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FEEDING BEHAVIOUR OF CALANOID COPEPODS AND ANALYSES OF THEIR FAECAL PELLETS

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GENERAL INTRODUCTION

Copepods occupy a key position in pelagic food webs. They transfer organic matter produced by the photosynthetic phytoplankton to higher trophic levels such as seabirds, marine mammals and pelagic fish stocks, thus finally to humans (Lenz 2000). Copepods are among the most abundant group of metazoans on Earth and dominate the zooplankton community in the open ocean (Boxshall 1998). The study of their feeding behaviour is crucial to understand and determine their impact on the fate of primary producers (Granéli et al. 1993, Banse 1995). A variable fraction of the energy consumed by copepods is assimilated and used for growth, respiration and reproduction while the fraction which is not utilized is egested as faecal pellet. These faecal pellets may be recycled in the upper water column (Smetacek 1980, González & Smetacek 1994) or sink towards the ocean floor (reviewed by Turner 2002). Studies of faecal pellets with emphasis on their contents give important information about the feeding behaviour of their producers as well as on their possible ecological role and biogeochemical impact (Turner 2002).

Feeding of calanoid copepods and analyses of the content of their faecal pellets will be the main focus of this thesis. It will address different aspects of copepod feeding with emphasis on species specific interactions as well as on algae survival and dissolution within faecal pellets. This section will therefore start with an introduction to the feeding techniques of calanoid copepods, and then investigate the role of copepod feeding and faecal pellets in the marine ecosystem. The last part will present different traditional methods commonly applied to estimate copepod grazing.

FEEDING TECHNIQUES OF CALANOID COPEPODS

The traditional view of planktonic calanoid copepods as relatively mechanical suspension-feeders has been replaced by the recognition that a wide variety of mechanisms exist to detect, pursue, capture and reject prey (Price 1988). In the early 80s, the paradigm of copepods as filter feeders was overturned. Direct visual behavioural observations of copepods by high speed micro-cinematography have demonstrated the ability to capture and handle individual algal cells (Alcaraz et al. 1980, Koehl & Strickler 1981, Paffenhöfer et al. 1982, Price et al.

1983, Paffenhöfer 1998). Among the predominantly herbivorous and omnivorous copepods we can distinguish between different feeding modes, namely the more passive suspension feeding and the raptorial feeding mode, but several organisms are able to switch between both (Schnack 1989, Kiørboe et al. 1996). The use of passive filtration or active capture of prey is dependent on the prey size (Price & Paffenhöfer 1986). The passive feeding mode is used to capture cells which are too small to be individually detected by the copepod. Generally it is known, that copepods prefer larger food items (Mullin 1963, Paffenhöfer & Knowles 1978, Paffenhöfer 1988), using the active feeding mode. Feeding is now recognized as a very active process involving three-dimensional flow fields (Koehl & Strickler 1981, Strickler 1982, Bundy & Vanderploeg 2002, Malkiel et al. 2003). For the creation of such flow fields, calanoid copepods have highly structured feeding appendages. They actively use five pairs of appendages when feeding (second antennae, mandibles, first and second maxillae and maxillipeds, Fig.1), while the first antenna may have sensorial function, detecting movements of the prey (Landry 1980), besides its function for copepod motility and quick locomotion (Alcaraz & Strickler 1988).

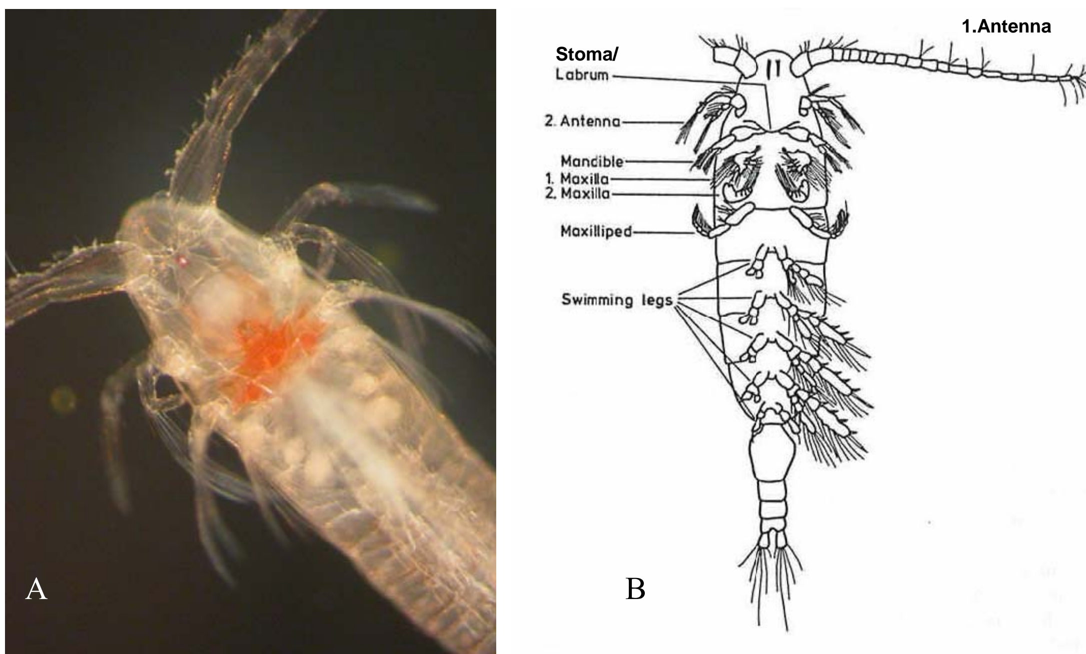

Fig. 1: (A) Photograph of *Calanus helgolandicus*, ventral view on the feeding appendages
(B) Schematic illustration of the ventral side of a copepod, showing the position of appendages (Schnack 1989)

The appendages create water currents on the ventral side of the copepod's body over the stationary second maxillae, which actively capture the food particles. Food particles are then transported to the stoma, where they are grabbed by the mandibular gnathobases (Fig. 2), minced if necessary, and ingested. The gnathobases of the copepod mandibles can be compared to the teeth in mammals and are designed for cutting, mincing and grinding. They can be used as an indicator if a copepod is primarily carnivore or herbivore/omnivore (Anraku & Omori 1963, Itoh, 1970, Schnack 1989, Michels & Schnack-Schiel 2005). Morphological differences of the mandibular gnathobases exist also between the sexes of some copepod species and between congener species from different geographical regions (Schnack 1989). The ingested food is transferred from the mouth via the oesophagus to the anterior region of the midgut. It accumulates there for 10 to 20 min (Arashkevich 1977) before part of it passes into the posterior region of the midgut where the faecal pellet is formed.

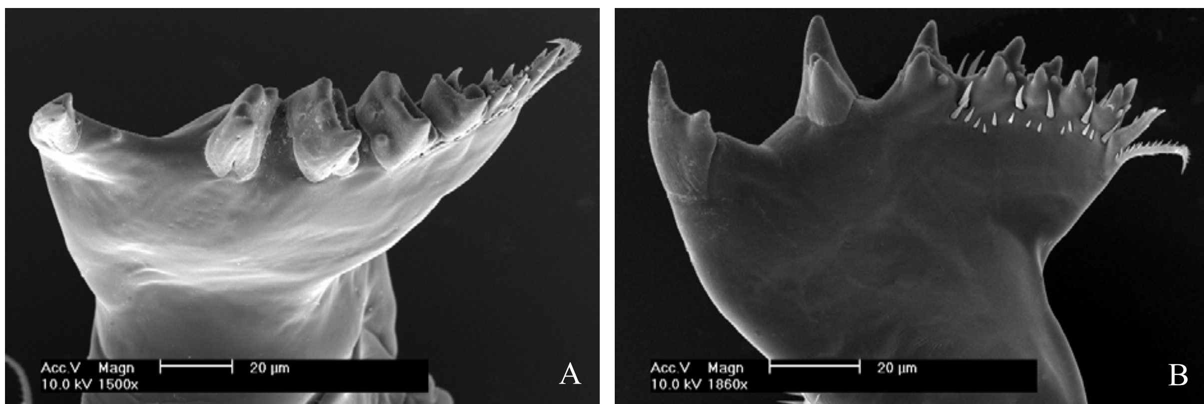

Fig. 2: Scanning electron micrograph of the mandibular gnathobases of the primarily herbivorous copepod species (A) *Calanus helgolandicus (right gnathobase)* and (B) *Temora longicornis (left gnathobase)*

The minimum food size copepods can feed on was for long time thought to be given by the distance between the setae of the second maxillae (Boyd 1976). However, studies including the influence of physical forces were able to show that copepods can graze on even smaller particles than given with the minimum distance of their setae (Price & Paffenhöfer 1986). Estimates on the thickness of the boundary layer around the setae and setules of the second maxillae suggest that the latter may function more as a solid paddle than an open sieve (Price & Paffenhöfer 1986, Schnack 1989). With this knowledge, there exists theoretically no minimum prey size (Hansen et al. 1994), but feeding efficiency decreases with decreasing food size. Hence, the question of the maximum food size arises. With the ability to mince

their prey, copepods are able to feed on particles extending their stoma size. However, observations of feeding on large prey items and possible maximum values are scarce in the current literature.

Large sized diatoms are frequently found in sediments and size as well as stability of the diatom frustules may present an effective protection against grazing (Smetacek 2000). Generally, the interaction between phytoplankton and their main predators leads to the evolutionary arms race (Smetacek 2001) and an unsuitable cell size, the stability of cell frustules or other morphological structures (like e.g. spines) as well as the production of toxins are discussed as possible phytoplankton defence strategies against copepod grazing (Wolfe 2000, Smetacek 2001, Hamm et al. 2003). Besides spending effort in defence mechanisms to avoid being eaten, another possible strategy is the development of mechanisms to survive the gut passage. Studies of species specific interactions among copepods and their prey are needed to improve the current understanding why certain phytoplankton cell structures are more beneficial than others.

THE CONSEQUENCES OF FEEDING FOR THE ECOSYSTEM AND THE COPEPOD ITSELF

Several studies have shown that large celled diatoms dominate the phytoplankton biomass during early spring blooms when nutrients are abundant (e.g. Eilertsen et al. 1989, Reigstad & Wassmann 1996), while dinoflagellates and coccolithopores are phytoplankton groups which usually become more important later in the season (Thierstein & Young 2004). The succession of phytoplankton blooms varies locally and is determined by physical factors such as light, mixing, advection, temperature and nutrient concentrations as well as biological factors such as competition and grazing (Eilertsen et al. 1989, Ziemann et al. 1991, Reigstad & Wassmann 1996). Hence, prediction which group will win the competition during the build up of a bloom is impossible. In most cases, copepod grazing activity is not able to suppress the occurrence of phytoplankton blooms due to the lower growth and reproduction rates of the copepods compared to their prey (but see: Halvorsen & Tande 1999). However, the ability of copepods to select between different prey items can influence the species composition and size distribution of phytoplankton in the oceans (Ryther & Sanders 1980, Granéli et al. 1993, Kiørboe 1997). Selective feeding on high quality food items may results in a species shift during ecosystem succession (Schultes 2004) and enhances the copepod reproduction. It is

known, that copepods are able to find patches of enhanced food concentration and even decrease their diurnal vertical migration in order to stay within patches of high food concentrations (Bainbridge 1953, RollwagenBollens & Landry 2000). Several dinoflagellate species are known to produce toxins which may serve as competitive advantage, decreasing their risk to be grazed. However, studies have shown that some copepods graze on toxic algae and toxins can be transferred through the food web from the phytoplankton via copepods to fish (White 1981, Teegarden & Cembella 1996, Tester et al. 2000). During grazing, the assimilation of food is sometimes incomplete (Marshall & Orr 1955, Conover 1966) and viable cells can occur in produced faecal pellets (Fowler & Fisher 1983). Such viable cells within faecal pellets may fuel the plankton community at a later stage of the bloom through recycling mechanisms, combined with mixing or upwelling.

Copepods feed in order to survive, grow and reproduce. Beside the importance of feeding to meet its own energy requirements, copepod females need to gain energy for gonad development and egg production (Mauchline 1998). Especially for the predominantly herbivorous zooplankton living at high latitudes the problem of long periods with shortage of food arises. Some copepods such as *Calanus* spp. undergo diapause during winter to save energy through inactivity, fasting and reduced respiration (Hirche 1998). Additionally, copepods have adapted to this highly seasonal food availability, by storing lipid reserves (Albers et al. 1996, Lee et al. 2006). Copepods are able to mobilize their lipid stores for gonad development and egg production (e.g. Hagen & Schnack 1996, Lee et al. 2006), making egg production in some high latitude species more or less independent from ambient food concentrations (Smith 1990, Hagen & Schnack 1996, Mauchline 1998). However, it is still questionable, which species are spawning on lipid reserves and which species are able to react directly to enhanced food concentrations, especially among the high latitude species (Hagen & Schnack 1996).

THE IMPORTANCE OF FAECAL PELLETS; WHY TO STUDY THEM?

The undigested food remains of calanoid copepods are enclosed by a peritrophic membrane (Gauld 1957) resulting in a compact faecal pellet (Fig. 3). Faecal pellets have been studied for a long period of time (e.g. Moore 1931, Gauld 1957, Schrader 1971, Martens 1978) and have been of interest in studies of biogeochemical cycling for the last decades (e.g. Fowler 1977,

Lee & Fisher 1994, Turner 2002, Xu & Wang 2003). With the high abundance of copepods present in the oceans (Huys & Boxshall 1991) and commonly reported faecal pellet production rates of 100 – 150 faecal pellets copepod^{-1} day^{-1} (Mauchline 1998), it becomes obvious that these faecal pellets must play an important role for the elemental cycling.

