomass. The relative composition of the nematode community inhabiting the mats is highly variable over time. By manipulating the atmosphere in enclosures set atop the hydrothermal water, we were able to simulate the unique physicochemical conditions of the cave. A model system was established that allows laboratory investigation of the mat community. The observed strong fluctuations in nematode community structure may reflect a succession of decreasing food conditions in the aging mats. An equilibrium situation leading to competitive exclusion of some species is probably not achieved; a cycle of microbial mat development induces successive development of nematode populations. Previous reports on the occurrence of nematodes surviving and reproducing under complete anoxia could not be confirmed. Interpretations of the female-bias in the nematode populations of the cave require further experiments. As evidenced by findings of a presumably epigean gnat, Movile Cave may not be as isolated from the surface as previously assumed. The described experimental set-up offers a wealth of new possibilities to investigate the functioning of Movile's ecosystem.

Acknowledgements

This investigation was financially supported by the German Research Foundation (TR 445/3-1). Mihai Baciu and Alexandra Hillebrand from the Group for Underwater and Speleological Exploration (GESS), Bucharest, Romania, assisted in the set-up of the experiment, obtaining the samples and providing laboratory space. Walter Sudhaus, Berlin, Germany, confirmed nematode species determination.

References

Barrett, J. 1991. Parasitic helminths, p. 146-164. *In* C. Bryant [ed.], Metazoan life without oxygen. Chapman and Hall.

Fenchel, T., and R. J. Riedl. 1970. The sulfide system: A new biotic community underneath the oxidized layer of marine sands bottoms. Mar. Biol. **7:** 255-268.

Giere, O. 2009. Meiobenthology - The microscopic motile fauna of aquatic sediments. Springer.

Gilboa-Garber, N. 1971. Direct spectrophotometric determination of inorganic sulfide in biological materials and in other complex mixtures. Anal. Biochem. **43:** 129-133.

Hardin, G. 1960. The competitive exclusion principle. Science **131 :** 1292-1297.

Huettel, R. N. 2004. Reproductive behaviour, p. 127-150. *In* R. Gaugler [ed.], Nematode behaviour. CABI Publishing.

Hutchens, E., S. Radajewski, M. G. Dumont, I. R. McDonald, and J. C. Murrell. 2004. Analysis of methanotrophic bacteria in Movile Cave by stable isotope probing. Environ. Microbiol. **6:** 111-120.

Jokela, J., C. M. Lively, M. F. Dybdahl, and J. A. Fox. 1997. Evidence for a cost of sex in the freshwater snail *Potamopyrgus antipodarum*. Ecology **78:** 452-460.

Lascu, C. 1989. Paleogeographical and hydrogeological hypothesis regarding the origin of a peculiar cave fauna. Misc. speol. Rom. **1:** 13-18.

Marin, C., and T. Nicolescu. 1993. The geochemistry of groundwater from southeastern Dobrogea, Romania. Trav. Inst. Spéol. "Emile Racovitza" **32:** 229-247.

Michiels, I., and W. Traunspurger. 2005. Benthic community patterns and the composition of feeding types and reproductive modes in freshwater nematodes. Nematology **7:** 21-36.

Muschiol, D., M. Markovic, I. Threis, and W. Traunspurger. 2008b. Predatory copepods can control nematode populations: A functional-response experiment with *Eucyclops subterraneus* and bacterivorous nematodes. Fundam. Appl. Limnol. **172:** 317-324.

Muschiol, D., M. Markovic, I. Threis, and W. Traunspurger. 2008a. Predator-prey relationship between the cyclopoid copepod *Diacyclops bicuspidatus* and a free-living bacterivorous nematode. Nematology **10:** 55-62.

Muschiol, D., and W. Traunspurger. 2008. Life at the extreme: meiofauna from three unexplored lakes in the caldera of the Cerro Azul volcano, Galápagos Islands, Ecuador. Aquat. Ecol. **[doi: 10.1007/s10452-008-9202-y]**.

Muschiol, D., and W. Traunspurger. 2007. Life cycle and calculation of the intrinsic rate of natural increase of two bacterivorous nematodes, *Panagrolaimus* sp. and *Poikilolaimus* sp. from chemoautotrophic Movile Cave, Romania. Nematology **9:** 271-284.

Pfannkuche, O., and H. Thiel 1988. Sample processing, p. 134-145. *In* R. P. Higgins and H. Thiel [eds.], Introduction to the study of meiofauna. Smithsonian Institution Press.

Poinar, G. O. Jr., and S. M. Sarbu. 1994. *Chronogaster troglodytes* sp.n. (Nematoda, Chronogasteridae) from Movile Cave, with a review of cavernicolous nematodes. Fund. Appl. Nematol. **17 :** 231-237.

Popa, R., and S. M. Sarbu. 1991. Trophic structure in an aquatic troglobiotic community based upon chemoautotrophic carbon fixation. Trav. Inst. Spéol. "Emile Racovitza" **30:** 51-58.

Powell, E. N., M. A. Crenshaw, and R. M. Rieger. 1980. Adaptations to sulfide in sulfide-system meiofauna. Endproducts of sulfide detoxification in three turbellarians and a gastrotrich. Mar. Ecol. Prog. Ser. **2:** 169-177.

Riess, W., O. Giere, O. Kohls, and S. M. Sarbu. 1999. Anoxic thermomineral cave waters and bacterial mats as habitat for freshwater nematodes. Aquat. Microb. Ecol. **18:** 157-164.

Rohwerder, T., W. Sand, and C. Lascu. 2003. Preliminary evidence for a sulphur cycle in Movile Cave, Romania. Acta Biotechnol. **23:** 101-107.

Sarbu, S. M., and T. C. Kane. 1995. A subterranean chemoautotrophically based ecosystem. NSS Bull. **57:** 91-98.

Sarbu, S. M., T. C. Kane, and B. K. Kinkle. 1996. A chemoautotrophically based cave ecosystem. Science **272:** 1953-1955.

Sarbu, S. M., B. K. Kinkle, L. Vlasceanu, T. C. Kane, and R. Popa. 1994. Microbiological characterisation of a sulfide-rich groundwater ecosystem. Geomicrobiol. J. **12:** 175-182.

Sarbu, S. M., and R. Popa 1992. A unique chemoautotrophically based cave ecosystem, p. 637-666. *In* A. I. Camacho [ed.], The natural history of biospeleology. Museo Nacional de Ciencias Naturales.

Seinhorst, J. W. 1962. On the killing, fixation and transferring to glycerin of nematodes. Nematologica **8:** 29-32.

Shurin, J. B., and E. G. Allen. 2001. Effects of competition, predation, and dispersal on species richness at local and regional scales. Am. Nat. **158:** 624-637.

Stiuca, E., and H. Ilinca. 1995. Pleistocene mammals (Rodentia) from Movile Cave (Romania). Theor. Appl. Karstol. **8:** 157-161.

Traunspurger, W. 1991. Fischbiologie des Königssees. Forschungsbericht 22, Nationalpark Berchtesgaden.

Traunspurger, W. 1997. Bathymetric, seasonal and vertical distribution of the feeding-types of nematodes in an oligotrophic lake. Vie Milieu **47:** 1-7.

Traunspurger, W., I. Michiels, and A. Eyualem 2006. Composition and distribution of free-living freshwater nematodes: Global and local perspectives, p. 46-76. *In* A. Eyualem, W. Traunspurger, and I. Andrássy [eds.], Freshwater nematodes: Ecology and taxonomy. CABI Publishing.

Triantaphyllou, A. C. 1973. Environmental sex differentiation of nematodes in relation to pest management. Annu. Rev. Phytopathol. **11:** 441-462.

11.2. Manuscript II

Muschiol, D. & Traunspurger, W., 2007: **Life cycle and calculation of the intrinsic rate of natural increase of two bacterivorous nematodes, *Panagrolaimus* sp. and *Poikilolaimus* sp. from chemoautotrophic Movile Cave, Romania**. – *Nematology* **9:** 271-284.

Nematology, 2007, Vol. 9(2), 271-284

Life cycle and calculation of the intrinsic rate of natural increase of two bacterivorous nematodes, *Panagrolaimus* sp. and *Poikilolaimus* sp. from chemoautotrophic Movile Cave, Romania

Daniel MUSCHIOL * and Walter TRAUNSPURGER

University Bielefeld, Animal Ecology, Morgenbreede 45, 33615 Bielefeld, Germany

Received: 21 November 2006; revised: 22 January 2007
Accepted for publication: 23 January 2007

Summary – The life cycle and somatic growth of two bacterivorous nematodes, *Panagrolaimus* sp. and *Poikilolaimus* sp., isolated from chemoautotrophic microbial mats in Movile Cave, Romania, were studied in monoxenic cultures at 20°C with *Escherichia coli* as the food source. A method is described that allows simultaneous investigation of the somatic growth pattern, age-specific fecundity, and age-specific mortality of single individuals with high accuracy. Somatic growth curves of the species are presented. During juvenile development, both species showed a strict linear increase in body length, whereas body weight increased exponentially. Growth was continuous without lag phases. The relationships between fresh weight, W (μg), and body length, L (mm), were $W = 1.6439L^{2.7672}$ for *Poikilolaimus* sp. and $W = 0.2085L^{4.0915}$ for *Panagrolaimus* sp. Life tables and fecundity schedules for the two species are presented. In addition, demographic parameters were calculated. For *Panagrolaimus*, the intrinsic rate of natural increase (r_m), calculated according to the Lotka equation, was 0.309, the net reproductive rate (R_0) 64, the mean generation time (T) 13.8 days and the minimum generation time (T_{min}) 9.5 days. The corresponding values for *Poikilolaimus* were $r_m = 0.165$, $R_0 = 108$, $T = 26.2$ and $T_{min} = 19.5$. *Panagrolaimus* produced fewer progeny than *Poikilolaimus* during its life but exhibited faster population growth due to its faster maturation. It showed a distinct post-reproductive period, whereas *Poikilolaimus* remained fertile until death.

Keywords – age-specific fecundity, biofilm, culture medium, generation time, life tables, microbial mat, population growth, somatic growth.

On the continental earth, 97% of all unfrozen freshwater is subsurface, whereas lakes and rivers represent less than 2% (Gilbert & Deharveng, 2002). Our ecological knowledge of groundwater ecosystems lags far behind that of lakes and rivers (Boulton *et al*., 2003). Conditions in aquifers can be relatively harsh for life, with low concentrations of nutrients and carbon, limited dissolved oxygen, an absence of light and restricted free space (Hancock *et al*., 2005). Thus, subterranean ecosystems have long been considered as extreme environments, inhabited only by a few specialised species. This paradigm is now being revised as many studies have shown that this environment harbours diverse animal communities (Gilbert & Deharveng, 2002). For example, Botosaneanu (1986) listed a known (minimal) species diversity of 322 aquatic subterranean nematode species. Since subterranean ecosystems are protected against surface environmental changes – in contrast to most surface ecosystems (rivers, wetlands), which are short-lived – they may persist relatively unchanged for millions of years.

In 1986, a natural cave passage later called Pestera Movile (Constantinescu, 1989; Lascu, 1989) was discovered in the proximity of Mangalia, southeastern Dobrogea, Romania. Apart from the sealed artificial entrance, Movile Cave is isolated from the surface. Situated 25 m below the earth's surface, a small lake (3 m^2) allows access to the lower submerged level of the cave (Fig. 1). Specific for the cave is the redox interface between the atmosphere and the completely anoxic hydrothermal waters (21°C) that contain significant amounts of H_2S, CH_4 and NH_4^+ (Sarbu & Kane, 1995), all of which are potential sources of energy for aerobic bacteria. Analogous to bacteria in deep-sea hydrothermal vents, the physicochemical characteristics of Movile Cave have produced a peculiar groundwater ecosystem that is supported by *in situ* chemoautotrophic production. In addi-

* Corresponding author, e-mail: daniel.muschiol@uni-bielefeld.de

Also available online - www.brill.nl/nemy

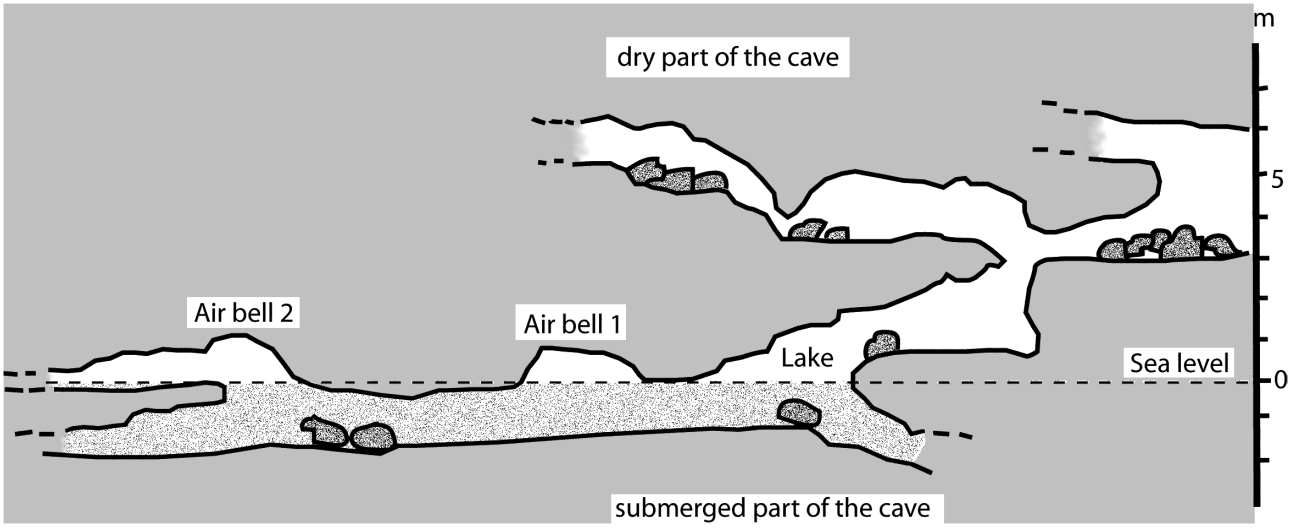

Fig. 1. *Cross-sectional map of the Movile Cave system. Redrawn from Sarbu* et al. *(1994).*

tion, the cave appears to lack any input of allochthonous photoautotrophically-based food (Sarbu & Kane, 1995). It is this situation that distinguishes the cave from the majority of the world's cave streams and groundwater ecosystems, which depend principally on energy derived from surface detritus as dissolved organic matter (DOM) and coarse particulate organic matter (CPOM) (Hancock *et al*., 2005). Microbial primary productivity acts as the food base for 48 species of cave-adapted terrestrial and aquatic invertebrates, of which 33 are endemic to Movile Cave.

Several air pockets ('air bells') are present in the lower level of the cave (Fig. 1). The atmosphere of the air bells is poor in oxygen (7-10%), rich in carbon dioxide (2-3.5%) and contains 1-2% methane (Sarbu & Kane, 1995; Sarbu *et al*., 1996). Thick (1-3 mm) microbial mats float on the water surface of air bell 2. The brownish mats are kept afloat by gas bubbles containing higher CH_4 concentrations (up to 13.5%). Methanotroph and sulphur oxidising bacteria were identified as primary producers in the mats (Rohwerder *et al*., 2003; Hutchens *et al*., 2004). Associated with these chemoautotrophic mats, at least 20 aquatic invertebrates have been found, including Heteroptera, Isopoda, Amphipoda, Harpacticoida, Cyclopida, Ostracoda, Gastropoda, Rotatoria, Annelida, Turbellaria and Nematoda (Sarbu & Kane, 1995; Riess *et al*., 1999). The metazoan fauna of the mats is clearly dominated by five nematode species that belong to the genera *Chronogaster*, *Panagrolaimus*, *Poikilolaimus*, *Udonchus* and *Monhystrella* (Riess *et al*., 1999; Muschiol, unpubl.). At least one of them, *Chronogaster troglodytes*, seems to be restricted to the specialised microbial-mat habitat of this unique cave (Poinar & Sarbu, 1994). All nematode species described thus far from Movile Cave are bacterial grazers. Based on their high abundance and biomass, it is clear that, as consumers of chemoautotrophic bacteria and as a food base for higher trophic levels, they play a major role in the cave's food web. Nevertheless, the literature contains very little information on these nematodes (see Poinar & Sarbu, 1994; Riess *et al*., 1999) and several key questions remain unanswered. For example, as the nematodes coexist in a quite homogeneous and unchanging environment and compete for the same food resources, mechanisms to reduce interspecific competition (niche differentiation, r-selection *vs* K-selection, interspecific interactions) and thus allow coexistence should be present. The paucity of food, which is characteristic for the overwhelming number of groundwater ecosystems, accounts for the slow metabolism, long life, slow growth and few young of most stygobite (*i.e*., obligatory hypogean) species (Gilbert & Deharveng, 2002); however, it is not known whether this is also true for the nematodes of the highly productive Movile Cave.

Our understanding of the dynamics of natural nematode populations suffers from the very restricted knowledge of life-cycle parameters in nematodes. Such data are essential for a meaningful interpretation of observed community structures. In the present investigation, we conducted a classical life-cycle experiment with the two most frequent species of Movile Cave's microbial mats, *Panagrolaimus* sp. and *Poikilolaimus* sp., in order to

assess the general biological features of the two species and to create a basis for further ecological experiments. The specific aims of this study were to estimate total life span, somatic growth curves, and a regression relating body weight and length to simplify future calculations of body mass. Furthermore, we aimed to produce complete life tables and fecundity schedules for the calculation of generation time, population doubling time, intrinsic rate of increase (r_m), net reproductive rate (R_0) and total fertility rate (TFR) of the two species.

Materials and methods

Laboratory cultures of *Panagrolaimus* sp. and *Poikilolaimus* sp. were established from floating bacterial mat from the Movile Cave in southeastern Dobrogea, Romania. The mat was collected in air bell 2 (Fig. 1) during a dive on 7 May 2004. Agnotobiotic cultures (*sensu* Dougherty, 1960) were established from small pieces of mat placed in the centre of Petri dishes containing 0.4-1.5% agar with yeast extract and/or peptone, NaCl, $CaCl_2$, $MgSO_4$, KH_2PO_4 and cholesterol in varying concentrations. As long as bacterial/fungal growth was not too strong due to the nutrient enrichments, most of the tested culture media proved to be suitable for cultivation of the two nematode species.

Modification of culture medium

Monoxenic cultures were established on NG (nematode growth) media seeded with OP50 (an uracil-requiring mutant of *Escherichia coli*), as described by Brenner (1974), with the only modification being that Bacto-agar was replaced by 1.5 g gellan gum (Gelrite; Merck, Kelco Division, San Diego, CA, USA) per litre medium (Eyre & Caswell, 1991). This modified medium, referred to here as NGG (nematode growth Gelrite), can be used as customary for the cultivation of nematodes; its consistency is comparable to 0.4% agar. The high transparency of the gel facilitates visual screening of culture plates. As the strength of the gel is determined by the concentration of divalent cations, the chelating agent ethylenediaminetetraacetic acid (EDTA) breaks the bonds responsible for the gel matrix, resulting in a liquid suspension that easily passes through sieves with a mesh size down to 10 μm. This method allows the nematodes to be readily extracted in virtually infinite numbers from cultures and without adhering residues of culture medium (Ferris *et al*., 1995). Preliminary experiments in our laboratory showed that exposure of the cultures to 0.01 M EDTA for 5 min influenced the growth and reproduction of neither *Panagrolaimus* sp. nor *Poikilolaimus* sp. A concentration of 0.005 M EDTA for 30 s proved to be sufficient to fluidify NGG when shaken. For this investigation, 250 ml bottles of freshly prepared autoclaved and still hot NGG were placed on a magnetic stirrer for several hours. During the cooling period, $CaCl_2$, $MgSO_4$, KH_2PO_4 and cholesterol (Brenner, 1974) were added aseptically. When the gel was constantly stirred until it reached room temperature, it failed to rigidify completely and retained a semi-fluid consistency. This semi-fluid gel proved to be perfectly suited for life-cycle experiments. It can be stored in the refrigerator for several months if contamination is avoided.

Food source

In the life-cycle experiments described here, an OP50 culture grown overnight in LB medium (1% peptone, 0.5% yeast extract, 1% NaCl) at 37°C served as the nematode food source. The food dependence of biosynthetic processes (somatic growth, egg production) follows a hyperbolic form, which can be described by the Michaelis-Menten function (Schiemer, 1982a). Accordingly, minor fluctuations in food concentration have a negligible influence on nematode population growth as long as they occur close to the concentration of optimal growth. Preliminary experiments indicated an optimal population growth at bacterial densities of around 10^9 cells ml^{-1}. Each time fresh food medium was prepared, the cell density of an overnight culture was checked spectrophotometrically with reference to a previously determined absorption (OD_{600}) *vs* cell density curve. The curve was generated by determining cell densities with epifluorescence microscopy after staining with DAPI (4′,6-diamidino-2-phenylindole), as described by Schallenberg *et al*. (1989). Known volumes of an overnight OP50 culture were washed in sterile K Medium (3.1 g NaCl l^{-1}, 2.4 g KCl l^{-1}; 2000 *g*, 15 min) and resuspended in semi-fluid NGG on a vortex mixer. This is a refinement of the method of Schiemer *et al*. (1980), who used a glass homogeniser to distribute bacteria evenly in 0.35% agar in order to obtain known food densities. As the size, weight, and nutritional value of bacterial cells change considerably during population growth in batch cultures (Schiemer, 1982b), cell density alone is not an appropriate measure for the nutritional value of a food medium and it can vary with time. Thus, we used NGG with peptone, which enabled the bacteria to cover their metabolic costs and, due to the

low temperature of the medium, allowed restricted bacterial growth during the experiment. Freshly prepared food medium that was not used immediately was kept in a refrigerator for up to 4 days.

Experimental set-up

Individuals of the monosexual species were kept in a hanging drop (10 μl) in a wet chamber, a set-up adopted from Schiemer *et al*. (1980). Perfectly suited for this were 12-well multiwell plates (Greiner 665102; Greiner Bio-One, Germany), the bottoms of which were covered with approximately 0.1 g (dry weight) cellulose that had been wetted with 500 μl of tap water. Each multiwell provides space for up to 12 individuals, each in its own drop of NGG (Fig. 2). When prepared in this way, the viscosity of the medium prevents the bacterial cells from accumulating at the bottom of the drop but allows the nematodes to move freely. To reduce evaporation, the multiwells were sealed with paraffin wax film. The transparent lid allows biological observation such as pharyngeal pumping rates without disturbance, the counting of eggs and juveniles, and photographic documentation of somatic growth.

All experiments were conducted in the dark at 20°C ($\pm$ 0.2°C). From monoxenic cultures in exponential growth, several gravid females of *Panagrolaimus* sp. and *Poikilolaimus* sp. that had acclimated to these conditions were transferred to drops of NGG containing *E. coli* (OP50; 10^9 cells ml^{-1}). After 24 h, the newborn juveniles, with an assumed average age of 12 h, were used for the experiment. The experiment was started with 24 juveniles of each species. Every 24 h, each individual was transferred to a fresh drop of NGG, making sure the nematodes never suffered food limitation. Half of the cohort was not transferred to the new drop at once but instead was transiently placed in a Petri dish containing relatively firm gellan gum (2.0 g Gelrite l^{-1}, ions as in usual NGG) covered by a thin film of water. In this set-up, surface tension forced the worms into a strict horizontal position that allowed more precise growth measurements than in either the hemispheric NGG drop or the set-up described by Woombs and Laybourn-Parry (1984). A high-resolution digital image was taken at maximal possible magnification, depending on the actual size of the animal, under a dissection microscope (Zeiss Stemi SV11 Apo, Nikon CoolPix 990). Body length and maximal width were determined using image analysis software (ImageJ v1.34s). The software is not subject to copyright protection and measurements can easily be made in all common digital image formats after calibration. Body weight was estimated using Andrássy's (1956) formula. Dead individuals were recorded. Individuals that were lost during handling or whose death was caused by accidental drying up were excluded from the life-table data. The use of an applicator stick with an eyelash mounted on the tip reduced accidental mortality to a minimum. Following transfer of the nematodes to the fresh drops, 10 μl of an aqueous solution of Rose Bengal (300 μg ml^{-1}) was added to the food drops in the inverted multiwell lid. The samples were covered with a circular 18-mm diam. cover slip and examined under a dissecting microscope at 40-fold magnification. An underlying grid facilitated counting of the juveniles. The experiment was conduced until the last animal died (day 72).

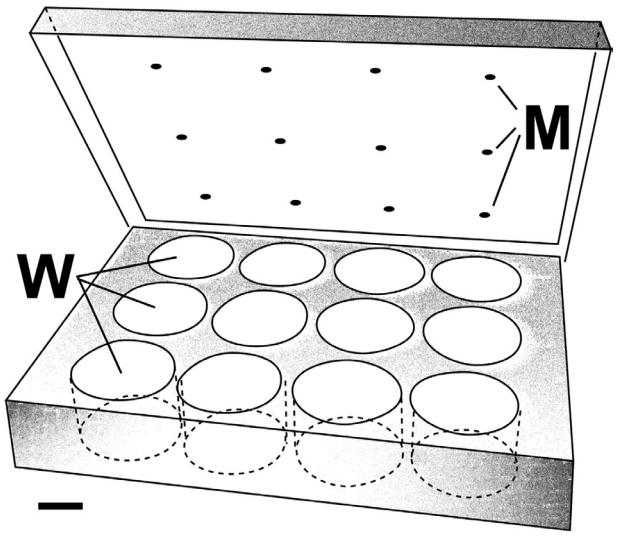

Fig. 2. *Multiwells as used for determination of life-cycle parameters. M = drops of 10 μl NGG medium, containing one individual each; W = wells containing wet cellulose. (Scale bar equals 10 mm.)*

Voucher specimens of the two nematode species were deposited in the W. Traunspurger Nematode Collection.

Data processing

Life tables and fecundity schedules are difficult to interpret on their own because they hide the dynamic behaviour of a population behind a mass of detail (Caughley & Gunn, 1996). For this reason, the data were summarised according to the upper-order parameters generation length, doubling time, and intrinsic rate of natural in-

crease (r_m) using the fundamental equation of population dynamics:

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

r_m = intrinsic rate of natural increase
x = time (d)
l_x = age-specific survival probability
m_x = age-specific fecundity

This equation is called the 'Euler equation' (see Charlesworth, 1994) and is also frequently referred to as the 'Lotka equation' after Lotka (1924) who applied it to human demography. As the equation does not lend itself to a direct solution, it has to be estimated by iteration (substituting successive trial values of r_m in the equation until the left-hand side sums to 1). The high time resolution of 24 h per age class and the consequential extensive life tables of this study made it necessary to determine r_m using a Microsoft® Visual Basic (6.0) macro in Excel (2002 SP3), which may be obtained from the authors on request.

The intrinsic rate of natural increase (r_m) is the growth rate of a population that has a stable age distribution and grows in an unlimited environment. The net reproductive rate ($R_0 = \sum l_x m_x$) is defined as the average number of offspring that an individual in a population will produce in its lifetime. Unlike the total fertility rate (TFR), R_0 depends on age-specific mortality rates. Since the concept of generation time is considered as rather arbitrary and slippery in the context of age-structured populations, several alternative measures have been proposed (see Charlesworth (1994) for a general survey). Here, we compare the values T_0, T_1 and T given by the following equations:

$$T_0 = \sum x l_x m_x \Big/ \sum l_x m_x$$
$$T_1 = (\ln R_0)/r_m$$
$$T = \sum x e^{-r_m x} l_x m_x$$

r_m = intrinsic rate of natural increase
x = time (d)
l_x = age-specific survival probability
m_x = age-specific fecundity
R_0 = net reproductive rate

T_0 (also referred to as T_c, the cohort generation time) is the mean age at reproduction of a cohort of females. T_1 refers to that period of time necessary for a population growing at a constant rate r_m to increase by the factor R_0. T is the mean age of the mothers of a set of new-born individuals in a population with a stable age distribution.

Results

Somatic growth

Figure 3 shows the somatic growth curves of the two investigated species. During juvenile development, both species showed a strict linear increase in body length. The onset of reproduction coincided clearly with the end of the linear growth phase (day 9.5 in *Panagrolaimus* and day 19.5 in *Poikilolaimus*). The relationships between body length L (μm) and age d (days) until the onset of reproduction were $L_{Pan.} = 214.0 + 159.4d$ ($r^2 = 0.97$) and $L_{Poi.} = 155.6 + 46.6d$ ($r^2 = 0.91$). An exponential growth curve, $W = W_0 e^{bt}$ (t = age in days; W = wet weight in μg), was fitted to the juvenile somatic growth data, as proposed by Herman and Vranken (1988). It can readily be seen that the body weight growth of juveniles of both species was exponential ($r^2 > 0.92$, see Fig. 6). However, as soon as the animals matured growth slowed down. The instantaneous growth rate of the juveniles of *Panagrolaimus* equalled 0.461 d^{-1}, that of *Poikilolaimus* 0.228 d^{-1}. These values corresponded to a body-weight doubling time ($t = \ln(2)/b$) of 36 h for *Panagrolaimus* and 73 h for *Poikilolaimus*. An observation that is not apparent in Figure 6 is that the nematodes, especially *Poikilolaimus*, showed pronounced body shrinkage 1 or 2 days before death. This shrinkage was always accompanied by reduced activity and resulted in higher standard deviations and a slight falling of the curve towards the end of the experiment.

As proposed by Ferris *et al.* (1995), we described the relationship between body width and length for each nematode species by a cubic function ($D = b_0 + b_1 L + b_2 L^2 + b_3 L^3$; D = width, L = length, values in μm) based on daily length and width measurements. The results are given together with data from Ferris *et al.* (1995) in Table 1 and Fig. 4*. The strong correlation of body length and width ($r^2 = 0.95$, see Table 1) for the two species, and the fact that Andrássy's (1956) formula for

* The original data for *Panagrolaimus detritophagus* result in unrealistic values. This erratum has to be corrected by reducing the b_2 coefficient for the species by one decimal to $2.44E^{-5}$ (Ferris, pers. comm.).