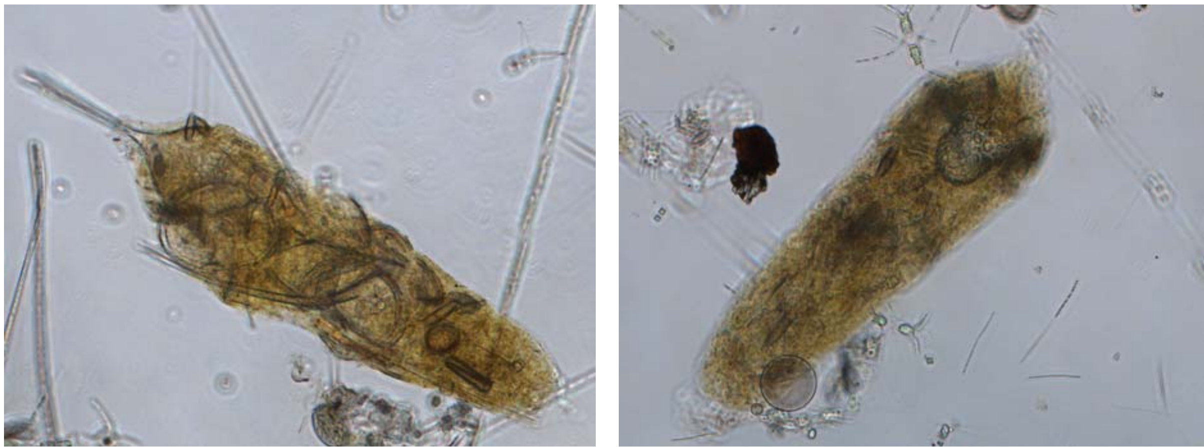

Fig. 3: Faecal pellets produced by the copepod *Pleuromamma robusta*, grazing on the natural plankton community. Pictures were taken at 160x magnification.

While single cells of phytoplankton typically have sinking rates of a few meters per day (Smayda 1970, 1974), densely packed zooplankton faecal pellets can sink several hundred meter per day (Smayda 1969, Small et al. 1979, Komar et al. 1981). This implies that faecal pellets may be of importance for vertical flux of organic material. However, numerous studies have shown that the contribution of copepod faecal pellets to the total material flux is highly variable ranging from <1% to 99% (Smetacek 1980, Bathmann et al. 1987, Viitasalo et al. 1999, WexelsRiser et al. 2001, Turner 2002, Sampei et al. 2004), with the majority of the studies reporting relatively low faecal pellet export, as fraction of the total particulate organic carbon (POC). The relative importance of faecal pellets for recycling or vertical flux depends on various factors, which include zooplankton abundance and species composition as well as faecal pellet abundance, size, density, chemical and particulate contents (Turner 2002). Mechanisms such as coprorphagy, coprorchaly or coprorhexy are thought to be important for the recycling of the pellets within the euphotic zone (Lampitt et al. 1990, Noji et al. 1991, Viitasalo et al. 1999, Wexels Riser et al. 2001). The fate of material stemming from faecal pellets recycled in the photic zone depends on the condition of the material enclosed within the faecal pellets. Microscopic analysis of copepod faecal pellets can give valuable qualitative

and semi-quantitative information on the copepods diet and the degree of dissolution within the pellets, since the surrounding membrane is more or less transparent (Gauld 1957). Main phytoplankton groups, such as diatoms, dinoflagellates and coccolithophores can easily be identified within faecal pellets because of their mineral skeletons (Turner 1984, Turner 1991). Diatom silica as well as coccolith calicite are important pools in the global silica, carbon and calcium cycles, respectively (Tréguer et al. 1995, Milliman et al. 1999, Ragueneau et al. 2000, Thierstein & Young 2004). And copepod feeding may have substantial effect on calcite dissolution (Harris 1994, Jansen & Wolf-Gladrow 2001) as well as on silica dissolution (Jansen 2002, Schultes 2004), but further investigations are needed for better understanding how grazing transforms the ingested phytoplankton cells. Signs and dynamics of dissolution can be revealed by use of scanning electron microscopy (SEM, Jansen 2002). This thesis therefore refers to microscopic techniques in order to clarify the role of grazing and faecal pellet quality in the pelagic environment.

TRADITIONAL TECHNIQUES TO MEASURE GRAZING

As copepods are known to be the main link between primary producers and higher trophic levels, it is of great importance to be able to estimate their grazing pressure on phytoplankton and microzooplankton in a qualitative and quantitative way. A number of different methods are commonly used to measure copepod feeding (Båmstedt et al. 2000). Depending on the question asked, there are positive and negative aspects and possible mistakes coming along with all methods available at present. In this chapter the most widely used methods applied to estimate zooplankton grazing will be briefly discussed.

A traditional method, widely used to measure grazing is the incubation (or bottle) experiment, introduced several decades ago by Frost (1972). A defined number of grazers are incubated in bottles filled with water including the prey. From direct cell counts before and after the incubation, it can be calculated what has been removed by the grazers. Control bottles, without grazers, allow correcting for algal growth during the experiment. Usually these experiments last for 24 hours to account for possible diurnal grazing cycles of the copepods. This method with direct cell counts is very time consuming, particularly when running field experiments with natural sea water including various different prey species. It is also unclear how well results from these incubations reflect reality, since the grazing

behaviour within the bottles most likely change due to different turbulence regimes and disturbance of the hydromechanic detection ability of copepods (Roman & Rublee 1980, Visser 2001). It appears that bottle incubations are a useful method, particularly when copepods are grazing on monocultures, but when working in the field, trophic cascading may mask the grazing impact of the incubated copepods (Frost 1972, Nejstgaard et al. 2001).

Microscopic gut or faecal pellet content analysis excludes some of the problems described for the incubation method, since direct observations of the gut or faecal pellet content can serve as a "fingerprint" of what has been grazed by the copepod (Turner 1984, Bathmann et al. 1987, Turner 1991). Examination of the gut and/or the faecal pellet content seems to be a relatively easy and accurate way to investigate the prey of copepods in the field, but different problems may arise when applying this method. While faecal pellet content can be analysed without much expenditure of time, the preparation of the gut may again be very time consuming. Additionally, ingestion can easily be underestimated, due to a variable but mostly significant proportion of unidentified organic matter within the guts and faecal pellets. Some organisms are less liable to digestion than others and problems arise when copepods eat only parts of their prey or suck out their soft parts only (Elbrächter 1973). Therefore, only semi-quantitative and qualitative conclusions can be drawn from this method.

The gut fluorescence method is considered to be another *in situ* method of determining zooplankton feeding rates (Mackas & Bohrer 1976, Head 1986). It is a rapid and cheap method to get a rough grazing estimate through the measurement of chlorophyll *a* and pheopigment content in the copepod gut. The method can be suitable during bloom conditions when autotrophic plankton is dominating, but is less suitable during non bloom scenarios since feeding on prey items without chlorophyll (heterotrophic organisms) is not considered. The total grazing impact will therefore mostly be underestimated. Another limitation using this method is that nothing can be said about selective grazing on prey species and about observed variable amounts of pigment destruction-losses (Conover et al. 1986, Wang & Conover 1986).

Faecal pellet production is directly related to ingestion and grazing estimates can therefore be made based on assumed or previously determined assimilation rates. The number and sizes of faecal pellets produced can be converted into carbon and ingestion rates can be calculated (Mauchline 1998 and references therein). This is a relatively robust method providing an estimate of total carbon ingestion. However, this method can also give no

information about possible food preferences, selection or which species have actually been grazed.

The egg production rate of some copepod species is also related to their ingestion (Dagg & Grill 1980, Kiørboe et al. 1985, Peterson & Dam 1996). But problems arise if copepods spawn on stored lipids (Mayzaud et al. 2002a). It is also known that starvation affects egg production only after a number of days which implies that past feeding conditions affect the present egg production rate (Tester & Turner 1990, Rey-Rassat et al. 2002).

A wide variety of different methods exist in literature (Båmstedt et al. 2000) to address the problem of quantification and qualification of copepod ingestion. Above, only some of the more common ones (partly used in this dissertation) are presented. Since each method has its specific limitations, scientists often combine several of them (Zeldis 2001, Mayzaud et al. 2002a, Mayzaud et al. 2002b) and they are working to develop new, improved methods to estimate *in situ* grazing impact of calanoid copepods. A promising approach seems to be offered by molecular methods, revealing the true genetic “fingerprint” of ingested prey determined from the *in situ* gut content or faecal pellets of copepods (Nejstgaard et al. 2003).

The manuscripts presented in this dissertation intend to give answers to some crucial questions related to copepod feeding behaviour as described in the aims and outlines of this thesis.

AIMS AND OUTLINE OF THIS THESIS

Feeding of calanoid copepods and the quality of their faecal pellets are in the focus of this thesis. Emphasis is put on the possible ecological impact of feeding and on the possible ecological impact of the produced faecal pellets.

In detail, the aims of the presented thesis were…

- …to estimate the ecological importance of copepod feeding in the field (in terms of calcite dissolution, toxin transfer through the food web and egg production in relation to food).
- …to analyse different possible defence strategies of phytoplankton against copepod grazing (toxin production, stability, chain formation, spines, cell size or the ability to survive digestion).
- …to combine established methods and develop and apply new techniques to qualify and quantify ingestion of different copepod species.

The importance of calcifying algae in the marine carbon and calcium cycles is widely known. Copepods as the main predators of these algae may have an important impact in this cycle due to calcite dissolution in their guts (Harris 1994, Milliman et al. 1999). **Manuscript I** is dealing with this topic and tries to shed light on the actual amount of calcite dissolving in copepod guts. Dissolution experiments were performed using the calcifying algae *Calcidiscus leptoporus* and the morphological changes of the coccoliths were studied by means of scanning electron microscope (SEM) analysis. These data were used for content interpretation of freshly produced faecal pellets in feeding experiments with the common North Sea copepods *Calanus helgolandicus* and *Temora longicornis*.

Manuscripts II & III are based on data from a field study in the North Sea. The aim of these studies was to evaluate the importance of mesozooplankton grazing as a biological loss factor of harmful algal blooms (HABs) under natural conditions. Experiments were conducted during an autumn bloom of the dinoflagellates *Dinophysis norvegica* and *Ceratium furca* to study whether or not copepods graze on *Dinophysis* sp. and *Ceratium* sp. under natural conditions. **Manuscript II** is focussing on faecal pellet production experiments conducted with *C. helgolandicus*, while **manuscript III** includes three more copepod species (*T. longicornis*, *Centropages* sp., *Acartia clausi*) and their egg production, faecal pellet production and grazing impact on the natural phytoplankton community. In these studies, the question arises, whether or not algae may survive the gut passage of copepods. The goal of **manuscript IV** was therefore to test a new method to determine the ability of different phytoplankton species to survive passing through the gut of a copepod. Until recently, evidences of algal survival after being grazed by copepods relied on incubations of freshly produced faecal pellets in medium and later analysis of the growing algae. This method has its limitations due to possible contamination and the fact that several algae species are difficult or even impossible to keep in culture, as known for *Dinophysis* sp. for instance (Sampayo 1993). Microscopic observations of faecal pellet content, chlorophyll auto-fluorescence as well as fluorescence from cells stained with the viability stain fluorescein diacetate (FDA) provided a new possibility for direct observation of freshly produced faecal pellets and analysis whether or not the ingested cells survives the gut passage. During the laboratory experiments presented in **manuscript IV** it was also studied if certain structures may protect the algae from being grazed upon. Three diatom- and one dinoflagellate species were chosen to study different morphological structures with the focus on possible defence strategies of the algae and the results are presented in **manuscript V.** The ability of copepods to graze on very large phytoplankton cells, which may in first glance look like unsuitable prey for the copepod was in the focus. Behavioural observations were carried out with the copepods *T. longicornis* and *A. clausi* grazing on the *Coscinodiscus wailesii*, the largest diatom commonly found in the North Sea. Results showed that traditional methods to determine grazing may sometimes be unsuitable and that new methods need to be developed. The new promising approach of genetical gut content analysis (Nejstgaard et al. 2003) to qualify and quantify the grazing impact of copepods was therefore tested in a study presented in **manuscript VI** and provided promising results.

Leaving the North Sea, the last manuscript is based on a study, conducted in the Southern Ocean in the frame of the European iron fertilization experiment (EIFEX). Large scale iron fertilization experiments are a useful tool to study *in situ* interactions within the ecosystem. Six major iron fertilization experiments have been conducted so far, with four of them in the Southern Ocean. All of them produced massive phytoplankton blooms, dominated by diatoms. In **manuscript VII** the ecological impact of food availability and feeding on reproduction was studied for the copepod *Rhincalanus gigas* which is known as a dominant species in Antarctic and circumpolar waters (Ommanney 1936, Atkinson 1991). EIFEX provided a good opportunity to follow the reproductive response of this copepod species during the build up of a diatom dominated phytoplankton bloom.

The manuscripts will be followed by a final discussion, where main results of these studies will be summarized and discussed.

LIST OF MANUSCRIPTS

This thesis is based on the following manuscripts, partly submitted or already published. They are referred to in the text by their Roman numbers. The contribution of the authors is specified on the following page.

I. Langer, G., G. Nehrke, **S. Jansen** (Submitted to Marine Ecology Progress Series) Dissolution of *Calcidiscus leptoporus* coccoliths in copepod guts? – A morphological study

II. Wexels Riser, C., **S. Jansen**, U. Bathmann, P. Wassmann (2003) Grazing of *Calanus helgolandicus* on *Dinophysis norvegica* during bloom conditions in the North Sea: evidence from investigations of faecal pellets. Marine Ecology Progress Series 256: 301-304.

III. **Jansen, S.**, C. Wexels Riser, P. Wassmann, U. Bathmann (2006) Copepod feeding behaviour and egg production during a dinoflagellate bloom in the North Sea. Harmful Algae 5: 102-112.

IV. **Jansen, S.** and U. Bathmann (Submitted to Marine Ecology Progress Series) Algae viability within copepod faecal pellets- Evidence from microscopic observations

V. **Jansen, S.** (Draft) Copepods grazing on *Coscinodiscus wailesii* – a question of size?

VI. Töbe, K., **S. Jansen**, U. John (Draft) Copepods grazing on Phytoplankton – First steps of identification using molecular tools

VII. **Jansen, S.,** C. Klaas, S. Krägefsky, L. von Harbou, U. Bathmann (2006) Reproductive response of the copepod *Rhincalanus gigas* to an iron-induced phytoplankton bloom in the Southern Ocean. Polar Biology DOI 10.1007/s00300-006-0147-0

Contribution of the authors:

Manuscript I
The concept was developed by all authors. Copepod feeding experiments were conducted by S. Jansen, while additional dissolution experiments were conducted by G. Langer and G. Nehrke. The manuscript was prepared by S. Jansen in close cooperation with the co-authors.