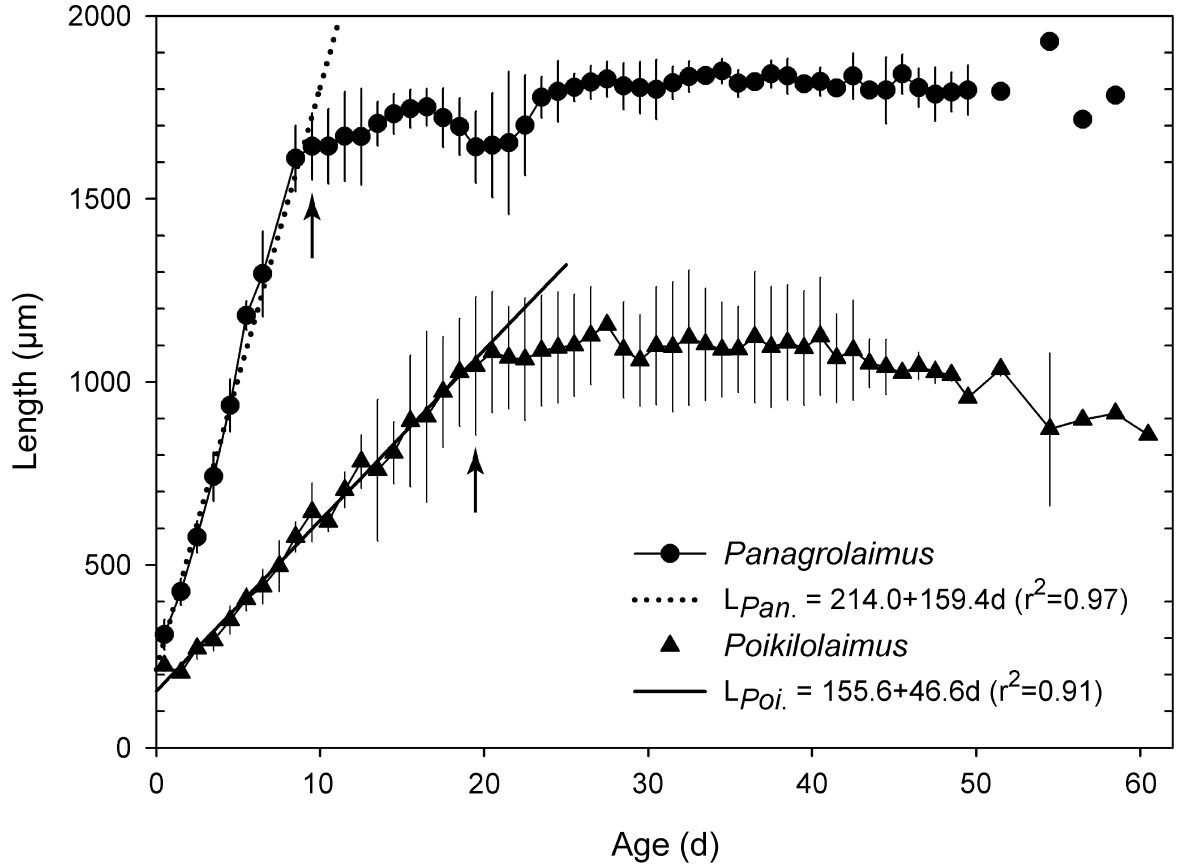

Fig. 3. *Somatic length-based growth curves of the two nematode species during the life-cycle experiment; 331 measurements of* Panagrolaimus *and 154 of* Poikilolaimus *were made. Bars indicate standard deviation of the arithmetic mean. Arrows indicate onset of reproduction (days 9.5 and 19.5). Regressions are related to somatic growth until the onset of reproduction. Only body lengths are shown, as body width proved to be strongly correlated with length (Table 1). Derived wet weights are shown in Figure 6.*

Table 1. *Coefficients of the cubic relationship* $D = b_0 + b_1L + b_2L^2 + b_3L^3$ *between body width (D, µm) and body length (L, µm) of seven species of bacterial-feeding nematodes, and the upper (L_{max}, µm) and lower (L_{min}, µm) bounds of the relationships.*

Species	b_0	b_1	b_2	b_3	L_{min}	L_{max}	r^2	n
*Panagrolaimus detritophagus**	6.51E+00	2.34E−02	2.44E−05	−6.34E−09	179	1240	0.93	229
Panagrolaimus sp.	6.84E+00	1.49E−02	−5.28E−06	4.90E−09	222	1961	0.95	331
*Caenorhabditis tripartitum**	1.40E+01	2.00E−03	6.01E−05	−1.98E−08	179	1890	0.95	322
Poikilolaimus sp.	1.76E+01	−3.35E−02	1.19E−04	−5.18E−08	189	1252	0.95	154
*Acrobeloides buetschlii**	2.04E+01	−8.37E−02	3.43E−04	−2.42E−07	179	775	0.92	200
*Caenorhabditis elegans**	3.17E+01	−1.41E−01	2.84E−04	−1.21E−07	298	1260	0.90	354
*Rhabditis cucumeris**	4.38E+01	−1.94E−01	3.68E−04	−1.54E−07	328	1260	0.96	314

Data designated with asterisks were taken from Ferris *et al*. (1995).

the calculation of nematode wet weight is based solely on length and width data, made it reasonable to create a regression relating body weight W and length L ($W = aL^b$, Fig. 5) using units of µg fresh weight and mm length, as proposed by Schiemer *et al*. (1980). For *Poikilolaimus* sp., the relationship was $W = 1.6439L^{2.7672}$ ($n = 153$, $r^2 = 0.948$); for *Panagrolaimus* sp., $W = 0.2085L^{4.0915}$ ($n = 329$, $r^2 = 0.934$).

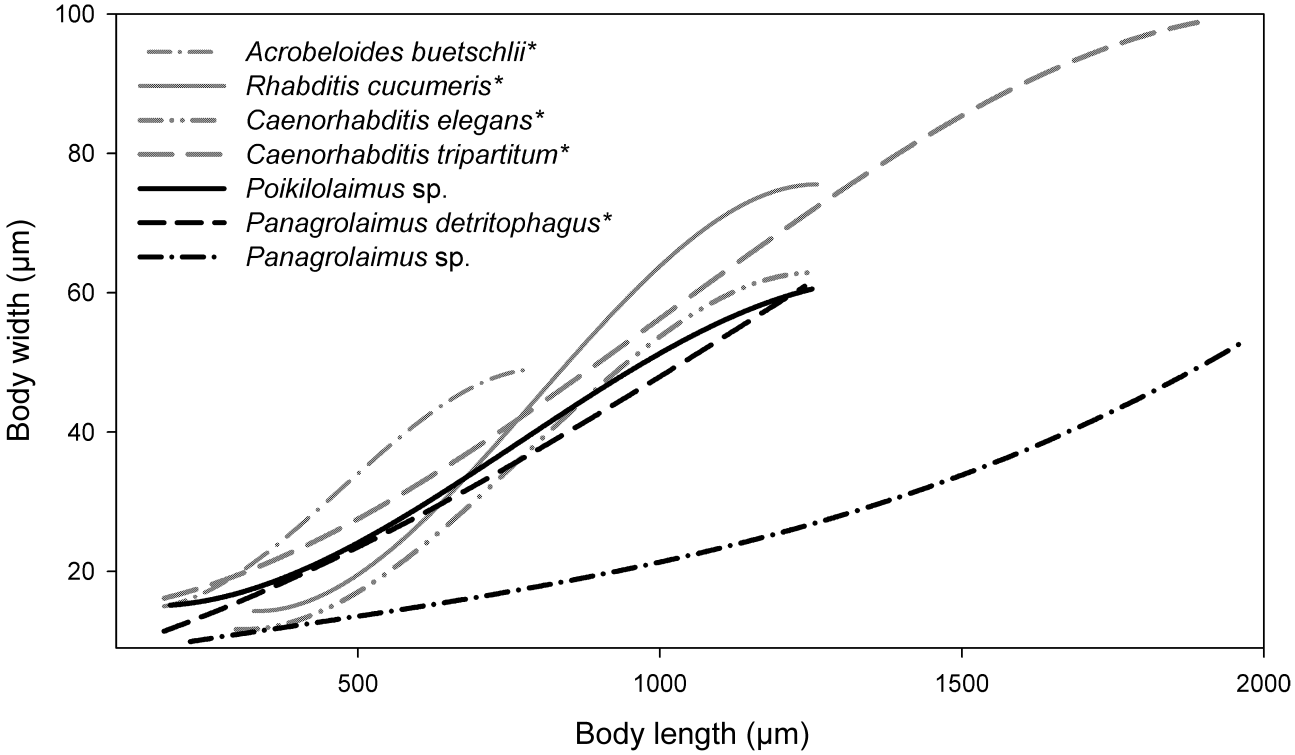

Fig. 4. *Illustration of the cubic relationship* $D = b_0 + b_1L + b_2L^2 + b_3L^3$ *between body width D and length L for seven species of bacterial-feeding nematodes within the upper and lower bounds of the relationships, as given in Table 1. Data from species designated with an asterisk were taken from Ferris* et al. *(1995).*

LIFE CYCLE

At the beginning of the reproductive period, *Panagrolaimus* sp. cultured at 20°C had an average age of 9.5 days, a body length of 1688 μm, a body width of 38.1 μm, and a biomass of 1.53 μg (medians). In contrast, *Poikilolaimus* sp. needed twice as long to start reproduction (19.5 days) and was much shorter (1133 μm), but considerably thicker and heavier (52.1 μm and 1.92 μg, respectively). Vranken and Heip (1985) found a correlation between minimum generation time T_{min} (in days) and weight at sexual maturity M_s (in ng dry weight) for free-living marine nematodes at 20°C. They described this relationship with the allometric function $T_{min} = 1.530M_s^{0.647}$ (see Heip *et al*. (1985) for a slightly different equation). When we applied this function to our experimental data, assuming a dry-weight content of 20% fresh weight (Duncan *et al*., 1974; Grootaert & Maertens, 1976; Grootaert & Small, 1982), minimum generation times of 62 days (observed 9.5 days) for *Panagrolaimus* and 72 days (observed 19.5 days) for *Poikilolaimus* were predicted. The values of the three alternative measures of mean generation time, T_0, T_1 and T, are given in Table 2. According to Charlesworth (1994), T is preferred over other measures of generation time. It is the mean age of the mothers of a set of newborn individuals in a population with stable age distribution. The T value was 13.8 days for *Panagrolaimus* and 26.2 days for *Poikilolaimus*.

Life tables and fecundity schedules for the reproductive period of the two investigated species are given in Table 3. The total fertility rate (TFR, *i.e*., the total number of offspring a female would have, on average, if individuals were to live to the maximum age) of *Panagrolaimus* was 77, that of *Poikilolaimus* 187. The net reproductive rate (R_0, which depends on age-specific mortality rates) of *Panagrolaimus* was 64, and that of *Poikilolaimus* 108.

The reproductive output of the two species during their lifetime is shown in Figure 6. The most conspicuous difference between the two life cycles is that *Panagrolaimus* started reproduction early (day 9.5) and stayed fecund for 18 days at the maximum, with a pronounced post-reproductive period. By contrast, *Poikilolaimus* needed longer to mature (19.5 days) but stayed fertile for 51 days, *i.e*., until death.

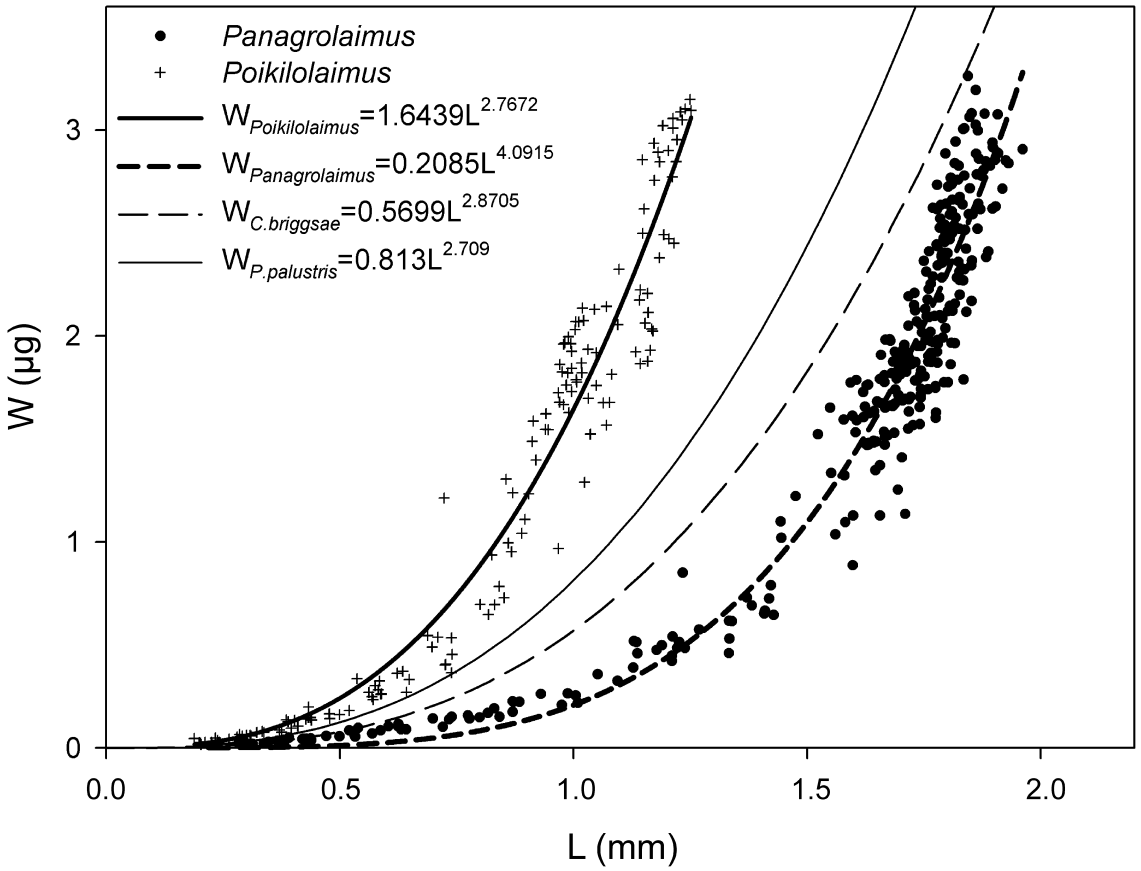

Fig. 5. *Illustration of the regressions relating body weight and length ($W = aL^b$) for* Panagrolaimus *and* Poikilolaimus*. Scatter diagrams show the body weights, as computed from original body lengths and widths, after Andrássy's (1956) formula. Thin lines in the middle illustrate published regressions for* Caenorhabditis briggsae *(Schiemer, 1982a) and* Plectus palustris *(Schiemer* et al.*, 1980).*

Table 2. *Alternative measures of mean generation time: T_0, T_1 and T.*

Species	T_0	T_1	T
Panagrolaimus sp.	14.4	13.5	13.8
Poikilolaimus sp.	33.7	28.4	26.2

Calculation of the intrinsic rate of natural increase (r_m) provided a value of 0.309 for *Panagrolaimus* and 0.165 for *Poikilolaimus*. These values correspond to a population doubling time ($t = \ln(2)/r_m$) of 2.24 days (54 h) for *Panagrolaimus* and 4.21 days (101 h) for *Poikilolaimus*.

Discussion

Methods

To our knowledge, Eyre and Caswell (1991) were the first to use gellan gum instead of agar in the cultivation of nematodes. We believe that gellan gum offers many advantages in ecological investigations of nematodes and other meiofauna. The most striking is that media prepared with gellan gum can be fluidified with low concentrations of EDTA in less than a minute. EDTA is non-toxic and no harmful effects on growth and reproduction of the nematodes were observed. After fluidification of the medium, nematodes can easily be extracted in virtually infinite numbers from cultures and are free of adhering residues of culture medium. The separation of developmental stages by size fractionation in stacked sieves of different mesh sizes is also possible and yields large numbers of individuals from each stage. Additionally, we had good results with membrane filters made of cellulose nitrate or mixed esters of cellulose with pore sizes between 0.2 and 8 μm. A modification of a mounting method proposed by Skibbe (1994) for protists enabled us to simplify the preparation of permanent

Table 3. *Life table and fecundity schedule for the reproductive period of* Poikilolaimus *sp. and* Panagrolaimus *sp.*

Poikilolaimus sp.				*Panagrolaimus* sp.			
x	l_x	m_x	$l_x m_x$	x	l_x	m_x	$l_x m_x$
18.5	0.73	2.7	2.0	7.5	1.00	0.1	0.1
19.5	0.73	4.1	3.0	8.5	1.00	0.0	0.0
20.5	0.73	5.2	3.8	9.5	1.00	3.8	3.8
21.5	0.73	5.5	4.0	10.5	0.95	8.4	8.0
22.5	0.73	5.9	4.3	11.5	0.95	7.8	7.4
23.5	0.73	5.7	4.2	12.5	0.95	7.7	7.3
24.5	0.73	5.2	3.8	13.5	0.86	8.6	7.3
25.5	0.73	5.5	4.1	14.5	0.86	3.7	3.2
26.5	0.73	6.3	4.6	15.5	0.86	5.6	4.8
27.5	0.73	4.8	3.5	16.5	0.71	8.1	5.8
28.5	0.67	6.0	4.0	17.5	0.71	6.7	4.8
29.5	0.67	5.6	3.7	18.5	0.71	7.2	5.1
30.5	0.60	6.4	3.9	19.5	0.71	4.9	3.5
31.5	0.60	6.0	3.6	20.5	0.71	2.6	1.9
32.5	0.60	6.2	3.7	21.5	0.71	0.9	0.7
33.5	0.60	6.0	3.6	22.5	0.52	0.2	0.1
34.5	0.60	5.9	3.5	23.5	0.52	0.3	0.1
35.5	0.60	5.3	3.2	24.5	0.43	0.3	0.1
36.5	0.60	6.1	3.7	25.5	0.43	0.3	0.1
37.5	0.60	5.3	3.2	$\sum$		77	64
38.5	0.60	5.3	3.2				
39.5	0.60	4.4	2.7				
40.5	0.60	5.9	3.5				
41.5	0.53	4.9	2.6				
42.5	0.53	5.6	3.0				
43.5	0.53	4.1	2.2				
44.5	0.47	4.7	2.2				
45.5	0.47	4.7	2.2				
46.5	0.47	3.9	1.8				
47.5	0.47	3.4	1.6				
48.5	0.47	3.9	1.8				
49.5	0.47	2.7	1.3				
50.5	0.47	3.0	1.4				
52.0	0.47	1.9	0.9				
54.0	0.47	2.4	1.1				
56.0	0.47	2.1	1.0				
58.0	0.40	1.4	0.6				
60.0	0.33	1.4	0.5				
62.0	0.27	1.4	0.4				
63.5	0.13	1.5	0.2				
65.5	0.13	2.3	0.3				
67.5	0.07	4.5	0.3				
69.5	0.07	2.0	0.1				
$\sum$		187	108				

x = pivotal age in days; l_x = age-specific survival probability; m_x = age-specific fecundity. The sum of all m_x values is the total fertility rate (TFR); the sum of all $l_x m_x$ values is the net reproductive rate (R_0).

mounts of nematodes (Muschiol *et al*., unpubl.), such as are needed in interspecies competition experiments.

Somatic growth

Nematodes can increase in size between moults and after the final moult. It has not been fully verified whether juvenile growth in nematodes is generally a continuous or discontinuous process. The nematode growth curves reported by various authors (Jantunen, 1964; Laybourn, 1979; Woombs & Laybourn-Parry, 1984) indicated continuous growth. The general nematode growth curves given by Malakhov *et al*. (1994, p. 171) suggested continuous, yet variable, growth rates during development. According to Yeates and Boag (2003), nematode growth curves suggest continuous growth during development, with saltatorial (stepwise) growth of the stylet or buccal cavity between stages. Nonetheless, a period of lethargus, when little or no growth occurs, is characteristic of many species of nematodes just before and during moulting (Lee, 2002). Lag phases have been noted in nematode growth curves reported by a number of investigators (Sommerville, 1960; Chuang, 1962; Thomas, 1965; Sohlenius, 1968, 1973). Wilson (1976) showed that discontinuities in the growth curves of *Caenorhabditis elegans* and *Panagrellus redivivus* occur at moulting. Our data on *Panagrolaimus* sp. and *Poikilolaimus* sp. suggest that juvenile growth is a continuous process. However, a slight asynchrony in the development of the investigated cohorts might have hidden the details. Hence, we plotted growth curves for each individual but could not detect a systematic pattern. A time resolution of 24 h is probably too long to detect minor fluctuations in growth rates, as each moult may take place relatively quickly; for example, those of *Panagrellus silusiae* and *C. elegans* take only 3-4 h (Lee, 2002 and references therein).

Generally, juvenile growth in nematodes is thought to be exponential (Malakhov *et al*., 1994)*. In conflict with these findings are recent studies on *C. elegans* (Knight *et al*., 2002) indicating that the apparent exponential growth between hatching and adulthood comprises a series of linear phases, one per juvenile stage, with the linear growth rate increasing at successive moults. In the two species of this study, juvenile body length growth was

* From published measurements, Yeates and Boag (2002, 2003) reviewed the increase in body volume during successive developmental stages of nematodes. As they used data that are stage-based rather than time-based, their findings are difficult to compare with our data.

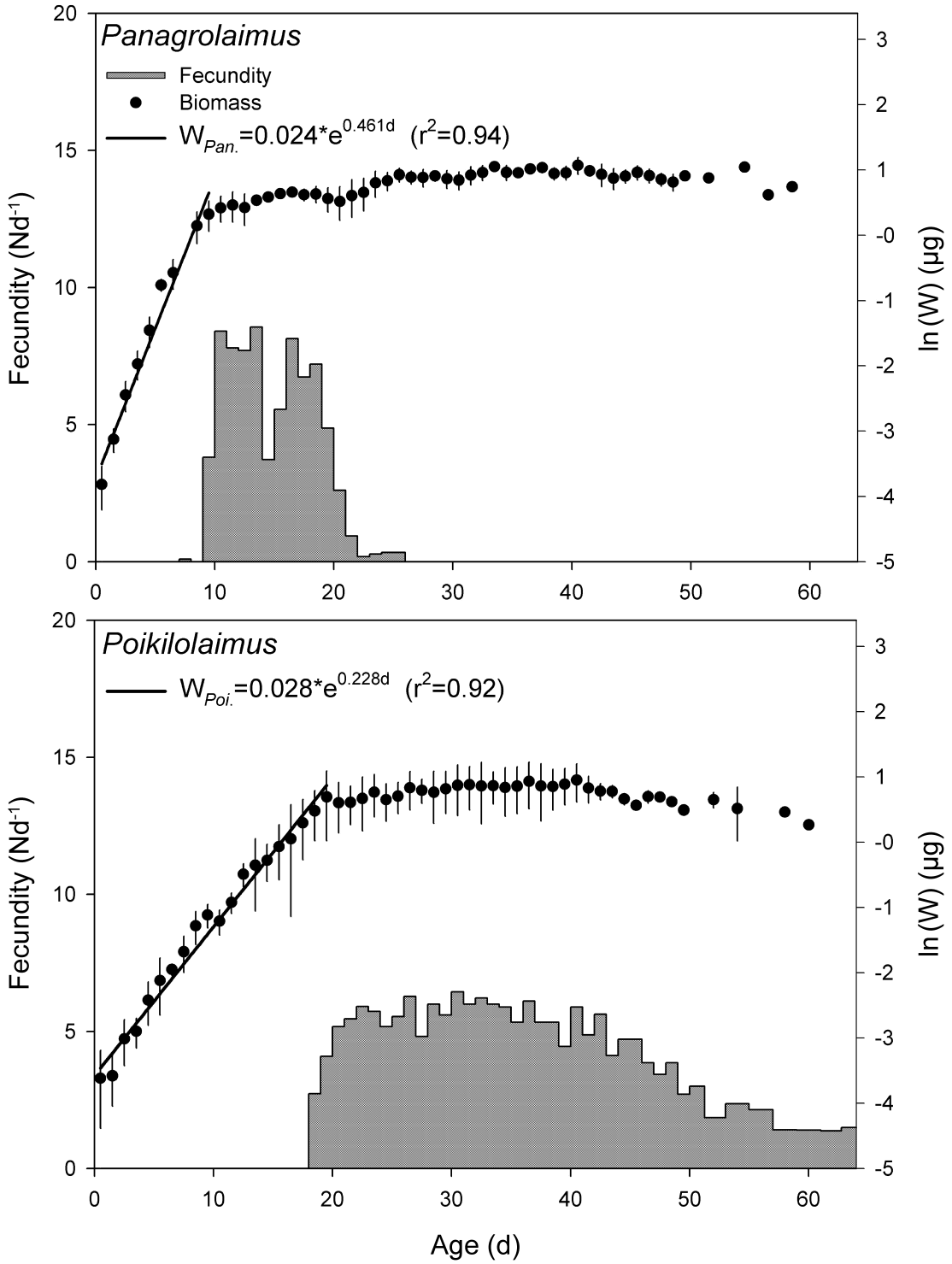

Fig. 6. *Biomass W and fecundity of* Panagrolaimus *sp. and* Poikilolaimus *sp. in relation to age. Note that* Panagrolaimus *starts reproduction earlier in its life-cycle (day 9.5) and shows a pronounced post-reproductive period whereas* Poikilolaimus *starts reproduction later (day 19.5) but stays fertile until death.*

linear (Fig. 3) and weight gain was exponential (Fig. 6). However, as our time resolution (24 h) was much longer than the 35 min used by Knight *et al*. (2002), we cannot exclude the possibility that a greater temporal resolution would indeed reveal a series of linear phases. Nonetheless, the good fit of our exponential regressions ($r^2 > 0.92$) appears to be sufficiently accurate for most ecological purposes.

Generally, the growth of *Panagrolaimus* sp. ($b = 0.461$) and *Poikilolaimus* sp. ($b = 0.228$) was comparatively slow. Herman and Vranken (1988) reported an instantaneous growth rate of 0.61 d^{-1} for *Monhystera disjuncta* cultured at 17°C, whereas Woombs and Laybourn-Parry (1984) even found values between 0.82 and 1.1 for females of three nematode species that had been isolated from sewage treatment plants and cultured at 20°C. Schiemer *et al*. (1980) investigated growth and reproduction of the benthic freshwater nematode *Plectus palustris* when cultured at 20°C. At high food density, young juveniles had an instantaneous growth rate of 0.41 which lies between the rates of our two species.

Slenderness of *Panagrolaimus* sp.

The illustration of the relationship between body width and length, shown in Figure 4, reveals an atypical growth pattern of *Panagrolaimus* sp. from Movile Cave. During its entire postembryonic ontogenesis, *Panagrolaimus* sp. is conspicuously more slender than typical bacterivorous nematodes. Even closely related *Panagrolaimus detritophagus* is distinctly stouter. This observation should be considered in the context of the work of Jensen (1986, 1987a, b) and of others stating that slenderness of the nematode body correlates with the amount of dissolved sulphide in the environment (8-12 mg l^{-1} in Movile Cave). It is known that the sizes and proportions of nematode species fed on different foods may vary (*e.g*., Townshend & Blackith, 1975). For *Panagrolaimus davidi*, Wharton (1998) compared field-collected and cultured nematodes and found that females from cultures were 10% longer and 7% narrower. However, during ecological experiments we examined more than 600 individual *Panagrolaimus* sp. under the microscope at 1000-fold magnification. These nematodes had not been cultured in the laboratory but came from bacterial mats growing on cave water (Muschiol & Traunspurger, unpubl.). Although most of the nematodes were not measured systematically, their morphometrics appeared very similar to those of cultured individuals. Accordingly, we regard the slenderness of *Panagrolaimus* sp. as characteristic for the species. Meiofauna living under sulphidic conditions is often extremely long and slender (Boaden, 1974, 1975). Body elongation, *i.e*., a higher proportion of body surface area per unit body volume, has been suggested to be an adaptation to low oxygen partial pressure in the environment as well as to the trans-epidermal uptake of dissolved organic matter (DOM) as additional nourishment (mixotrophy) for thiobiotic nematodes (Jensen, 1986, 1987b). Observations on the investigated *Panagrolaimus* sp. from Movile Cave are consistent with this suggestion (Fig. 4). The slenderness of these nematodes, even when they are compared to closely related *P. detritophagus*, appears to be an adaptation to the unique conditions of their natural habitat. According to Schiemer *et al*. (1990), for symbiotic stilbonematids, a high surface:volume ratio may be advantageous for a worm that grows potential food on its body surface. However, until now, internal or external symbionts of nematodes from Movile Cave could not be detected, as discussed by Poinar and Sarbu (1994) for *Chronogaster troglodytes* from this cave.

Life cycle

This study is the first to present life-cycle data for a species of the genus *Poikilolaimus*. For the genus *Panagrolaimus*, life-cycle data on four species cultivated at 20°C are available.

Fertility

For *Panagrolaimus australis*, Yeates (1970) reported a fertility of 195.6 on *Bacillus cereus*, and Brown *et al*. (2004) a fertility of 118 (value estimated from their Fig. 2) for *Panagrolaimus davidi* on unidentified bacteria. Both values are notably higher than the R_0 of our species of *Panagrolaimus* (64).

Development time

Published values of T_{min} are 8.8 days for *Panagrolaimus australis* (Yeates, 1970), 9.0 days for *P. davidi* (Brown *et al*., 2004), 7 days for *P. detritophagus*, and 8 days for *P. superbus* (Sohlenius, 1988). Our species of *Panagrolaimus* ($T_{min} = 9.5$ days) is (slightly) slower than all of them.

Population growth rate

Venette and Ferris (1997) reported a population growth rate of 0.148 for *P. detritophagus*, but this value was only an approximation of the true value of r_m. For *P. davidi* (an antarctic species), Brown *et al*. (2004) reported an intrinsic rate of natural increase (r_m) of 0.278, which

is slightly lower than our value for *Panagrolaimus* sp. (0.309).

Generally, the interpretation of our results suffers from the very restricted knowledge of life-cycle parameters in nematodes. Of all published population growth rates within the Nematoda, to the best of our knowledge, only six were based on complete life tables and fecundity schedules and the results of the Lotka equation (Schiemer, 1982b, 1983; Vranken & Heip, 1983; Vranken *et al*., 1988; Procter, 1986; Brown *et al*., 2004). Only three of those growth rates were based on the same cultivation temperature as used in this investigation. Within the range of published data, the two species from Movile Cave exhibited comparably low r_m values; *Poikilolaimus* had the lowest growth rate at 20°C ever published. However, it is known that some nematode species reproduce much slower. For example, Wieser and Kanwisher (1960) report a generation time of one year for *Enoplus communis*. Due to the weak data pool and the fact that most free-living nematodes cannot be cultured up to now, we are convinced that the two species from highly productive Movile Cave (population doubling times of 2.24 and 4.21 days, respectively) belong to the faster growing representatives of their order.

Acknowledgements

Mihai Baciu and Alexandra Hillebrand from the Group for Underwater and Speleological Exploration (GESS), Bucharest, Romania helped to obtain the samples. Marén Bökamp carried out large parts of the experimental work. Species determination was confirmed by Walter Sudhaus, Berlin. We thank two anonymous reviewers for their valuable comments on the manuscript. This investigation was financially supported by the Deutsche Forschungsgemeinschaft.

References

ANDRÁSSY, I. (1956). Die Rauminhalts- und Gewichtsbestimmung der Fadenwürmer (Nematoda). *Acta Zoologica Hungarica* 2, 1-15.

BOADEN, P.J.S. (1974). Three new thiobiotic Gastrotricha. *Cahiers de Biologie Marine* 15, 367-378.

BOADEN, P.J.S. (1975). Anaerobiosis, meiofauna and early metazoan evolution. *Zoologica Scripta* 4, 21-24.

BOTOSANEANU, L. (1986). *Stygofauna mundi: a faunistic, distributional, and ecological synthesis of the world fauna inhabiting subterranean waters (including the marine interstitial)*. Leiden, The Netherlands, Brill, 740 pp.

BOULTON, A.J., HUMPHREYS, W.F. & EBERHARD, S.M. (2003). Imperilled subsurface waters in Australia: Biodiversity, threatening processes and conservation. *Aquatic Ecosystem Health & Management* 6, 41-54.

BRENNER, S. (1974). The genetics of *Caenorhabditis elegans*. *Genetics* 77, 71-94.

BROWN, I.M., WHARTON, D.A. & MILLAR, R.B. (2004). The influence of temperature on the life history of the Antarctic nematode *Panagrolaimus davidi*. *Nematology* 6, 883-890.

CAUGHLEY, G. & GUNN, A. (1996). *Conservation biology in theory and practice*. Cambridge, MA, USA, Blackwell Science, 459 pp.

CHARLESWORTH, B. (1994). *Evolution in age-structured populations*. Cambridge, UK, Cambridge University Press, 320 pp.

CHUANG, S.H. (1962). The embryonic and postembryonic development of *Rhabditis teres* (A. Schneider). *Nematologica* 7, 317-330.

CONSTANTINESCU, T. (1989). Considérations sur la zone karstique de "La Movile" (Mangalia, Dobrogea du sud, Roumanie). *Miscellanea Speologica Romanica* 1, 7-12.

DOUGHERTY, E.C. (1960). Cultivation of aschelminthes, especially rhabditid nematodes. In: Sasser, J.N. & Jenkins, W.R. (Eds). *Nematology. Fundamentals and recent advances, with emphasis on plant parasitic soil forms.* Chapel Hill, NC, USA, University of North Carolina Press, pp. 297-318.

DUNCAN, A., SCHIEMER, F. & KLEKOWSKI, R.Z. (1974). A preliminary study of feeding rates on bacterial food by adult females of a benthic nematode, *Plectus palustris* de Man 1880. *Polskie Archiwum Hydrobiologii [Polish archives of Hydrobiology]* 21, 237-255.

EYRE, M.J. & CASWELL, E.P. (1991). Sterile culture of *Rotylenchulus reniformis* on tomato root with gellan gum as a supporting medium. *Journal of Nematology* 23, 229-231.

FERRIS, H., LAU, S. & VENETTE, R.C. (1995). Population energetics of bacterial-feeding nematodes: respiration and metabolic rates based on CO_2 production. *Soil Biology and Biochemistry* 27, 319-330.

GILBERT, J. & DEHARVENG, L. (2002). Subterranean ecosystems: A truncated functional biodiversity. *Bioscience* 52, 473-481.

GROOTAERT, P. & MAERTENS, D. (1976). Cultivation and life cycle of *Mononchus aquaticus*. *Nematologica* 22, 173-181.

GROOTAERT, P. & SMALL, R.W. (1982). Aspects of the biology of *Labronema vulvapillatum* (Meyl), (Nematoda, Dorylaimidae) in laboratory culture. *Biologische Jaarboek* 50, 135-148.

HANCOCK, P.J., BOULTON, A.J. & HUMPHREYS, W.F. (2005). Aquifers and hyporheic zones: Towards an ecological understanding of groundwater. *Hydrogeology Journal* 13, 98-111.

HEIP, C., VINCX, M. & VRANKEN, G. (1985). The ecology of marine nematodes. *Oceanography and Marine Biology* 23, 399-489.

HERMAN, P.M.J. & VRANKEN, G. (1988). Studies of the life-history and energetics of marine and brackish-water nematodes. II. Production, respiration and food uptake by *Monhystera disjuncta*. *Oecologia* 77, 457-463.

HUTCHENS, E., RADAJEWSKI, S., DUMONT, M.G., MCDONALD, I.R. & MURRELL, J.C. (2004). Analysis of methanotrophic bacteria in Movile Cave by stable isotope probing. *Environmental Microbiology* 6, 111-120.

JANTUNEN, R. (1964). Moulting of *Caenorhabditis briggsae* (Rhabditidae). *Nematologica* 10, 419-424.

JENSEN, P. (1986). Nematode fauna in the sulphide-rich brine seep and adjacent bottoms of the East Flower Garden, NW Gulf of Mexico. IV. Ecological aspects. *Marine Biology* 92, 489-503.

JENSEN, P. (1987a). Differences in microhabitat, abundance, biomass and body size between oxybiotic and thiobiotic free-living marine nematodes. *Oecologia* 71, 564-567.

JENSEN, P. (1987b). Feeding ecology of free-living aquatic nematodes. *Marine Ecology Progress Series* 35, 187-196.

KNIGHT, C.G., PATEL, M.N., AZEVEDO, R.B.R. & LEROI, A.M. (2002). A novel mode of ecdysozoan growth in *Caenorhabditis elegans*. *Evolution and Development* 4, 16-27.

LASCU, C. (1989). Paleogeographical and hydrogeological hypothesis regarding the origin of a peculiar cave fauna. *Miscellanea spéologica Romanica* 1, 13-18.

LAYBOURN, J. (1979). The effects of temperature on the respiration and production of the freshwater nematode *Anonchus* sp. *Oecologia* 41, 329-337.

LEE, D.L. (2002). Cuticle, moulting and exsheathment. In: Lee, D.L. (Ed.). *The biology of nematodes.* London, UK, Taylor & Francis, pp. 171-210.

LOTKA, A.J. (1924). *Elements of physical biology*. Baltimore, MD, USA, Williams & Wilkins, 460 pp.

MALAKHOV, V.V., HOPE, D. & BENTZ, G.V. (1994). *Nematodes: structure, development, classification, and phylogeny*. Washington, DC, USA, Smithsonian Institution Press, 286 pp.

POINAR JR, G.O. & SARBU, S.M. (1994). *Chronogaster troglodytes* sp.n. (Nematoda, Chronogasteridae) from Movile Cave, with a review of cavernicolous nematodes. *Fundamental and Applied Nematology* 17, 231-237.

PROCTER, D.L.C. (1986). Fecundity, reproductive effort, age-specific reproductive tactics and intrinsic rate of natural increase of a High Arctic nematode belonging to the genus *Chiloplacus*. *Holarctic Ecology* 9, 104-108.

RIESS, W., GIERE, O., KOHLS, O. & SARBU, S.M. (1999). Anoxic thermomineral cave waters and bacterial mats as habitat for freshwater nematodes. *Aquatic Microbial Ecology* 18, 157-164.

ROHWERDER, T., SAND, W. & LASCU, C. (2003). Preliminary evidence for a sulphur cycle in Movile Cave, Romania. *Acta Biotechnologica* 23, 101-107.

SARBU, S.M. & KANE, T.C. (1995). A subterranean chemoautotrophically based ecosystem. *National Speleological Society Bulletin* 57, 91-98.

SARBU, S.M., KINKLE, B.K., VLASCEANU, L., KANE, T.C. & POPA, R. (1994). Microbiological characterisation of a sulfide-rich groundwater ecosystem. *Geomicrobiology Journal* 12, 175-182.

SARBU, S.M., KANE, T.C. & KINKLE, B.K. (1996). A chemo-autotrophically based cave ecosystem. *Science* 272, 1953-1955.

SCHALLENBERG, M., KALFF, J. & RASMUSSEN, J.B. (1989). Solutions to problems in enumerating sediment bacteria by direct counts. *Applied and Environmental Microbiology* 55, 1214-1219.

SCHIEMER, F. (1982a). Food dependence and energetics of freeliving nematodes I. Respiration, growth and reproduction of *Caenorhabditis briggsae* (Nematoda) at different levels of food supply. *Oecologia* 54, 108-121.

SCHIEMER, F. (1982b). Food dependence and energetics of freeliving nematodes II. Life history parameters of *Caenorhabditis briggsae* (Nematoda) at different levels of food supply. *Oecologia* 54, 122-128.

SCHIEMER, F. (1983). Comparative aspects of food dependence and energetics of freeliving nematodes. *Oikos* 41, 32-42.

SCHIEMER, F., DUNCAN, A. & KLEKOWSKI, R.Z. (1980). A bioenergetic study of a benthic nematode, *Plectus palustris* de Man 1880, throughout its life cycle. II. Growth, fecundity and energy budgets at different densities of bacterial food. *Oecologia* 44, 205-212.

SCHIEMER, F., NOVAK, R. & OTT, J.A. (1990). Metabolic studies on thiobiotic free-living nematodes and their symbiotic microorganisms. *Marine Biology* 106, 129-137.

SKIBBE, O. (1994). An improved quantitative protargol stain for ciliates and other planctonic protists. *Archiv für Hydrobiologie* 130, 339-347.

SOHLENIUS, B. (1968). Influence of microorganisms and temperature upon some rhabditid nematodes. *Pedobiologia* 8, 137-145.

SOHLENIUS, B. (1973). Growth and reproduction of a nematode *Acrobeloides* sp. cultivated on agar. *Oikos* 24, 64-72.

SOHLENIUS, B. (1988). Interactions between two species of *Panagrolaimus* in agar cultures. *Nematologica* 34, 208-217.

SOMMERVILLE, R.I. (1960). The growth of *Cooperia curticei* (Giles, 1892) a nematode parasite of sheep. *Parasitology* 50, 261-267.

THOMAS, P.R. (1965). Biology of *Acrobeles complexus* Thorne, cultivated on agar. *Nematologica* 11, 395-408.

TOWNSHEND, J.L. & BLACKITH, R.E. (1975). Fungal diet and the morphometric relationships in *Aphelenchus avenae*. *Nematologica* 21, 19-25.

VENETTE, R.C. & FERRIS, H. (1997). Thermal constraints to population growth of bacterial-feeding nematodes. *Soil Biology and Biochemistry* 29, 63-74.

VRANKEN, G. & HEIP, C. (1983). Calculation of the intrinsic rate of natural increase, rm, with *Rhabditis marina* Bastian 1865 (Nematoda). *Nematologica* 29, 468-477.

VRANKEN, G. & HEIP, C. (1985). Aspects of the life cycle of free-living marine nematodes. In: VanGrieken, R.E. & Wollast, R. (Eds). *Progress in Belgian oceanographic research.* Antwerpen, Belgium, Belgian Center of Oceanography, pp. 267-278.

VRANKEN, G., HERMAN, P.M.J. & HEIP, C. (1988). Studies of the life-history and energetics of marine and brackish-water nematodes I. Demography of *Monhystera disjuncta* at different temperature and feeding conditions. *Oecologia* 77, 296-301.

WHARTON, D.A. (1998). Comparison of the biology and freezing tolerance of *Panagrolaimus davidi*, an Antarctic nematode, from field samples and cultures. *Nematologica* 44, 643-653.

WIESER, W. & KANWISHER, J. (1960). Growth and metabolism in a marine nematode *Enoplus communis* Bastian. *Zeitschrift für Vergleichende Physiologie* 43, 29-36.

WILSON, P.A.G. (1976). Nematode growth patterns and moulting cycle: population growth profile. *Journal of Zoology* 179, 135-151.

WOOMBS, M. & LAYBOURN-PARRY, J. (1984). Growth, reproduction and longevity in nematodes from sewage treatment plants. *Oecologia* 64, 168-172.

YEATES, G.W. (1970). Studies on laboratory cultures of dune sand nematodes. *Journal of Natural History* 4, 119-136.

YEATES, G.W. & BOAG, B. (2002). Post-embryonic growth in longidorid nematodes. *Nematology* 4, 883-889.

YEATES, G.W. & BOAG, B. (2003). Growth and life histories in Nematoda with particular reference to environmental factors. *Nematology* 5, 653-664.

11.3. Manuscript III

Muschiol, D., Schroeder, F. & Traunspurger, W., 2009: **Life cycle and population growth rate of *Caenorhabditis elegans* studied by a new method**. – *BMC Ecology* **9:** 14.

Life cycle and population growth rate of *Caenorhabditis elegans* studied by a new method

DANIEL MUSCHIOL, FABIAN SCHROEDER, and **WALTER TRAUNSPURGER**

Abstract

Background. The free-living nematode *Caenorhabditis elegans* is the predominant model organism in biological research, being used by a huge number of laboratories worldwide. Many researchers have evaluated life-history traits of *C. elegans* in investigations covering quite different aspects such as ecotoxicology, inbreeding depression and heterosis, dietary restriction/supplement, mutations, and ageing. Such traits include juvenile growth rates, age at sexual maturity, adult body size, age-specific fecundity/mortality, total reproduction, mean and maximum lifespan, and intrinsic population growth rates. However, we found that in life-cycle experiments care is needed regarding protocol design. Here, we test a recently developed method that overcomes some problems associated with traditional cultivation techniques. In this fast and yet precise approach, single individuals are maintained within hanging drops of semi-fluid culture medium, allowing the simultaneous investigation of various life-history traits at any desired degree of accuracy. Here, the life cycles of wild-type *C. elegans* strains N2 (Bristol, UK) and MY6 (Münster, Germany) were compared at 20°C with 5×10^9 *Escherichia coli* ml^{-1} as food source.

Results. High-resolution life tables and fecundity schedules of the two strains are presented. Though isolated 700 km and 60 years apart from each other, the two strains barely differed in life-cycle parameters. For strain N2 ($n = 69$), the intrinsic rate of natural increase (r_m d^{-1}), calculated according to the Lotka equation, was 1.375, the net reproductive rate (R_0) 291, the mean generation time (T) 90 h, and the minimum generation time (T_{min}) 73.0 h. The corresponding values for strain MY6 ($n = 72$) were $r_m = 1.460$, $R_0 = 289$, $T = 84$ h, and $T_{min} = 67.3$ h. Peak egg-laying rates in both strains exceeded 140 eggs d^{-1}. Juvenile and early adulthood mortality was negligible. Strain N2 lived, on average, for 16.7 d, while strain MY6 died 2 days earlier; however, differences in survivorship curves were statistically non-significant.

Conclusions. We found no evidence that adaptation to the laboratory altered the life history traits of *C. elegans* strain N2. Our results, discussed in the light of earlier studies on *C. elegans*, demonstrate certain advantages of the hanging drop method in investigations of nematode life cycles. Assuming that its reproducibility is validated in further studies, the method will reduce the inter-laboratory variability of life-history estimates and may ultimately prove to be more convenient than the current standard methods used by *C. elegans* researchers.

Background

Since 1965, when Sydney Brenner chose the free-living nematode *Caenorhabditis elegans* (Maupas, 1900) Dougherty, 1953 as the model animal in which to investigate development and function in a simple nervous system, an enormous amount of work has been done on "the worm". Despite the practical challenge of being tiny to work with at about 1 mm in length, *C. elegans* is easy to breed and maintain, in addition to having a short generation time and lifespan. These features have made *C. elegans* the predominant model organism in biological research. Today, the depth of understanding of the genetics, anatomy, and developmental biology of this organism probably exceeds that of any other animal [1] and it remains the only metazoan in which the entire cell lineage (959 cells) has been traced, from egg to adult [2].

A wide variety of studies have reported life-history traits (LHTs) of *C. elegans*. Such traits include juvenile growth rates, age at sexual maturity, adult body size, age-specific fecundities, total reproduction, generation time, age-specific mortality, mean and maximum lifespan, and the intrinsic rate of natural increase [r_m: 3]. Investigations evaluating the LHTs of *C. elegans* have covered many different aspects, such as inbreeding depression and heterosis [4-7], dietary restriction/supplement [8-12], mutations [13-17], ecotoxicology [18-21], and ageing [review in 22].

In reviewing the relevant literature, we found that the many excellent studies on *C. elegans* make use of quite different approaches in the design of life-cycle experiments. But the high diversity of protocol designs (cultivation temperature, solid vs. liquid media, food quality and quantity, way of data acquisition) hampers comparisons of data from different sources. For example, probably as a result of differing protocol designs, values of *C. elegans* wild-type total fecundity in the literature differ by a factor of up to 8.6 [327 vs. 38: 10,23]. Moreover, data collected under obviously suboptimal culture conditions may be difficult to reproduce.

In an attempt to join the advantages of liquid and solid media in the cultivation of nematodes, we recently developed an easy, fast, and yet precise method to perform life-cycle experiments [24]. In the present work, we adopted this "hanging-drop" method to studies of the wild-type *C. elegans* strain N2. The method's convenience and reliability make its application of interest in many *C. elegans*-related research fields.

Additionally, we aimed to iterate a recent study by Chen et al. [25] which provided evidence that strain N2 has adapted to laboratory conditions with respect to many important demographic parameters. This *C. elegans* strain had been in continuous laboratory culture since the 1940s [6] before it became the geneticists' reference wild-type strain N2 [26]. The influence of inadvertent selection and genetic drift on *C elegans* strains kept in culture is unclear [25]. Since, we compared the N2 strain to a recently isolated strain, MY6, from Münster (Northwest Germany) in order to determine whether the two strains, isolated 700 km and 60 years apart from each other, differed with respect to several LHTs. The specific aim of the study was to supplement existing data on *C. elegans* with complete high-resolution life tables and fecundity schedules and to assess the nematode's age at sexual maturity, mean and

maximum lifespan, net reproductive rate (R_0), total fertility rate (*TFR*), generation time, population doubling time, and intrinsic rate of increase (r_m).

Methods

Two wild-type isolates of *Caenorhabditis elegans*, MY6 and N2, were obtained from the Caenorhabditis Genetics Center (University of Minnesota, St Paul) on NGM [Nematode Growth Medium: 27] agar plates spotted with OP50 (a uracil-requiring mutant of *E. coli*). Strain MY6 had been isolated in July 2002 from a compost heap in Roxel, Münster (Northwest Germany) and frozen within five generations after isolation by H. Schulenberg (Westphalia Wilhelm's University, Münster). Strain N2, isolated from mushroom compost near Bristol (UK) by L.N. Staniland, is the canonical "wild-type" *C. elegans* strain used in laboratories throughout the world. It has been maintained in the laboratory (interrupted by periods of freezing) for about 60 years.

EXPERIMENTAL SET-UP. Three weeks prior to the life-cycle experiments, the two *C. elegans* strains were transferred to NGG [Nematode Growth Gelrite: 24] culture plates seeded with OP50 in order to remove the influence of maternal effects. The preparation and ingredients of NGG are analogous to those of standard NGM, the only modification being the replacement of Bacto-agar by 1.5 g l^{-1} gellan gum, a bacterial exopolysaccharide (Gelrite, Merck & Co., Kelco Division) [28]. The two strains were kept in the exponential growth phase by sub-culturing them onto fresh culture plates every 6 days. Stocks were kept at 20.0°C and life-cycle experiments were carried out at the same temperature. All manipulations were done at room temperature (20±1°C).

The original description of the experimental procedures used to record the reproductive output and lifespan of individual worms can be found in Muschiol & Traunspurger [24]. Briefly, synchronous stage 1 juveniles (J1) of *C. elegans* strains N2 and MY6 ($n = 72$ per strain) were kept individually in hanging 8-µl drops of food medium in the lid of 12-well multiwell plates (Greiner 665102). The food medium consisted of washed *E. coli* OP50 cells resuspended in semi-fluid NGG. The gel-like consistency of the medium was achieved by constant stirring of freshly autoclaved NGG on a magnetic stirrer during the cooling period. This food medium is perfectly suited for life-cycle experiments as its viscosity permits the nematodes to move freely but prevents bacterial cells from accumulating at the bottom of the drop.

Bacterial density was set to 5×10^9 cells ml^{-1} with reference to a previously determined absorption (OD_{600}) vs. cell density curve [24]. The chosen cell density was well-considered, as it is critical in life-cycle experiments to preclude food limitation. Since Schiemer [29], working with *Caenorhabditis briggsae*, reported a somewhat reduced fecundity at food concentrations of 10^9 *E. coli* cells ml^{-1}, we chose to provide a higher concentration of bacteria. However, very high food concentrations may have a detrimental effect, as shown by Johnson et al. [30], who reported a dramatically reduced mean life expectancy of *C. elegans* at *E. coli* densities of 10^{10} cells ml^{-1}.

As soon as the ovaries of the experimental animals began to develop (J4), each individual was transferred to a fresh drop of food medium every 6–24 h (see below) while the previous drop was checked for produced offspring. In *C. elegans*, it is not sufficient to determine offspring in terms of produced eggs because self-progeny brood sizes are determined by the number of self-sperm and the additional oocytes produced are laid unfertilized unless the hermaphrodite is mated [23]. In order to distinguish between sterile and fertile eggs, each transfer of a maternal worm to a fresh food drop was followed by a 24-h-incubation of the previous drop. After this period, all fertile eggs had hatched. The juveniles were relaxed by heating the drop to 80°C and then fixed and stained by the addition of an 8-µl drop of 37% formaldehyde and Rose Bengal (300 µg ml^{-1}). Then, the fixed samples were covered with a circular 18-mm cover slip and counted under a dissecting microscope at 40-fold magnification, using an underlying grid to facilitate counting. The experiment was conducted until the last adult died (day 32). A worm was scored as dead if it ceased to respond to light touch with an eyelash mounted on the tip of an applicator stick and showed a loss of turgor. Dead worms were kept for an additional 24 h, after which they were checked for offspring hatched within the mothers' carcasses. Individuals that were lost during handling were excluded from the data set (n = 3 or 2.1% of total individuals).

TIME RESOLUTION. A species' intrinsic rate of population increase is determined to a much greater extent by the rate of oviposition in the first days of adult life than by the total number of eggs laid in the lifespan of the adult. With each successive day, the contribution of eggs laid to the value of r_m is less [3]. Accordingly, in the experimental determination of oviposition rates, those measured in early adult life should be the most accurate. When reproduction is continuous, as it is in *C. elegans* populations, the continua of time t and age x can be divided into discrete intervals of arbitrary length, thus approximating a continuous-time model to any desired degree of accuracy by a discrete age-class model [31: p. 14]. In nematodes, the length of each age class is usually set to 1 day [e.g., 29]. Since *C. elegans* is an extremely fast-developing species that matures in less than 72 h, age classes of 1 day appeared quite broad to us. Thus, instead, we increased the accuracy by determining oviposition rates every 6 h during early adult life. In order to keep workload on a reasonable scale (576 drops of food medium had to be fixed and checked for offspring daily), temporal resolution was reduced to 12 h after age-class x = 128.5 (h) and to 24 h after x = 293.5. Additionally, the number of replicates was reduced from 72 to 36 per strain after x = 137.5, i.e., when more than 90% of total reproduction was completed.

CREATION OF SYNCHRONOUS COHORTS. To obtain precise fecundity schedules, it is crucial to perform life-cycle experiments with a cohort of highly synchronous individuals. Then, age-specific fecundities are determined between two identical stages of successive generations (egg-egg/J1-J1). In *C. elegans*, however, the time of egg deposition is a poor indicator of the egg's developmental stage, as the time from egg fertilization to egg-laying can vary considerably depending on the physiological condition and ontogenetic stage of the animal to be assayed [32: p. 157]. Egg-laying can even be delayed to the extent that hatching occurs

within the uterus ['bagging' or matricidal hatching: 33] or instantly after the eggs have been laid. Accordingly, the time of hatching rather than the time of egg deposition is a better indicator of ontogenetic age [4]. Thus, we started our experiment with cohorts of juveniles that had hatched within a narrow time span (less than 4 h). However, the experimental set-up applied in this investigation recorded fecundity in terms of fertile eggs per time interval (see above). In order to account for this discrepancy (start of the experiment with juveniles, but offspring counted as eggs), we determined the average time required by a freshly laid egg to hatch (T_{hatch}). The (virtual) starting point of our experiment (age $x = 0$) was then defined as the hatching time of the examined juveniles minus T_{hatch}.

ESTIMATION OF HATCHING TIME T_{HATCH}. Adults and stage 4 juveniles of both *C. elegans* strains were randomly picked from exponentially growing NGG culture plates and transferred to 20-µl drops of semi-fluid NGG containing OP50 at a density of 5×10^9 cells ml^{-1}. Ten individuals were pooled in one drop; ten drops per strain were prepared and incubated at 20.0 °C. After 15 h, the drops were fixed, stained, and examined under a dissecting microscope at 40-fold magnification. An underlying grid facilitated counting of the eggs (N_{egg}) and hatched juveniles (N_{juv}). The average hatching time was calculated as the proportion of unhatched eggs to total eggs laid multiplied by the experimental time ($T = 15$ h): $N_{egg}/(N_{egg}+N_{juv})\times T$. If, for example, one third of all laid eggs had been hatched, it was assumed that these hatched eggs had been laid, on average, during the first third of the 15 h whereas all eggs laid in the last two thirds did not have enough time to hatch – resulting in an average hatching time of 10 h. T_{hatch} was calculated separately for each strain as the arithmetic mean of the 10 drops per strain.

DATA PROCESSING. Life tables and fecundity schedules are difficult to interpret on their own because they hide the dynamic behaviour of a population behind a mass of detail [34: p. 151]. For this reason, the data were summarized according to the upper-order parameters generation length, doubling time, and intrinsic rate of natural increase (r_m) using the fundamental equation of population dynamics:

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

r_m = intrinsic rate of natural increase
x = time [d]
l_x = age-specific survival probability
m_x = age-specific fecundity

This equation is called the Euler equation, [31: p. 23] and is also frequently referred to as the Lotka equation, after Lotka [35], who applied it to human demography. As the equation does not lend itself to a direct solution, it has to be estimated by iteration (substituting successive trial values of r_m in the equation until the left-hand side sums to 1).

The intrinsic rate of natural increase (r_m) is the growth rate of a population that has a stable age distribution and grows in an unlimited environment. Since r_m integrates the entire age schedules of survival and fertility into a single measure, it measures fitness in age-structured populations [31]. In this study, the high time resolution of 6–24 h per age-class and the consequential extensive life tables made it necessary to determine r_m using a Microsoft®

Visual Basic (6.0) macro in Excel (2007), which may be obtained from the authors on request. The net reproductive rate ($R_0 = \sum l_x m_x$) is defined as the average number of offspring that an individual in a population will produce in its lifetime. Unlike the total fertility rate (*TFR*), R_0 depends on age-specific mortality rates. Since the concept of generation time is considered as rather arbitrary and slippery in the context of age-structured populations, several alternative measures have been proposed [see 31 for a general survey]. Here, the values T_0, T_1, and T were compared, as defined by the following equations:

$$T_0 = \sum x l_x m_x / \sum l_x m_x$$
$$T_1 = (\ln R_0)/r_m$$
$$T = \sum x e^{-r_m x} l_x m_x$$

r_m = intrinsic rate of natural increase
x = time [d]
l_x = age-specific survival probability
m_x = age-specific fecundity
R_0 = net reproductive rate

T_0 (also referred to as T_c, the cohort generation time) is the mean age at reproduction of a cohort of females. T_1 refers to that period of time necessary for a population growing at a constant rate r_m to increase by the factor R_0. T is the mean age of the mothers of a set of newborn individuals in a population with a stable age distribution.

To analyse discrete and non-paired data, a non-parametric Mann–Whitney U-test or, when appropriate, parametric T-test was used. The assumption of homogeneous variances in the T-test was confirmed with Brown & Forsythe's test. The survival of the two *C. elegans* strains was compared using the log-rank test. Total numbers of fertile eggs were correlated with total lifespan using Spearman's rank correlation coefficient. All statistical analyses were carried out using the Statistica software package (v7.0, StatSoft Inc. 2004).

Results

HATCHING. The comparison of hatching times (time from egg deposition to hatch) revealed a small but significant (*d.f.* = 38, t = -5.2, $p < 0.001$) difference between the two *C. elegans* strains: At 20.0°C, freshly laid eggs of strain N2 needed, on average, 7.3 h to hatch (range 5.0 – 10.6; Table 1) while eggs of strain MY6 needed 9.9 h (range 6.8 – 11.9).

SURVIVORSHIP. Juvenile and early adulthood mortality was negligible in both strains: All 141 juveniles observed in this investigation reached sexual maturity and 99% of them reached an age older than 1 week (173.5 h, Table 2). At that point of time, reproduction was mostly completed, as 93.4% (N2) and 97.5% (MY6) of total reproductive output had already been laid. Plotting survivorship against time (Figure 1) generated type I survivorship curves [36; see ecology textbooks] typical of species with low mortality rates until near the end of the lifespan. During most of the experiment, strain N2 evinced a somewhat lower mortality than strain MY6, as reflected by a slower flattening of its survivorship curve. However, the log rank test revealed no significant difference between them (z = 1.68; p = 0.09).

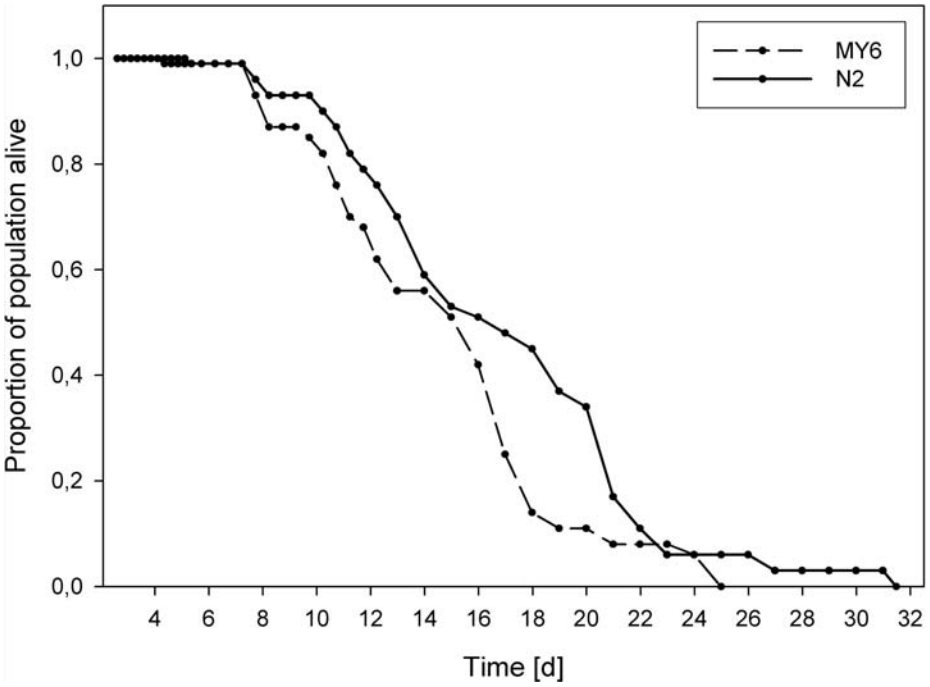

Figure 1 – Hermaphrodite survivorship curves of *C. elegans* strains MY6 and N2. Lines represent fraction surviving at the given interval after egg deposition (age x = 0). Note that mortality among juveniles and young adults was virtually zero. Population sizes were n = 72 (MY6) and n = 69 (N2) until age 5.7 d and n = 36 (MY6 and N2) thereafter.

Although maximum lifespan is a poor statistical variable, it is included here because of its wide use as a species-specific parameter in the literature on ageing [37]. The longest-lived individual of strain N2 died on day 32, while individuals of strain MY6 reached a maximum age of 25 days. In contrast to maximum lifespan, mean lifespan is a reliable quantitative marker for the study of mutations and genetic lines that extend life in *C. elegans* [5, and references therein]. In the present study, strain N2 lived for 16.7 days, while strain MY6 died 2 days earlier (14.7; Table 1); this difference was statistically non-significant ($d.f.$ = 70, t = -1.62, p = 0.11).

REPRODUCTION. A total of 2832 data points were obtained from the experiment, and a total of 39,075 offspring were counted to obtain the productivity data. Figure 2 shows the fecundities of both *C. elegans* strains at 20.0°C. Strain N2 laid the first eggs at a mean age of 73.0 h (range 68.5 – 80.5; n = 69), whereas with a minimum generation time of only 67.3 h (range 62.5 – 74.5; n = 72), strain MY6 matured 6 h faster (Table 1). The difference between these minimum generation times [T_{min}: 38] was statistically significant (U = 897; p < 0.001).

Table 1 - Life-cycle parameters of *C. elegans* strains MY6 and N2. Values represent arithmetic means ± s.d. All time intervals were measured from the moment of egg deposition.