Manuscript II
The experiments have been planned, processed and examined by C. Wexels Riser. SEM analysis was conducted by S. Jansen. S. Jansen and C. Wexels Riser wrote the manuscript in cooperation with the other co-authors.

Manuscript III
The concept was developed by S. Jansen. Feeding and egg production experiments were processed and examined by S. Jansen, while C. Wexels Riser provided the faecal pellet production data. The manuscript was written by S. Jansen in cooperation with the co-authors.

Manuscript IV
The experiments have been planned, processed and examined by S. Jansen. The manuscript was written by S. Jansen in discussion with U. Bathmann.

Manuscript V
The experiments have been planned, processed and examined by S. Jansen. The manuscript was written by S. Jansen.

Manuscript VI
The concept was developed by all authors. Copepod grazing experiments were planned and conducted by S. Jansen. K. Töbe developed the species specific primers and carried out the genetical analysis. The manuscript was written with contributions of all authors.

Manuscript VII
The concept was developed by S. Jansen. Experimental work and determination of the gonad developmental stages was carried out by S. Jansen. Chlorophyll *a* data were provided by C. Klaas. Zooplankton sampling was conducted by all authors. The manuscript was written by S. Jansen in cooperation with the co-authors.

MANUSCRIPT I

Dissolution of *Calcidiscus leptoporus* coccoliths in copepod guts? A morphological study

Gerald Langer, Gernot Nehrke, Sandra Jansen

Submitted to Marine Ecology Progress Series

Dissolution of *Calcidiscus leptoporus* coccoliths in copepod guts? A morphological study

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Abstract

In a dissolution experiment using the calcifying algae *Calcidiscus leptoporus* the morphological changes of the coccoliths were followed by means of SEM analysis. The decrease in coccolith calcite mass during this experiment was quantified. These data were used for the interpretation of the coccolith morphologies within faecal pellets produced in additionally conducted feeding experiments with the calanoid copepods *Calanus helgolandicus* and *Temora longicornis*. Linking the observed coccolith morphologies from the coccolith dissolution experiments with the coccolith morphologies observed within the faecal pellets it can be concluded that the coccolith dissolution during copepod gut passage is well below 8 weight (wt.) %.

Key words: calcite dissolution, copepods, *Calcidiscus leptoporus*, faecal pellets, SEM

Introduction

The production and dissolution of biogenic marine calcite constitutes an important part of the marine carbon and calcium cycle (Milliman 1993, De La Rocha & DePaolo 2000). In the photic zone where coccoliths, the calcite platelets of coccolithophores, are produced no dissolution of calcite can occur, since ocean surface waters are supersaturated with respect to calcite (Zeebe & Wolf-Gadrow 2001). However, calcite dissolution could occur in a micro-environment undersaturated with respect to calcite. The gut of copepods feeding on coccolithophores for example could provide such an undersaturated environment.

In an experimental study the mass of coccolith calcite ingested by copepods was compared to the calcite mass egested and it was shown that up to 73 wt. % of the coccolith mass was dissolved during gut passage (Harris 1994). Jansen & Wolf-Gladrow (2001) on the other side, predicted no significant dissolution to occur under the same experimental conditions (constant feeding). For other grazing conditions like alternating grazing and non-grazing periods dissolution of up to 25 wt. % was predicted by the authors (Jansen & Wolf-Gladrow 2001).

Several authors (Roth et al. 1975, Honjo 1976, Honjo & Roman 1978, Bathmann et al. 1987) made detailed analyses of copepod faecal pellets by means of scanning electron microscopy (SEM), which did not reveal signs of coccolith dissolution. From this observation they concluded that the copepod gut is not acidic. The assumption of a non-acidic copepod gut was experimentally confirmed by Pond et al. (1995), who measured gut pH by means of pH sensitive fluorescent dye. Using this method they reported pH values of ~7 for starved specimens and ~8 for specimens fed with coccolithophores. Even though several authors investigated coccolith dissolution during copepod gut passage, the methods used are diverse making it difficult to compare results directly. The interpretation of SEM images with respect to coccolith dissolution is difficult since no data are available relating morphological data to numbers in terms of wt. % calcite dissolution.

To fill this gap we have performed a dissolution experiment with coccospheres of the widespread species *Calcidiscus leptoporus*. Research so far has focused on *Emiliania huxleyi*, a bloom forming species. Data from copepod feeding experiments (Roth et al. 1975, Harris 1994, Nejstgaard et al. 1994, Nejstgaard et al. 1997) as well as a dissolution experiment showing graded dissolution of coccoliths (McIntyre & McIntyre 1971) are available.

However, morphological data were not related to calcite mass loss. *C. leptoporus* is at least as important as *E. huxleyi* in terms of calcite export production (Sprengel et al. 2000, Baumann et al. 2004). Moreover, *C. leptoporus* is more resistant to dissolution than *E. huxleyi* (McIntyre & McIntyre 1971, Berger 1973). Hence data on dissolution of *C. leptoporus* coccoliths are desirable. In our study the morphological changes during *C. leptoporus* coccolith dissolution were followed by means of SEM analysis and correlated to the observed mass loss. These data were used for the interpretation of the coccolith morphologies within faecal pellets produced in additionally conducted feeding experiment with the copepods *Calanus helgolandicus* and *Temora longicornis*.

Material and Methods

Dissolution experiments using *Calcidiscus leptoporus*

A monospecific culture of *Calcidiscus leptoporus* (strain AC365, from South Atlantic off South Africa, CODENET culture collection, ALGOBANK http://www.nhm.ac.uk/hosted_sites/ina/CODENET/caencultures.htm) was grown in sterile filtered (0.2 µm) seawater enriched with 100 µM nitrate, 6.25 µM phosphate, trace metals and with vitamins according to F/2 (Guillard & Ryther 1962) The incident photon flux density was 350 µmol m^{-2} s^{-1} and a 12/12 hour light/dark cycle was applied. Cells were grown at optimum growth temperature, i.e. 20°C. For determination of cell density samples were counted immediately after sampling using a Sedgwick Rafter counting cell. Cells were grown to a density of ~2500 cells ml^{-1} and then put in an oven at 45°C for 10 hours in order to kill the cells. Previous tests showed that cell density remained constant after heat-treatment and SEM observations showed that the morphology of the coccoliths did not change.

At the start and end of the experiment alkalinity and dissolved inorganic carbon (DIC) were measured. Total alkalinity was calculated from linear Gran plots (Gran 1952) after duplicate potentiometric titration (Bradshaw et al. 1981, Brewer et al. 1986) and DIC was measured in triplicate using a Shimadzu TOC 5050A analyzer. For alkalinity measurements samples were sterile filtered (0.2 µm), and stored in 300 ml borosilicate flasks at 0°C. DIC samples were sterile filtered and stored in 6 ml borosilicate flasks free of air bubbles at 0°C.
The carbonate system was calculated from temperature, salinity, and the concentrations of DIC, total alkalinity and phosphate, using the program CO_2sys (Lewis & Wallace 1998).

Equilibrium constants of Mehrbach et al. (1973) refitted by Dickson & Millero (1987) were chosen.

The calcite saturation state (Ω = IAP K^{-1}, where IAP is the ion activity product of Ca^{2+} and CO_3^{2-} and K the solubility constant of calcite) was adjusted by adding calculated amounts of 1 M HCl to the medium. The total volume of cell culture was then subdivided into separate flasks. In order to prevent gas exchange with the atmosphere 0.6 l borosilicate flasks were filled without headspace and closed with Teflon lined screw caps. The flasks were continuously rotated on a plankton wheel at 15°C in order to prevent sedimentation. At distinct points in time the contents of one flask was filtered on pre-weighted 25-mm polycarbonate (PC) filters with a pore-size of 0.8 µm. The PC-filters were dried in a drying cabinet at 60°C for 12 hours and then weighed on a Mettler Toledo® UMX2 micro-balance. The reduction in total cell mass per filter was used as a measure for calcite loss through dissolution. Therefore it is required, that cell densities can be related linearly to cell mass. This was tested by preparing solutions with different cell densities in triplicate. The result of this test is presented in Figure 1. Besides calcite mass total cell mass includes also protoplast mass. In case organic components were destroyed as well during calcite dissolution a part of the observed reduction in total cell mass per filter could be attributed to loss of organic material. In order to estimate the mass percentage of calcite and organic material, we measured particulate organic carbon (POC, ~60 pg $cell^{-1}$) and particulate inorganic carbon (PIC, ~140 pg $cell^{-1}$) per cell. Samples for determination of total particulate carbon (TPC) and particulate organic carbon (POC) were filtered on precombusted (12 hours, 500°C) GF/F-filters (0.6 µm) and stored at -20°C until further handling. Prior to analysis, the POC filters were fumed for two hours with a saturated HCl solution to remove all inorganic carbon. TPC and POC were subsequently measured on a Carlo Erba® NA-1500 Analyzer. Particulate inorganic carbon (PIC) was calculated as the difference between TPC and POC. Assuming that 50 wt. % of the protoplast dry mass is carbon mass we calculated the mass of the organic material (~120 pg $cell^{-1}$). In that case 90 wt. % of total cell mass is calcite. It has to be noted however, that this means that our numbers of wt. % calcite dissolved are to be regarded as maximum values.

The filters used for the determination of the mass loss during dissolution were used for SEM analysis. SEM-micrographs of the filtered samples were taken using a Quanta 200F /FEI®. Therefore the filters were cut into smaller pieces and fixed with double sided carbon

tape on aluminium stubs. The stubs were coated with Platinum/Palladium using an Emscope® SC500 sputter coater.

Faecal pellet production experiments

Faecal pellet production experiments were carried out, using a mixture of *C. leptoporus* and *Thalassiosira weissflogii* as food. *C. leptoporus* and *T. weissflogii* were grown in F/2 medium (Guillard & Ryther 1962) in a temperature controlled room at 20 and 15°C, respectively. For faecal pellet production experiments, algae were mixed to achieve an end concentration of 1300 µg C liter^{-1}. While a mixture of 1:3 regarding to cell numbers was chosen, corresponding to1390 cells *C. leptoporus* ml^{-1} and 4590 cells *T. weissflogii* ml^{-1}.

Copepods were collected close to Helgoland with a 180 µm net, slowly towed from 15 m to surface. After retrieval, copepods were diluted in 10 liter of surface water and adult females of *Calanus helgolandicus* and *Temora longicornis* were sorted out in a temperature controlled room at 15°C. Animals were transferred to 1 liter bottles containing the experimental algae concentration for over-night acclimatisation on a plankton wheel. After 16 hours, females were carefully transferred to 250 ml beakers filled with the same algae mixture used during the acclimatisation. For both copepod species *C. helgolandicus* and *T. longicornis*, 5 parallels were set up including 2 or 3 animals each, respectively. Faecal pellet production experiments were carried out for a total of 6 hours, while beakers were checked once an hour for faecal pellets, which were directly enumerated and picked out to prevent coprophagy. Faecal pellet production rates were calculated and faecal pellets prepared for analysis with the SEM (Wexels Riser et al. 2003).

Results and Discussion

Dissolution data

Dead cells of *Calcidiscus leptoporus* were exposed to seawater undersaturated with respect to calcite ($\Omega = 0.5$) for different time spans. The decrease in calcite mass during this experiment is shown in Figure 2. The morphologies present at distinct points in time (circled points in Figure 2) are presented in Figure 3. At the start of the experiment coccoliths display, apart from the usual culture artefacts, a normal morphology. It is well known that malformations are more abundant in culture samples than in oceanic samples.

Since the general reader might not be familiar with coccolith morphology we would like to briefly explain the morphology of *C. leptoporus* coccoliths. A coccolith is comprised of two shields, the proximal and the distal shield (Figure 4A). The coccosphere, i.e. the shell of the coccolithophore cell, is build of roughly 20 interlocking coccoliths (Figure 4B). For detailed description of terminology see Young et al. (1997).

The typical coccolith morphology present at the onset of the experiment (t_0, Figure 2) is shown in Figure 3A while a more detailed view of this morphology (Figure 3B) shows a coccolith in proximal view and one in distal view. With a weight loss of 8 % (t_1, Figure 2), the detachment of distal and proximal shield can be observed throughout the whole sample and is therewith representative for this early stage of dissolution (Figure 3C and 3D). As dissolution proceeds (15 to 33 wt. %) the detached proximal and distal shields themselves fall apart (Figure 3E and 3F). Roth & Berger (1975) reported coccoliths of *C. leptoporus* in surface sediments, some of them lacking proximal shields. They interpreted the detachment of the shields as a sign of heavy dissolution. We regard this characteristic feature as an indicator of an early dissolution stage, which appears already in dissolution below 8 wt. %. In the following discussion of the copepod faecal pellet production experiments we will therefore put special emphasis on single shields in faecal pellets.

Dissolution of *C. leptoporus* within copepod faecal pellets?

Calanus helgolandicus and *Temora longicornis* feeding on a monoculture of *C. leptoporus* (~6,000 cells ml^{-1}) resulted in faecal pellets that were notably fragile. Only one faecal pellet produced by *C. helgolandicus* could be prepared for SEM analysis (Figure 5A and 5B). It is not clear whether the peritrophic membrane of the faecal pellets is lacking when copepods feed on *C. leptoporus* monoculture, but a lack or instability of the peritrophic membrane could possibly explain the observed fragility of the faecal pellets. To increase the stability of the faecal pellets, experiments were repeated with a mixture of *C. leptoporus* and the diatom *Thalassiosira weissflogii*. Faecal pellets remained stable and complete during further handling.

SEM observations of the faecal pellets produced were used to compare coccolith morphologies from dissolution experiments (Figure 3) with the coccoliths within the faecal pellets (Figure 5). Pictures show coccoliths in a faecal pellet of *C. helgolandicus*, feeding on a monoculture of *C. leptoporus* (Figure 5B) and from the same species feeding on a mixture of

C. leptoporus and *T. weissflogii* (Figure 5C). The coccoliths in neither faecal pellet display detached shields. It has to be noted that especially in Figure 5B all coccoliths in lateral view clearly are comprised of both distal and proximal shield. The same holds true for faecal pellets produced by *T. longicornis* (Figure 5D). In general all coccoliths observed were comprised of both shields. However, some coccoliths show signs of damage, which can be clearly interpreted as mechanical destruction caused by the mandibles of the copepod. This can safely be concluded from the following observations: partially dissolved coccoliths show detached shields while the shields themselves are not broken (Figures 3). In mechanically destroyed coccoliths on the other hand the shields are broken and mostly not detached from one another (Figure 5). This interpretation is supported by copepod feeding experiments carried out by Roth et al. (1975), where also mechanically destroyed coccoliths were described.