	N2	MY6	p-level
T_{hatch} [h]	7.3 ± 1.6	9.9 ± 1.5	< 0.001
lifespan [d]	16.7 ± 5.8	14.7 ± 4.9	0.11
T_{min} [h]	73.0 ± 4.4	67.3 ± 3.1	< 0.001
T_{rate} [h]	108.2 ± 9.0	105.5 ± 9.2	0.08
rate [N d^{-1}]	141 ± 19	144 ± 22	0.42
$T_0/T_1/T$ [h]	115/99/90	106/93/84	-
r_m d^{-1}	1.375	1.460	-
PDT [h]	12.1	11.4	-

T_{hatch} = time from egg deposition to hatching; T_{min} = age at first egg deposition; T_{rate} = age at maximum rate of egg-laying; **rate** = maximum rate of egg-laying; **total** = total number of eggs produced by individual worms; T_0, T_1, T = alternative measures of generation time; r_m = intrinsic rate of natural increase; **PDT** = population doubling time

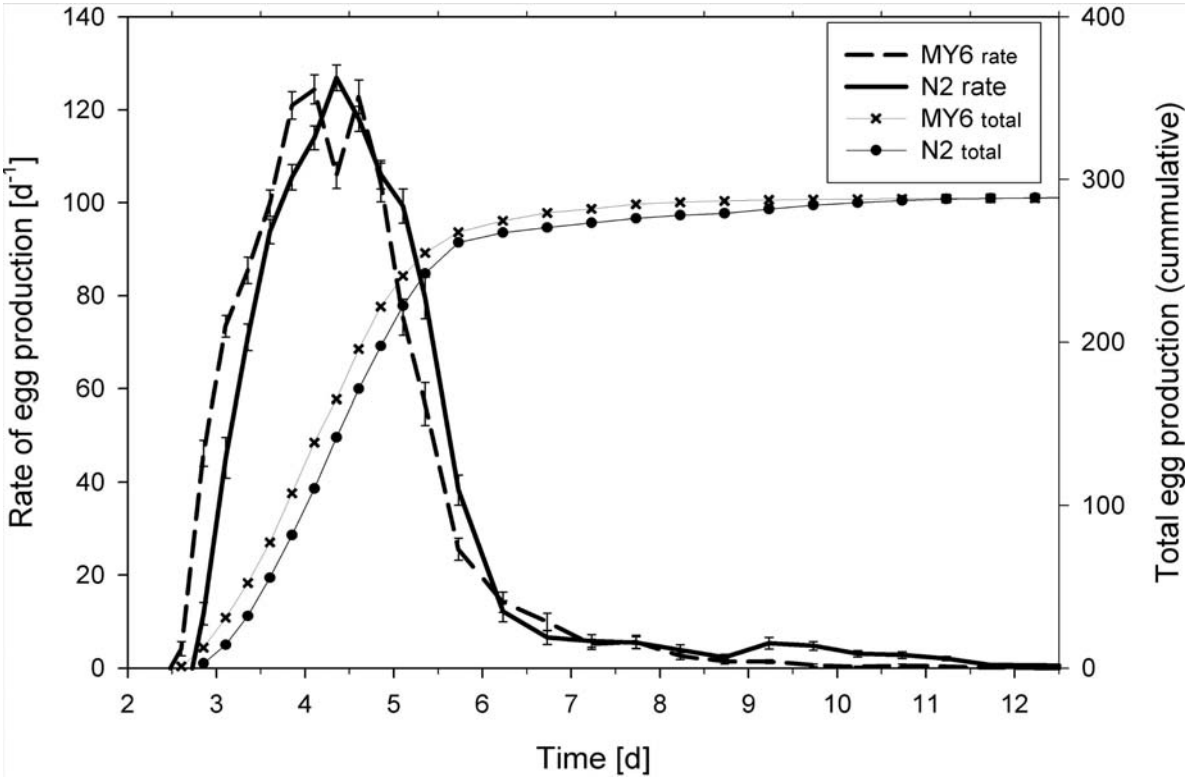

Figure 2 - Fecundities of *C. elegans* strains MY6 and N2. Population sizes were n = 72 (MY6) and n = 69 (N2) until age 5.7 d and n = 36 (MY6 and N2) thereafter (s.e. bars).

Once reproduction had begun, the egg-laying rate steeply increased, reaching a maximum at an average age of 108.2 h (N2) and 105.5 h (MY6) (Table 1). The maximum egg-laying rates observed in this investigation were 141 (N2) and 144 (MY6) eggs d^{-1}. Neither maximum egg-laying rate (*d.f.* = 139, t = 0.80, p = 0.42) nor the age of the nematode when the former was reached (*d.f.* = 139, t = -1.75, p = 0.08) differed significantly between the two strains. Interestingly, the egg-laying rate of strain MY6 showed a bipartite maximum, interrupted by a slightly decreased egg-laying rate (Figure 2). The total number of fertile eggs produced during the entire lifespan was 291 for strain N2 and 289 for strain MY6. As suggested by Davies & Hart [10], the total number of produced eggs was plotted against total lifetime in order to estimate whether the two measures were related to each other. In both strains, the distribution appeared rather random (not shown here). In strain N2, Spearman's rank correlation coefficient was not significantly different from zero (r_s = 0.15, p = 0.38), indicating neither positive nor negative correlation. In strain MY6, a weak but significant positive correlation was found (r_s = 0.44, $p < 0.01$).

The values of the three alternative measures of mean generation time, T_0, T_1, and T, are given in Table 1. According to Charlesworth [31], T is preferred over other measures of generation time. It is the mean age of the mothers of a set of newborn individuals in a population with stable age distribution. The T value was 90 h for strain N2 and 84 h for strain MY6.

Life tables and fecundity schedules for the reproductive period of the two investigated *C. elegans* strains are given in Table 2. The total fertility rate (*TFR*, i.e., the total number of

offspring a hermaphrodite would have, on average, if individuals were to live to the maximum age) of strain N2 was 295, and that of strain MY6 290. The net reproductive rate (R_0, which depends on age-specific mortality rates) of N2 was 289, and that of MY6 291. This minute difference between the *TFR* and R_0 values of the two strains is the result of the negligible juvenile and young adult mortalities (Table 2). Calculation of the intrinsic rate of natural increase (r_m) yielded a value of 1.375 for strain N2 and 1.460 for strain MY6, corresponding to a population doubling time ($t = \ln(2)/r_m$) of 12.1 and 11.4 h, respectively.

Discussion

METHOD. Our study not only supplements previous investigations but also, more importantly, provides a comfortable approach to establish large cohort life tables at any desired temporal resolution. The hanging drop method overcomes several inconveniences associated with other methods employed in nematode life-cycle studies.

Solid Media**.** In the majority of life-cycle studies, *C. elegans* is maintained on NGM agar plates spotted with *E. coli* OP50. This is the standard method for cultivating *C. elegans*, but it holds several disadvantages. First of all, it is very difficult to control bacterial density on solid media. In the light of the obvious dependence of LHTs on food density, this appears rather unsatisfactory. One approach to overcome this limitation was developed by Tain et al. [9], who spread OP50 cells at different concentrations on NGM plates and killed them by exposure to UV light after varying time intervals. However, this approach only allowed a rough estimate of food conditions ("excess, high, and low"). Additionally, the effect of a diet consisting of dead bacteria is unclear. At least, *C. elegans* showed considerably reduced pharyngeal pumping rates when fed heat-killed OP50 [39]. The method used in the present investigation provides a fast and accurate method to adjust the medium to any desired food concentration. Moreover, when the influence of drugs, toxicants, or other compounds is to be tested, the test substance can be added to the medium immediately at the beginning of the experiment, thus minimizing its incorporation and metabolization by the bacteria.

A further difficulty in life-cycle experiments on solid media is the poor visibility of eggs and small juveniles on the uneven bacterial lawn, especially along the edge of a plate. Accordingly, Peters et al. [40] were confronted with a large error when counting eggs, which led them to conclude that egg counts on plates are likely to be substantial underestimates. The poor visibility of small juveniles within the bacterial lawn on agar plates is the probable reason that in most studies on the fecundity of *C. elegans* offspring was counted at the late juvenile to adult stage [e.g., 16,17,41]. However, early juvenile mortality and juveniles entering the dauer stage remain very difficult to record.

The problem can be reduced to some extent by the substitution of agar with gellan gum in the preparation of solid media [42]. The high transparency of such media considerably facilitates visual screening of culture plates. Moreover, as the strength of NGG media is determined by the concentration of divalent cations, low concentrations of the nontoxic

chelating agent ethylenediaminetetraacetic acid (EDTA) break the bonds responsible for the gel matrix, yielding a liquid suspension that easily passes through sieves with a mesh size down to 10 µm. This method permits the liquefaction of whole culture plates such that the nematodes can be readily extracted in virtually infinite numbers from cultures without adhering residues of culture medium [24]. However, in life-cycle experiments, the hanging drop method still has the advantage that all reproductive output is concentrated in one tiny drop of food medium that can be scanned quickly and accurately at high magnification.

Table 2 - Abbreviated life table and fecundity schedule of the reproductive periods of *C. elegans* strains MY6 and N2. Pivotal time intervals x are related to the average time of egg deposition. Population sizes were n = 69 (N2) and n = 72 (MY6) until x = 137.5 h and n = 36 (MY6 and N2) thereafter.

		N2			MY6		
x	D	l_x	m_x	l_x*m_x	l_x	m_x	l_x*m_x
62.5	6				1.00	1.04	1.04
68.5	6	1.00	2.93	2.93	1.00	11.56	11.56
74.5	6	1.00	11.30	11.30	1.00	18.36	18.36
80.5	6	1.00	17.77	17.77	1.00	21.36	21.36
86.5	6	1.00	23.45	23.45	1.00	24.99	24.99
92.5	6	1.00	26.38	26.38	1.00	30.24	30.24
98.5	6	1.00	28.49	28.49	1.00	31.10	31.10
104.5	6	0.99	32.04	31.72	1.00	26.47	26.47
110.5	6	0.99	29.81	29.51	1.00	30.69	30.69
116.5	6	0.99	26.78	26.51	1.00	26.10	26.10
122.5	6	0.99	25.07	24.82	1.00	18.85	18.85
128.5	6	0.99	20.09	19.89	0.99	14.34	14.20
137.5	12	0.99	19.35	19.16	0.99	12.92	12.79
149.5	12	0.99	6.17	6.11	0.99	7.17	7.10
161.5	12	0.99	3.34	3.31	0.99	5.03	4.98
173.5	12	0.99	2.94	2.91	0.99	2.60	2.57
185.5	12	0.96	2.85	2.74	0.93	3.03	2.82
197.5	12	0.93	2.12	1.97	0.87	1.48	1.29
209.5	12	0.93	1.27	1.18	0.87	0.81	0.70
221.5	12	0.93	2.88	2.68	0.87	0.81	0.70
233.5	12	0.93	2.58	2.40	0.85	0.37	0.31
245.5	12	0.90	1.72	1.55	0.82	0.15	0.12
257.5	12	0.87	1.61	1.40	0.76	0.37	0.28
269.5	12	0.82	1.28	1.05	0.70	0.24	0.17
504.6			3.09	1.84		0.10	0.06
∑			295	291		290	289

x = pivotal age in hours; *D* = length of age class in hours; l_x = age-specific survival probability; m_x = age-specific fecundity. $\sum m_x$ = total fertility rate (*TFR*); $\sum l_x*m_x$ = net reproductive rate (R_0).

In life- cycle studies carried out on agar plates, a substantial proportion of the population is usually lost or killed unintentionally during transfer or dies by desiccation after the worms crawl up the wall of the plate. This ‘lost’ component may account for up to 58% of total

replicates [43: note 29]. But high artificial mortalities bias estimates of average lifespan, since long-living individuals have a higher probability of being censored from the survivorship data. In the present investigation, death by desiccation never occurred because the worms cannot overcome the hanging drop's surface tension. In total, only 2.1% (n = 3) of the total individuals were accidentally lost during handling, which is very low compared to the more than 3000 individual transfers conducted.

Liquid Media**.** Some of the inconveniences that accompany life-cycle experiments on solid media can be avoided by using liquid media, an approach that was established by Johnson & Wood [6]. Although the authors found that the lifespan of different stocks covaried, which they ascribed to "uncontrolled environmental effects", their approach established a basis for numerous seminal studies. Yet, the problem of environmental effects causing significant variation in fecundity and/or lifespan between replicates persisted [e.g., 5,44]. Friedman & Johnson [45] were forced to include a reference strain in all experiments due to this variation. Keightley et al. [17] additionally observed significant measurer effects, which they ascribed to differences in the relative levels of experience among the measurers carrying out the worm assays. It is known that the fecundity of *C. elegans* in liquid culture is generally lower than on solid media [5], a problem that Brooks [44] related to the lower oxygen availability in liquid. Other observations have included a far more rapid undulation behaviour [46, after 47] and reduced food consumption [48] in liquid media. Shook & Johnson [47] even found no correlation at all between survival on solid media and previous measures of survival in liquid media. In our laboratory, we have noted that it is difficult to adjust bacterial density in liquid culture because both bacteria and nematodes accumulate at the bottom of the vial, resulting in a higher *de facto* cell concentration in the nematodes' surroundings. In our opinion, the extreme conditions present within the slurry bacterial sediment might explain many of the difficulties associated with liquid culture.

As a matter of course, the high reproducibility of data obtained with the hanging drop method remains to be demonstrated. Yet, the fact that many of the above-listed problems associated with life-cycle experiments on solid or liquid media can be avoided in semi-fluid NGG make it a promising approach.

TEMPORAL RESOLUTION – Concerning the required temporal resolution of life-cycle studies, our data demonstrate the necessity of sufficient small age classes, at least during the first days of reproduction. In fast-reproducing species like *C. elegans*, minor differences in maturation time and other LHTs may be overlooked if the temporal resolution is set to one day or even longer. This can be illustrated with a simple example, taking as the basis the presented life table of strain N2 (Table 2), with the only modification being a slightly coarser temporal resolution at the very beginning of the reproductive period. Therefore, we pooled the fecundities of four 6-h age-classes (pivotal ages 56.5–74.5 h) into a single 24-h-age-class (pivotal age 65.5 h; net reproductive rate 14.2) and kept everything else equal. A calculation of the intrinsic growth rate based on this only slightly coarser life table yielded a value of r_m = 1.406, corresponding to a 0.26-h underestimate of strain N2's population doubling time. This

difference may initially be negligible but the difference between predicted and observed population sizes reaches 13% after only 4 days of unlimited growth. There are several scenarios thinkable in which a coarse temporal resolution in a life-cycle experiment will result in even more substantial errors of r_m. Systematic errors that lead to over- or underestimation of the age of the cohort, such as may result from inaccurate estimates of hatching times, are even more problematic: When we introduced a systematic 12-h error into the life table of strain N2, r_m rose to 1.640, resulting in a 189% difference between predicted and observed population sizes after 4 days of unlimited growth.

Table 3 - Exemplary studies reporting on lifespan and/or fecundity of *C. elegans*. All listed studies were conducted at 20°C with E. coli OP50 as food source. Underlined values indicate lifespans determined in liquid media.

	Strain	Medium	Lifespan[d]	Fecundity
Keightley et al. [17]	N2	solid	13.4	248
Halligan et al. [16]	N2	MYOB	11.9	258
Keightley & Caballero [59]	N2	MYOB	14.0	255[a]
Chen et al. [58]	N2	NGM	14.8	285.6
Tain et al. [9][b]	N2	NGM	15	244
Shook & Johnson [47]	N2	NGM	13.3	287
Johnson & Hutchinson [5]	N2	NGM	–	255
Hodgkin & Barnes [23]	N2	NGM	–	327
this investigation	N2	NGG	16.7[c]	291
Shook et al. [51][d]	N2	NGM / S	15.7	254
Friedman & Johnson [45][d]	N2	NGM / S	20.5 – 24.1	104 – 342
Johnson & Hutchinson [5]	N2	S	17.9	115
Brooks [44]	N2	S	15.2 – 20.6	94 – 238
Johnson & Wood [6][e]	N2	S	18.2 – 22.7	–
Johnson & Hutchinson [5]	6 WI	S	14.8 – 20.8	–
Hodgkin & Barnes [23]	15 WI	NGM	–	235 – 353
Dolgin et al. [4][f]	10 WI	NGM	13.3 – 14.9	228 – 262
this investigation	MY6	NGG	14.7[c]	289

MYOB = Modified Youngren's only bactopeptone (solid); **NGM** = nematode growth medium (solid); **S** = S basal medium plus cholesterol (liquid); **WI** = wild isolates, other than N2;
[a] value interpolated from Figure 2; [b] values interpolated from Figs. 1A, 1C; [c] includes time from egg deposition to hatching, see Table 1; [d] fecundity was assayed on NGM, survival in S medium; [e] *E. coli* B/r or OP50 used as food; [f] values interpolated from Figs. 2A, 2C.

DIFFERENCES BETWEEN STRAINS N2 AND MY6. An interesting result of this study is that strains N2 and MY6, isolated 700 km and 60 years apart from each other, do not differ with respect to most life-cycle parameters. No significant differences between the strains were found in key parameters such as total fecundity, lifespan, as well as age at and magnitude of maximum fecundity (Table 1). Likewise, the general shape of the two survivorship curves (Figure 1) was very similar, the minute differences being statistically non-significant. This finding is consistent with results from Johnson & Hutchinson [5: Table 1], who compared the lifespan of seven different *C. elegans* wild-type strains in liquid culture and concluded that all seven strains had similar life expectancies. Similarly, Sutphin & Kaeberlein [12] found no evidence that adaptation to the laboratory has altered the survival of *C. elegans* strain N2

compared to five different *C. elegans* wild-type strains grown on NGM agar. In the present study, the only significant differences between strains N2 and MY6 were a somewhat later deposition of the first egg (T_{min}) and a somewhat faster hatching time (T_{hatch}: Table 1) in N2. A longer hatching time, however, does not necessarily imply differences in the time span between fertilization and hatching. Instead, it may simply be due to the fact that strain N2 retains its eggs longer, depositing them at a later ontogenetic stage. In contrast, differences in T_{min} are biologically important since time was measured between identical stages (freshly deposited eggs, see Methods) of successive generations. The observed 5.7-h-later time at which N2 first reproduced accounts for the calculated difference in the strains' population doubling times, 12.1 h for N2 and 11.4 h for MY6. This difference is quite small, especially when compared to the population doubling times of other free-living nematodes [e.g., 101 h in *Poikilolaimus*: 24]. However, we can calculate the ratio of population sizes after some time *t*, given that both N2 and MY6 start with the same population size [49: p. 37]:

$$\frac{N_{MY6}(t)}{N_{N2}(t)} = e^{(r_{MY6} - r_{N2})t}$$

It is clear that, as time progresses, the above ratio will increase, with MY6 becoming numerically more and more dominant in a theoretically combined population. After one week of unlimited growth, the ratio will be 1.8, rising to 3.3 after 2 weeks and 12.8 after one month. However, the assumption of extended periods of unlimited growth is unrealistic for natural populations: Within one month, an exponentially growing *C. elegans* MY6 population in stable age distribution (r_m = 1.460) is theoretically capable of increasing ($N_{(t)} = N_{(0)}e^{rt}$) by a factor of 10^{19}. Converted into wet weight production, this means that, after one month, the progeny of a single *C. elegans* individual (wet weight roughly 1 µg) could potentially have a wet weight of 10^7 metric tons.

Why are the differences between the two strains negligible? As the Bristol variety of *C. elegans* had been in continuous laboratory culture for decades before being used to found the reference wild-type strain N2 [26], we expected distinct differences between this laboratory strain and a recently isolated natural strain such as MY6.

A possible explanation for the finding that strains N2 and MY6 nevertheless barely differed in their life histories can be found in the worm's particular androdioecious breeding system. Wild-type populations reproduce almost exclusively as self-fertilizing hermaphrodites, especially since the mating efficiency of the rare males is poor compared to a congeneric dioecious species [7]. In contrast to populations of outbreeding organisms, which maintain variant loci by selection for heterozygotes, selfing *C. elegans* populations are driven to homozygosity and consequently face strong selection against less-fit variants. This selection purges the population of deleterious recessive alleles [50] and consequently, wild-type *C. elegans* populations are already homozygous at most or all loci. Under these circumstances, it is plausible that selection for laboratory conditions and/or bottleneck effects act weaker in *C. elegans* than one would expect in genetically more diverse species. Indeed,

laboratory populations of *C. elegans* face no or negligible inbreeding depression and crosses between laboratory strains show no heterosis (hybrid vigor) effects [4,5,7]. The homozygosity of the mainly self-fertilizing hermaphrodite *C. elegans* therefore well explains our finding that 60 years of laboratory culture apparently have not left a distinct trace in the Bristol strain's life cycle.

In conflict with our results, Chen et al. [25] found significant differences in several important demographic properties between strain N2 and a wild-caught *C. elegans* isolated from snails. Both total fertility and early survival of the wild-caught worm were lower; consequently, it obtained a considerably lower intrinsic growth rate than strain N2 ($r_m = \ln(\lambda)$ = 1.249 vs. 1.348). The authors concluded that their results support the hypothesis that N2 has adapted to laboratory conditions. Apparently, there is a need for further studies, involving other strains from different geographic regions, to satisfactorily answer the question whether the Bristol strain N2 has undergone laboratory evolution.

In this context one should also mention that Johnson & Hutchinson [5] presented experimental evidence that the Berg BO (var. Bergerac) strain of *C. elegans* reproduces better in liquid culture than on solid agar plates. The authors argued that this characteristic might be the result of laboratory evolution, with an increased fecundity arising from the conditions under which this special stock was maintained i.e., axenic liquid culture. This interesting observation may offer opportunities to investigate the evolution of LHTs in the controlled environment of the laboratory.

COMPARISON TO EARLIER LIFE-CYCLE STUDIES. The results presented in this investigation are in good agreement with previous studies on *C. elegans*: The values for LHTs such as age at sexual maturity, mean and maximum lifespan, and total reproduction lie within the range reported by previous studies, as is discussed in detail below. However, a comprehensive review of the enormous number of studies dealing with various aspects of the life cycle of *C. elegans* is far beyond the scope of this paper. The vast majority of researchers in *C. elegans* are geneticists, and some 200 or more genes have now been found to cause hypomorphic (reduced function) mutations that extend life in the worm. According to Henderson et al. [22], *C. elegans* ageing studies have been summarized in more than 50 reviews alone, omitting other aspects of the *C. elegans* life cycle. We restrict the discussion of our results to a few general conclusions, keeping the focus on wild-type LHTs.

Survivorship – The survivorship curves of strains N2 and MY6 (Figure 1) did not differ significantly from each other and are strikingly similar to those presented by Johnson et al. [30: Figure 1A]. On average, the two *C. elegans* strains in this study lived for somewhat longer than 2 weeks (Table 1), a lifespan that lies within the range reported by earlier studies. However, published lifespan estimates (Table 3) have suggested that *C. elegans* strain N2 tends to live longer in liquid (about 18.9 d) than on solid media (about 13.7 d). Our estimate (16.7 d) lies in the middle. Yet, there seems to be no simple relationship between culture medium and lifespan, since recombinant-inbred (RI) strains of *C. elegans* even showed a highly significant longer survival on agar than in liquid [47,51]. Generally, a long life is not

necessarily an indicator of favourable conditions. For example, dietary restriction reduces fecundity and growth, but increases longevity in many organisms including *C. elegans* [9]. The reason for the relatively large variation in lifespan estimates between different studies is unclear, but may at least partly be related to suboptimal culture conditions and methodical problems, as discussed above.

Although average and maximum lifespan estimates of *C. elegans* are naturally of tremendous importance in ageing research, they are relatively tedious from the ecologist's point of view because of the negligible juvenile and early adulthood mortality of *C. elegans*. All 141 juveniles observed in this investigation reached sexual maturity and 99% of them became older than 1 week (173.5 h, Table 2), i.e., until reproduction was essentially completed (Figure 2). But the intrinsic rate of natural increase (r_m) is independent of lifespan as long as death occurs after the reproductive period. Life history theory suggests that the absence of significant selective pressure allows the accumulation of mutations that increase the probability of death beyond the reproductive period. In other words, selection favours those alleles that allow the individual to survive just long enough to reproduce competitively [disposable soma theory of ageing: see 52]. The evolution of genes influencing ageing is strongly dependent on, and in fact may be largely an accidental by-product of, selection of other LHTs [47].

Total fecundity and egg-laying rate. As Birch [3] pointed out, the intrinsic rate of population increase is determined to a much greater extent by the rate of oviposition at the very beginning of adult life than by the total number of eggs laid in the lifespan of the adult. With each successive day, the contribution of eggs laid to the value of r_m decreases. In order to maximize population growth, a shortening of maturation time can thus be more advantageous than an increase in total egg production. Protandric *C. elegans* hermaphrodites face a trade-off between maturation time and total egg-production. Sperm is produced exclusively at the very beginning of the reproductive period and is subsequently used to self-fertilize oocytes. If the hermaphrodites do not mate, they face sperm limitation because many more oocytes than sperm are produced [53]. But an increase in sperm production is necessarily associated with a delay in the onset of egg production, which offsets the benefit of higher total fecundity. This trade-off was investigated by Hodgkin & Barnes [23], who provided empirical evidence that a mutant producing 50% more sperm is outcompeted by wild-type worms. The authors additionally investigated brood sizes of 15 recent natural isolates of *C. elegans* obtained from a variety of geographical locations. The strains were that similar in fecundity (Table 3) that the authors concluded that "a brood size of about 300 self-progeny is a universal optimum" for *C. elegans* populations all over the world. As a matter of fact, total fecundities of the two strains investigated in this study were very close to this number (N2 = 291, MY6 = 289).

Shook & Johnson [47: Table 2] found that *C. elegans* strain N2 needed 67.1 h from hatch to first reproduction, a value that differs by only 1.4 h from our results (Table 1: T_{min} - T_{hatch} = 65.7 h). However, strain MY6 only needed 57.4 h from hatch to first reproduction, an

advantage that was only partly counterbalanced by its longer hatching time. As the two strains have almost identical total fecundities, the earlier onset of reproduction in MY6 cannot be the result of reduced sperm production. Interestingly, the egg-laying rate of strain MY6 showed a bipartite maximum, interrupted by a slightly decreased egg-laying rate (Figure 2). Considering the high number of replicates (n = 72), this temporary decrease cannot be regarded as an artefact and one might speculate that an adaptation of resource allocation in MY6 is somehow related to its faster maturation.

The maximum egg-laying rates observed in this investigation were somewhat above 140 eggs d^{-1} (Table 1). Similar rates were reported by Tain et al. [9: Figure 1B] and Hodgkin & Barnes [23: p. 22]. However, Johnstone et al. [54: Table 1a] reported considerably higher rates of 194 eggs d^{-1}. The reason for this substantial difference may well be a behavioural peculiarity of *C. elegans* which we observed during our experiments: When a gravid worm is touched in order to remove it from the culture medium, it instantly empties its uterus and lays up to a dozen eggs. This 'stress deposition' potentially causes substantial overestimates of egg-laying rates, especially if only a single short time interval is investigated [3 h in 54]. We found that the use of an eyelash mounted on the tip of an applicator stick is very useful to reduce this source of error. With some practice, the worm is usually caught at the first attempt; eggs are thereby laid within the food medium adhering to the eyelash and transferred together with the worm.

POPULATION GROWTH – Despite the wealth of excellent studies of survival, and the smaller number of studies of reproduction, there are almost no estimates of the population growth rate of *C. elegans* [25, p. 1060]. However, as shown in the seminal paper of Hodgkin & Barnes [23, see above], the intrinsic population growth rate is a far more useful statistic than lifespan or fecundity alone, since r_m integrates the entire age schedules of survival and fertility into a single measure. Intrinsic growth rates are also indispensable for reliable estimates of nematode biomass production, such as are needed in food-web analyses [55].

Vassilieva & Lynch [15: Table 1] calculated an intrinsic population growth rate of 1.35 for *C. elegans* (strain N2 on NGM, 20°C, OP50). However, this estimate may have been biased as the authors reported total fecundities of 170, which is less than 60% of the ~ 300 offspring usually produced by *C. elegans* [23, this investigation]. A subsequent publication [56] had the same limitation ($r_m \approx 1.28$; fecundity ≈ 175, interpolated from control line regressions in Figure 2). It was therefore surprising that the estimates of r_m nonetheless differed only slightly from our calculations (N2: 1.375).

Keightley & Eyre-Walker [57: Figure 2a] also determined intrinsic growth rates of *C. elegans* strain N2. Unfortunately, their data are difficult to compare to our estimate because r_m values were presented in terms of a frequency distribution of individual strains. However, their r_m values were consistently higher than our estimate and in many of the lines (~ 40%) the intrinsic growth rates clustered around 1.53. In subsequent publications, Keightley and co-workers [e.g., 16,17,40] also calculated intrinsic growth rates but did not present them because r_m values were further transformed into the upper-order parameter "relative fitness."

Although this fitness measure has certain advantages, it makes comparison between different studies impossible.

By using a Leslie matrix, Shook & Johnson [47: Table 2] estimated the expected population growth of *C. elegans* strain N2. From their derived factor "population growth" = e^{100r} = 686, the daily population growth rate can be calculated as ln(686)/100×24 = 1.567. This value is somewhat higher than our r_m (1.375) and corresponds to a 1.5-h-shorter population doubling time. However, it was not clear whether the authors accounted for hatching times—apparently fecundity was determined as the number of fertile eggs laid within a certain time interval since hatching of the mother.

The most extensive study of *C. elegans*' LHTs conducted thus far was by Chen et al. [58], who monitored survival and reproduction of 1000 individuals of strain N2, maintained at 20°C on NGM seeded with OP50. The intrinsic growth rate ($r_m = \ln(\lambda) = 1.348$) reported by the authors was quite close to our estimate (1.375).

In summary, the hanging drop method, in which nematodes are cultured in a drop of semi-fluid NGG, appears to be a useful strategy to assess the LHTs of the investigated strains. This approach has considerable potential in studies of other nematode species and in benthic meiofauna in general. Unpublished experiments in our laboratory indicate a high reproducibility; moreover, the method is simple enough to be applied by unpractised researchers. Given that further studies can validate the high reproducibility of data obtained with the hanging drop method, it may considerably simplify work in many *C. elegans*-related research fields.

Authors' contributions

DM and FS contributed equally to the conception, design and accomplishment of the study. DM conducted statistical analysis and drafted the manuscript. WT conceived of the study, and participated in its design and coordination and revised the manuscript.

All authors read and approved the final manuscript.

Acknowledgements

Nematode strains used in this work were provided by the Caenorhabditis Genetics Center. This investigation was financially supported by the German Research Foundation (TR 445/3-1).

References

1. Riddle DL, Blumenthal T, Meyer BJ, Priess JR: *C. elegans II.* Plainview, NY: Cold Spring Harbor Laboratory Press; 1997.
2. Sulston JE, Schierenberg E, White JG, Thomson JN: **The embryonic cell lineage of the nematode *Caenorhabditis elegans*.** *Dev Biol* 1983, **100(1)**:64-119.
3. Birch LC: **The intrinsic rate of natural increase of an insect population.** *J Anim Ecol* 1948, **17**:15-26.
4. Dolgin ES, Charlesworth B, Baird SE, Cutter AD: **Inbreeding and outbreeding depression in *Caenorhabditis* nematodes.** *Evolution* 2007, **61**:1339-1352.
5. Johnson TE, Hutchinson EW: **Absence of strong heterosis for life span and other life history traits in *Caenorhabditis elegans*.** *Genetics* 1993, **134(2)**:465-474.
6. Johnson TE, Wood WB: **Genetic analysis of life span in *Caenorhabditis elegans*.** *Proc Natl Acad Sci USA* 1982, **79(21)**:6603-6607.
7. Chasnov JR, Chow KL: **Why are there males in the hermaphroditic species *Caenorhabditis elegans*?** *Genetics* 2002, **160(3)**:983-994.
8. Houthoofd K, Braeckman BP, Johnson TE, Vanfleteren JR: **Life extension via dietary restriction is independent of the Ins/IGF-1 signalling pathway in *Caenorhabditis elegans*.** *Exp Gerontol* 2003, **38(9)**:947-954.
9. Tain LS, Lozano E, Sáez AG, Leroi AM: **Dietary regulation of hypodermal polyploidization in *C. elegans*.** *BMC Dev Biol* 2008, **8**:28.
10. Davies KG, Hart JE: **Fecundity and lifespan manipulations in *Caenorhabditis elegans* using exogenous peptides.** *Nematology* 2008, **10(1)**:103-112.
11. Larsen PL, Clarke CF: **Extension of life-span in *Caenorhabditis elegans* by a diet lacking coenzyme Q.** *Science* 2002, **5552**:120-123.
12. Sutphin GL, Kaeberlein M: **Dietary restriction by bacterial deprivation increases life span in wild-derived nematodes.** *Exp Gerontol* 2008, **43(3)**:130-135.
13. Azevedo RBR, Keightley PD, Lauren-Maatta C, Vassilieva LL, Lynch M, Leroi AM: **Spontaneous mutational variation for body size in *Caenorhabditis elegans*.** *Genetics* 2002, **162(2)**:755-765.
14. Estes S, Ajie BC, Lynch M, Phillips PC: **Spontaneous mutational correlations for life-history, morphological and behavioral characters in *Caenorhabditis elegans*.** *Genetics* 2005, **170(2)**:645-653.
15. Vassilieva LL, Lynch M: **The rate of spontaneous mutation for life-history traits in *Caenorhabditis elegans*.** *Genetics* 1999, **151(1)**:119-129.
16. Halligan DL, Peters AD, Keightley PD: **Estimating numbers of EMS-induced mutations affecting life history traits in *Caenorhabditis elegans* in crosses between inbred sublines.** *Genet Res* 2003, **82(3)**:191-205.
17. Keightley PD, Davies EK, Peters AD, Shaw RG: **Properties of ethylmethane sulfonate-induced mutations affecting life-history traits in *Caenorhabditis elegans* and inferences about bivariate distributions of mutation effects.** *Genetics* 2000, **156(1)**:143-154.
18. Donkin SG, Williams PL: **Influence of developmental stage, salts and food presence on various end-points using *Caenorhabditis elegans* for aquatic toxicity testing.** *Environ Toxicol Chem* 1995, **14(12)**:2139-2147.
19. Höss S, Juttner I, Traunspurger W, Pfister G, Schramm KW, Steinberg CEW: **Enhanced**

growth and reproduction of *Caenorhabditis elegans* (Nematoda) in the presence of 4-nonylphenol. *Environ Pollut* 2002, **120(2)**:169-172.

20. Traunspurger W, Haitzer M, Höss S, Beier S, Ahlf W, Steinberg C: **Ecotoxicological assessment of aquatic sediments with *Caenorhabditis elegans* (Nematoda) - A method for testing liquid medium and whole-sediment samples.** *Environ Toxicol Chem* 1997, **16(2)**:245-250.
21. Williams PL, Dusenbery DB: **Aquatic toxicity testing using the nematode *Caenorhabditis elegans*.** *Environ Toxicol Chem* 1990, **9(10)**:1285-1290.
22. Henderson ST, Rea SL, Johnson TE: **Dissecting the process of aging using the nematode *Caenorhabditis elegans*.** In *Handbook of the biology of aging*. Edited by Masoro EJ, Austad SN. Amsterdam: Elsevier Academic Press; 2006:360-399.
23. Hodgkin J, Barnes TM: **More is not better: Brood size and population growth in a self-fertilizing nematode.** *Proc R Soc Lond B* 1991, **246(1315)**:19-24.
24. Muschiol D, Traunspurger W: **Life cycle and calculation of the intrinsic rate of natural increase of two bacterivorous nematodes, *Panagrolaimus* sp. and *Poikilolaimus* sp. from chemoautotrophic Movile Cave, Romania.** *Nematology* 2007, **9(2)**:271-284.
25. Chen J, Lewis EE, Carey JR, Caswell H, Caswell-Chen EP: **The ecology and biodemography of *Caenorhabditis elegans*.** *Exp Gerontol* 2006, **41(10)**:1059-1065.
26. Brenner S: **The genetics of *Caenorhabditis elegans*.** *Genetics* 1974, **77**:71-94.
27. Sulston J, Hodgkin J: **Methods.** In *The nematode Caenorhabditis elegans*. Edited by Wood WB. Plainview, NY: Cold Spring Harbor Laboratory Press; 1988:587-606.
28. Eyre MJ, Caswell EP: **Sterile culture of *Rotylenchulus reniformis* on tomato root with gellan gum as a supporting medium.** *J Nematol* 1991, **23(2)**:229-231.
29. Schiemer F: **Food dependence and energetics of freeliving nematodes II. Life history parameters of *Caenorhabditis briggsae* (Nematoda) at different levels of food supply.** *Oecologia* 1982, **54(1)**:122-128.
30. Johnson TE, Friedman DB, Foltz N, Fitzpatrick PA, Shoemaker JE. **Genetic variants and mutations of *Caenorhabditis elegans* provide tools for dissecting the aging processes.** Harrison DE. *Genetic effects on aging II*. 101-127. 1990. Caldwell, NJ, Telford.
31. Charlesworth B: *Evolution in age-structured populations.* Cambridge: Cambridge University Press; 1994.
32. Hope IA: *C. elegans: A practical approach.* Oxford: Oxford University Press; 1999.
33. Luc M, Taylor DP, Netscher C: **Endotokia matricida and intra-uterine development and hatching in nematodes.** *Nematologica* 1979, **25(2)**:268-274.
34. Caughley G, Gunn A: *Conservation biology in theory and practice.* Cambridge, Mass.: Blackwell Science; 1996.
35. Lotka AJ: *Elements of physical biology.* Baltimore, MD: Williams & Wilkins; 1924.
36. Pearl R: *The rate of living: being an account of some experimental studies on the biology of life duration.* London: University of London Press; 1928.
37. Masoro EJ, Austad SN: *Handbook of the biology of aging.* Amsterdam: Elsevier Academic Press; 2006.
38. Vranken G, Heip C: **Calculation of the intrinsic rate of natural increase, r_m, with *Rhabditis marina* Bastian 1865 (Nematoda).** *Nematologica* 1983, **29(4)**:468-477.
39. Wood JG, Rogina B, Lavu S, Howitz K, Helfand SL, Tatar M, Sinclair D: **Sirtuin activators mimic caloric restriction and delay ageing in metazoans.** *Nature* 2004,

430:686-689.

40. Peters AD, Halligan DL, Whitlock MC, Keightley PD: **Dominance and overdominance of mildly deleterious induced mutations for fitness traits in *Caenorhabditis elegans*.** *Genetics* 2003, **165(2)**:589-599.
41. Knight CG, Azevedo RBR, Leroi AM: **Testing life-history pleiotropy in *Caenorhabditis elegans*.** *Evolution* 2001, **55(9)**:1795-1804.
42. Ferris H, Lau S, Venette RC: **Population energetics of bacterial-feeding nematodes: respiration and metabolic rates based on CO_2 production.** *Soil Biol Biochem* 1995, **27(3)**:319-330.
43. Arantes-Oliveira N, Apfeld J, Dillin A, Kenyon C: **Regulation of life-span by germ-line stem cells in *Caenorhabditis elegans*.** *Science* 2002, **5554**:502-504.
44. Brooks A: **Genetic specification of life span and self-fertility in recombinant-inbred strains of *Caenorhabditis elegans*.** *Heredity* 1991, **67(1)**:19-28.
45. Friedman DB, Johnson TE: **A mutation in the age-1 gene in *Caenorhabditis elegans* lengthens life and reduces hermaphrodite fertility.** *Genetics* 1988, **118(1)**:75-86.
46. Croll NA: **Components and patterns in the behavior of the nematode *Caenorhabditis elegans*.** *J Zool* 1975, **176**:159-176.
47. Shook DR, Johnson TE: **Quantitative trait loci affecting survival and fertility-related traits in *Caenorhabditis elegans* show genotype-environment interactions, pleiotropy and epistasis.** *Genetics* 1999, **153(3)**:1233-1243.
48. Avery L, Thomas JH: **Feeding and defecation.** In *C. elegans II*. Edited by Riddle DL. Plainview, NY: Cold Spring Harbor Laboratory Press; 1997:679-716.
49. Roff DA: *The evolution of life histories: Theory and analysis.* New York: Chapman & Hall; 1992.
50. Lande R, Schemske DW: **The evolution of self-fertilization and inbreeding depression in plants. I. Genetic models.** *Evolution* 1985, **39(1)**:24-40.
51. Shook DR, Brooks A, Johnson TE: **Mapping quantitative trait loci affecting life history traits in the nematode *Caenorhabditis elegans*.** *Genetics* 1996, **142(3)**:801-817.
52. Kirkwood TBL: **The disposable soma theory of aging.** In *Genetic effects on aging II*. Edited by Harrison DE. Caldwell, NJ: Telford; 1990:9-20.
53. Ward S, Carrel JS: **Fertilization and sperm competition in the nematode *Caenorhabditis elegans*.** *Dev Biol* 1979, **73**:304-321.
54. Johnstone DB, Wei AG, Butler A, Salkoff L, Thomas JH: **Behavioral defects in *C. elegans* egl-36 mutants result from potassium channels shifted in voltage-dependence of activation.** *Neuron* 1997, **19(1)**:151-164.
55. Muschiol D, Markovic M, Threis I, Traunspurger W: **Predatory copepods can control nematode populations: A functional-response experiment with *Eucyclops subterraneus* and bacterivorous nematodes.** *Fundam Appl Limnol* 2008, **172(4)**:317-324.
56. Vassilieva LL, Hook AM, Lynch M: **The fitness effects of spontaneous mutations in *Caenorhabditis elegans*.** *Evolution* 2000, **54(4)**:1234-1246.
57. Keightley PD, Eyre-Walker A: **Terumi Mukai and the riddle of deleterious mutation rates.** *Genetics* 1999, **153(2)**:515-523.
58. Chen JJ, Senturk D, Wang JL, Müller HG, Carey JR, Caswell H, Caswell-Chen EP: **A demographic analysis of the fitness cost of extended longevity in *Caenorhabditis elegans*.** *Journals of Gerontology Series A -Biological Sciences and Medical Sciences* 2007, **62(2)**:126-135.
59. Keightley PD, Caballero A: **Genomic mutation rate for lifetime reproductive output and lifespan in *Caenorhabditis elegans*.** *Proc Natl Acad Sci USA* 1997, **94**:3823-3827.

11.4. Manuscript IV

Muschiol, D., Marković, M., Threis, I. & Traunspurger, W., 2008: **Predator-prey relationship between the cyclopoid copepod *Diacyclops bicuspidatus* and a free-living bacterivorous nematode**. – *Nematology* **10:** 55-62.

Nematology, 2008, Vol. 10(1), 55-62

Predator-prey relationship between the cyclopoid copepod *Diacyclops bicuspidatus* and a free-living bacterivorous nematode

Daniel MUSCHIOL *, Mirjana MARKOVIĆ, Ilka THREIS and Walter TRAUNSPURGER

University Bielefeld, Animal Ecology, Morgenbreede 45, 33615 Bielefeld, Germany

Received: 7 May 2007; revised: 10 August 2007
Accepted for publication: 14 August 2007

Summary – The potential of copepods as predators of free-living nematodes was tested by presenting *Diacyclops bicuspidatus*, a common holarctic cyclopoid copepod, with *Panagrolaimus* sp. as prey in the laboratory. *Diacyclops bicuspidatus* readily fed on nematodes of all size classes, including prey longer than itself. No size preference was observed. Handling times varied between a few seconds and several minutes, depending on the size of the prey. At different prey densities, the feeding rates of *D. bicuspidatus* followed a type II functional response. Starved *D. bicuspidatus* consumed up to 45.1 nematodes in 2 h, equivalent to 43.5% of the copepod's body mass. Indications that nematophagous nutrition is common among freshwater copepods are discussed.

Keywords – benthos, food chain, foraging behaviour, functional response, Holling type, invertebrate predators, meiofauna prey, *Panagrolaimus*.

Copepods are found in a wide variety of aquatic environments, ranging from the benthic, littoral and pelagic waters of lakes and oceans to swamps, large rivers, temporary ponds, phytotelmata and interstitial and subterranean systems (Williamson & Reid, 2001). They have also been collected from many semi-terrestrial habitats, such as moist soil and arboreal mosses (Reid, 1986). At present, over 11 500 species of copepods have been described (Humes, 1994). Free-living freshwater representatives are found in four (Harpacticoida, Cyclopoida, Calanoida and Gelyelloida) of the ten 'orders'. In Europe, Gelyelloida are known from only two species that are found in karstic systems (Galassi *et al.*, 2002). Calanoids are primarily planktonic, whilst cyclopoids and harpacticoids are generally associated with substrates in littoral or benthic habitats. However, a few species of cyclopoids are planktonic and may contribute substantially to the zooplankton biomass of many lakes and ponds (Williamson & Reid, 2001). The Cyclopoida comprise 12 'families', of which Cyclopidae is well represented by the 900 species and subspecies found in freshwater habitats (Dole-Olivier *et al.*, 2000). Due to their high abundance and wide distribution, copepods play a pivotal role in aquatic food webs, both as primary and secondary consumers as well as a major source of food for many larger invertebrates and vertebrates. Copepods display a large variety of feeding behaviours and food requirements, and some species can modulate their diet depending on food availability. Cyclopoids are grasping feeders that generally eat larger foods than calanoids. Most copepods are omnivorous to some extent, with foods ranging from detritus and pollen to algae, bacteria, ciliates, rotifers, other small crustaceans, dipteran larvae, and even larval fish (Williamson & Reid, 2001, and references therein). Many species are cannibalistic (Brandl, 1998, and references therein).

Copepods develop from fertilised eggs from which a larval stage called a nauplius hatches. There are six naupliar stages followed by six copepodid stages. Of the latter, the last is the adult, which does not continue to moult. The greatest change in morphology occurs between the last naupliar and first copepodid stage. Although the associated niche shift is still not understood (Galassi *et al.*, 2002), nauplii and early copepodids are generally more herbivorous (Williamson & Reid, 2001). The high morphological variation in mouthparts among closely related copepod species suggests that trophic diversification is important in niche separation (Hopp & Maier, 2005). Most studies on copepod diets have focused on marine habitats (*e.g.*, Lampitt, 1978; Zhang *et al.*, 2006) or planktonic communities of lentic freshwater bodies (review in Brandl, 1998). By contrast, little is known on copepods that feed in benthic, interstitial and

* Corresponding author, e-mail: daniel.muschiol@uni-bielefeld.de

Also available online - www.brill.nl/nemy

subsurface habitats (Galassi *et al.*, 2002). This is somewhat surprising, as copepods constitute a major portion of the biomass and productivity of freshwater systems (Williamson & Reid, 2001). In the benthic communities of lakes, it is not uncommon to find densities of copepods in excess of 10 000 and, occasionally, as high as 250 000 individuals/m^2 (Strayer, 1985; Sarvala, 1998). Such meiofaunal communities are regularly dominated by nematodes in terms of species number and abundance (Traunspurger, 2002). Although nematodes and copepods co-occur in large numbers in many natural habitats, possible predator-prey interactions have been widely disregarded (Lehman & Reid, 1992).

Encouraged by scattered reports in the literature of copepods foraging on free-living nematodes (Marcotte, 1977; Rocha & Bjornberg, 1988; Lehman & Reid, 1992; Reversat *et al.*, 1992), we conducted a classical predator-prey experiment with *Diacyclops bicuspidatus*, a common Holarctic cyclopoid copepod, and *Panagrolaimus* sp., a free-living bacterivorous nematode. *Diacyclops bicuspidatus* appeared particularly suited for this kind of investigation because it has already been proposed as a predator of benthic animals due to its epi- or endobenthic way of living (Maier, 1990). The following questions were addressed: *i*) is *Diacyclops bicuspidatus* a potential predator of nematodes and, if so, what is its maximum consumption rate? and *ii*) how are these consumption rates linked to prey density?

Materials and methods

Predator and prey source

Monoxenic laboratory cultures of the bacterivorous nematode *Panagrolaimus* sp., originally extracted from Movile Cave, Romania, were established on NGG media seeded with OP50 (a uracil-requiring mutant of *Escherichia coli*) as described in Muschiol and Traunspurger (2007). The nematodes were extracted from exponentially growing culture plates after fluidification of the medium with ethylenediaminetetraacetic acid (EDTA; 0.005 M for 30 s) by the use of sieves with a mesh size of 5 μm. As a potential copepod predator, *D. bicuspidatus* Claus, 1857 were obtained from a small eutrophic artificial pond (diam. 2 m; N052°02.20′ E008°29.45′) in the vicinity of University Bielefeld, north-west Germany. Species determination revealed that the investigated copepod population did not belong to one of the two European varieties/subspecies *D. b. odessanus* or *D. b. limnobius* (Glatzel, pers. comm.). Only females retained on a mesh size of 200 μm were used for the experiments.

Experimental procedures

In order to determine a suitable exposure time for functional response experiments and to find the appropriate prey-density range, prey in various densities and sizes were presented and consumption was directly observed under a dissection microscope at 30× magnification. The functional response experiments were conducted at 20°C in 12-well multiwell plates (Greiner Bio-one, Kremsmuenster, Austria). As Stemberger (1986) estimated a gut passage time of 7-8 h for *Diacyclops*, the copepods were starved for 3 days prior to the experiment to ensure a uniform hunger level. To avoid cannibalism, they were maintained individually. Each well was filled with 3 ml of water from the organism's natural habitat (1.2 μm filtered). Actively moving nematodes were accurately counted and transferred into the wells with a micropipette connected by rubber tubing to a mouthpiece. A single starved copepod was then added to each arena. The experiment was terminated with a hot (60°C) mixture of formaldehyde (4% final concentration) and Rose Bengal (300 μg ml^{-1}). The copepod was then removed from the multiwell, mounted onto a glass slide, and its biomass was estimated. The remaining nematodes were transferred to a Petri dish and recounted under 30× magnification. An underlying grid facilitated counting. Partially devoured nematodes were classified by size according to the residual proportion of the body (90, 80, 70%, *etc*.).

Functional response experiments

The consumption rate of adult *D. bicuspidatus* (body length: 980.2 ± 80.0 μm; n = 34) was investigated with respect to prey density by offering seven different densities of *Panagrolaimus* sp. (10, 25, 50, 75, 100, 150, 200; n = 5 each). Five wells of each nematode density without copepods served as controls. The experiments were terminated after 120 min. To take into account the number of nematodes lost during handling and recounting, the number of nematodes recovered after exposure to the predator was deducted from the average number of nematodes recovered in the controls. At each prey density, Grubbs' test (1969) was used to determine upper outliers in the resulting predation rates; these were excluded from further calculations (n = 1 or 2.9% of total replicates). As a model

of the predator's functional response, Holling's (1959a, b) disc equation was applied:

$$N_e = aTN_0/(1 + aT_hN), \qquad \text{(Equation 1)}$$

where N_e is the number of prey consumed, a is the attack rate, T is the total time prey were exposed to predation, N_0 is the number of prey presented, and T_h is the time required to handle the prey. Non-linear curve fitting with least squares regression (Statistica v7.0, StatSoft Inc., 2004) was used to fit the data to Holling's disc equation and to estimate the values of a and T_h. The effect of prey density on the consumption rate was tested by means of non-parametric rank-based Kruskal-Wallis one-way ANOVA (Statistica v7.0, StatSoft, 2004).

Biomass calculations

Randomly chosen representatives of the nematodes (n = 60), as well as all of the copepods used in the experiments, were fixed in 4% formaldehyde and high-resolution digital images were taken under a dissection microscope (Jenoptik ProgRes C12plus; Zeiss Stemi SV11 Apo). Body lengths were determined using calibrated image-analysis software (ImageJ v1.34s). The lengths of the nematodes were measured by assuming the worm's tail to be an idealised cone, as specified by Andrássy (1956). Nematode biomasses were then calculated according to a published regression relating body weight W and length L ($W = aL^b$), with units of μg fresh weight and mm length: $W = 0.2085L^{4.0915}$ (Muschiol & Traunspurger, 2007). The dry weight of the nematodes was assumed to be 20% of their wet weight (Sohlenius, 1979). Copepod body lengths (n = 34) were measured from the anterior of the cephalothorax to the base of the caudal furca. Copepod biomasses were then determined according to the regression of Dumont *et al.* (1975) for adult female Cyclopoida without eggs: $W = 1.1 \times 10^{-7}L^{2.59}$ (W = μg dry weight; L = μm body length).

Results

Direct observations of predator-prey interactions showed that female *D. bicuspidatus* readily fed on nematodes of all sizes and without a size preference. Small nematodes (length < 0.6 mm) were generally eaten in 1-2 s, in a manner similar to sucking in spaghetti, whereas the consumption of large nematodes (length up to 1.7 mm) sometimes took as long as several minutes, interrupted by periods of inactivity. Thus, it was difficult to determine an average handling time based on direct observations. The efficiency of capturing, overwhelming and devouring the nematodes suggested that this type of feeding is not uncommon. The ability of *D. bicuspidatus* to overwhelm and consume adult nematodes of considerable size, often longer than its own body length, was quite remarkable.

The number of *Panagrolaimus* sp. consumed by *D. bicuspidatus* increased curvilinearly with prey density. The more prey individuals presented to the predator, the shorter the searching time, until the latter was relatively short compared to the handling time and a plateau was reached (Fig. 1). The effect of prey density on the predation rate was significant (Kruskal-Wallis ANOVA: df = 6; H = 26.8; $P < 0.001$). The maximum number of *Panagrolaimus* eaten per 120 min in any microcosm was 45.1 for the 100 density and 43.8 for the 150 density. Very high prey densities reduced consumption rates, as the averaged predation rate at the 200 density was lower than that at the 150 density. Consumption rates at different prey densities are best described by Holling's (1959b) type II functional response (Fig. 1). Parameter estimates of the attack rate (a) and the handling time (T_h) in Equation 1 were 1.874×10^{-4} and 174.2 s, respectively. Estimation of the average dry weight of *Panagrolaimus* yielded a value of 0.060 ($\pm$ 0.038) μg. The highest observed biomass consumption was thus 2.71 μg dry weight for the 100 density. This value corresponds to 43.5% of the average dry weight estimated for *D. bicuspidatus* (6.23 $\pm$ 1.35 μg).

Discussion

Although nematodes and copepods co-occur in high numbers in many natural habitats, reports of nematophagous copepods are extremely scarce. In a review of the invertebrate predators of nematodes, Small (1988) did not mention copepods. Likewise, in their review on trophic relationships in freshwater meiofauna, Schmid and Schmid-Araya (2002, Appendix 1) did not report copepods as predators of nematodes. However, in the present investigation, *D. bicuspidatus* proved to be a voracious predator of free-living nematodes in the laboratory. In nature, the species is very common throughout Europe – in ponds, puddles, and the littoral of lakes (Einsle, 1993). Lapesa *et al*. (2002) investigated the predatory behaviour of *D. bicuspidatus odessanus*, and classified it as a cruising predator specialised in attacking slow-moving prey. Maier (1990) investigated a *D. bicuspidatus* population from a shallow eutrophic lake in southern Germany

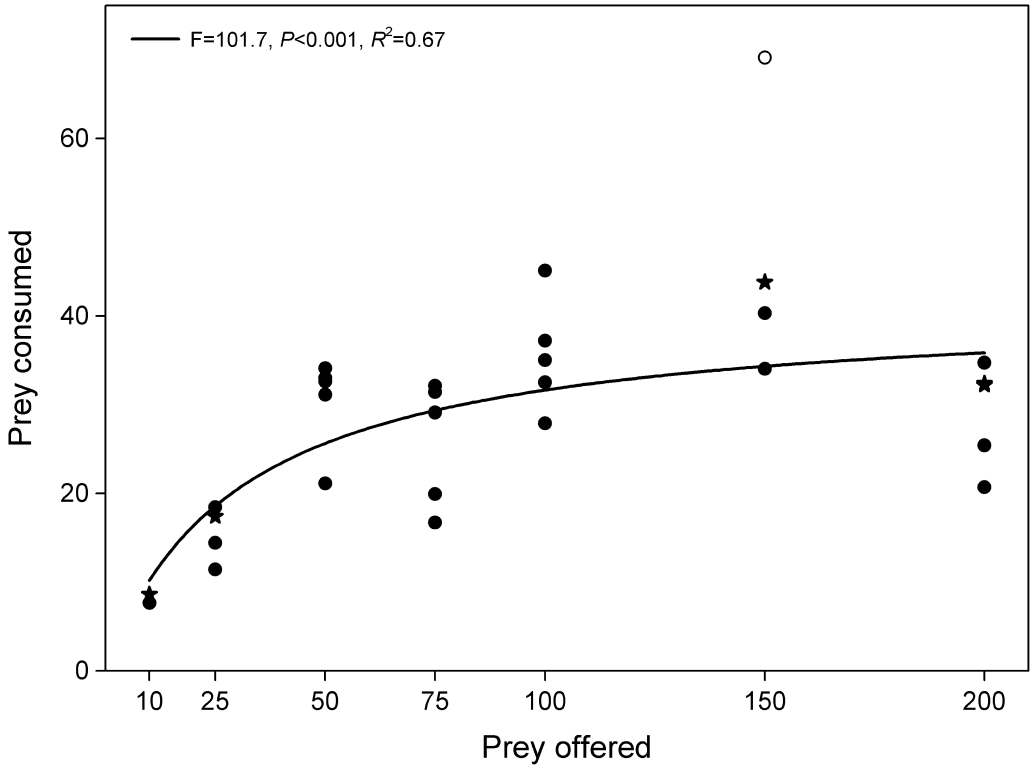

Fig. 1. *Functional response of* Diacyclops bicuspidatus *feeding on* Panagrolaimus *sp. Each replicate (●) is shown. Stars indicate same consumption rate in two or more replicates. One replicate (○) was defined as an outlier and excluded from further calculations. The regression line was fitted by non-linear least squares regression using Equation 1, given in the text.*

and found that a great majority of the adults live in the mud or close above it and, thus, "may prefer benthic living prey". Our results suggest that this assumption is correct. Moreover, as most cyclopoids and harpacticoids are omnivorous to some extent and are generally associated with substrates in littoral or benthic habitats, the consumption of nematodes might be more common within the Copepoda than generally assumed.

Why are there so few reports on copepods feeding on nematodes? A probable explanation is that much of our knowledge on the nutrition of copepods originates from examinations of their gut contents. Two seminal papers using this approach were published 50 years ago by Fryer (1957a, b), who carefully examined the gut contents and described the details of mouthparts of both carnivorous and herbivorous littoral and bottom-dwelling copepod species. The author reported the occurrence of unidentifiable "mush" whose colour varied from grey to a rich brown in carnivorous species. He suggested that these properties derived from the consumption of soft-bodied and quickly disintegrating animals. Elbourn (1966) reported that *Cyclops strenuus* fed only on the soft parts of dead adult females of *Daphnia* by squeezing out their skeletal parts. Therefore, although these predators were carnivorous, "they would show no distinguishable animal remains in their guts". In the soft-bodied and transparent nematodes, the situation is probably analogous. Thus, we regard it unlikely that examination of gut contents is an appropriate technique to reveal nematophagous nutrition within the copepods. Moreover, it is well known that gut content examination can be misleading, as stressed copepods show a tendency to regurgitate and defecate, which may result in a large proportion of empty stomachs (Elbourn, 1966), and because food items have different passage times in the gut (Zhang *et al.*, 2006). Clarke (1978) emphasised the limitations of gut analysis in assessing diet. Probably as a consequence of these difficulties, the only reports on nematophagous copepods known to the authors have derived from behavioural observations in the laboratory.

Several publications have dealt with the ingestion of parasitic mermithid nematodes by copepods (*e.g.*, Platzer

& Mackenzie-Graham, 1980; Achinelly *et al.*, 2003). However, these observations may not be generally applicable to non-parasitic nematodes, since mermithids are good swimmers that actively search their host in the water column and thus are more exposed to predation (Dang *et al.*, 2005). Moreover, the larval stages of other parasitic nematodes, such as *Camallanus trispinosus*, or *Dracunculus medinensis*, enter the alimentary canal of their intermediate copepod hosts *via* the mouth without damage and as a normal feature of their life history (Moorthy, 1938; Fryer, 1957a). To the best of our knowledge, the literature contains only four reports of copepod predation on free-living or plant-parasitic nematodes and only two of them focused on cyclopoids:

1. *Phyllognathopus viguieri* is a widely distributed harpacticoid copepod reported to inhabit numerous freshwater and semi-terrestrial habitats. Lehman and Reid (1992) found that this species is capable of preying on seven species of plant-parasitic nematodes as well as on *Steinernema scapterisci*, *Rhabditis* sp. and *Butlerius* sp. Predation rates were up to 6.9 second-stage juveniles of *Meloidogyne incognita* per hour.

2. *Tisbe furcata*, a marine benthic harpacticoid, can be a voracious carnivore capable of devouring juvenile nematodes (Marcotte, 1977). The author concluded that the organism's carnivorous habit may help to explain the inverse relationships that have been reported between the densities of *T. furcata* and nematodes. Moreover, Dahms and Pottek (1992) related the elongate mandible of *T. furcata* to it supposed predatory feeding behaviour.

3. Rocha and Bjornberg (1988) reported that a continental soil- and leaf litter-dwelling cyclopoid, *Allocyclops silvaticus*, feeds on nematodes in culture dishes.

4. Another continental copepod, the epibenthic pond-dwelling cyclopoid *Paracyclops affinis*, was observed to be a predator of juveniles of *Heterodera sacchari* (Reversat *et al.*, 1992). The rate of ingestion was as high as 375 juveniles per copepod per day. However, the authors concluded that this predatory behaviour was due to experimental conditions and is not common in nature.

Density dependence of feeding rates

In the majority of investigations (*e.g.*, Lampitt, 1978; Brandl & Fernando, 1978; Stemberger, 1986; Williamson, 1986), the intensity of foraging by copepods increased with prey density (but see Landry, 1978). This general pattern turned out to be the case also for *D. bicuspidatus* foraging on *Panagrolaimus* sp. By contrast, Anderson (1970) pointed out that feeding rates of predacious cyclopoids were influenced more by hunger than by prey abundance. While this is certainly true (a well-fed predator is unlikely to hunt), starvation of the copepod prior to the functional response experiments in this study ensured a uniform level of hunger, and the experiment was sufficiently short largely to exclude satiation effects. The ingestion rates at the highest prey density (200) were significantly lower than those at the 150 density. Based on our direct observations, this finding may have indicated that very high prey densities confused the copepods or disturbed their feeding on captured prey. Similarly, Jamieson (1980) reported that the predation rates of *Mesocyclops leuckarti* (Cyclopoida) were extremely variable at high prey densities. Also, high densities of the nematode *Pelodera teres* seemed to confuse the predatory tardigrade *Macrobiotus richtersi* and led to a decreased consumption rate (Hohberg & Traunspurger, 2005). Nonetheless, observations made under artificial laboratory conditions may not be relevant in nature. In order to clarify the role of nematode predation through copepods, future experiments under more natural conditions should be helpful. In particular, it has been shown (Beier *et al.*, 2004) that sediments of different grain sizes alter the effectiveness of invertebrate predation on nematodes.