From the calcite mass loss curve (Figure 2) in combination with our SEM analysis we can conclude that less than 8 wt. % of *C. leptoporus* calcite is dissolved in the gut of the investigated copepod species *C. helgolandicus* and *T. longicornis* at the given faecal pellet production rates. *C. helgolandicus* produced 2.9 ± 0.3 faecal pellets female^{-1} hour^{-1}, while production rate of *T. longicornis* was calculated to be 4.1 ± 0.5 faecal pellets female^{-1} hour^{-1}. The faecal pellet production rate can be used to estimate gut passage times. Assuming that the gut volume is equivalent to two faecal pellets, gut passage time is twice the interval between the production of a single pellet (Mauchline 1998). Therefore, estimated gut passage times from *C. helgolandicus* and *T. longicornis* in the present study were about 30 and 40 minutes, respectively. Gut passage times of 30 to 40 minutes were also observed in a number of other species (Arashkevich 1977) and Jansen & Wolf-Gladrow (2001) chose 30 minutes as a representative value in their model. We conclude that our experiments investigate the calcite loss at standard natural food conditions considering gut passage time.

In a number of studies faecal pellets were analysed by means of SEM, most of them containing well preserved coccoliths which show no signs of dissolution (Roth et al. 1975, Honjo 1976, Honjo & Roman 1978, Bathmann et al. 1987). The question remained, however, at which dissolution stage (i.e. wt. % calcite dissolved) partial dissolution of coccoliths can be recognized in SEM-images. Since different coccolithophore species were used in the above mentioned studies it is not straightforward to apply our correlation between mass loss and morphology to these data directly. Most experimental and field studies focused on the

coccolithophore species *Emiliania huxleyi.* McIntyre & McIntyre (1971) showed that *E. huxleyi* coccoliths display partially dissolved central areas in a very early dissolution stage. Unfortunately they did not quantify the mass loss associated with the changing morphologies. However, since *C. leptoporus* is more resistant to dissolution than *E. huxleyi* (Berger 1973), and 8 wt. % dissolution is clearly visible in our experiments, it is likely that this holds true for other species, especially for more soluble ones like *E. huxleyi*. Therewith, we conclude that coccolithophore calcite dissolution in the gut of copepods in general is below 8 wt. %. This assumption is supported by a numerical model by Jansen & Wolf-Gladrow (2001) stating that under the conditions comparable to those in our experiments (constant feeding) no significant dissolution should occur.

To our knowledge only one study states that significant coccolith dissolution occurs during copepod gut passage (Harris 1994). In the latter study, where *Pseudocalanus elongates* and *C. helgolandicus* were grazing on *E. huxleyi* 50 to 73 wt. % of the ingested coccoliths were not egested. The author concludes that this considerable loss is due to acid digestion in the copepod gut. Harris states that the high mass loss he reports is in conflict with SEM observations presented in the literature (Harris 1994). The author gives three possible explanations for this discrepancy: firstly intact coccoliths observed by means of SEM are only those which have escaped dissolution, secondly incomplete quantitative recovery of faecal pellets (i.e. underestimation of egestion) and thirdly "sloppy feeding" (i.e. overestimation of ingestion). Since our dissolution experiment showed that dissolution proceeds homogeneously throughout the whole sample, i.e. affecting all coccolith simultaneously, we would expect to find partially dissolved coccoliths in a faecal pellet if significant amounts of coccoliths were dissolved in copepod guts. Therefore we are not conform with the first explanation of Harris (1994). The fact that the author used a monoculture as food, however, could render the second explanation likely, because in our feeding experiments we could hardly recover one faecal pellet containing exclusively *C. leptoporus* coccoliths, since they were fragile. If this holds true also for *E. huxleyi* monoculture it could possibly explain the high apparent dissolution in the study of Harris (1994). The third explanation given by Harris (1994), i.e. "sloppy feeding" should result in considerable amounts of loose coccoliths floating in the bottles. Large amounts of loose coccoliths are generally not observed in *C. leptoporus* cultures, because this species does not shed coccoliths like *E. huxleyi*; meaning that only complete coccospheres are usually present. We could not find high numbers of loose coccoliths on the filters containing

the faecal pellets. However, this observation is not sufficient to exclude “sloppy feeding”, since the volume of water on theses filters is not representative (see Material and Methods).

The latter renders it most likely that the massive dissolution reported by Harris (1994) represents an overestimation. Considering our findings in context with other studies (see above) there are good reasons to assume that only insignificant dissolution of coccoliths during copepod gut passage occurs. It might be concluded that calcite dissolution above the lysocline is generally not mediated by copepods.

Conclusion

SEM observations on the dissolution of *Calcidiscus leptoporus* showed that the detachment of the proximal and the distal shield of a coccolith is an early stage in the dissolution process which is readily observable at 8 wt. % dissolution.

This result was used to interpret the morphology of *C. leptoporus* coccoliths within freshly produced faecal pellets of the copepods *Calanus helgolandicus* and *Temora longicornis.* Since no detached shields were found in faecal pellets it was concluded that less than 8 wt. % of *C. leptoporus* calcite was dissolved during gut passage of the investigated copepod species.

Acknowledgments

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Figure captions:

Figure 1: Dependence of *Calcidiscus leptoporus* cell mass on cell density.

Figure 2: Development of *Calcidiscus leptoporus* mass loss in the course of the dissolution experiment. The circled dots mark the points in time at which the coccolith morphologies where investigated by means of SEM (Figure 3).

Figure 3: Morphological changes during the dissolution of *Calcidiscus leptoporus* at distinct points in time marked in Figure 2. (A) At time t_0 the sample is dominated by complete coccospheres with coccolith showing no signs of dissolution (slightly elongated spheres result from the filtration procedure). (B) Detailed view of a typical coccolith at t_0 in proximal (left) and distal view (right). (C) At time t_1 all proximal and distal shields are detached. (D) Close-up of the marked area in the previous picture, showing detached proximal and distal shields. A few shields are already disintegrated (example in the lower right). SEM-micrographs E and F taken at time t_2 and t_5 respectively show later stages of dissolution characterised by more distinct disintegration of detached shields.

Figure 4: General morphology of *Calcidiscus leptoporus*. (A) Side view of a coccolithophore lith (coccolith) build up by distal and proximal shield. (B) An open coccosphere, i.e. the shell of the coccolithophore cell, build of roughly 20 interlocking coccoliths

Figure 5: (A) Faecal pellet produced by *Calanus helgolandicus* while feeding on a monoculture of *Calcidiscus leptoporus*. (B) Detailed view of the coccoliths within the faecal pellets shown in the previous image. Faecal pellet of (C) *Calanus helgolandicus* and (D) *Temora longicornis* containing *Thalassiosira weissflogii* and *Calcidiscus leptoporus*. It has to be noted that all coccoliths in lateral view clearly are comprised of both distal and proximal shield.

Figure 1:

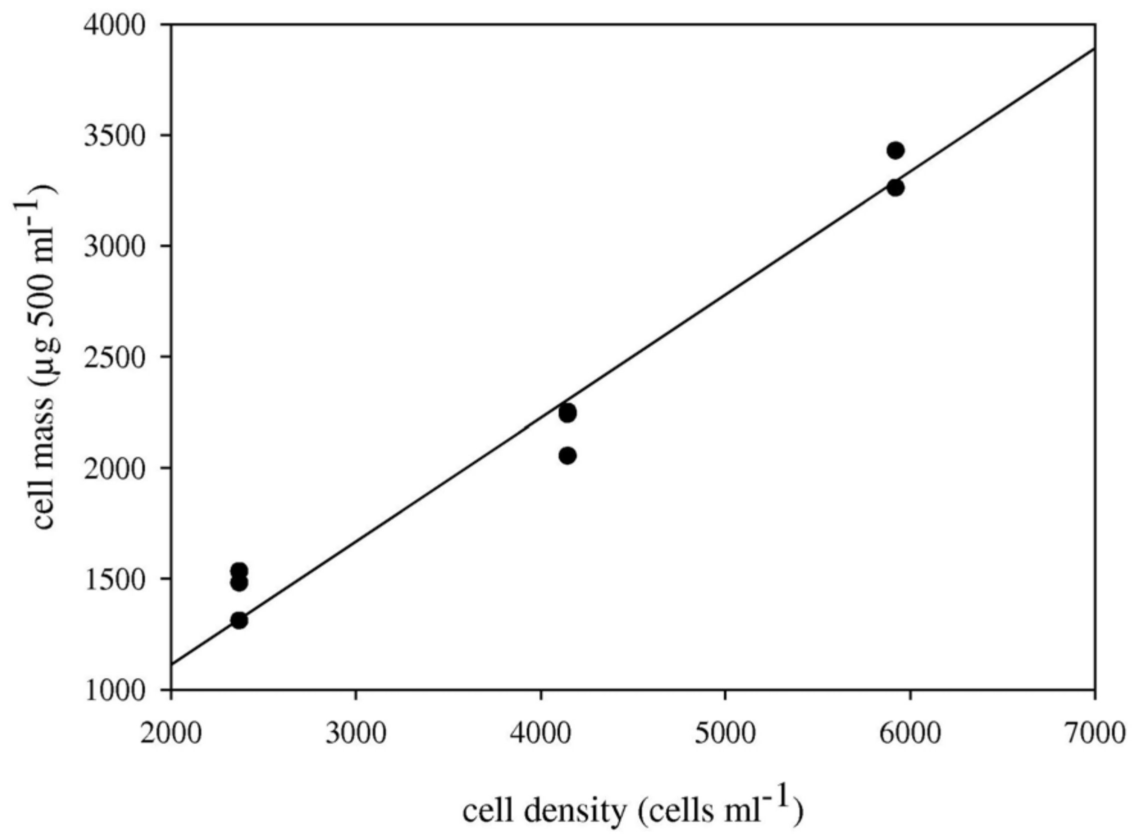
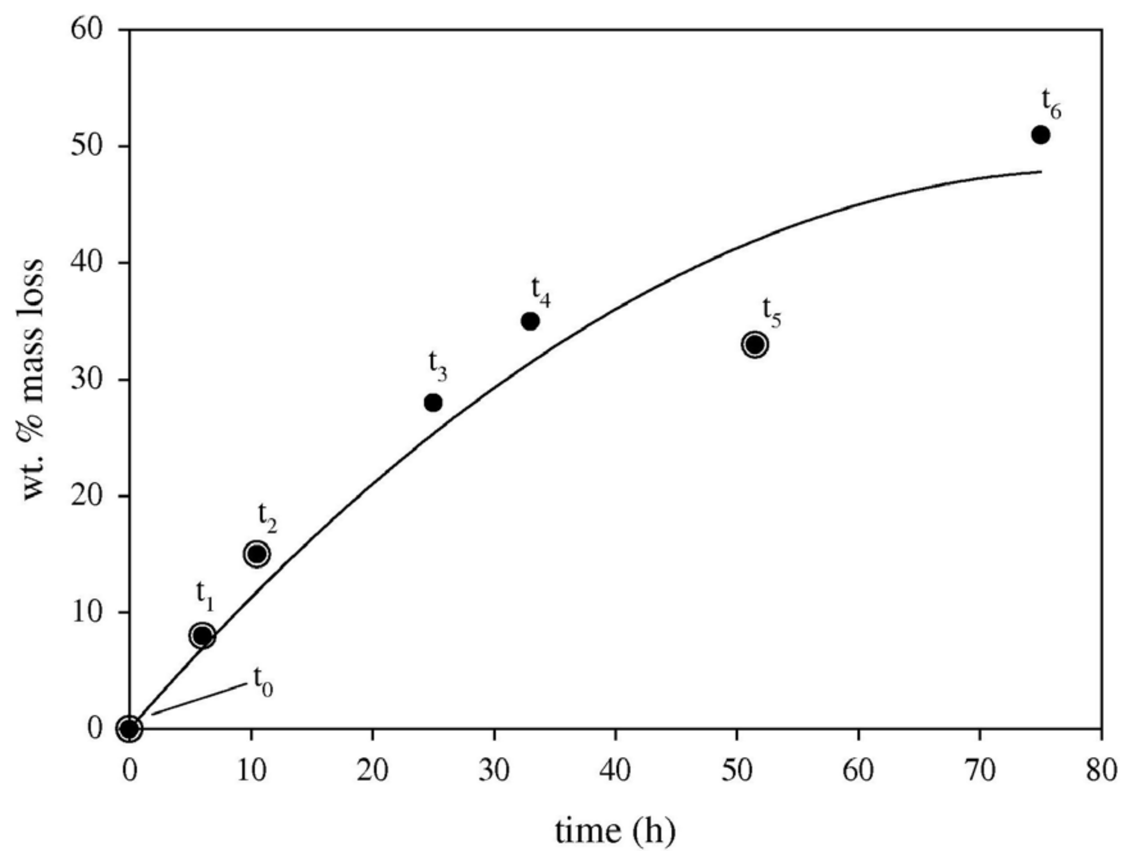


Figure 2:



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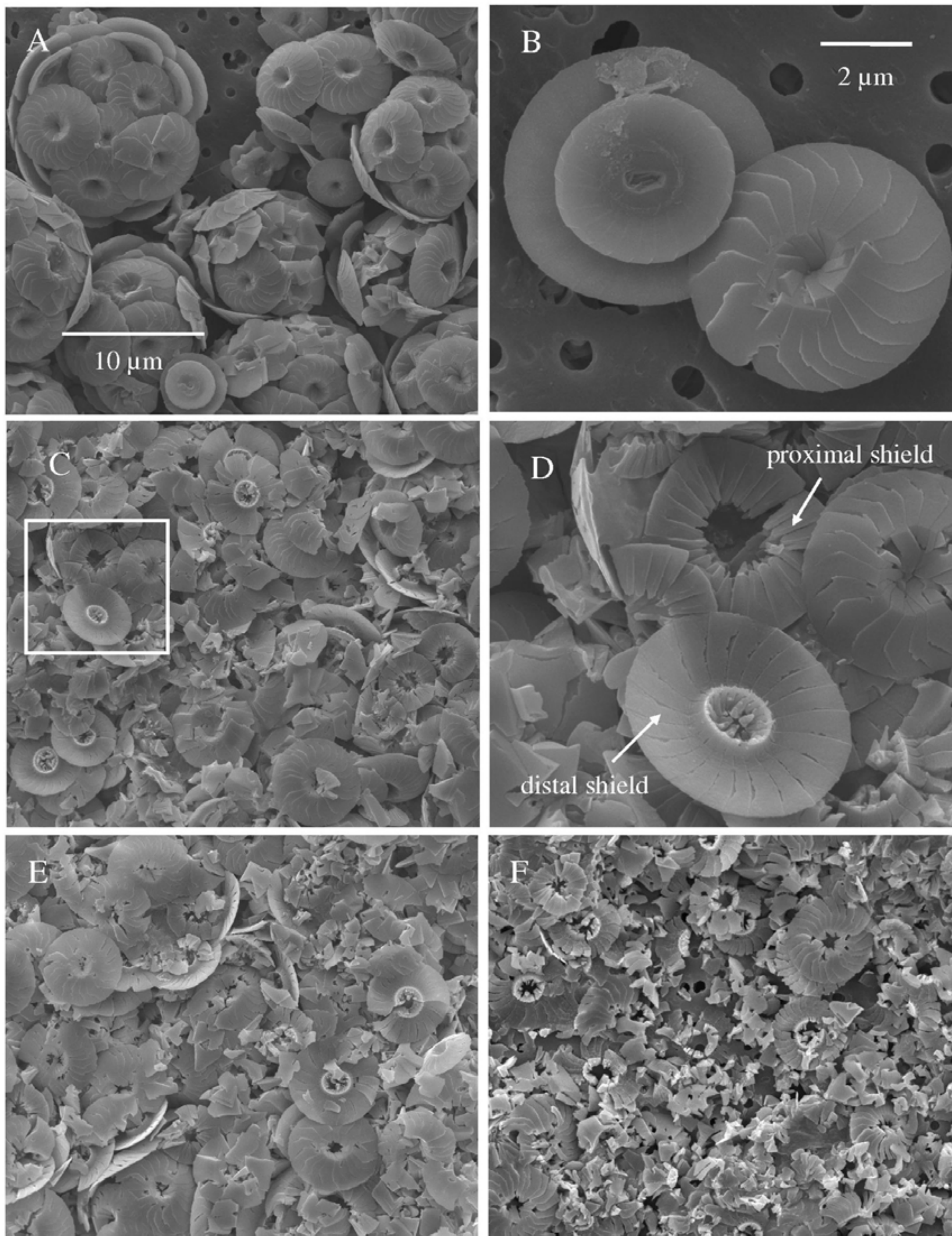


Figure 4:

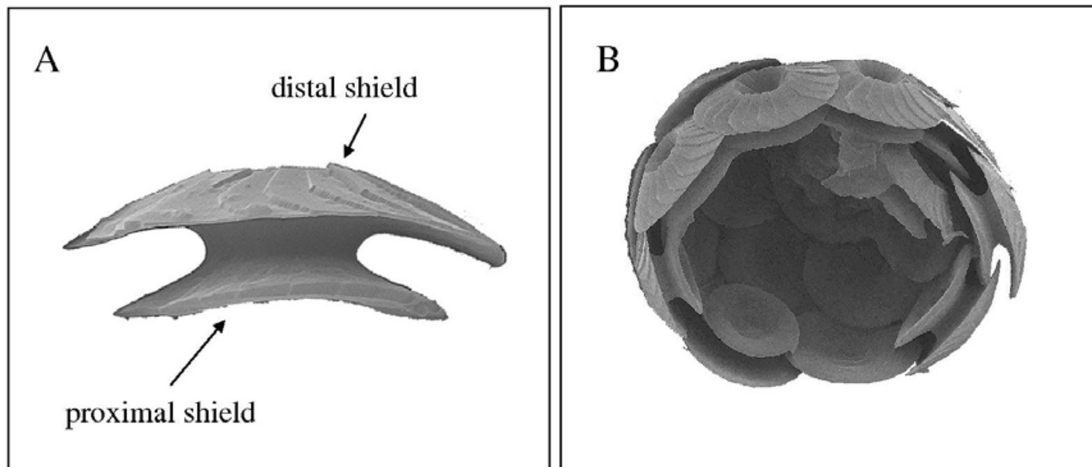
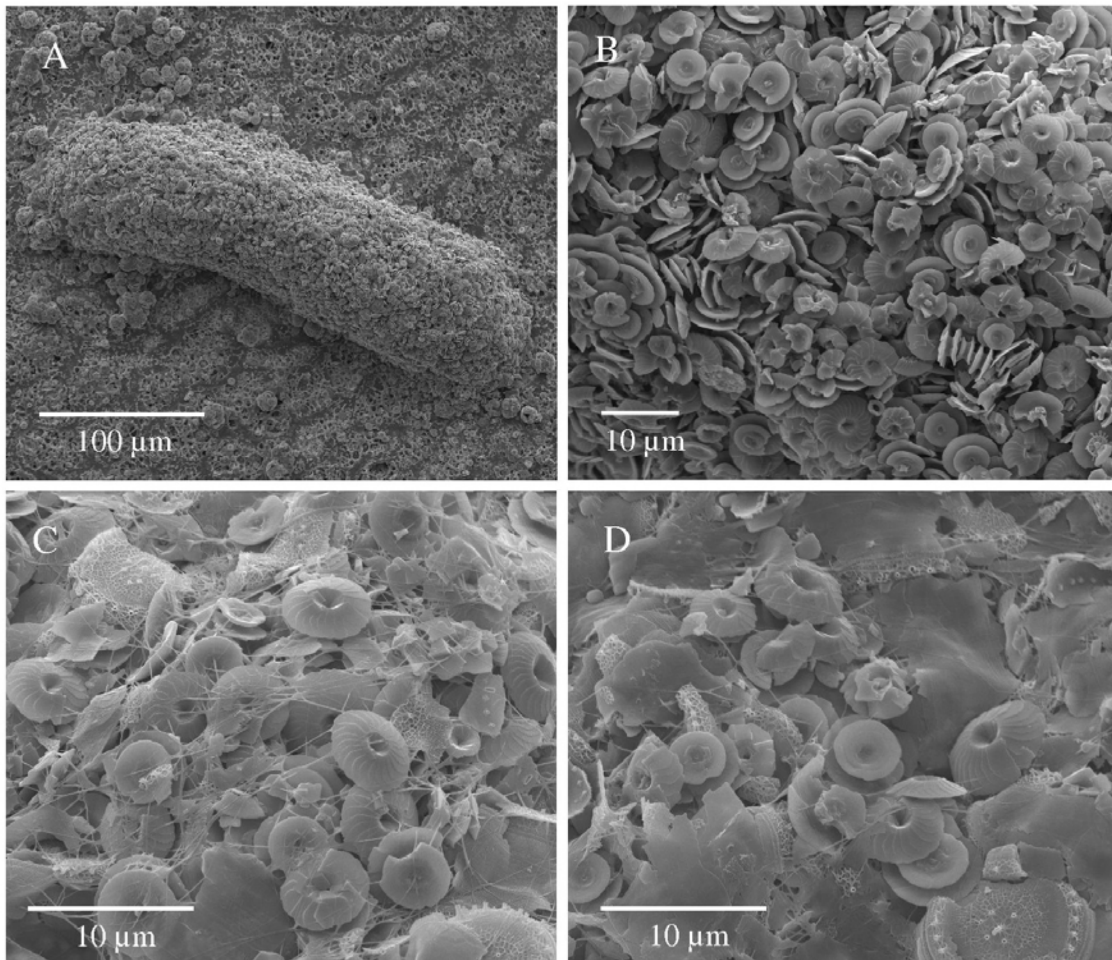


Figure 5:



MANUSCRIPT II

Grazing of *Calanus helgolandicus* on *Dinophysis norvegica* during bloom conditions in the North Sea: evidence from investigations of faecal pellets

Christian Wexels Riser, Sandra Jansen, Ulrich Bathmann, Paul Wassmann

Marine Ecology Progress Series 256: 301-304

MANUSCRIPT III

Copepod feeding behaviour and egg production during a dinoflagellate bloom in the North Sea

Sandra Jansen, Christian Wexels Riser, Paul Wassmann, Ulrich Bathmann

Harmful Algae 5: 102-112

MANUSCRIPT IV

Algae viability within copepod faecal pellets-

Evidence from microscopic observations

Sandra Jansen and Ulrich Bathmann

Submitted to Marine Ecology Progress Series

Note:

Algae viability within copepod faecal pellets - Evidence from microscopic examinations

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Abstract

The viability of different phytoplankton species within copepod faecal pellets was tested. Monocultures of the dinoflagellate *Scrippsiella trochoida* and the diatoms *Fragilariopsis kerguelensis, Proboscia alata* and *Chaetoceros dichaeta*, respectively, were fed to *Calanus helgolandicus*, *Temora longicornis* and *Centropages typicus*. Microscopic observations of faecal pellet content, chlorophyll auto-fluorescence and the fluorescence from cells stained with the viability stain fluorescein diacetate revealed that some *S. trochoida* cells survived the gut passage of *T. longicornis* and *C. typicus*. Viable cells of *F. kerguelensis* were found in faecal pellets of all copepod species investigated. The two diatom species *P. alata* and *C. dichaeta* were reduced to small fragments within all faecal pellets and could thereby not survive grazing by any of the three copepod species.

Keywords: algal viability, faecal pellets, copepods, FDA

Introduction

Copepod faecal pellets are of interest not only in zooplankton ecology, but also in studies of biogeochemical cycles and export fluxes in the sea (Turner 2002). Copepods reduce the vertical flux of phytoplankton through grazing, but may also enhance the vertical flux of phytoplankton-derived material through production of fast sinking faecal pellets. Faecal pellet production rates are directly related to the amount of food consumed by the copepods (e.g. Marshall and Orr 1955). Before egestion, the digested as well as the undigested food is packed together and surrounded by a peritrophic membrane (Gauld 1957; Honjo and Roman 1978; Turner and Ferrante 1979). In general, faecal pellets produced by zooplankton species have sinking velocities of up to several hundred meters per day (Turner and Ferrante 1979; Komar et al. 1981; Cadée et al. 1992). Their contribution to the total material flux is highly variable, between <1% up to 99% (Smetacek 1980; Bathmann et al. 1987; Viitasalo et al. 1999; Wexels Riser et al. 2001; Turner 2002), but the majority of the studies report relatively low faecal pellet export rates as a fraction of the total particulate organic carbon (POC) content in the water, even when copepods are present in high abundances. Low faecal pellet export rates suggest that the faecal material is recycled within the euphotic zone rather than exported (Lampitt et al. 1990; Viitasalo et al. 1999; Wexels Riser et al. 2001).

The fate of material stemming from faecal pellets recycled in the upper ocean depends on the condition of the material enclosed within the faecal pellets. Thus, it is crucial to know how grazing affects the ingested phytoplankton cells. The question if phytoplankton cells can survive grazing or if grazing results in a certain death is one of the central ones in this context. Not all food items undergo the same degree of fragmentation during feeding by copepods (Honjo and Roman 1978; Turner and Ferrante 1979) and several studies reported signs of intact (Turner 1984a; Turner 1984b; Bathmann and Liebezeit 1986; Wexels Riser et al. 2003; Jansen et al. 2006) and in some cases even viable cells inside faecal pellets (Fowler and Fisher 1983). Most of these studies used scanning electron microscopy (SEM) to look at faecal pellet contents. These images give good qualitative information on the degree of fragmentation, but observations of intact cells within faecal pellets give no information on the viability of these cells.

The aim of the present work was to study the fate of different algae species within faecal pellets using the viability stain fluorescein diacetate (FDA) to answer the question if

cells may survive grazing and digestion, possibly being protected against physical or chemical destruction.

Material and Methods

Algae collection and cultivation

The chain-forming diatoms *Fragilariopsis kerguelensis*, *Proboscia alata* and *Chaetoceros dichaeta* were isolated in austral summer 2004 during the Polarstern cruise ANT XXI/3 to the Atlantic sector of the Southern Ocean (approximately 49°S, 02°E). The diatoms were isolated from samples collected with a plankton net and cultivated at 4°C. *F. kerguelensis* was choosen in the present study for its heavily silicified frustules. Cells of *P. alata* are of elongated, cylindrical shape with weakly silicified frustules. *C. dichaeta* is characterised by long hollow spines that protrude from the four corners of the rectangular cells and with respect to the other two species its frustules exhibit an intermediate degree of silification.

The dinoflagellate *Scrippsiella trochoida* was isolated in August 2001, during the Heincke cruise HE152 to the North Sea. It was grown in a temperature controlled room at 15°C. *S. trochoida* live as single, motile cells. Instead of the silicified frustule from the diatoms, this dinoflagellate is covered by a theca, which is a complicated construction consisting of an outer membrane with underlying membrane vesicles containing thecal plates. All phytoplankton cultures were grown in sterile filtered seawater, enriched with nutrients according to F/2 medium (Guillard and Ryther 1962).

Copepod collection and experimental setup

Copepods were collected with a 160 µm mesh zooplankton net, in November 2005 at the permanent station Helgoland Roads in the North Sea (54°11´N, 7°54´E). After retrieval, copepods were kept in 10 l of filtered seawater and stored cold and dark until further handling in the laboratory. After some hours, females of the abundant copepod species *Calanus helgolandicus*, *Temora longicornis* and *Centropages typicus* were sorted out under the binocular. Animals were acclimated for 8-12 hours in 1 l beakers, containing one of the four algae species in monoculture. After acclimatisation, copepods were transferred to 1 l bottles, containing the same algae species as before and mounted on a slowly rotating plankton wheel.