Handling time

Direct observation of predator-prey interactions revealed highly variable handling times depending on the size of the captured nematode. This observation is consistent with literature reports. Handling times reaching 30 min or more for large prey were reported by Fryer (1957a), Gilbert and Williamson (1978), Lampit (1978) and Williamson (1980). Relatively small prey were reported to be consumed very fast, between 2 and 7 s (Williamson, 1980), or so fast that "capture and ingestion appear to occur in one movement" (Lampitt, 1978). The amount of time needed by the copepod to consume slender nematodes in this investigation (1-2 s) was often so short that it seemed as if prey were ingested mostly unchewed. The considerable length of these quickly consumed prey (300 μm and more), which exceeded the length of the copepod's stomach, did not seem to hinder consumption. In this context, Fryer's (1957b) observation of 380 μm-long filamentous algae in the stomach of *Eucyclops macruroides* (body length 1.2 mm) is relevant. Due to the highly variable handling times, the estimated handling time (174.2 s) in Equation 1 should be considered as an approximate average.

CONSUMED BIOMASS

The amount of prey killed in this investigation – up to 43.5% of the copepod's dry mass in 2 h – was astonishingly large. However, this finding is consistent with literature reports. For example, Fryer (1957a) stressed the enormous amount of food that can be crammed into a cyclopoid stomach. The biomass of prey killed daily by various carnivorous copepods may range from 0.1 to more than one times the predator's biomass (review in Brandl & Fernando, 1975). On a mixture of algal and invertebrate prey, *Cyclops kolensis* even ingested a daily dry mass equivalent to 143% of its body mass (Adrian & Frost, 1992). However, one has to consider that not all killed prey is necessarily ingested (sloppy feeding) and not all ingested prey is assimilated. Copepods feeding on large prey lose large amounts of dissolved organic carbon (DOC) by sloppy feeding, while no DOC is produced when the prey is small relative to the copepod and thus swallowed whole (Møller, 2007, and references therein).

In summary, the functional response of *D. bicuspidatus* indicated that this predator is able to consume large numbers of nematodes. As nematodes and copepods can be quite frequent in soils and are generally dominant components of the majority of marine and freshwater meiobenthic communities, this finding is of interest for the construction of carbon and energy budgets. If a trophic linkage between nematodes and copepods is more common in aquatic systems than generally assumed, it might provide an important benthic-pelagic coupling between microbial carbon, endobenthic nematodes, epibenthic copepods, and pelagic fish predators.

Acknowledgements

Species determination of the copepod was confirmed by Thomas Glatzel, Oldenburg. Determination of the nematode was confirmed by Walter Sudhaus, Berlin. This investigation was financially supported by the Deutsche Forschungsgemeinschaft (TR 445/3-1).

References

ACHINELLY, M.F., MICIELI, M.V. & GARCÍA, J.J. (2003). Pre-parasitic juveniles of *Strelkovimermis spiculatus* Poinar & Camino, 1986 (Nematoda: Mermithidae) predated upon by freshwater copepods (Crustacea: Copepoda). *Nematology* 5, 885-888.

ADRIAN, R. & FROST, T.M. (1992). Comparative feeding ecology of *Tropocyclops prasinus mexicanus* (Copepoda, Cyclopoida). *Journal of Plankton Research* 14, 1369-1382.

ANDERSON, R.S. (1970). Predator-prey relationship and predation rates for crustacean zooplankters from some lakes in Western Canada. *Canadian Journal of Zoology* 48, 1229-1240.

ANDRÁSSY, I. (1956). Die Rauminhalts- und Gewichtsbestimmung der Fadenwürmer (Nematoda). *Acta Zoologica Hungarica* 2, 1-15.

BEIER, S., BOLLEY, M. & TRAUNSPURGER, W. (2004). Predator-prey interactions between *Dugesia gonocephala* and free-living nematodes. *Freshwater Biology* 49, 77-86.

BRANDL, Z. (1998). Feeding strategies of planktonic cyclopoids in lacustrine ecosystems. *Journal of Marine Systems* 15, 87-95.

BRANDL, Z. & FERNANDO, C.H. (1975). Investigations on the feeding of carnivorous cyclopoids. *Internationale Vereinigung für Theoretische und Angewandte Limnologie: Verhandlungen* 19, 2959-2965.

BRANDL, Z. & FERNANDO, C.H. (1978). Prey selection by cyclopoid copepods *Mesocyclops edax* and *Cyclops vicinus*. *Internationale Vereinigung für Theoretische und Angewandte Limnologie: Verhandlungen* 20, 2505-2510.

CLARKE, N.V. (1978). The food of adult copepods from Lake Kainji. *Freshwater Biology* 8, 321-326.

DAHMS, H.-U. & POTTEK, M. (1992). *Metahuntemannia* Smirnov, 1946 and *Talpina* gen. nov. (Copepoda, Harpacticoida) from the deep-sea of the high Antarctic Weddell Sea with a description of eight new species. *Microfauna Marina* 7, 7-78.

DANG, G.T.T., PACHECO-PEREZ, R. & PLATZER, E. (2005). Surface swimming behavior of infectious juveniles of *Romanomermis culicivorax*. *Journal of Nematology* 37, 364.

DOLE-OLIVIER, M.J., GALASSI, D.M.P., MARMONIER, P. & CREUZÉ DES CHÂTELLIERS, M. (2000). The biology and ecology of lotic microcrustaceans. *Freshwater Biology* 44, 63-91.

DUMONT, H.J., VAN DE VELDE, I. & DUMONT, S. (1975). The dry weight estimate of biomass in a selection of Cladocera, Copepoda and Rotifera from plankton, periphyton and benthos of continental waters. *Oecologia* 19, 75-97.

EINSLE, U. (1993). *Süßwasserfauna von Mitteleuropa: Vol. 8.4.1: Calanoida und Cyclopoida*. Stuttgart, Germany, Fischer, 208 pp.

ELBOURN, C.A. (1966). Some observations on the food of *Cyclops strenuus strenuus* (Fischer). *Annals and Magazine of Natural History* 9 (3rd Series), 227-231.

FRYER, G. (1957a). The feeding mechanism of some freshwater cyclopoid copepods. *Proceedings of the Zoological Society of London* 129, 1-25.

FRYER, G. (1957b). The food of some fresh-water cyclopoid copepods and its ecological significance. *Journal of Animal Ecology* 26, 263-286.

GALASSI, D., MARMONIER, P., DOLE-OLIVIER, M.J. & RUNDLE, S.D. (2002). Microcrustacea. In: Rundle, S.D., Robertson, A. & Schmid-Araya, J.M. (Eds). *Freshwater meiofauna: biology and ecology.* Leiden, The Netherlands, Backhuys Publishers, pp. 135-175.

GILBERT, J.J. & WILLIAMSON, C.E. (1978). Predator-prey behavior and its effect on rotifer survival in associations of *Mesocyclops edax*, *Asplanchna girodi*, *Polyarthra vulgaris*, and *Keratella cochlearis*. *Oecologia* 37, 13-22.

GRUBBS, F.E. (1969). Procedures for detecting outlying observations in samples. *Technometrics* 11, 1-21.

HOHBERG, K. & TRAUNSPURGER, W. (2005). Predator-prey interaction in soil food web: functional response, size-dependent foraging efficiency, and the influence of soil texture. *Biology and Fertility of Soils* 41, 419-427.

HOLLING, C.S. (1959a). Some characteristics of simple types of predation and parasitism. *Canadian Entomologist* 91, 385-398.

HOLLING, C.S. (1959b). The components of predation as revealed by a study of small mammal predation of the European pine sawfly. *Canadian Entomologist* 91, 293-320.

HOPP, U. & MAIER, G. (2005). Implication of the feeding limb morphology for herbivorous feeding in some freshwater cyclopoid copepods. *Freshwater Biology* 50, 742-747.

HUMES, A.G. (1994). How many copepods? *Hydrobiologia* 292-293, 1-7.

JAMIESON, C.D. (1980). The predatory feeding of copepodid stages III to adult *Mesocyclops leuckarti* (Claus). In: Kerfoot, W.C. (Ed.). *Evolution and ecology of zooplankton communities.* Hanover, NH, USA, University Press of New England, pp. 518-537.

LAMPITT, R.S. (1978). Carnivorous feeding by a small marine copepod. *Limnology and Oceanography* 23, 1228-1231.

LANDRY, M.R. (1978). Predatory feeding behavior of a marine copepod *Labidocera trispinosa*. *Limnology and Oceanography* 23, 1103-1113.

LAPESA, S., SNELL, T.W., FIELDS, D.M. & SERRA, M. (2002). Predatory interactions between a cyclopoid copepod and three sibling rotifer species. *Freshwater Biology* 47, 1685-1695.

LEHMAN, P.S. & REID, J.W. (1992). *Phyllognatopus viguieri* (Crustacea: Harpacticoida), a predaceous copepod of phytoparasitic, entomopathogenic, and free-living nematodes. *Proceedings of the Soil and Crop Science Society of Florida* 52, 78-82.

MAIER, G. (1990). Reproduction and life history of a Central European, planktonic *Diacyclops bicuspidatus* population. *Archiv für Hydrobiologie* 117, 485-495.

MARCOTTE, B.M. (1977). An introduction to the architecture and kinematics of harpacticoid (Copepoda) feeding: *Tisbe furcata* (Baird, 1837). *Mikrofauna des Meeresbodens* 61, 183-196.

MØLLER, E.F. (2007). Production of dissolved organic carbon by sloppy feeding in the copepods *Acartia tonsa*, *Centropages typicus*, and *Temora longicornis*. *Limnology and Oceanography* 52, 79-84.

MOORTHY, V.N. (1938). Observations on the development of *Dracunculus medinensis* larvae in *Cyclops*. *American Journal of Hygiene* 27, 436-464.

MUSCHIOL, D. & TRAUNSPURGER, W. (2007). Life cycle and calculation of the intrinsic rate of natural increase of two bacterivorous nematodes, *Panagrolaimus* sp. and *Poikilolaimus* sp. from chemoautotrophic Movile Cave, Romania. *Nematology* 9, 271-284.

PLATZER, E.G. & MACKENZIE-GRAHAM, L.L. (1980). *Cyclops vernalis* as a predator of the pre-parasitic stages of *Romanomermis culicivorax*. *Journal of the American Mosquito Control Association: Mosquito News* 40, 252-257.

REID, J.W. (1986). Some usually overlooked cryptic copepod habitats. *Syllogeus* 58, 594-598.

REVERSAT, G., GARCIA, N. & DUSSART, B. (1992). The copepod *Paracyclops affinis* as a predator of plant parasitic nematodes. *Fundamental and Applied Nematology* 15, 183-191.

ROCHA, C.E.F.D. & BJORNBERG, M.H.G.D.C. (1988). *Allocyclops silvaticus* sp. n. (Copepoda, Cyclopoida, Cyclopidae), the first representative of the genus in South America. *Hydrobiologia* 167/168, 445-448.

SARVALA, J. (1998). Ecology and role of benthic copepods in northern lakes. *Journal of Marine Systems* 15, 75-86.

SCHMID, P.E. & SCHMID-ARAYA, J.M. (2002). Trophic relationships in temporary and permanent freshwater meiofauna. In: Rundle, S.D., Robertson, A. & Schmid-Araya, J.M. (Eds). *Freshwater meiofauna: biology and ecology.* Leiden, The Netherlands, Backhuys Publishers, pp. 295-319.

SMALL, R.W. (1988). Invertebrate predators. In: Poinar, G.O. & Jansson, H.-B. (Eds). *Diseases of nematodes, volume II.* Boca Raton, FL, USA, CRC Press, pp. 73-92.

SOHLENIUS, B. (1979). A carbon budget for nematodes, rotifers and tardigrades in a Swedish coniferous forest soil. *Holarctic Ecology* 2, 30-40.

STEMBERGER, R.S. (1986). The effects of food deprivation, prey density and volume on clearance rates and ingestion rates of *Diacyclops thomasi*. *Journal of Plankton Research* 8, 243-251.

STRAYER, D. (1985). The benthic micrometazoans of Mirror Lake, New Hampshire. *Archiv für Hydrobiologie, Supplement* 72, 287-426.

TRAUNSPURGER, W. (2002). Nematoda. In: Rundle, S.D., Robertson, A. & Schmid-Araya, J.M. (Eds). *Freshwater meiofauna: biology and ecology.* Leiden, The Netherlands, Backhuys Publishers, pp. 63-104.

WILLIAMSON, C.E. (1980). The predatory behavior of *Mesocyclops edax*: Predator preferences, prey defenses, and starvation-induced changes. *Limnology and Oceanography* 25, 903-909.

WILLIAMSON, C.E. (1986). The swimming and feeding behavior of *Mesocyclops*. *Hydrobiologia* 134, 11-19.

WILLIAMSON, C.E. & REID, J.W. (2001). Copepoda. In: Thorp, J.H. & Covich, A.P. (Eds). *Ecology and classification of North American freshwater invertebrates.* San Diego, CA, USA, Academic Press, pp. 915-954.

ZHANG, G.T., LI, C.L., SUN, S., ZHANG, H.Y., SUN, J. & NING, X.R. (2006). Feeding habits of *Calanus sinicus* (Crustacea: Copepoda) during spring and autumn in the Bohai Sea studied with the herbivore index. *Scientia Marina* 70, 381-388.

11.5. Manuscript V

Muschiol, D., Marković, M., Threis, I. & Traunspurger, W., 2008: **Predatory copepods can control nematode populations: A functional-response experiment with *Eucyclops subterraneus* and bacterivorous nematodes**. – *Fundamental and Applied Limnology* **172:** 317-324.

Fundamental and Applied Limnology
Archiv für Hydrobiologie
Vol. 172/4: 317–324, September 2008

Predatory copepods can control nematode populations: A functional-response experiment with *Eucyclops subterraneus* and bacterivorous nematodes

Daniel Muschiol*, Mirjana Marković, Ilka Threis and **Walter Traunspurger**[1]

Bielefeld University

With 3 figures and 1 table

Abstract: A classical functional-response experiment was conducted in order to test the potential of copepods as predators of free-living nematodes. The cyclopoid copepod *Eucyclops subterraneus scythicus* readily fed on the two offered bacterivorous nematode species, *Panagrolaimus* sp. and *Poikilolaimus* sp. In nature, the investigated organisms are members of a very simply structured community occurring in floating microbial mats in the chemo-autotrophic groundwater ecosystem of Movile Cave, Romania. At different prey densities, the copepod's feeding rates followed type II functional responses. Within 20 min, the maximum number of consumed nematodes was 25.8 *Panagrolaimus* sp. or 37.5 *Poikilolaimus* sp., corresponding to 28.7 % and 31.1 % of the predator's average dry weight. Within 24 h, the predator's mean consumption corresponded to 264.4 % of its own dry weight. Using the estimated copepod feeding rates and known population growth rates of the mat's nematodes, we calculated that *E. s. scythicus* has the potential to top-down control exponentially growing nematode populations. This study is the first attempt to quantify a predator-prey relationship between coexisting copepods and nematodes at the population level and sheds light on a trophic link that could be relevant in many marine, limnic, and semi-terrestrial habitats.

Key words: microbial mat, predator-prey, meiofauna, invertebrate predators, food chain, top-down control.

Introduction

Due to their high abundance and wide distribution, copepods play a pivotal role in aquatic food webs, serving both as primary and secondary consumers and as a major source of food for many larger invertebrates and vertebrates. More than 11,500 species of copepods have been described (Humes 1994) from a wide variety of aquatic environments, ranging from the benthic, littoral, and pelagic waters of lakes and oceans to swamps, large rivers, temporary ponds, phytotelmata, and interstitial and subterranean systems (Williamson & Reid 2001). They have also been collected from many semi-terrestrial habitats, such as moist soil and arboreal mosses (Reid 1986). Copepods can be quite abundant; in the benthic communities of lakes, it is not uncommon to find densities of copepods in excess of 10,000 and, occasionally, as high as 250,000 individuals m^{-2} (Strayer 1985, Sarvala 1998). However, in terms of species number and abundance, such meiofaunal communities are regularly dominated by nematodes, which can exceed 10^7 individuals m^{-2} (Traunspurger et al. 2006).

Although nematodes and copepods coexist in large numbers in many natural habitats, nematophagous copepods have been reported only sporadically (Marcotte 1977, Rocha & Bjornberg 1988, Lehman & Reid 1992, Reversat et al. 1992). This is somewhat surpris-

[1] **Authors' address:** Bielefeld University, Animal Ecology, Morgenbreede 45, 33615 Bielefeld, Germany.

* Corresponding author; e-mail: daniel.muschiol@uni-bielefeld.de

DOI: 10.1127/1863-9135/2008/0172-0317 1863-9135/08/0172-0317 $ 2.00

ing, as it is known that copepods consume a large variety of foods, ranging from detritus and pollen to algae, bacteria, ciliates, rotifers, other small crustaceans, dipteran larvae, and even larval fish (Williamson & Reid 2001, and references therein). Based on predator-prey experiments with the common Holarctic cyclopoid copepod *Diacyclops bicuspidatus* (Muschiol et al. 2008), we proposed that the consumption of nematodes is more common among freshwater copepods than generally assumed. Provided that this trophic linkage is of relevant magnitude in nature, it would constitute an important benthic-pelagic coupling between microbial carbon, endobenthic nematodes, epibenthic copepods, and pelagic predators such as young fishes. However, no attempt has been made to quantify this relationship or to answer the question whether predatory copepods might even top-down control natural nematode populations. The reason for this is obvious: Most ecosystems are far too complex to apply simple predator-prey experiments carried out in the laboratory, as dozens of nematode species and invertebrate predators interact in an unpredictable manner. In this context, the relatively simple food web of Movile Cave appears particularly suited to raise questions about possible predator-prey interactions between copepods and nematodes.

Discovered in 1986 in south-eastern Romania, Movile Cave has been completely isolated from the surface since preglacial times (Sarbu et al. 1996). The cave's peculiar ecosystem is supported solely by in situ chemoautotrophic production. Hydrothermal anoxic water in the lower level of the cave contains significant amounts of H_2S and CH_4, both of which are oxidized by thick (1–3 mm) microbial mats growing on the water surface (Rohwerder et al. 2003, Hutchens et al. 2004). Unlike the vast majority of cave communities, which do not contain producers and are thus oligotrophic systems, the large biomass of the microbial mats present in Movile Cave supports rich aquatic and terrestrial communities. Around 20 aquatic species have been found associated with the mats, including copepods in high abundances; thus far, one harpacticoid (*Parapseudoleptomesochra italica* Pesce & Petkovski, 1980) and two cyclopoid copepods (*Eucyclops subterraneus scythicus* Plesa, 1989 and *Tropocyclops prasinus* Fischer, 1860) (Plesa 1989, Popa & Sarbu 1991, Sarbu & Kane 1995) have been described. However, in terms of metazoan biomass and abundance, the mats are overwhelmingly dominated by nematodes. A first study on the mat's nematode fauna (Riess et al. 1999) reported total densities of 9.8×10^6 individuals m^{-2}, comprising five species. The two most frequent species among them, *Panagrolaimus* sp. and *Poikilolaimus* sp., are characterized by short generation times and high fecundities, resulting in population doubling times as low as 54 hours under optimal food conditions (Muschiol & Traunspurger 2007). Feeding-type analysis of 749 nematodes collected from the mats during three dives in 2004 and 2005 revealed that all nematodes in the mats were bacterial feeders; no predatory nematodes were found (Muschiol & Traunspurger, unpubl.). Based on their high abundance, biomass, and turnover rates, nematodes should play a pivotal role in the cave's food web as consumers of chemoautotrophic bacteria. Yet, whether the nematodes themselves serve as food for invertebrate predators or represent an energetic dead end within Movile Cave's food web is still an unanswered question. Although a preliminary study on the cave's food web was carried out by Popa & Sarbu (1991), to date there is no evidence whether any of the taxa (e.g., planarians, amphipods, copepods, ostracods) recorded in Movile Cave's microbial mats use the huge nematode standing-stock as a food resource to any significant extent. Based on our own observations, cyclopoid copepods are sufficiently abundant within the mats to potentially control the nematodes.

The peculiar ecosystem of Movile Cave provides unique opportunities to investigate possible top-down control of nematode populations by copepods: First of all, important nematophagous taxa common in the majority of freshwater and marine habitats are either completely missing (e.g., chironomids, tardigrades, and predatory nematodes) or numerically negligible (e.g., ostracods and acarids). Secondly, the nematode community of the cave's microbial mats consists of only five species, thus being rather simply structured compared to the complex communities found in benthic marine and freshwater habitats, which usually contain >50 nematode species (e.g., Michiels & Traunspurger 2005). Last but not least, life cycle experiments with dominant nematodes from Movile Cave permit a reliable estimation of nematode production, a necessary basis for the evaluation of top-down effects (Muschiol & Traunspurger 2007).

In the present study, we conducted a classical predator-prey experiment with two dominant nematode species of Movile Cave's microbial mats, *Panagrolaimus* sp. and *Poikilolaimus* sp., as prey and Movile's most numerous copepod, *Eucyclops subterraneus scythicus*, as predator. We aimed (1) to evaluate the copepod's potential role as a predator of nematodes and (2) to determine whether top-down control of nematode populations by predatory copepods is possible.

Material and methods

Predator and prey sources

Monoxenic laboratory cultures of *Panagrolaimus* sp. and *Poikilolaimus* sp. from floating microbial mat of Movile Cave were established on NGG (nematode growth Gelrite) medium seeded with OP50 (a uracil-requiring mutant of *Escherichia coli*), as described in Muschiol & Traunspurger (2007). Sieves with a mesh size of 5 µm were used to extract the nematodes from exponentially growing cultures after liquefaction of the medium with ethylenediaminetetraacetic acid (EDTA; 0.005M) for 30 sec (Muschiol & Traunspurger 2007).

Late copepodid stages and adults (>670 µm body length) of *Eucyclops subterraneus scythicus* were extracted from their natural habitat by sieving water from the cave lake (200-µm mesh size). Since attempts to cultivate the species failed, all individuals were used for the experiment within 4 days after sampling from the lake.

Functional-response experiments

The experiments were conducted at 20 °C in 12-well multiwell plates (Greiner 665102). Each well was filled with 1.5 ml cave water (0.2-µm filtered). Actively moving nematodes were accurately counted and transferred into the wells with a micropipette connected by rubber tubing to a mouthpiece. The optimal exposure time and prey density range for the functional-response experiments were determined in a preliminary experiment: Various sizes and densities of the two nematode species were presented to the copepod and consumption was continuously observed for up to one hour under a dissecting microscope (30-fold magnification; N = 7). Attack rates, handling times, and behavioral observations were recorded on a voice recorder.

Based on these observations, seven different densities of each nematode species (5, 10, 25, 50, 75, 100, 150 nematodes per well) were used in the main experiment (N = 6 for each combination of prey species and prey density). Five wells of each density without copepods served as controls. To ensure a uniform hunger level, the copepods were starved for 24 h prior to the experiment. The experiments started with the addition of a single copepod to each arena and were terminated after 20 min with a hot (60 °C) mixture of formaldehyde (4 % final concentration) and Rose Bengal (300 µg ml^{-1}). The copepod was then removed from the arena, and its biomass was estimated (see below), while the remaining nematodes were transferred to a Petri dish and recounted under 30-fold magnification. An underlying grid facilitated counting. Estimated consumption rates were corrected based on the recovery rate of the controls. As a certain proportion of the killed nematodes had been consumed only partially by the copepods, we classified fragmented nematodes by size according to the residual proportion of the body (90, 80, 70 %, etc). For example, three fragments of 30, 50, and 70 % (= 150) were considered to represent 1.5 unconsumed nematodes and included as such into the number of recovered nematodes. From the resulting predation rates, those corresponding to less than 5 % of the offered prey number were defined as zero and excluded from further calculations (N = 9 or 10.6 % of total replicates). This was a reasonable assumption since preliminary observations showed that a certain proportion of the copepod population was non-feeding.

As a model of the predators' functional response, Holling's (1959a, 1959b) disc equation was applied:

$$N_e = aTN_0/(1 + aT_hN) \quad \text{(Eq. 1)}$$

where N_e is the number of prey consumed, a is the attack rate, T is the total time the prey was exposed to predation, N_0 is the number of prey presented, and T_h is the time required to handle the prey. Non-linear curve-fitting with least squares regression for each prey species (Statistica v7.0, StatSoft Inc. 2004) was used to fit the data to Holling's disc equation and to estimate the values of a and T_h.

The effects of prey species and prey density on the consumption rate in terms of consumed prey numbers and prey biomass were tested by means of a two-way ANOVA and Tukey-HSD post-hoc test (Statistica v7.0, StatSoft Inc. 2004).

24-h experiment

In order to estimate total consumption during 24 h at high prey density, Petri dishes (diameter 5.2 cm) were filled with 20 ml cave water (0.2-µm filtered). Eight-hundred *Panagrolaimus* sp. were counted accurately with a micropipette into each of three replicates containing a single predator and three controls (without predator). The densities in the Petri dishes corresponded to a density of approximately 60 nematodes in the 12-well multiwell plates used for the functional-response experiments. The copepods in this experiment were not starved prior to the experiment.

Biomass calculations

Randomly chosen representatives of the copepods (N = 85) and the two nematode species (N = 60 each) used in the experiment were fixed in 4 % formaldehyde. High-resolution digital images were then taken under a dissection microscope (Jenoptik ProgRes C12plus; Zeiss Stemi SV11 Apo). Body lengths were determined using calibrated image-analysis software (ImageJ v1.34s; freeware: http://rsb.info.nih.gov/ij/). The lengths of the nematodes were measured by assuming the worm's tail to be an idealized cone, as specified by Andrássy (1956). Nematode biomass was calculated according to the published regressions relating body weight W and length L ($W = aL^b$) using units of µg fresh weight and mm length: For *Poikilolaimus* sp., the relationship was $W = 1.6439L^{2.7672}$; for *Panagrolaimus* sp., $W = 0.2085L^{4.0915}$ (Muschiol & Traunspurger 2007). The nematodes' dry weight was assumed to be 20 % of their wet weight (Sohlenius 1979).

The copepods' body lengths were measured from the anterior of the cephalothorax to the base of the caudal furca. Copepod biomass was determined according to the regression of Dumont et al. (1975) for adult female Cyclopoida without eggs: $W = 1.1 * 10^{-7}L^{2.59}$ (W = µg dry weight; L = µm body length).

Results

Eucyclops subterraneus scythicus efficiently fed on both prey species. Direct observations revealed that small nematodes were generally ingested in 1–2 seconds, in a manner resembling sucking in a spaghetti strand, whereas the consumption of large nematodes took up to several minutes, interrupted by periods of inactivity. If a capture attempt failed, the copepod usu-

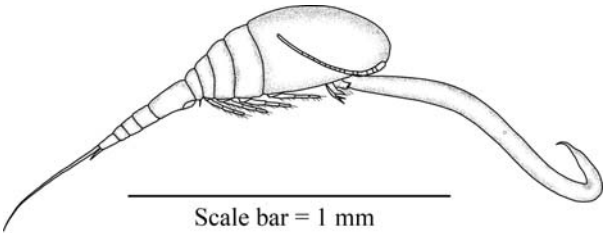

Fig. 1. *Eucyclops* from Movile Cave feeding on adult *Poikilolaimus*. Note the remarkable prey size relative to the predator.

ally swam in rapid, tight, horizontal loops to recover the prey. The efficiency with which the nematodes, often of remarkable size in relation to the predator (Fig. 1), were captured, overwhelmed, and ingested provided evidence that this type of feeding is not uncommon.

Prey density significantly affected predation rates ($p < 0.001$; Table 1), and the number of consumed nematodes increased curvilinearly with prey density

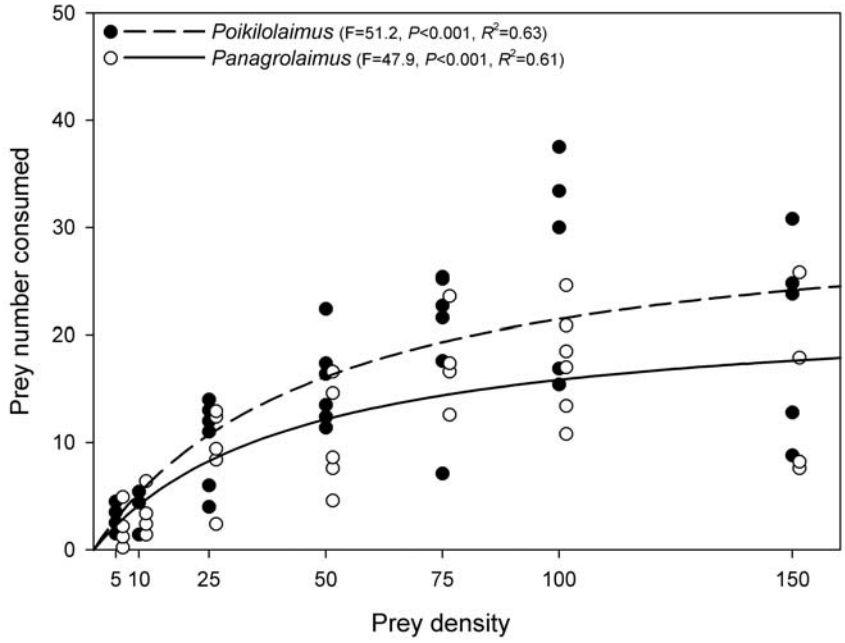

Fig. 2. Functional-response curves of *Eucyclops subterraneus scythicus* feeding for 20 min on *Poikilolaimus* sp. (-----) and *Panagrolaimus* sp. (—–). The lines were fitted by non-linear regression using equation (1). Each replicate of *Poikilolaimus* (●) and *Panagrolaimus* (○) is shown. Both species were offered in corresponding densities; for clarity, the data points were slightly displaced laterally.

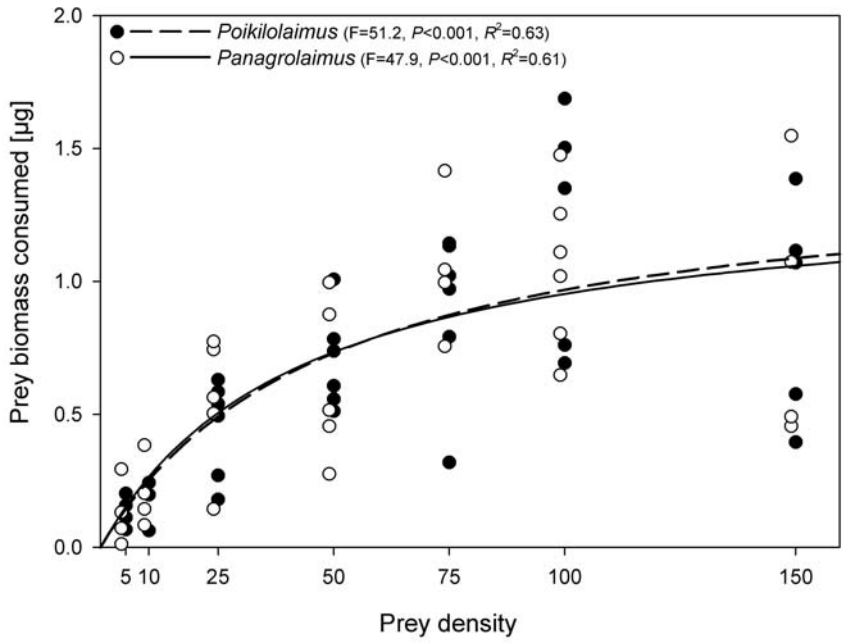

Fig. 3. Dry prey biomass consumed in 20 min by *Eucyclops subterraneus scythicus* at various prey densities. Designations as in Fig. 2.

Table 1. Results of the two-way ANOVA on the predation rates with prey species and prey density as factors. The $\log_e(x)$-transformed predation rates were homoscedastic.

	Consumed prey number				Consumed prey biomass			
Factor	d.f.	MS	F	*p*	d.f.	MS	F	*p*
Species	1	1.987	6.608	<0.05	1	0.028	0.095	0.759
Density	6	9.066	30.142	<0.001	6	9.066	30.142	<0.001
Species × Density	6	0.093	0.309	0.930	6	0.093	0.309	0.930
Error	62	0.301			62	0.301		

(Fig. 2). The more prey individuals presented to the predator, the shorter the searching time, until the latter was small compared with the handling time and a plateau was reached. In both prey species, predation on the two lowest prey densities differed significantly from predation on the three highest densities (Tukey-HSD: $p < 0.05$). However, the predation rate at the 150 density appeared to remain slightly lower than that at the 100 density, a finding that was consistent with behavioral observations: Very high prey densities seemed to disturb the copepods, e.g., copepods often interrupted feeding on a captured prey when they were touched by another moving nematode. Copepods of comparable size differed in feeding behavior: After the consumption of 10–15 nematodes, copepods usually rested motionless for 5–30 min. However, some individuals continued to attack and kill nematodes but did not ingest them. Approximately 10–15 % of the population did not feed at all, probably because they were moulting, senescent, or stressed by the laboratory conditions.

The prey species significantly affected predation rates ($p < 0.05$; Table 1), a finding that was also consistent with behavioral observations: In contrast to *Panagrolaimus*, which showed little response to attack, *Poikilolaimus* responded with vigorous undulation. While this had no effect on the consumption of juveniles, the copepods regularly failed to overwhelm forceful undulating stout adults. However, *Panagrolaimus* turned out to be an excellent swimmer and dissipated homogeneously in the water column whereas *Poikilolaimus* instantly sunk to the bottom of the arena, the preferred hunting ground of *E. subterraneus scythicus*. The maximum number of nematodes consumed during 20 min in any microcosm was 25.8 *Panagrolaimus* and 37.5 *Poikilolaimus*.

Consumption rates at different prey densities were adequately described by Holling's (1959b) type II functional response (Fig. 2). Parameter estimates of attack rate (a) and handling time (T_h) in Eq. (1) were 4.39×10^{-4} and 52.9 sec, respectively, for *Panagrolaimus*, 5.44 $\times 10^{-4}$ and 37.4 sec, respectively, for *Poikilolaimus*. Averaged dry-weight estimates of the two prey species were 0.045 µg (± 0.028 µg) for *Poikilolaimus* and 0.060 µg (± 0.038 µg) for *Panagrolaimus*. Using these values, we recalculated the observed predation rates as units of consumed biomass (Fig. 3). Again, the two-way ANOVA showed a significant effect of prey density on consumption rate ($p < 0.001$); however, the effect of prey species became non-significant ($p = 0.759$; Table 1). Accordingly, regressions using Eq. (1) were very similar (Fig. 3). The highest observed biomass consumption was 1.55 µg *Panagrolaimus* and 1.68 µg *Poikilolaimus*. These values correspond to 28.7 % and 31.1 % of the average dry weight estimated for *E. subterraneus scythicus* (5.41 ± 1.02 µg).

24-h experiment

The mean consumption by a non-starving single copepod during 24 h was 238.2 (± 62.8) nematodes, corresponding to 14.29 µg (± 3.77 µg) dry weight. This equals 264.4 % of the average copepod's dry weight per 24 h, or 11.0 % per hour.

In order to assess whether this consumption rate could control the mat's nematode populations, we estimated the theoretically possible daily nematode production. For this, we assumed that growth of the nematodes within the mats is (hypothetically) unlimited, i.e., it follows their intrinsic rate of natural increase (r_m). A continuously reproducing population has a stable age structure, so that r_m can be expressed in units such as number of organisms, biomass, or energy (Fenchel 1974). While intrinsic growth rates are unknown for the vast majority of nematode species, they have been determined for both *Poikilolaimus* ($r_m = 0.165$) and *Panagrolaimus* ($r_m = 0.309$) inhabiting Movile Cave by Muschiol & Traunspurger (2007). If we assume that the highest nematode density reported for the cave's microbial mats (9.8×10^6 nematodes m^{-2}: Riess et al. 1999) consists exclusively of the fast growing *Panagrolaimus* sp. ($r_m = 0.309$; average dry weight: 0.06 µg), then the population's daily dry bio-

mass production m^{-2} (P_d) can be estimated as:

$$P_d = 9.8 \times 10^6 \times 0.060\,\mu g \times e^{0.309} - 9.8 \times 10^6 \times 0.060\,\mu g = 212893\,\mu g$$

As an average copepod consumed 14.29 µg nematode dry mass per day in our experiment, then 14898 feeding copepods m^{-2} could eliminate the mat's daily nematode production.

Discussion

Reports of nematode predation by copepods are extremely scarce in the literature, although the two groups co-occur in high numbers in many natural habitats. However, considering the fact that most copepods are omnivorous to some extent (Williamson & Reid 2001 and references therein), it appears unlikely that particularly epi- and endobenthic copepods do not exploit ubiquitous nematodes as a food source. This discrepancy was pointed out by Muschiol et al. (2008), who compiled evidence that the consumption of nematodes is more common among freshwater copepods than generally assumed.

In our laboratory experiment, *E. subterraneus scythicus* readily and efficiently consumed two different nematode species and we see no reason to doubt that nematode predation also takes place in the copepod's natural habitat, the unique ecosystem of Movile Cave. While starved copepods consumed up to one third of their own biomass in 20 min, non-starved copepods consumed 11 % of their own biomass per hour during 24 hours. As Stemberger (1986) estimated the gut passage time of *Diacyclops thomasi* to be 7–8 h, we regard this feeding rate as the steady state. Our calculations showed that, based on the three assumptions: (1) that the highest nematode density reported from Movile Cave consisted (2) exclusively of the fast growing *Panagrolaimus* sp., growing (3) without density effects, i.e., with unlimited growth potential, a density of 17,527 (14,898 plus 15 % non-feeding members of the population) *E. subterraneus scythicus* m^{-2} mat would suffice to eliminate the mat's daily nematode biomass production. Unfortunately, copepod population densities have never been estimated in Movile Cave. The reason for this became obvious during our own sampling efforts: Sampling of natural-grown mats from the air bells requires diving equipment and is extremely difficult, as the diver enters the air bells from below and instantly destroys the fragile mats due to both the air bubbles generated by his breathing and water turbulences. A reliable estimation of copepod population densities per area under these circumstances is not possible. Accordingly, all publications describing the cave's copepod populations only vaguely report "large populations" (e.g., Sarbu & Kane 1995). However, densities of 17,527 copepods m^{-2} are by no means unrealistically high compared to published data from other habitats. Densities in excess of 10,000 copepods m^{-2} are regularly found in the benthic communities of lakes (e.g., Strayer 1985, Traunspurger 1996), and occasionally much higher densities are reached (Sarvala 1998). Moreover, preliminary results of experiments in Movile Cave that are currently in progress suggest that the nematode density of 9.8×10^6 nematodes m^{-2}, reported by Riess et al. (1999), should be considered as an upper limit (Muschiol & Traunspurger, unpubl.). However, the finding that *E. subterraneus scythicus* has the potential to exert efficient top-down control on Movile Cave's nematode populations does not imply that bottom-up effects might not (temporarily) decrease nematode growth rates nor that nematodes constitute the sole food item for the cave's copepods. In contrast to calanoid copepods and cladocerans, which are restricted to a rather narrow food spectrum, cyclopoid copepods probably switch between various food sources and may even utilize bacterial aggregations and detritus as availability changes within their habitat (Santer et al. 2006). This opportunistic feeding strategy presumably contributes considerably to the success of these organisms in many habitats. For the future, the analysis of carbon and nitrogen stable isotope ratios (SIRA), as already successfully applied to freshwater cyclopoid copepods (Santer et al. 2006), offers a promising approach to shed further light on Movile's fascinating food web.

Ingestion rates

In the majority of investigations (e.g., Confer 1971, Brandl & Fernando 1978, Lampitt 1978, Stemberger 1986, Williamson 1986), the intensity of foraging in copepods was found to increase with prey density (but see: Landry 1978). This general picture is also true for *Eucyclops subterraneus scythicus* foraging on bacterivorous nematodes from Movile Cave. Consumption rates increased curvilinearly with prey density and were best described by Holling's (1959b) type II functional response (Fig. 2). However, the picture is not always quite so simple, as Williamson (1986) pointed out. That author found that the ingestion rates of *Mesocyclops* were a complex function of prey behavior, morphology, and size, in addition to prey density. Prey behaviour (vigorous undulation of *Poikilolaimus* and

swimming of *Panagrolaimus*) and size (particularly body width) indeed influenced the ingestion rates in this investigation (Fig. 2). Interestingly, the differences turned out to be negligible when prey density was related to consumed biomass (Fig. 3). We interpret this observation as a result of a trade-off between searching time, success rate, handling time, and prey biomass: On average, *Panagrolaimus* was larger, resulting in a prolonged handling time, and thus leaving less time to search for additional prey; however, a successful attack yielded more biomass to consume compared to that provided by *Poikilolaimus*.

The ingestion rates at the highest prey density (150) tended to be lower than those at the next highest (100) density (Fig. 2). Based on our direct observations, we interpret this as a result of the fact that very high prey densities confused the copepods or disturbed feeding on captured prey. Consistent with this assumption, Jamieson (1980) reported that predation rates of *Mesocyclops leuckarti* (Cyclopoida) varied greatly at high prey densities. In a similar experimental set-up to investigate the predatory tardigrade *Macrobiotus richtersi*, high densities of the nematode prey *Pelodera teres* also reduced the consumption rates of the predator (Hohberg & Traunspurger 2005).

Handling time

Direct observation of predator-prey interactions revealed highly variable handling times depending on the size of the captured nematode. This observation is consistent with literature reports on carnivorous copepods. Handling times of 30 min or more for large prey items of various taxa were reported by Fryer (1957), Gilbert and Williamson (1978), Lampitt (1978), and Williamson (1980). Relatively small prey was reported to be consumed very quickly, between 2 and 7 sec (Williamson 1980), or even so fast that "capture and ingestion appear to occur in one movement" (Lampitt 1978). The short time in which slender nematodes were consumed (1–2 sec) strongly suggested that they were ingested mostly unchewed.

In conclusion, this study is the first attempt to quantify a trophic connection between co-occurring copepods and nematodes on the population level. Using known population growth rates, we were able to show that, at moderate densities (below 2×10^4 copepods m^{-2}), copepods have the potential to top-down control exponentially growing nematode populations with densities around 10^7 nematodes m^{-2}. Confirmation of the trophic linkage between copepods and nematodes by studies carried out in other habitats would establish an important benthic-pelagic coupling between microbial carbon, endobenthic nematodes, epibenthic copepods, and pelagic predators such as young fishes.

Acknowledgements

Mihai Baciu and Alexandra Hillebrand from the Group for Underwater and Speleological Exploration (GESS), Bucharest, Romania assisted in obtaining the samples. Species determination was confirmed by Thomas Glatzel, Oldenburg. This investigation was financially supported by the Deutsche Forschungsgemeinschaft (TR 445/3-1).

References

Andrássy, I., 1956: Die Rauminhalts- und Gewichtsbestimmung der Fadenwürmer (Nematoda). – Acta Zool. Hung. **2:** 1–15.

Brandl, Z. & Fernando, C. H., 1978: Prey selection by cyclopoid copepods *Mesocyclops edax* and *Cyclops vicinus*. – Verh. Internat. Verein. Limnol. **20:** 2505–2510.

Confer, J. L., 1971: Intrazooplankton predation by *Mesocyclops edax* at natural prey densities. – Limnol. Oceanogr. **16:** 663–666.

Dumont, H. J., Van de Velde, I. & Dumont, S., 1975: The dry weight estimate of biomass in a selection of Cladocera, Copepoda and Rotifera from plankton, periphyton and benthos of continental waters. – Oecologia **19:** 75–97.

Fenchel, T., 1974: Intrinsic rate of natural increase: The relationship with body size. – Oecologia **14:** 317–326.

Fryer, G., 1957: The feeding mechanism of some freshwater cyclopoid copepods. – Proc. Zool. Soc. Lond. **129:** 1–25.

Gilbert, J. J. & Williamson, C. E., 1978: Predator-prey behavior and its effect on rotifer survival in associations of *Mesocyclops edax*, *Asplanchna girodi*, *Polyarthra vulgaris*, and *Keratella cochlearis*. – Oecologia **37:** 13–22.

Hohberg, K. & Traunspurger, W., 2005: Predator-prey interaction in soil food web: Functional response, size-dependent foraging efficiency, and the influence of soil texture. – Biol. Fert. Soils **41:** 419–427.

Holling, C. S., 1959a: Some characteristics of simple types of predation and parasitism. – Can. Entomol. **91:** 385–398.

– 1959b: The components of predation as revealed by a study of small mammal predation of the European pine sawfly. – Can. Entomol. **91:** 293–320.

Humes, A. G., 1994: How many copepods? – Hydrobiologia **292–293:** 1–7.

Hutchens, E., Radajewski, S., Dumont, M. G., McDonald, I. R. & Murrell, J. C., 2004: Analysis of methanotrophic bacteria in Movile Cave by stable isotope probing. – Environ. Microbiol. **6:** 111–120.

Jamieson, C. D., 1980: The predatory feeding of copepodid stages III to adult *Mesocyclops leuckarti* (Claus). – In: Kerfoot, W. C. (ed.): Evolution and ecology of zooplankton communities. – University Press, New England, pp. 518–537.

Lampitt, R. S., 1978: Carnivorous feeding by a small marine copepod. – Limnol. Oceanogr. **23:** 1228–1231.

Landry, M. R., 1978: Predatory feeding behavior of a marine copepod *Labidocera trispinosa*. – Limnol. Oceanogr. **23:** 1103–1113.

Lehman, P. S. & Reid, J. W., 1992: *Phyllognatopus viguieri* (Crustacea: Harpacticoida), a predaceous copepod of phytoparasitic, entomopathogenic, and free-living nematodes. – Soil Crop. Sci. Soc. Fla. Proc. **52:** 78–82.

Marcotte, B. M., 1977: An introduction to the architecture and kinematics of harpacticoid (Copepoda) feeding: *Tisbe furcata* (Baird, 1837). – Mikrofauna Meeresbodens **61:** 183–196.

Michiels, I. & Traunspurger, W., 2005: Benthic community patterns and the composition of feeding types and reproductive modes in freshwater nematodes. – Nematology **7:** 21–36.

Muschiol, D., Markovic, M., Threis, I. & Traunspurger, W., 2008: Predator-prey relationship between the cyclopoid copepod *Diacyclops bicuspidatus* and a free-living bacterivorous nematode. – Nematology **10:** 55–62.

Muschiol, D. & Traunspurger, W., 2007: Life cycle and calculation of the intrinsic rate of natural increase of two bacterivorous nematodes, *Panagrolaimus* sp. and *Poikilolaimus* sp. from chemoautotrophic Movile Cave, Romania. – Nematology **9:** 271–284.

Plesa, C., 1989: Étude préliminaire des Cyclopides (Crustacea, Copepoda) de la grotte 'Pestera de la Movile', Mangalia (Roumanie). – Miscellanea spéologica Romanica **1:** 39–45.

Popa, R. & Sarbu, S. M., 1991: Trophic structure in an aquatic troglobiotic community based upon chemoautotrophic carbon fixation. – Travaux de l'Institut de Spéologie Emile Racovitza **30:** 51–58.

Reid, J. W., 1986: Some usually overlooked cryptic copepod habitats. – Syllogeus **58:** 594–598.

Reversat, G., Garcia, N. & Dussart, B., 1992: The copepod *Paracyclops affinis* as a predator of plant parasitic nematodes. – Fund. Appl. Nematol. **15:** 183–191.

Riess, W., Giere, O., Kohls, O. & Sarbu, S. M., 1999: Anoxic thermomineral cave waters and bacterial mats as habitat for freshwater nematodes. – Aquat. Microb. Ecol. **18:** 157–164.

Rocha, C. E. F. d. & Bjornberg, M. H. G. d. C., 1988: *Allocyclops silvaticus* sp. n. (Copepoda, Cyclopoida, Cyclopidae), the first representative of the genus in South America. – Hydrobiologia **167/168:** 445–448.

Rohwerder, T., Sand, W. & Lascu, C., 2003: Preliminary evidence for a sulphur cycle in Movile Cave, Romania. – Acta Biotechnol. **23:** 101–107.

Santer, B., Sommerwerk, N. & Grey, J., 2006: Food niches of cyclopoid copepods in eutrophic Plußsee determined by stable isotope analysis. – Arch. Hydrobiol. **167:** 301–316.

Sarbu, S. M. & Kane, T. C., 1995: A subterranean chemoautotrophically based ecosystem. – Bull. Nation. Speleolog. Soc. **57:** 91–98.

Sarbu, S. M., Kane, T. C. & Kinkle, B. K., 1996: A chemoautotrophically based cave ecosystem. – Science **272:** 1953–1955.

Sarvala, J., 1998: Ecology and role of benthic copepods in northern lakes. – J. Mar. Syst. **15:** 75–86.

Sohlenius, B., 1979: A carbon budget for nematodes, rotifers and tardigrades in a Swedish coniferous forest soil. – Holarctic Ecol. **2:** 30–40.

Stemberger, R. S., 1986: The effects of food deprivation, prey density and volume on clearance rates and ingestion rates of *Diacyclops thomasi*. – J. Plankton Res. **8:** 243–251.

Strayer, D., 1985: The benthic micrometazoans of Mirror Lake, New Hampshire. – Arch. Hydrobiol. Suppl. **72:** 287–426.

Traunspurger, W., 1996: Distribution of benthic nematodes in the littoral of an oligotrophic lake (Königssee, National Park Berchtesgaden, FRG). – Arch. Hydrobiol. **135:** 393–412.

Traunspurger, W., Michiels, I. & Eyualem-Abebe, 2006: Composition and distribution of free-living freshwater nematodes: Global and local perspectives. – In: Eyualem-Abebe, Traunspurger, W. & Andrássy, I. (eds): Freshwater nematodes: Ecology and taxonomy. – CABI Publishing, Oxfordshire, pp. 46–76.

Williamson, C. E., 1980: The predatory behavior of *Mesocyclops edax*: Predator preferences, prey defenses, and starvation-induced changes. – Limnol. Oceanogr. **25:** 903–909.

– 1986: The swimming and feeding behavior of *Mesocyclops*. – Hydrobiologia **134:** 11–19.

Williamson, C. E. & Reid, J. W., 2001: Copepoda. – In: Thorp, J. H. & Covich, A. P. (eds): Ecology and classification of North American freshwater invertebrates. – Academic Press, San Diego, pp. 915–954.

Submitted: 4 January 2008; accepted: 15 June 2008.

12. References

Achinelly, M. F., Micieli, M. V. & García, J. J., 2003: **Pre-parasitic juveniles of *Strelkovimermis spiculatus* Poinar & Camino, 1986 (Nematoda: Mermithidae) predated upon by freshwater copepods (Crustacea: Copepoda)**. – *Nematology* **5:** 885-888.

Adrian, R. & Frost, T. M., 1992: **Comparative feeding ecology of *Tropocyclops prasinus mexicanus* (Copepoda, Cyclopoida)**. – *Journal of Plankton Research* **14:** 1369-1382.

Anderson, R. S., 1970: **Predator-prey relationship and predation rates for crustacean zooplankters from some lakes in Western Canada**. – *Canadian Journal of Zoology* **48:** 1229-1240.

Andrássy, I., 1992: **A short census of free-living nematodes**. – *Fundamental and Applied Nematology* **15:** 187-188.

Andrássy, I., 1956: **Die Rauminhalts- und Gewichtsbestimmung der Fadenwürmer (Nematoda)**. – *Acta Zoologica Hungarica* **2:** 1-15.

Arantes-Oliveira, N., Apfeld, J., Dillin, A. & Kenyon, C., 2002: **Regulation of life-span by germ-line stem cells in *Caenorhabditis elegans***. – *Science* **5554:** 502-504.

Avery, L. & Thomas, J. H., 1997: **Feeding and defecation**. - In: Riddle, D.L. (ed.): *C. elegans II*. – Cold Spring Harbor Laboratory Press, Plainview, NY, pp. 679-716.

Azevedo, R. B. R., Keightley, P. D., Lauren-Maatta, C., Vassilieva, L. L., Lynch, M. & Leroi, A. M., 2002: **Spontaneous mutational variation for body size in *Caenorhabditis elegans***. – *Genetics* **162:** 755-765.

Bagarinao, T., 1992: **Sulfide as an environmental factor and toxicant: tolerance and adaptations in aquatic organisms**. – *Aquatic Toxicology* **24:** 21-62.

Bandeili, B., 2006: **Bestimmung der Bakteriendiversität von Matten aus der Höhle Movile mit Hilfe der 16S rRNA-Gen Analyse. Bachelorarbeit an der Universität Bielefeld**.

Barrett, J., 1991: **Parasitic helminths**. - In: Bryant, C. (ed.): *Metazoan life without oxygen*. – Chapman and Hall, London, pp. 146-164.

Beier, S., Bolley, M. & Traunspurger, W., 2004: **Predator-prey interactions between *Dugesia gonocephala* and free-living nematodes**. – *Freshwater Biology* **49:** 77-86.

Birch, L. C., 1948: **The intrinsic rate of natural increase of an insect population**. – *Journal of Animal Ecology* **17:** 15-26.

Boaden, P. J. S., 1974: **Three new thiobiotic Gastrotricha**. – *Cahiers de Biologie Marine* **15:** 367-378.

Boaden, P. J. S., 1975: **Anaerobiosis, meiofauna and early metazoan evolution**. – *Zoologica Scripta* **4:** 21-24.

Botosaneanu, L., 1986: **Stygofauna mundi : a faunistic, distributional, and ecological synthesis of the world fauna inhabiting subterranean waters (including the marine interstitial)**. - Brill, Leiden.

Boulton, A. J., Humphreys, W. F. & Eberhard, S. M., 2003: **Imperilled subsurface waters in Australia: Biodiversity, threatening processes and conservation**. – *Aquatic Ecosystem Health & Management* **6:** 41-54.

Brandl, Z., 1998: **Feeding strategies of planktonic cyclopoids in lacustrine ecosystems**. – *Journal of Marine Systems* **15:** 87-95.

Brandl, Z. & Fernando, C. H., 1975: **Investigations on the feeding of carnivorous cyclopoids**. – *Verhandlungen. Internationale Vereinigung für theoretische und angewandte Limnologie* **19:** 2959-2965.

Brandl, Z. & Fernando, C. H., 1978: **Prey selection by cyclopoid copepods *Mesocyclops edax* and *Cyclops vicinus***. – *Verhandlungen. Internationale Vereinigung für theoretische und angewandte Limnologie* **20:** 2505-2510.

Brenner, S., 1974: **The genetics of *Caenorhabditis elegans***. – *Genetics* **77:** 71-94.

Brooks, A., 1991: **Genetic specification of life span and self-fertility in recombinant-inbred strains of *Caenorhabditis elegans***. – *Heredity* **67:** 19-28.

Brown, I. M., Wharton, D. A. & Millar, R. B., 2004: **The influence of temperature on the life history of the Antarctic nematode *Panagrolaimus davidi***. – *Nematology* **6:** 883-890.

Caughley, G. & Gunn, A., 1996: **Conservation biology in theory and practice**. - Blackwell Science, Cambridge, Mass.

Charlesworth, B., 1994: **Evolution in age-structured populations**. - Cambridge University Press, Cambridge.

Chasnov, J. R. & Chow, K. L., 2002: **Why are there males in the hermaphroditic species *Caenorhabditis elegans*?** – *Genetics* **160:** 983-994.

Chen, J., Lewis, E. E., Carey, J. R., Caswell, H. & Caswell-Chen, E. P., 2006: **The ecology and biodemography of *Caenorhabditis elegans***. – *Experimental Gerontology* **41:** 1059-1065.

Chen, J. J., Senturk, D., Wang, J. L., Müller, H. G., Carey, J. R., Caswell, H. & Caswell-Chen, E. P., 2007: **A demographic analysis of the fitness cost of extended longevity**

in *Caenorhabditis elegans*. – *Journals of Gerontology Series A -Biological Sciences and Medical Sciences* **62:** 126-135.

Chuang, S. H., 1962: **The embryonic and postembryonic development of *Rhabditis teres* (A. Schneider)**. – *Nematologica* **7:** 317-330.

Clarke, N. V., 1978: **The food of adult copepods from Lake Kainji**. – *Freshwater Biology* **8:** 321-326.

Confer, J. L., 1971: **Intrazooplankton predation by *Mesocyclops edax* at natural prey densities**. – *Limnology and Oceanography* **16:** 663-666.

Constantinescu, T., 1989: **Considérations sur la zone karstique de "La Movile" (Mangalia, Dobrogea du sud, Roumanie)**. – *Miscellanea spéologica Romanica* **1:** 7-12.

Croll, N. A., 1975: **Components and patterns in the behavior of the nematode *Caenorhabditis elegans***. – *Journal of Zoology* **176:** 159-176.

Dahms, H.-U. & Pottek, M., 1992: ***Metahuntemannia* Smirnov, 1946 and *Talpina* gen. nov. (Copepoda, Harpacticoida) from the deep-sea of the high Antarctic Weddell Sea with a description of eight new species**. – *Microfauna Marina* **7:** 7-78.

Dang, G. T. T., Pacheco-Perez, R. & Platzer, E., 2005: **Surface swimming behavior of infectious juveniles of *Romanomermis culicivorax***. – *Journal of Nematology* **37:** 364.

Davies, K. G. & Hart, J. E., 2008: **Fecundity and lifespan manipulations in *Caenorhabditis elegans* using exogenous peptides**. – *Nematology* **10:** 103-112.

Dole-Olivier, M. J., Galassi, D. M. P., Marmonier, P. & Creuzé Des Châtelliers, M., 2000: **The biology and ecology of lotic microcrustaceans**. – *Freshwater Biology* **44 :** 63-91.

Dolgin, E. S., Charlesworth, B., Baird, S. E. & Cutter, A. D., 2007: **Inbreeding and outbreeding depression in *Caenorhabditis* nematodes**. – *Evolution* **61:** 1339-1352.

Donkin, S. G. & Williams, P. L., 1995: **Influence of developmental stage, salts and food presence on various end-points using *Caenorhabditis elegans* for aquatic toxicity testing**. – *Environmental Toxicology and Chemistry* **14:** 2139-2147.

Dougherty, E. C., 1960: **Cultivation of aschelminthes, especially rhabditid nematodes**. - In: Sasser, J.N. & Jenkins, W.R. (eds.): *Nematology. Fundamentals and Recent Advances, with Emphasis on Plant Parasitic soil Forms*. – University of North Carolina Press, Chapel Hill, pp. 297-318.

Dumont, H. J., Van de Velde, I. & Dumont, S., 1975: **The dry weight estimate of biomass in a selection of Cladocera, Copepoda and Rotifera from plankton, periphyton and benthos of continental waters**. – *Oecologia* **19:** 75-97.

Duncan, A., Schiemer, F. & Klekowski, R. Z., 1974: **A preliminary study of feeding rates on bacterial food by adult females of a benthic nematode, *Plectus palustris* De Man 1880**. – *Polskie archiwum hydrobiologii = Polish archives of hydrobiology* **21:** 237-255.

Einsle, U., 1993: **Süßwasserfauna von Mitteleuropa: Vol. 8.4.1: Calanoida und Cyclopoida**. - Fischer, Stuttgart.

Elbourn, C. A., 1966: **Some observations on the food of *Cyclops strenuus strenuus* (Fischer)**. – *Annals and Magazine of Natural History* **9 (3.Ser.):** 227-231.

Estes, S., Ajie, B. C., Lynch, M. & Phillips, P. C., 2005: **Spontaneous mutational correlations for life-history, morphological and behavioral characters in *Caenorhabditis elegans***. – *Genetics* **170:** 645-653.

Eyre, M. J. & Caswell, E. P., 1991: **Sterile culture of *Rotylenchulus reniformis* on tomato root with gellan gum as a supporting medium**. – *Journal of Nematology* **23:** 229-231.

Fenchel, T., 1978: **The ecology of micro- and meiobenthos**. – *Annual review of ecology and systematics* **9:** 99-121.

Fenchel, T., 1974: **Intrinsic rate of natural increase: The relationship with body size**. – *Oecologia* **14:** 317-326.

Fenchel, T. & Riedl, R. J., 1970: **The sulfide system: A new biotic community underneath the oxidized layer of marine sands bottoms**. – *Marine Biology* **7:** 255-268.

Ferris, H., Lau, S. & Venette, R. C., 1995: **Population energetics of bacterial-feeding nematodes: respiration and metabolic rates based on CO_2 production**. – *Soil Biology and Biochemistry* **27:** 319-330.

Fisher, C. R., 1990: **Chemoautotrophic and methanotrophic symbiosis in marine invertebrates**. – *Rev. Aquat. Sci.* **2:** 399-463.

Friedman, D. B. & Johnson, T. E., 1988: **A mutation in the age-1 gene in *Caenorhabditis elegans* lengthens life and reduces hermaphrodite fertility**. – *Genetics* **118:** 75-86.

Fry, B., 2006: **Stable isotope ecology**. - Springer, New York.

Fryer, G., 1957a: **The feeding mechanism of some freshwater cyclopoid copepods**. – *Proceedings of the Zoological Society of London* **129:** 1-25.

Fryer, G., 1957b: **The food of some fresh-water cyclopoid copepods and its ecological significance**. – *Journal of Animal Ecology* **26:** 263-286.

Galassi, D., Marmonier, P., Dole-Olivier, M. J. & Rundle, S. D., 2002: **Microcrustacea**. - In: Rundle, S.D., Robertson, A.L. & Schmid-Araya, J.M. (eds.): *Freshwater meiofauna: biology and ecology*. – Backhuys Publishers, Leiden, pp. 135-175.

Giere, O., 2009: **Meiobenthology - The microscopic motile fauna of aquatic sediments**. - Springer, Berlin.

Gilbert, J. J. & Williamson, C. E., 1978: **Predator-prey behavior and its effect on rotifer survival in associations of *Mesocyclops edax*, *Asplanchna girodi*, *Polyarthra vulgaris*, and *Keratella cochlearis***. – *Oecologia* **37:** 13-22.

Gilbert, J. & Deharveng, L., 2002: **Subterranean ecosystems: A truncated functional biodiversity**. – *Bioscience* **52:** 473-481.

Gilboa-Garber, N., 1971: **Direct spectrophotometric determination of inorganic sulfide in biological materials and in other complex mixtures**. – *Analytical biochemistry* **43:** 129-133.

Gosse, P. H., 1859: **Evenings at the microscope**. - P.F. Collier & Son, New York.

Grootaert, P. & Maertens, D., 1976: **Cultivation and life cycle of *Mononchus aquaticus***. – *Nematologica* **22:** 173-181.

Grootaert, P. & Small, R. W., 1982: **Aspects of the biology of *Labronema vulvapillatum* (Meyl), (Nematoda, Dorylaimidae) in laboratory culture**. – *Biologisch Jaarboek* **50:** 135-148.