The experiments were conducted in a 15°C temperature controlled room using algae concentrations equal to ~800 µg C l^{-1}. This corresponds to 4800, 100, 3700 and 2000cells ml^{-1} for *F. kerguelensis*, *P. alata*, *C. dichaeta* and *S. trochoida* respectively (Menden-Deuer and Lessard 2000; Montresor et al. 2003). After 12-16 hours, the content of each bottle was gently sieved over a 30 µm mesh to collect the produced faecal pellets. Faecal pellet samples were prepared for the SEM (Wexels Riser et al. 2003) and micrographs were taken using a Quanta 200F/FEI. Replicate faecal pellet samples were observed directly after staining under a light microscope (Zeiss, Axiovert 200). No fixatives were used prior to microscopical observations. The viability of cells within the faecal pellets was monitored using the fluorescein diacetate (FDA) method (Garvey et al. submitted). Briefly, faecal pellets were carefully cleaned with filtered seawater, transferred to a 3 ml Utermöhl chamber and stained with FDA. After 10 minutes on ice, in darkness, the sample could be viewed under the inverted fluorescence microscope. The FDA staining primarily tests the integrity of the cell membrane, which is likely to be closely correlated with viability. Viable cells show a green fluorescence (wavelength 520-530 nm) after excitation with blue light of the wavelength 495 nm. In our samples the red chlorophyll *a* auto-fluorescence masked the green FDA fluorescence, which has been found to be true for many diatom species (Garvey et al. submitted). Red fluorescence is not an indicator of viability, and dead cells may autofluoresce for months after cell death. However, the use of filter set 44 by Zeiss (excitation: BP 475/40; beamsplitter: FT 500; emission: BP 530/50), remedies this problem, masking the red auto-fluorescence, making the green viability fluorescence visible. Figure 1b and 1d show a *F. kerguelensis* chain after FDA staining under fluorescent light (450-500 nm), where the green FDA fluorescence is masked by the red chlorophyll *a* auto-fluorescence. After additional use of Filter 44 only the green FDA fluorescence remains visible (Figs 1c, 1e). However, the green fluorescence was weak in most cases and faded rapidly, which is the reason why photographs of FDA stained cells in faecal pellets were not possible for all species, where the green fluorescence was observed.

Results

Faecal pellets were produced by all copepod species investigated. The size of the faecal pellets produced by the three copepod species ranged according to their body size, with

Calanus helgolandicus producing the largest faecal pellets, followed by *Temora longicornis* and *Centropages typicus*. Faecal pellet sizes were independent of the phytoplankton species grazed upon.

Figure 2 shows faecal pellets produced by *C. helgolandicus*, *T. longicornis* and *C. typicus* after grazing on the dinoflagellate *Scrippsiella trochoida*. Within faecal pellets produced by *C. helgolandicus*, *S. trochoida* cells appeared crushed and no green fluorescence was observed. These faecal pellets had a homogeneous content (Fig. 2a). Within the faecal pellets of *T. longicornis* and *C. typicus,* however, single intact cells of *S. trochoida* could be identified (Figs 2b, 2d). These cells looked intact, undigested and strongly fluoresced red from the chlorophyll *a* auto-fluorescence (Figs 2c, 2e). Green fluorescence from the FDA-stain was also clearly visible within faecal pellets of *T. longicornis* and *C. typicus* after grazing on *S. trochoida*, indicating that individual cells of this species can survive the gut passage.

When grazing on *Fragilariopsis kerguelensis* intact cells could be identified within faecal pellets produced by all three copepod species (Fig. 3). The SEM pictures show faecal pellets from *C. helgolandicus* and *T. longicornis* in detail (Fig. 4), where several broken frustules are visible within the faecal pellets, but also complete and undigested cells can be seen, especially within chains (Fig. 4). Green fluorescence from FDA staining could be seen in faecal pellets produced by all the copepod species grazing on *F. kerguelensis* (Fig. 5).

Grazing on monocultures of the remaining two diatoms, *Proboscia alata* and *Chaetoceros dichaeta* resulted in faecal pellets where no intact cells could be observed. Diatoms were reduced to small fragments during grazing and faecal pellets were exclusively packed with cell debris. Photos taken under the light microscope show faecal pellets with an indefinable, homogeneous, brownish content for all copepod species investigated (Figs 6, 7). However, more details can be seen in the SEM images, photos of faecal pellets produced by *C. helgolandicus* are shown as an example in figure 8. Faecal pellets produced by all species, containing *P. alata* were exclusively filled with flake-like cell debris (Fig. 8a). Within the faecal pellets containing *C. dichaeta*, cell debris was visible as well, but faecal pellets also contained many identifiable fragments of spines (Fig. 8b). Neither intact cells nor fluorescein fluorescence was observed in any of the faecal pellets containing *P. alata* or *C. dichaeta*.

Discussion

In this study we showed that cells of some phytoplankton species may survive copepod gut passage. The condition of the enclosed phytoplankton cells in faecal pellets is presumably unimportant if these sink to the deep ocean, but detrital faecal pellet content can serve as a source of nutrients while viable phytoplankton cells within the pellets can act as seeding material and provide an addition to the algal community, if conditions are suitable. Faecal pellets may be recycled within the euphotic zone as a result of a number of different processes, including microbial degradation or zooplankton activity (Turner 2002). Bacterial degradation is probably not contributing significantly to the recycling of copepod faecal pellets within the euphotic zone, since this process is too slow compared with the high sinking velocity and thereby low residence time of faecal pellets in the upper layer. On the other hand, grazing on faecal pellets by zooplankton may contribute significantly towards reducing vertical flux. Lampitt and co-workers found that copepods can be highly efficient breaking down their own faecal pellets while ingesting only a small proportion (Lampitt et al. 1990). The process of breaking faecal pellets is termed “coprorhexy”, distinct from coprophagy which refers to the ingestion of faecal material. The cyclopoid copepod *Oithona* spp. is one of the species thought to feed efficiently on faecal pellets (Gonzalez and Smetacek 1994; Reigstad et al. 2005). When recycled in the upper water column, part of the faecal pellet content may be kept in the system and is therefore important for most of the ongoing processes in the upper water column. As we showed, viable algae of some species can occur within faecal pellets, implying that these cells survive the grazing process and digestion itself. Such cells can potentially grow if released from the faecal pellets under favourable conditions (Fowler and Fisher 1983). The ability to survive grazing may be interpreted as an evolutionary defence strategy against zooplankton grazing. The interaction between phytoplankton and zooplankton has been suggested as one of the most important factors driving planktonic evolution in the oceans (Verity and Smetacek 1996). Zooplankton evolves more effective feeding strategies while phytoplankton in turn evolves more effective defence strategies against grazing. Diatom frustules are suggested to be such a defence strategy, since they may serve as effective mechanical protection against crushing by their predators due to their architecture and the material properties of the diatom silica (Hamm et al. 2003).

Copepods in turn have developed mandibles, which contain silica and are strong enough to crack these frustules to reach the nutritious content of the diatoms.

The frustules of *Fragilariopsis kerguelensis* are particularly thick. Some of the cells escaped digestion in the present experiments and stayed intact and even viable after gut passage. The SEM pictures revealed that many of the intact cells were part of chains, suggesting that this alga additionally may benefit from chain formation, increasing its chance of survival when it is grazed. By contrast, the chain forming, spiny diatom *Proboscia alata* did not escape digestion and the long spins of *Chaetoceros dichaeta* provided no efficient protection against copepod grazing. Both of these diatoms have thin frustules compared to *F. kerguelensis*. It appears that the combination of chain formation and strong frustules may avoid certain destruction of cells, allowing individual cells of *F. kerguelenis* to remain viable after gut passage. Since copepods were grazing on monocultures in the present study, it may be possible, that they avoid these algae because of their morphology in the field. However, North Sea copepods were evidently able to graze on the Antarctic diatoms under investigation. Antarctic copepods are generally larger and most likely stronger than the chosen copepods from the North Sea. Therefore it can be assumed that during grazing by Antarctic copepods indivual cells of *F. kerguelensis* will also escape destruction.

Among Dinoflagellates mostly the resting stages are assumed to serve as protection against grazing (Montresor et al. 2003), but there are also observations, that vegetative cells of *Dinophysis norvegica* may escape digestion due to their stable frustules (Wexels Riser et al. 2003; Jansen et al. 2006). In the present study, viable cells were found in faecal pellets produced by *Temora longicornis* and *Centropages typicus* when grazing on vegetative cells of *S. trochoida*. The faecal pellets produced by *Calanus helgolandicus* contained no viable *Scrippsiella trochoida* cells. Montresor and co-workers described faecal pellets of *Acartia clausi*, *C. typicus* and *T. stylifera* to contain a homogeneous and compact content, greenish and brownish in colour. Only a low number of apparently intact cells were recognized within faecal pellets of *T. stylifera* (Montresor et al. 2003). Digestion resistance in the present study may be due to rapid gut transit in animals grazing at high cell densities (~2000 cells ml^{-1}, corresponding to 800 µgC l^{-1}) compared to the low cell numbers (~150 cells ml^{-1}, corresponding to 50 µgC l^{-1}) used by Montresor et al. (2003). Whether the survival of individual *S.trochoida* cells in some but not in other copepods is due to different gut passage times or due to differences in handling between the copepod species, is uncertain and requires

further work on this topic, looking at faecal pellets produced by different species at different algal concentrations. The viability of algae within faecal pellets show possible pathways for phytoplankton cells to the deep sea or they may serve to reseed the phytoplankton community in the overlying surface waters with vegetative cells or resting spores, given a sufficient upward transport mechanism such as mixing and upwelling (Fowler and Fisher 1983).

Acknowledgements

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Figure captions:

Figure 1:

Fragilariopsis kerguelensis chain stained with fluorescein diacetate (FDA) under visible (a) and fluorescence light without (b, d) and with the use of filter set 44 from Zeiss (c, e). The green fluorescence from FDA staining is mostly masked by the red chlorophyll *a* auto-fluorescence, when no filter is used (b, d).

Figure 2:

Faecal pellets from *Calanus helgolandicus* (a), *Temora longicornis* (b, c) and *Centropages typicus* (d, e) after feeding on a monoculture of *Scrippsiella trochoida*. Photos (a, b) and (d) were taken with visible light, while (c) and (e) show the chlorophyll *a* auto-fluorescence masking the FDA-fluorescence of cells within the faecal pellets of (b) and (d), respectively. Scale bars are given.

Figure 3:

Faecal pellets from *Calanus helgolandicus* (a), *Temora longicornis* (b) and *Centropages typicus* (c) after feeding on a monoculture of *Fragilariopsis kerguelensis*.

Figure 4:

Scanning electron microscope pictures of faecal pellets produced by *Calanus helgolandicus* (a, b) and *Temora longicornis* (c, d) after feeding on a monoculture of *Fragilariopsis kerguelensis*. Arrows mark examples of apparently intact cells within chains.

Figure 5:

Faecal pellets from *Calanus helgolandicus* (a) and *Temora longicornis* (b) feeding on *Fragilariopsis kerguelensis*. The green fluorescence after staining with fluorescein diacetate indicates alive cells within the pellets.

Figure 6:
Faecal pellets from *Calanus helgolandicus* (a), *Temora longicornis* (b) and *Centropages typicus* (c) after feeding on a monoculture of *Proboscia alata*. Intact cells are visible in the background of photo (a) and (b). The content of all faecal pellets looks homogenous. For details of (a) see Figure 6a.

Figure 7:
Faecal pellets from *Calanus helgolandicus* (a), *Temora longicornis* (b) and *Centropages typicus* (c) after feeding on a monoculture of *Chaetoceros dichaeta*. For details of (a) see Figure 6b.

Figure 8:
Scanning electron microscope photos of faecal pellets produced by *Calanus helgolandicus*, grazing on (a) *Proboscia alata* and (b) *Chaetoceros dichaeta*. Pellets of *C.helgolandicus* are shown as an example, those of the other copepod species looked similar. Faecal pellets containing *P. alata* show flake-like cell debris of the diatom (a). *C. dichaeta* cells within the faecal pellets are also reduced to small pieces (b), and fragments of the spines from the diatoms are visible (see arrow).

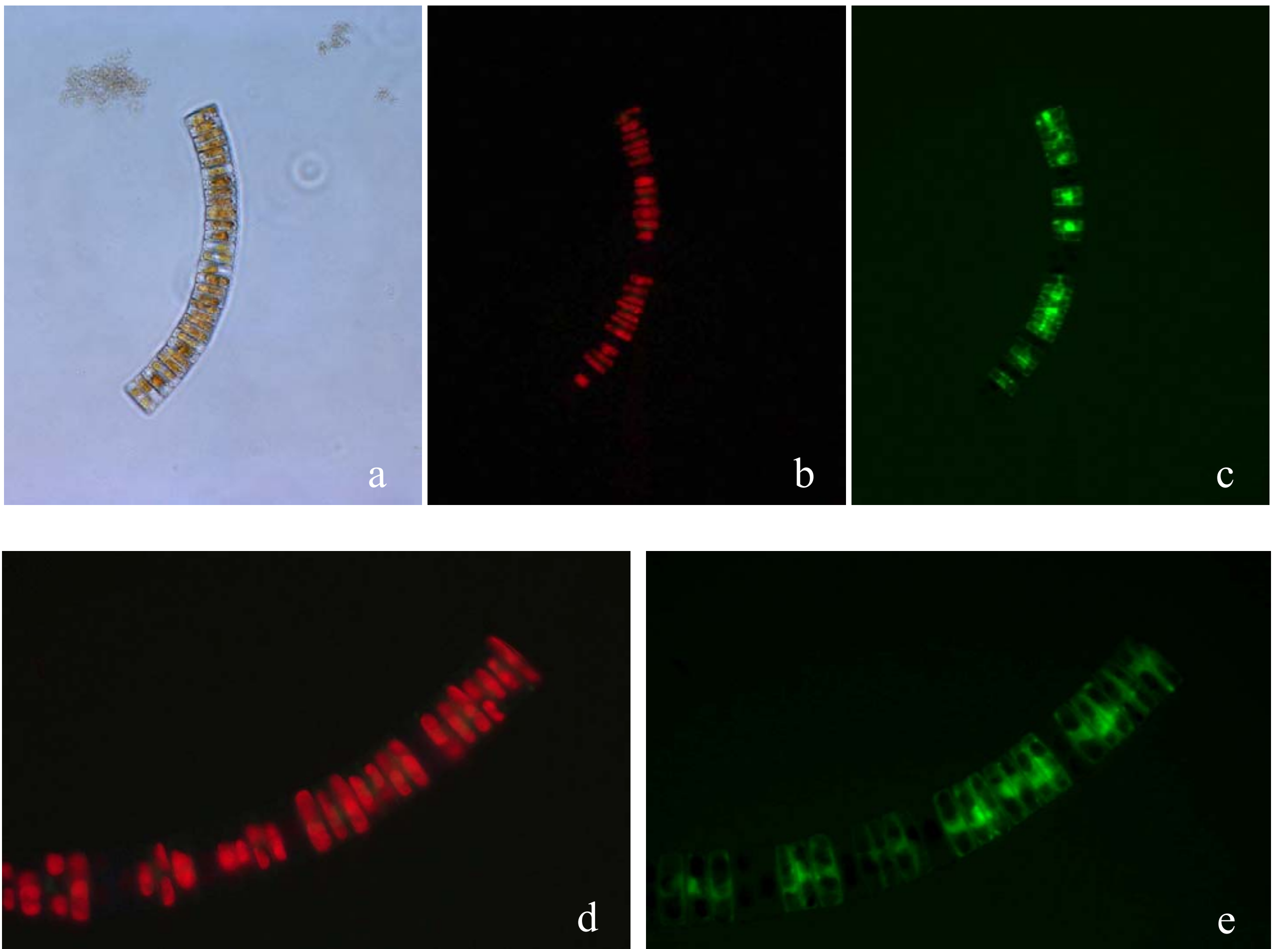

Figure 1

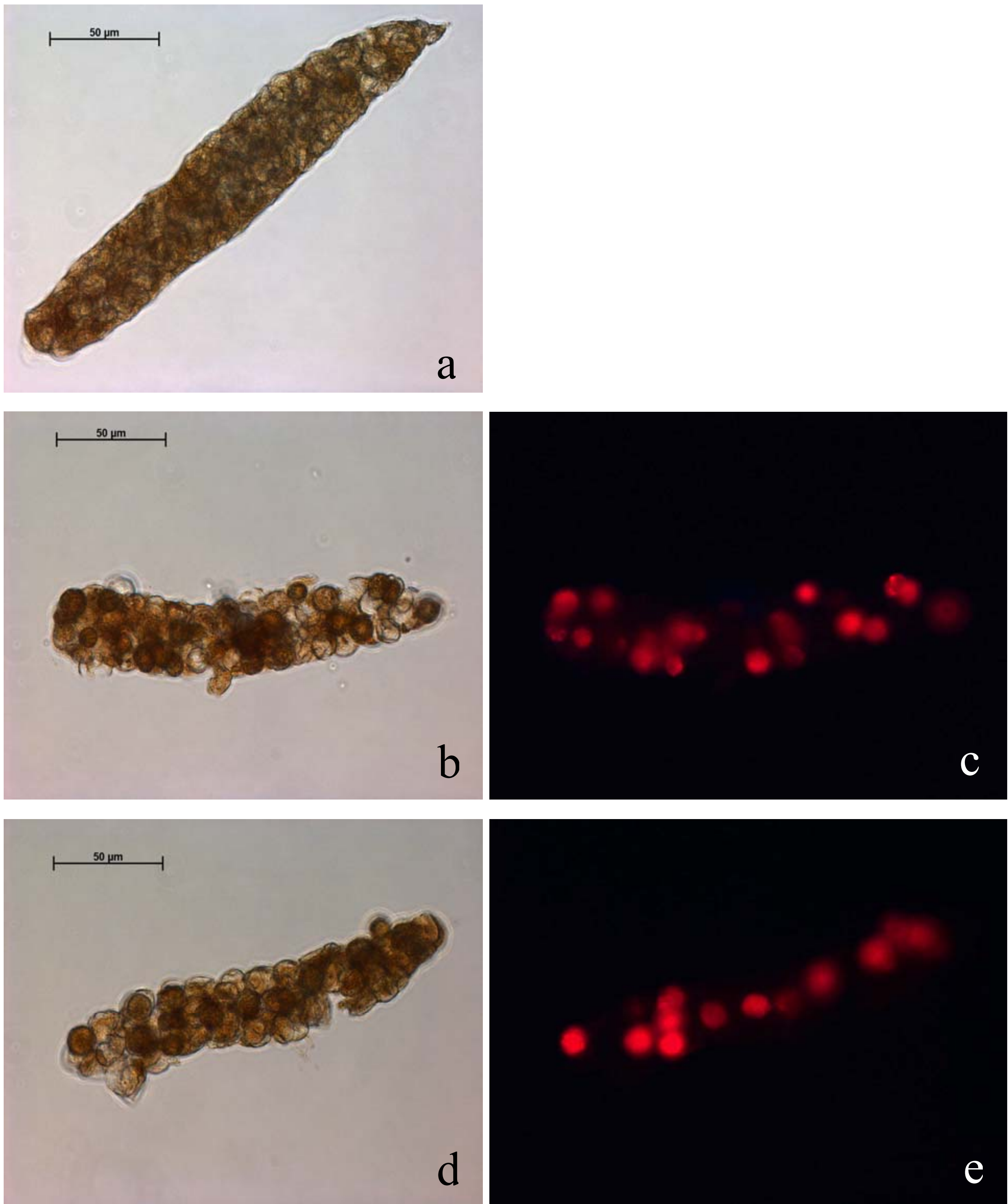

Figure 2

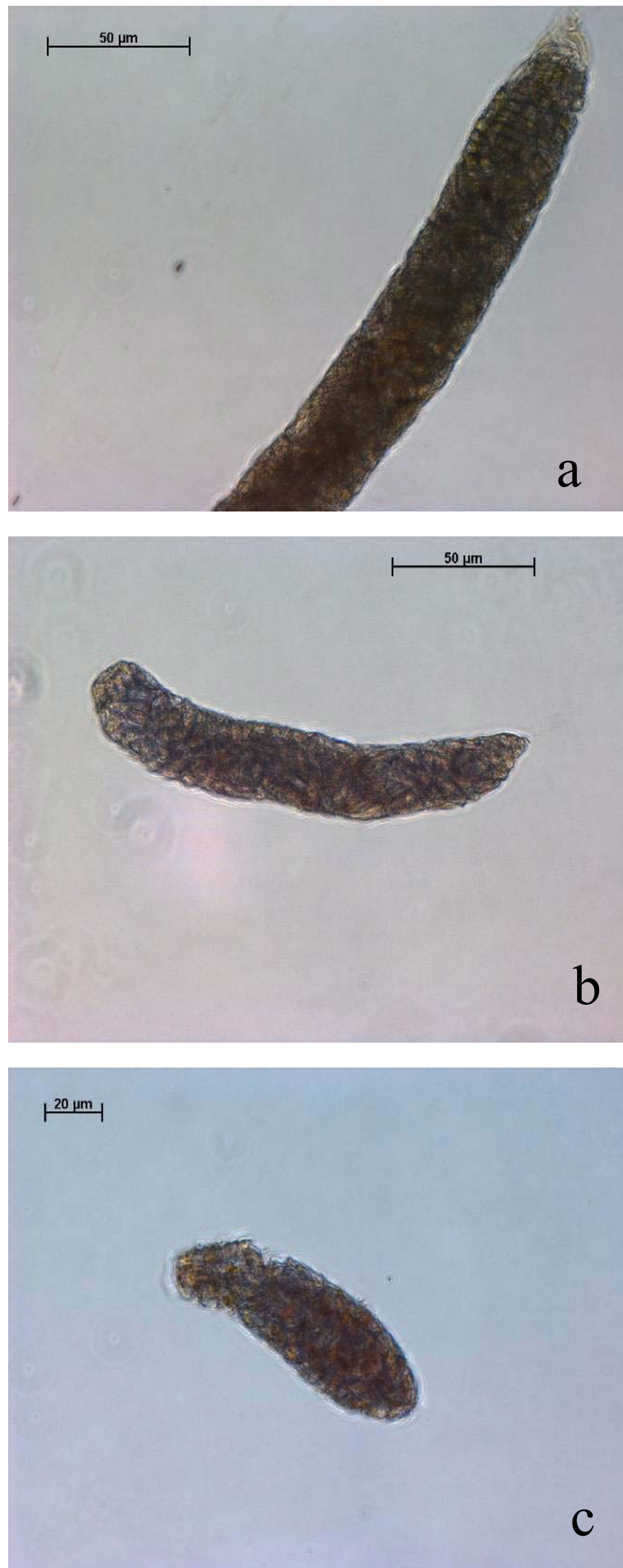

Figure 3

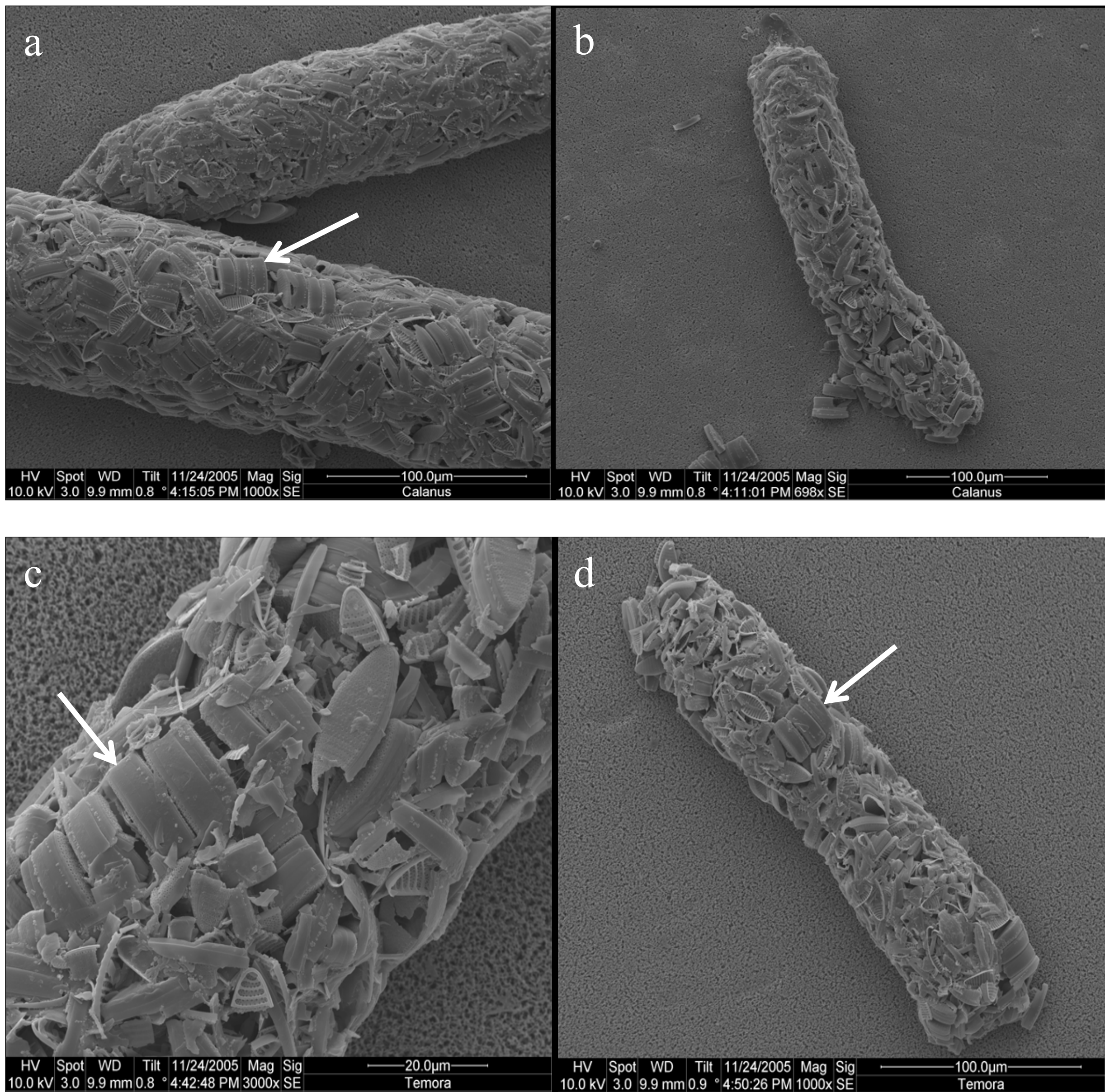

Figure 4

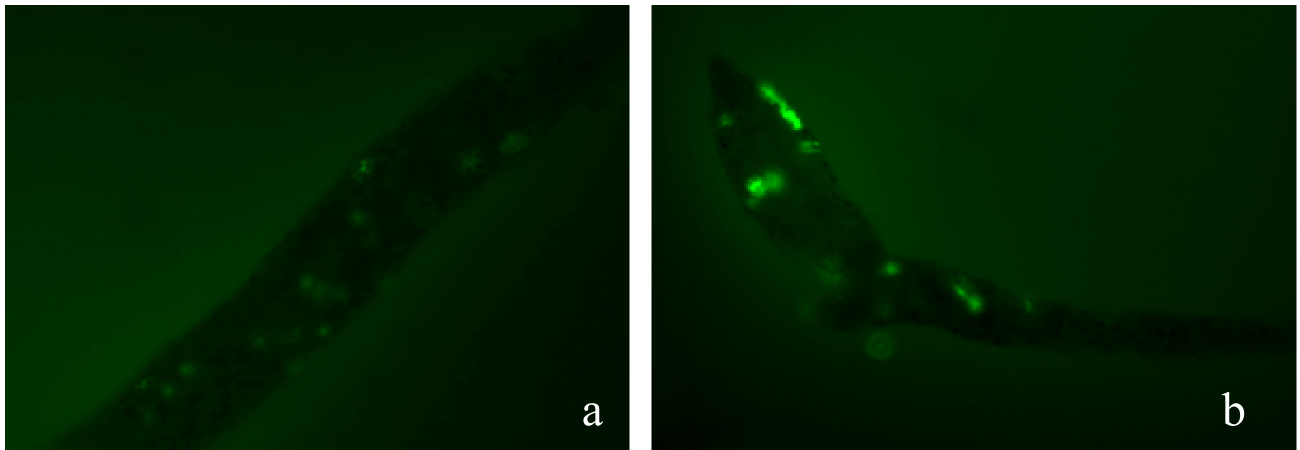

Figure 5

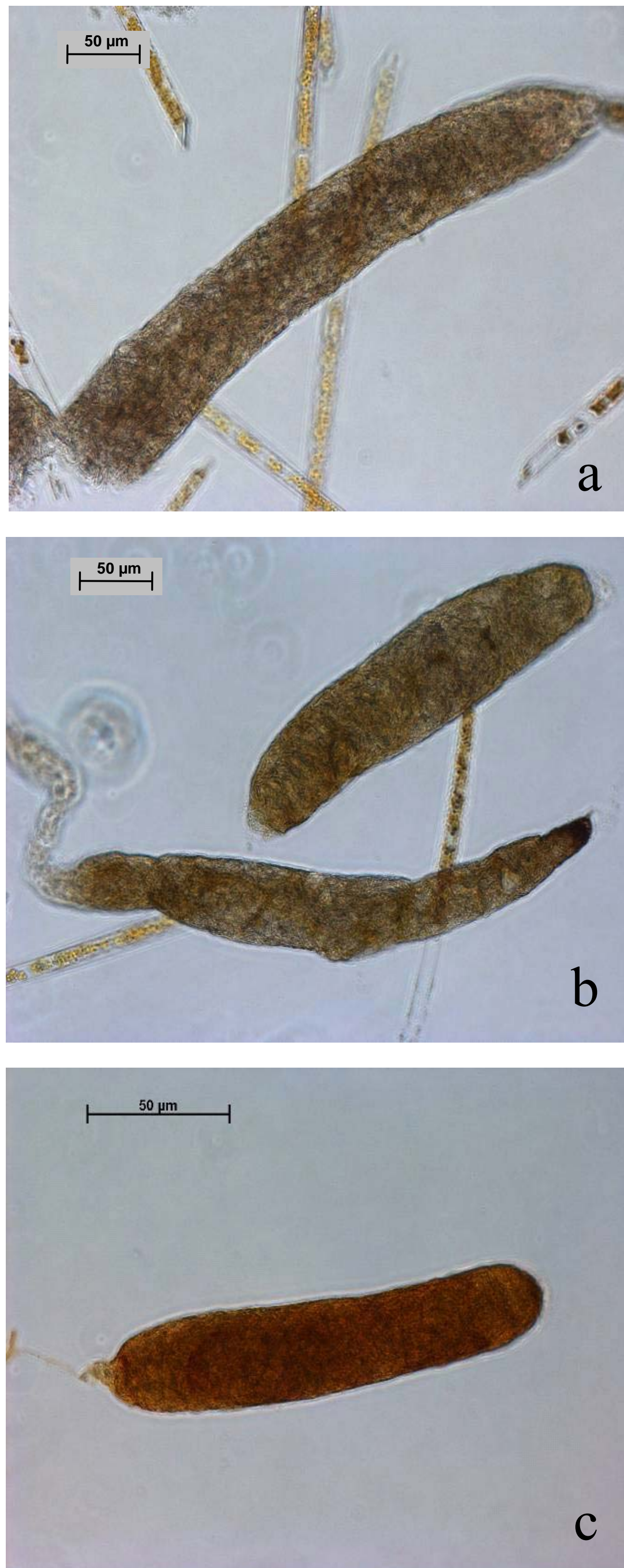

Figure 6

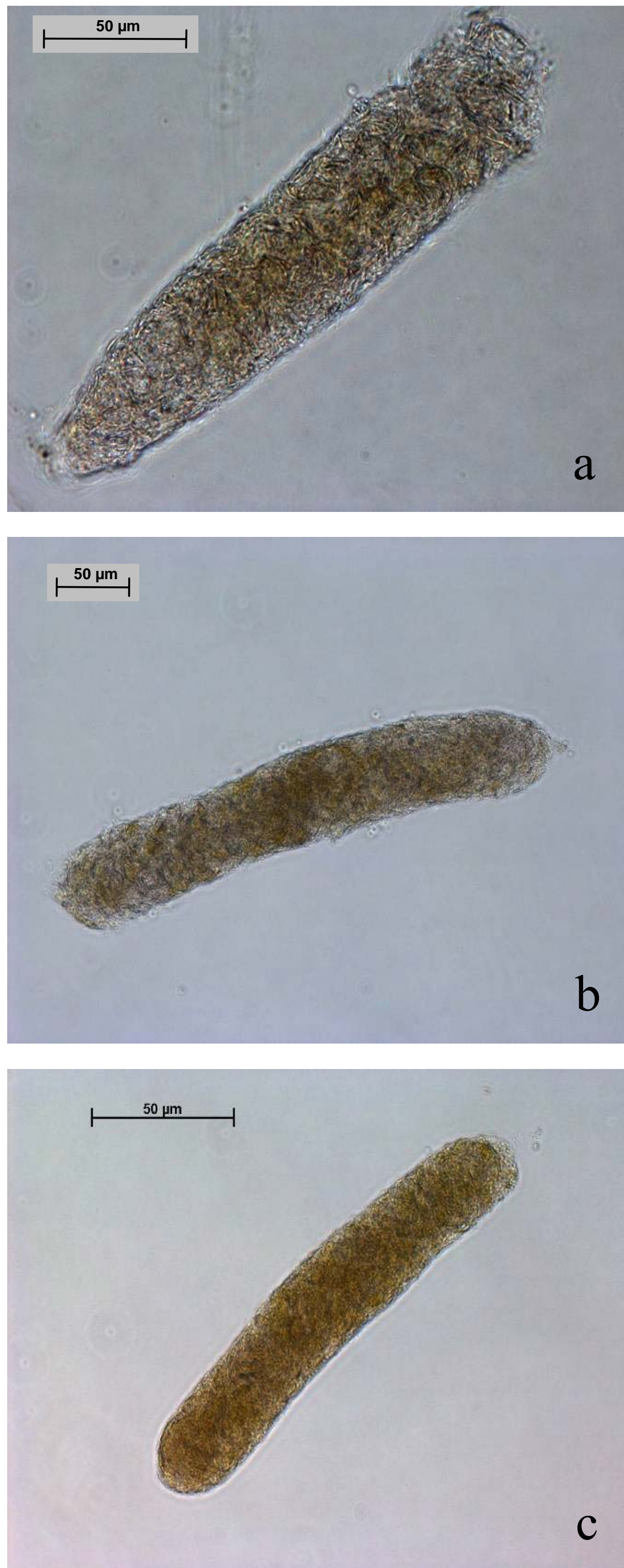

Figure 7

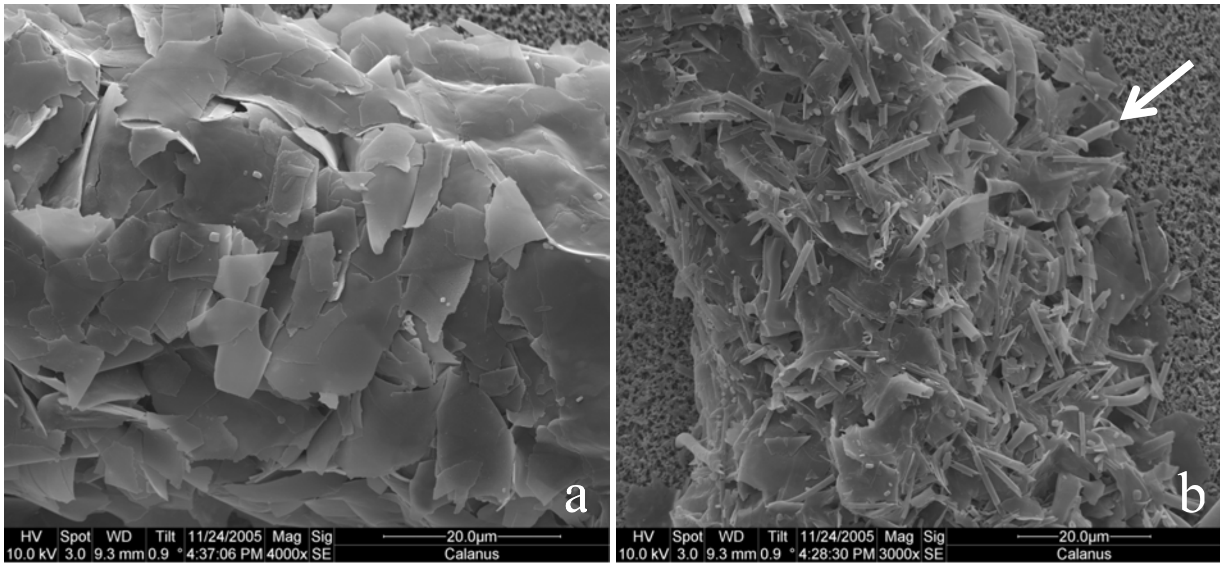

Figure 8

MANUSCRIPT V

Copepods grazing on *Coscinodiscus wailesii* -

A question of size?

Sandra Jansen

Draft

Copepods grazing on *Coscinodiscus wailesii* - A question of size?

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Abstract

Grazing experiments performed with adult *Acartia clausi* and *Temora longicornis* showed that *A. clausi* could not feed on *Coscinodiscus wailesii*, while *T. longicornis* females did feed on this large centric diatom. *T. longicornis* showed a skilful handling of the large algae. The cells were not completely ingested but copepods bit a small piece out of the silica wall and then ingested only the cell content. Faecal pellet analyses showed only little remains of the silica walls in the faecal pellets, and most of the content could only be identified as undefined matter. Therefore the general analyses of faecal pellet and gut content of copepods from the field must be interpreted with caution. To avoid possible misinterpretations, genetic techniques may be an upcoming useful approach in studies of copepod ingestion.

Keywords: North Sea, Copepods, Grazing, Feeding strategies

Introduction

Copepods are selective feeders and their mode of feeding has important implications for the structure of pelagic food webs (Kiørboe et al. 1996). The traditional view of copepods as relatively mechanical suspension-feeders has been replaced by the recognition that a wide variety of mechanisms exist to detect, pursue, capture and reject prey (Price 1988). The ability and skill in handling individual food items was mainly discovered by direct video observations (Alcaraz et al. 1980, Koehl & Strickler 1981). The use of passive filtration or active capture of prey is dependent on the prey size grazed upon. The passive feeding mode is used to capture cells which are too small to be individually detected by the copepod. Generally it is known that copepods prefer larger food items (Mullin 1963, Paffenhöfer 1988), using the active feeding mode. Hansen et al. (1994) evaluated the assumption of a fixed size ratio between pelagic predators and their prey on the basis of available literature data and presented an optimum ratio of 18:1 for copepods. When grazing at the upper end of the prey size spectrum, an inefficient feeding behaviour may occur due to sloppy feeding (e.g. Roy et al. 1989) which have a strong impact on the food web, as dissolved organic matter (DOC) is released to the water with increasing amounts as function of prey size (Friis Møller 2005).
The present study gives insights into the individual handling of extremely large diatoms by copepods. The feeding behaviour of the common North Sea copepods *Acartia clausi* and *Temora longicornis* was studied under laboratory condition with the largest centric North Sea diatom *Coscinodiscus wailesii* provided as food. Emphasis was on behavioural observations of the copepods confronted with the large centric diatoms.

Material and Methods

Phytoplankton collection