Grubbs, F. E., 1969: **Procedures for detecting outlying observations in samples**. – *Technometrics* **11:** 1-21.

Hakenkamp, C. C., Morin, A. & Strayer, D. L., 2002: **The functional importance of freshwater meiofauna**. - In: Rundle, S.D., Robertson, A.L. & Schmid-Araya, J.M. (eds.): *Freshwater meiofauna: biology and ecology*. – Backhuys Publishers, Leiden, pp. 321-335.

Halligan, D. L., Peters, A. D. & Keightley, P. D., 2003: **Estimating numbers of EMS-induced mutations affecting life history traits in *Caenorhabditis elegans* in crosses between inbred sublines**. – *Genetical Research* **82:** 191-205.

Hancock, P. J., Boulton, A. J. & Humphreys, W. F., 2005: **Aquifers and hyporheic zones: Towards an ecological understanding of groundwater**. – *Hydrogeology Journal* **13:** 98-111.

Hardin, G., 1960: **The competitive exclusion principle**. – *Science* **131 :** 1292-1297.

Heip, C., Vincx, M. & Vranken, G., 1985: **The ecology of marine nematodes**. – *Oceanography and Marine Biology* **23:** 399-489.

Henderson, S. T., Rea, S. L. & Johnson, T. E., 2006: **Dissecting the process of aging using the nematode *Caenorhabditis elegans***. - In: Masoro, E.J. & Austad, S.N. (eds.): *Handbook of the biology of aging*. – Elsevier Academic Press, Amsterdam, pp. 360-399.

Hentschel, U., Berger, E. C., Bright, M., Felbeck, H. & Ott, J. A., 1999: **Metabolism of nitrogen and sulfur in ectosymbiotic bacteria of marine nematodes (Nematoda, Stilbonematinae)**. – *Marine Ecology Progress Series* **183:** 149-158.

Herman, P. M. J. & Vranken, G., 1988: **Studies of the life-history and energetics of marine and brackish-water nematodes. II. Production, respiration and food uptake by *Monhystera disjuncta***. – *Oecologia* **77:** 457-463.

Hodgkin, J. & Barnes, T. M., 1991: **More is not better: Brood size and population growth in a self-fertilizing nematode**. – *Proceedings of the Royal Society of London - Series B: Biological Sciences* **246:** 19-24.

Hohberg, K. & Traunspurger, W., 2005: **Predator-prey interaction in soil food web: Functional response, size-dependent foraging efficiency, and the influence of soil texture**. – *Biology and Fertility of Soils* **41:** 419-427.

Holling, C. S., 1959a: **Some characteristics of simple types of predation and parasitism**. – *Canadian Entomologist* **91:** 385-398.

Holling, C. S., 1959b: **The components of predation as revealed by a study of small mammal predation of the European pine sawfly**. – *Canadian Entomologist* **91:** 293-320.

Hope, I. A., 1999: ***C. elegans*: A practical approach**. - Oxford University Press, Oxford.

Hopp, U. & Maier, G., 2005: **Implication of the feeding limb morphology for herbivorous feeding in some freshwater cyclopoid copepods**. – *Freshwater Biology* **50:** 742-747.

Höss, S., Juttner, I., Traunspurger, W., Pfister, G., Schramm, K. W. & Steinberg, C. E. W., 2002: **Enhanced growth and reproduction of *Caenorhabditis elegans* (Nematoda) in the presence of 4-nonylphenol**. – *Environmental Pollution* **120:** 169-172.

Houthoofd, K., Braeckman, B. P., Johnson, T. E. & Vanfleteren, J. R., 2003: **Life extension via dietary restriction is independent of the Ins/IGF-1 signalling pathway in *Caenorhabditis elegans***. – *Experimental Gerontology* **38:** 947-954.

Huettel, R. N., 2004: **Reproductive behaviour**. - In: Gaugler, R. (ed.): *Nematode behaviour*. – CABI Publishing, Wallingford, pp. 127-150.

Humes, A. G., 1994: **How many copepods?** – *Hydrobiologia* **292-293:** 1-7.

Hutchens, E., Radajewski, S., Dumont, M. G., McDonald, I. R. & Murrell, J. C., 2004: **Analysis of methanotrophic bacteria in Movile Cave by stable isotope probing**. – *Environmental Microbiology* **6:** 111-120.

Jamieson, C. D., 1980: **The predatory feeding of copepodid stages III to adult *Mesocyclops leuckarti* (Claus)**. - In: Kerfoot, W.C. (ed.): *Evolution and ecology of zooplankton communities*. – University Press, New England, pp. 518-537.

Jantunen, R., 1964: **Moulting of *Caenorhabditis briggsae* (Rhabditidae)**. – *Nematologica* **10:** 419-424.

Jensen, P., 1986: **Nematode fauna in the sulphide-rich brine seep and adjacent bottoms of the East Flower Garden, NW Gulf of Mexico. IV. Ecological aspects**. – *Marine Biology* **92:** 489-503.

Jensen, P., 1987a: **Differences in microhabitat, abundance, biomass and body size between oxybiotic and thiobiotic free-living marine nematodes**. – *Oecologia* **71:** 564-567.

Jensen, P., 1987b: **Feeding ecology of free-living aquatic nematodes**. – *Marine Ecology Progress Series* **35:** 187-196.

Johnson, T. E., Friedman, D. B., Foltz, N., Fitzpatrick, P. A. & Shoemaker, J. E., 1990: **Genetic variants and mutations of *Caenorhabditis elegans* provide tools for dissecting the aging processes**. - In: Harrison, D.E. (ed.): *Genetic effects on aging II*. – Telford, Caldwell, NJ, pp. 101-127.

Johnson, T. E. & Hutchinson, E. W., 1993: **Absence of strong heterosis for life span and other life history traits in *Caenorhabditis elegans***. – *Genetics* **134:** 465-474.

Johnson, T. E. & Wood, W. B., 1982: **Genetic analysis of life span in *Caenorhabditis elegans***. – *Proceedings of the National Academy of Sciences of the United States of America* **79:** 6603-6607.

Johnstone, D. B., Wei, A. G., Butler, A., Salkoff, L. & Thomas, J. H., 1997: **Behavioral defects in *C. elegans* egl-36 mutants result from potassium channels shifted in voltage-dependence of activation**. – *Neuron* **19:** 151-164.

Jokela, J., Lively, C. M., Dybdahl, M. F. & Fox, J. A., 1997: **Evidence for a cost of sex in the freshwater snail *Potamopyrgus antipodarum***. – *Ecology* **78:** 452-460.

Keightley, P. D. & Caballero, A., 1997: **Genomic mutation rate for lifetime reproductive output and lifespan in *Caenorhabditis elegans***. – *Proceedings of the National Academy of Sciences of the United States of America* **94:** 3823-3827.

Keightley, P. D., Davies, E. K., Peters, A. D. & Shaw, R. G., 2000: **Properties of ethylmethane sulfonate-induced mutations affecting life-history traits in *Caenorhabditis elegans* and inferences about bivariate distributions of mutation effects**. – *Genetics* **156:** 143-154.

Keightley, P. D. & Eyre-Walker, A., 1999: **Terumi Mukai and the riddle of deleterious mutation rates**. – *Genetics* **153:** 515-523.

Kirkwood, T. B. L., 1990: **The disposable soma theory of aging**. - In: Harrison, D.E. (ed.): *Genetic effects on aging II*. – Telford, Caldwell, NJ, pp. 9-20.

Knight, C. G., Azevedo, R. B. R. & Leroi, A. M., 2001: **Testing life-history pleiotropy in *Caenorhabditis elegans***. – *Evolution* **55:** 1795-1804.

Knight, C. G., Patel, M. N., Azevedo, R. B. R. & Leroi, A. M., 2002: **A novel mode of ecdysozoan growth in *Caenorhabditis elegans***. – *Evolution and Development* **4:** 16-27.

Krebs, C. J., 1994: **Ecology: The experimental analysis of distribution and abundance**. - HarperCollins College Publishers, New York.

Lambshead, P. J., 1993: **Recent developments in marine benthic biodiversity research**. – *Oceanis* **19:** 5-24.

Lampitt, R. S., 1978: **Carnivorous feeding by a small marine copepod**. – *Limnology and Oceanography* **23:** 1228-1231.

Lande, R. & Schemske, D. W., 1985: **The evolution of self-fertilization and inbreeding depression in plants. I. Genetic models**. – *Evolution* **39:** 24-40.

Landry, M. R., 1978: **Predatory feeding behavior of a marine copepod *Labidocera trispinosa***. – *Limnology and Oceanography* **23:** 1103-1113.

Lapesa, S., Snell, T. W., Fields, D. M. & Serra, M., 2002: **Predatory interactions between a cyclopoid copepod and three sibling rotifer species**. – *Freshwater Biology* **47:** 1685-1695.

Larsen, P. L. & Clarke, C. F., 2002: **Extension of life-span in *Caenorhabditis elegans* by a diet lacking coenzyme Q**. – *Science* **5552:** 120-123.

Lascu, C., 1989: **Paleogeographical and hydrogeological hypothesis regarding the origin of a peculiar cave fauna**. – *Miscellanea spéologica Romanica* **1:** 13-18.

Laybourn, J., 1979: **The effects of temperature on the respiration and production of the freshwater nematode *Anonchus* sp.** – *Oecologia* **41:** 329-337.

Lee, D. L., 2002: **Cuticle, Moulting and Exsheathment**. - In: Lee, D.L. (ed.): *The biology of nematodes*. – Taylor & Francis, London, pp. 171-210.

Lehman, P. S. & Reid, J. W., 1992: ***Phyllognatopus viguieri* (Crustacea: Harpacticoida), a predaceous copepod of phytoparasitic, entomopathogenic, and free-living nematodes**. – *Soil and Crop Science Society of Florida - Proceedings* **52:** 78-82.

Lotka, A. J., 1924: **Elements of physical biology**. - Williams & Wilkins, Baltimore, MD.

Luc, M., Taylor, D. P. & Netscher, C., 1979: **Endotokia matricida and intra-uterine development and hatching in nematodes**. – *Nematologica* **25:** 268-274.

Maier, G., 1990: **Reproduction and life history of a Central European, planktonic *Diacyclops bicuspidatus* population**. – *Archiv für Hydrobiologie* **117:** 485-495.

Malakhov, V. V., Hope, D. & Bentz, G. V., 1994: **Nematodes: Structure, development, classification, and phylogeny**. - Smithsonian Institution Press.

Marcotte, B. M., 1977: **An introduction to the architecture and kinematics of harpacticoid (Copepoda) feeding: *Tisbe furcata* (Baird, 1837)**. – *Mikrofauna des Meeresbodens* **61:** 183-196.

Marin, C. & Nicolescu, T., 1993: **The geochemistry of groundwater from southeastern Dobrogea, Romania**. – *Travaux de l'Institut de Spéologie Emile Racovitza* **32:** 229-247.

Masoro, E. J. & Austad, S. N., 2006: **Handbook of the biology of aging**. - Elsevier Academic Press, Amsterdam.

McCutchan, J. H. Jr., Lewis, W. M. Jr., Kendall, C. & McGrath, C. C., 2003: **Variation in trophic shift for stable isotope ratios of carbon, nitrogen, and sulfur**. – *Oikos* **102:** 378-390.

McDowell, E. M., 1978: **Fixation and processing**. - In: Trump, B.F. & Jones, R.T. (eds.): *Diagnostic electronic microscopy*. – Wiley, New York, pp. 113-139.

Michiels, I. & Traunspurger, W., 2005: **Benthic community patterns and the composition of feeding types and reproductive modes in freshwater nematodes**. – *Nematology* **7:** 21-36.

Moens, T. & Vincx, M., 1998: **On the cultivation of free-living marine and estuarine nematodes**. – *Helgoländer Meeresuntersuchungen* **52:** 115-139.

Moletta, M., Delgenes, J.-P., Godon, J.-J., Delgenes, J. P. & Godon, J. J., 2007: **Differences in the aerosolization behavior of microorganisms as revealed through their transport by biogas**. – *Science of the total environment* **379:** 75-88.

Mølller, E. F., 2007: **Production of dissolved organic carbon by sloppy feeding in the copepods *Acartia tonsa*, *Centropages typicus*, and *Temora longicornis***. – *Limnology and Oceanography* **52:** 79-84.

Moorthy, V. N., 1938: **Observations on the development of *Dracunculus medinensis* larvae in *Cyclops***. – *American Journal of Hygiene* **27:** 436-464.

Muschiol, D., Giere, O. & Traunspurger, W.: **Thiobiotic meiofauna in a chemosynthetic subterranean freshwater ecosystem (Movile Cave, Romania)**. (*submitted*)

Muschiol, D., Marković, M., Threis, I. & Traunspurger, W., 2008a: **Predatory copepods can control nematode populations: A functional-response experiment with *Eucyclops subterraneus* and bacterivorous nematodes**. – *Fundamental and Applied Limnology* **172:** 317-324.

Muschiol, D., Marković, M., Threis, I. & Traunspurger, W., 2008b: **Predator-prey relationship between the cyclopoid copepod *Diacyclops bicuspidatus* and a free-living bacterivorous nematode**. – *Nematology* **10:** 55-62.

Muschiol, D., Schroeder, F. & Traunspurger, W., 2009: **Life cycle and population growth rate of *Caenorhabditis elegans* studied by a new method**. – *BMC Ecology* **9:** 14.

Muschiol, D. & Traunspurger, W., 2009: **Life at the extreme: meiofauna from three unexplored lakes in the caldera of the Cerro Azul volcano, Galápagos Islands, Ecuador**. – *Aquatic Ecology* **43:** 235-248.

Muschiol, D. & Traunspurger, W., 2007: **Life cycle and calculation of the intrinsic rate of natural increase of two bacterivorous nematodes, *Panagrolaimus* sp. and *Poikilolaimus* sp. from chemoautotrophic Movile Cave, Romania**. – *Nematology* **9:** 271-284.

Nuß, B., 1984: **Ultrastrukturelle und ökophysiologische Untersuchungen an kristalloiden Einschlüssen der Muskeln eines sulfidtoleranten limnischen Nematoden (*Tobrilus gracilis*)**. – *Veröffentlichungen des Instituts für Meeresforschung Bremerhaven* **20:** 3-15.

Oeschger, R. & Schmaljohann, R., 1988: **Association of various types of epibacteria with *Halicryptus spinulosus* (Priapulida)**. – *Marine Ecology Progress Series* **48:** 285-293.

Ott, J. A., Rieger, R. M. & Enderes, F., 1982: **New mouthless interstitials worms from the sulfide system: symbiosis with prokaryotes**. – *Marine Ecology* **3:** 313-333.

Pearl, R., 1928: **The rate of living: being an account of some experimental studies on the biology of life duration**. - University of London Press, London.

Peters, A. D., Halligan, D. L., Whitlock, M. C. & Keightley, P. D., 2003: **Dominance and overdominance of mildly deleterious induced mutations for fitness traits in *Caenorhabditis elegans***. – *Genetics* **165:** 589-599.

Pfannkuche, O. & Thiel, H., 1988: **Sample processing**. - In: Higgins, R.P. & Thiel, H. (eds.): *Introduction to the study of meiofauna*. – Smithsonian Institution Press, Washington, D.C., pp. 134-145.

Platzer, E. G. & Mackenzie-Graham, L. L., 1980: ***Cyclops vernalis* as a predator of the pre-parasitic stages of *Romanomermis culicivorax***. – *Journal of the American Mosquito Control Association : Mosquito news* **40:** 252-257.

Plesa, C., 1989: **Étude préliminaire des Cyclopides (Crustacea, Copepoda) de la grotte 'Pestera de la Movile', Mangalia (Roumanie)**. – *Miscellanea spéologica Romanica* **1:** 39-45.

Poinar, G. O. Jr. & Sarbu, S. M., 1994: ***Chronogaster troglodytes* sp.n. (Nematoda, Chronogasteridae) from Movile Cave, with a review of cavernicolous nematodes**. – *Fundamental and Applied Nematology* **17 :** 231-237.

Popa, R. & Sarbu, S. M., 1991: **Trophic structure in an aquatic troglobiotic community based upon chemoautotrophic carbon fixation**. – *Travaux de l'Institut de Spéologie Emile Racovitza* **30:** 51-58.

Powell, E. N., Crenshaw, M. A. & Rieger, R. M., 1980: **Adaptations to sulfide in sulfide-system meiofauna. Endproducts of sulfide detoxification in three turbellarians and a gastrotrich**. – *Marine Ecology Progress Series* **2:** 169-177.

Procter, D. L. C., 1986: **Fecundity, reproductive effort, age-specific reproductive tactics and intrinsic rate of natural increase of a High Arctic nematode belonging to the genus *Chiloplacus***. – *Holarctic Ecology* **9:** 104-108.

Reid, J. W., 1986: **Some usually overlooked cryptic copepod habitats**. – *Syllogeus* **58:** 594-598.

Reversat, G., Garcia, N. & Dussart, B., 1992: **The copepod *Paracyclops affinis* as a predator of plant parasitic nematodes**. – *Fundamental and Applied Nematology* **15:** 183-191.

Riddle, D. L., Blumenthal, T., Meyer, B. J. & Priess, J. R., 1997: ***C. elegans* II**. - Cold Spring Harbor Laboratory Press, Plainview, NY.

Riess, W., Giere, O., Kohls, O. & Sarbu, S. M., 1999: **Anoxic thermomineral cave waters and bacterial mats as habitat for freshwater nematodes**. – *Aquatic Microbial Ecology* **18:** 157-164.

Robertson, A. L., Rundle, S. D. & Schmid-Araya, J. M., 2000: **Putting the meio- into stream ecology: current findings and future directions for lotic meiofaunal research**. – *Freshwater Biology* **44 :** 177-183.

Rocha, C. E. F. d. & Bjornberg, M. H. G. d. C., 1988: ***Allocyclops silvaticus* sp. n. (Copepoda, Cyclopoida, Cyclopidae), the first representative of the genus in South America**. – *Hydrobiologia* **167/168:** 445-448.

Roff, D. A., 1992: **The evolution of life histories: Theory and analysis**. - Chapman & Hall, New York.

Rohwerder, T., Sand, W. & Lascu, C., 2003: **Preliminary evidence for a sulphur cycle in Movile Cave, Romania**. – *Acta Biotechnologica* **23:** 101-107.

Santer, B., Sommerwerk, N. & Grey, J., 2006: **Food niches of cyclopoid copepods in eutrophic Plußsee determined by stable isotope analysis**. – *Archiv für Hydrobiologie* **167:** 301-316.

Sarbu, S. M., Gheorghe, C., Popescu-Jarnea, V., Vlasceanu, L., Popa, R. & Lascu, C., 1995: **Stable isotope studies in Movile Cave**. – *Travaux de l'Institut de Spéologie Emile Racovitza* **34:** 99-102.

Sarbu, S. M. & Kane, T. C., 1995: **A subterranean chemoautotrophically based ecosystem**. – *Bulletin of the National Speleological Society* **57:** 91-98.

Sarbu, S. M., Kane, T. C. & Kinkle, B. K., 1996: **A chemoautotrophically based cave ecosystem**. – *Science* **272:** 1953-1955.

Sarbu, S. M., Kinkle, B. K., Vlasceanu, L., Kane, T. C. & Popa, R., 1994: **Microbiological characterisation of a sulfide-rich groundwater ecosystem**. – *Geomicrobiology Journal* **12:** 175-182.

Sarbu, S. M. & Popa, R., 1992: **A unique chemoautotrophically based cave ecosystem**. - In: Camacho, A.I. (ed.): *The natural history of biospeleology*. – Museo Nacional de Ciencias Naturales, Madrid, pp. 637-666.

Särkkä, J., 1995: **Profundal meiofauna in two large lakes: influence of pollution and bathymetric differences**. – *Archiv für Hydrobiologie* **132:** 453-493.

Sarvala, J., 1998: **Ecology and role of benthic copepods in northern lakes**. – *Journal of Marine Systems* **15:** 75-86.

Schallenberg, M., Kalff, J. & Rasmussen, J. B., 1989: **Solutions to problems in enumerating sediment bacteria by direct counts**. – *Applied and Environmental Microbiology* **55:** 1214-1219.

Schiemer, F., 1983: **Comparative aspects of food dependence and energetics of freeliving nematodes**. – *Oikos* **41:** 32-42.

Schiemer, F., 1982a: **Food dependence and energetics of freeliving nematodes I. Respiration, growth and reproduction of *Caenorhabditis briggsae* (Nematoda) at different levels of food supply**. – *Oecologia* **54:** 108-121.

Schiemer, F., 1982b: **Food dependence and energetics of freeliving nematodes II. Life history parameters of *Caenorhabditis briggsae* (Nematoda) at different levels of food supply**. – *Oecologia* **54:** 122-128.

Schiemer, F., Duncan, A. & Klekowski, R. Z., 1980: **A bioenergetic study of a benthic nematode, *Plectus palustris* de Man 1880, throughout its life cycle. II. Growth, fecundity and energy budgets at different densities of bacterial food**. – *Oecologia* **44:** 205-212.

Schiemer, F., Novak, R. & Ott, J. A., 1990: **Metabolic studies on thiobiotic free-living nematodes and their symbiotic microorganisms**. – *Marine Biology* **106:** 129-137.

Schmid, P. E. & Schmid-Araya, J. M., 2002: **Trophic relationships in temporary and permanent freshwater meiofauna**. - In: Rundle, S.D., Robertson, A.L. & Schmid-Araya, J.M. (eds.): *Freshwater meiofauna: biology and ecology*. – Backhuys Publishers, Leiden, pp. 295-319.

Seinhorst, J. W., 1962: **On the killing, fixation and transferring to glycerin of nematodes**. – *Nematologica* **8:** 29-32.

Shook, D. R., Brooks, A. & Johnson, T. E., 1996: **Mapping quantitative trait loci affecting life history traits in the nematode *Caenorhabditis elegans***. – *Genetics* **142:** 801-817.

Shook, D. R. & Johnson, T. E., 1999: **Quantitative trait loci affecting survival and fertility-related traits in *Caenorhabditis elegans* show genotype-environment interactions, pleiotropy and epistasis**. – *Genetics* **153:** 1233-1243.

Shurin, J. B. & Allen, E. G., 2001: **Effects of competition, predation, and dispersal on species richness at local and regional scales**. – *American Naturalist* **158:** 624-637.

Skibbe, O., 1994: **An improved quantitative protargol stain for ciliates and other planctonic protists**. – *Archiv für Hydrobiologie* **130:** 339-347.

Small, R. W., 1988: **Invertebrate predators**. - In: Poinar, G.O. & Jansson, H.-B. (eds.): *Diseases of nematodes. Volume II*. – CRC Press, Boca Raton, pp. 73-92.

Sohlenius, B., 1968: **Influence of microorganisms and temperature upon some rhabditid nematodes**. – *Pedobiologia* **8:** 137-145.

Sohlenius, B., 1973: **Growth and reproduction of a nematode *Acrobeloides* sp. cultivated on agar**. – *Oikos* **24:** 64-72.

Sohlenius, B., 1988: **Interactions between two species of *Panagrolaimus* in agar cultures**. – *Nematologica* **34:** 208-217.

Sohlenius, B., 1979: **A carbon budget for nematodes, rotifers and tardigrades in a Swedish coniferous forest soil**. – *Holarctic Ecology* **2:** 30-40.

Sommerville, R. I., 1960: **The growth of *Cooperia curticei* (Giles, 1892) a nematode parasite of sheep**. – *Parasitology* **50:** 261-267.

Stemberger, R. S., 1986: **The effects of food deprivation, prey density and volume on clearance rates and ingestion rates of *Diacyclops thomasi***. – *Journal of Plankton Research* **8:** 243-251.

Stiuca, E. & Ilinca, H., 1995: **Pleistocene mammals (Rodentia) from Movile Cave (Romania)**. – *Theoretical and Applied Karstology* **8:** 157-161.

Strayer, D., 1985: **The benthic micrometazoans of Mirror Lake, New Hampshire**. – *Archiv für Hydrobiologie, Supplement* **72:** 287-426.

Sulston, J. & Hodgkin, J., 1988: **Methods**. - In: Wood, W.B. (ed.): *The nematode Caenorhabditis elegans*. – Cold Spring Harbor Laboratory Press, Plainview, NY, pp. 587-606.

Sulston, J. E., Schierenberg, E., White, J. G. & Thomson, J. N., 1983: **The embryonic cell lineage of the nematode *Caenorhabditis elegans***. – *Developmental Biology* **100:** 64-119.

Sutphin, G. L. & Kaeberlein, M., 2008: **Dietary restriction by bacterial deprivation increases life span in wild-derived nematodes**. – *Experimental Gerontology* **43:** 130-135.

Tain, L. S., Lozano, E., Sáez, A. G. & Leroi, A. M., 2008: **Dietary regulation of hypodermal polyploidization in *C. elegans***. – *BMC Developmental Biology* **8:** 28.

Thiermann, F., Vismann, B. & Giere, O., 2000: **Sulphide tolerance of the marine nematode *Oncholaimus campylocercoides* - a result of internal sulphur formation?** – *Marine Ecology Progress Series* **193:** 251-259.

Thomas, P. R., 1965: **Biology of *Acrobeles complexus* Thorne, cultivated on agar**. – *Nematologica* **11:** 395-408.

Townshend, J. L. & Blackith, R. E., 1975: **Fungal diet and the morphometric relationships in *Aphelenchus avenae***. – *Nematologica* **21:** 19-25.

Traunspurger, W., 1991: **Fischbiologie des Königssees**. - Forschungsbericht 22, Nationalpark Berchtesgaden, Berchtesgaden, Germany.

Traunspurger, W., Haitzer, M., Höss, S., Beier, S., Ahlf, W. & Steinberg, C., 1997a: **Ecotoxicological assessment of aquatic sediments with *Caenorhabditis elegans* (Nematoda) - A method for testing liquid medium and whole-sediment samples**. – *Environmental Toxicology and Chemistry* **16:** 245-250.

Traunspurger, W., 1996: **Distribution ot benthic nematodes in the littoral of an oligotrophic lake (Königssee, National Park Berchtesgaden, FRG)**. – *Archiv für Hydrobiologie* **135:** 393-412.

Traunspurger, W., 1997: **Bathymetric, seasonal and vertical distribution of the feeding-types of nematodes in an oligotrophic lake**. – *Vie et Milieu* **47:** 1-7.

Traunspurger, W., 2002: **Nematoda**. - In: Rundle, S.D., Robertson, A.L. & Schmid-Araya, J.M. (eds.): *Freshwater meiofauna: biology and ecology*. – Backhuys Publishers, Leiden, pp. 63-104.

Traunspurger, W., Bergtold, M. & Goedkoop, W., 1997b: **The effects of nematodes on bacterial activity and abundance in a freshwater sediment**. – *Oecologia* **112:** 118-122.

Traunspurger, W., Michiels, I. & Eyualem, A., 2006: **Composition and distribution of free-living freshwater nematodes: Global and local perspectives**. - In: Eyualem, A., Traunspurger, W. & Andrássy, I. (eds.): *Freshwater nematodes: Ecology and taxonomy*. – CABI Publishing, Oxfordshire, pp. 46-76.

Triantaphyllou, A. C., 1973: **Environmental sex differentiation of nematodes in relation to pest management**. – *Annual Review of Phytopathology* **11:** 441-462.

Vander Zanden, M. J. & Rasmussen, J. B., 2001: **Variation in ä^{15}N and ä^{13}C trophic fractionation: Implications for aquatic food web studies**. – *Limnology and Oceanography* **46:** 2061-2066.

Vassilieva, L. L., Hook, A. M. & Lynch, M., 2000: **The fitness effects of spontaneous mutations in *Caenorhabditis elegans***. – *Evolution* **54:** 1234-1246.

Vassilieva, L. L. & Lynch, M., 1999: **The rate of spontaneous mutation for life-history traits in *Caenorhabditis elegans***. – *Genetics* **151:** 119-129.

Venette, R. C. & Ferris, H., 1997: **Thermal constraints to population growth of bacterial-feeding nematodes**. – *Soil Biology and Biochemistry* **29:** 63-74.

Vranken, G. & Heip, C., 1985: **Aspects of the life cycle of free-living marine nematodes**. - In: VanGrieken, R.E. & Wollast, R. (eds.): *Progress in Belgian oceanographic research*. – Belgian Center of Oceanography, Antwerpen, pp. 267-278.

Vranken, G. & Heip, C., 1983: **Calculation of the intrinsic rate of natural increase, r_m, with *Rhabditis marina* Bastian 1865 (Nematoda)**. – *Nematologica* **29:** 468-477.

Vranken, G., Herman, P. M. J. & Heip, C., 1988: **Studies of the life-history and energetics of marine and brackish-water nematodes I. Demography of *Monhystera disjuncta* at different temperature and feeding conditions**. – *Oecologia* **77:** 296-301.

Ward, S. & Carrel, J. S., 1979: **Fertilization and sperm competition in the nematode *Caenorhabditis elegans***. – *Developmental Biology* **73:** 304-321.

Wegener, C., 2000: **Symbiont oder Pathogen? Symbiontische Eigenschaften einer genetisch homogenen Gruppe von Luzerne-nodulierenden Rhizobien. Diss. Universität Bielefeld.:** -187.

Wharton, D. A., 1998: **Comparison of the biology and freezing tolerance of *Panagrolaimus davidi*, an Antarctic nematode, from field samples and cultures**. – *Nematologica* **44:** 643-653.

Wieser, W. & Kanwisher, J., 1960: **Growth and metabolism in a marine nematode *Enoplus communis* Bastian**. – *Zeitschrift für vergleichende Physiologie* **43:** 29-36.

Williams, P. L. & Dusenbery, D. B., 1990: **Aquatic toxicity testing using the nematode *Caenorhabditis elegans***. – *Environmental Toxicology and Chemistry* **9:** 1285-1290.

Williamson, C. E., 1986: **The swimming and feeding behavior of *Mesocyclops***. – *Hydrobiologia* **134:** 11-19.

Williamson, C. E., 1980: **The predatory behavior of *Mesocyclops edax*: Predator preferences, prey defenses, and starvation-induced changes**. – *Limnology and Oceanography* **25:** 903-909.

Williamson, C. E. & Reid, J. W., 2001: **Copepoda**. - In: Thorp, J.H. & Covich, A.P. (eds.): *Ecology and classification of North American freshwater invertebrates*. – Academic Press, San Diego, pp. 915-954.

Wilson, P. A. G., 1976: **Nematode growth patterns and molting cycle: population growth profile**. – *Journal of Zoology* **179:** 135-151.

Wood, J. G., Rogina, B., Lavu, S., Howitz, K., Helfand, S. L., Tatar, M. & Sinclair, D., 2004: **Sirtuin activators mimic caloric restriction and delay ageing in metazoans**. – *Nature* **430:** 686-689.

Woombs, M. & Laybourn-Parry, J., 1984: **Growth, reproduction and longevity in nematodes from sewage treatment plants**. – *Oecologia* **64:** 168-172.

Yeates, G. W., 1970: **Studies on laboratory cultures of dune sand nematodes**. – *Journal of Natural History* **4:** 119-136.

Yeates, G. W. & Boag, B., 2003: **Growth and life histories in Nematoda with particular reference to environmental factors**. – *Nematology* **5:** 653-664.

Zhang, G. T., Li, C. L., Sun, S., Zhang, H. Y., Sun, J. & Ning, X. R., 2006: **Feeding habits of *Calanus sinicus* (Crustacea : Copepoda) during spring and autumn in the Bohai Sea studied with the herbivore index**. – *Scientia Marina* **70:** 381-388.