C. wailesii is the largest centric diatom commonly found in the North Sea phytoplankton (Fig. 1A). Single cells were isolated from a water sample, taken in April 2005 at the permanent station Helgoland Roads in the North Sea (54°11´N, 7°54´E). Algae were cultured in F/2 medium (Guillard & Ryther 1962) in a temperature controlled room at 15°C and a light:dark cycle of 16:8 hours was applied. Mean cell size at the time of the grazing experiments was calculated after measurement of 50 cells under an inverted microscope (Zeiss Axiovert 200) at 160x magnification.

Zooplankton collection

Copepods were collected with a 160 µm mesh zooplankton net, in November 2005 at the permanent station Helgoland Roads in the North Sea (54°11´N, 7°54´E). After retrieval, the copepods were diluted in 10 l filtered seawater and stored dark at 15°C until further handling. Back in the laboratory, copepods were checked and healthy females of the abundant copepods *A. clausi* and *T. longicornis* were picked out for experiments. *A. clausi* is a small neritic copepod. The carapace length of an adult female is approximately 1.15 -1.22 mm (Rose 1970), with an equivalent spherical diameter (ESD) of 388 µm. *T. longicornis* females can reach a carapace length of 1.5 mm (Rose 1970), with an estimated ESD of 612 µm (Møller 2005). Animals were kept in a temperature controlled room in 1 l glass bottles on a slowly rotating plankton wheel. Prior to the experiments, the copepods were fed a mixture of *Rhodomonas baltica* and *Thalassiosira weissflogii*.

Grazing observations

A. clausi and T. longicornis females were kept in 100 ml beakers, while grazing observations were made under the binocular in a temperature controlled room (15°C). *C. wailesii* was provided in a concentration of ~3 cells ml^{-1}, equivalent to about 800 µg C l^{-1} (Tada et al. 2000). Handling time was defined as the time from first capture of a cell until the ingestion or rejection. The mean handling time was estimated based on 20 single observations. Faecal pellets were picked out of the beakers at the end of the observation and examined under an inverted light microscope (Zeiss Axiovert 200) where micrographs were taken (Zeiss Axiocam MRc5).

Results and Discussion

At the time of the experiments the diameter of *C. wailesii* was 379 ± 1.5µm (n = 50). With an assumed height of this cylindrical-shaped diatom of 190 µm, the estimated average ESD value was ~345 µm. Thus, the predator-prey size ratios were 1.1:1 and 1.8:1 for *A. clausi* and *T. longicornis*, respectively. Both ratios are extremely low, keeping in mind that the optimum ratio for the body size of predatory copepods to their prey is thought to be ~18:1, ranging from 10:1 to 30:1 (Hansen et al. 1994). Another indication of the relative sizes of

grazers and algae is given by the fact that the diameter of *C. wailesii* represents about 50% of the carapace length of *A. clausi* and 30% of the body length of *T. longicornis*. The size of the algae is several times larger then the stoma size of both species and can not be ingested as whole, because of the stable frustules of the diatoms. When using a needle to crush a *C. wailesii* frustule on one side of the cell, the protoplasma concentrated on the opposite side of where the cell was destructed (Fig. 1B).

Behavioural observations

A. clausi was not grazing on *C. wailesii* during the present study. Therefore, all other analyses could only be made for the remaining copepod species investigated. *T. longicornis* fed on the diatom and the handling time ranged from 20 to 259 sec with a mean of 107 sec (n = 20). Within the beakers of *T. longicornis,* several empty cells were found which showed only small signs of destruction on two (mostly opposed) sides of the cell (Fig. 2). During the direct observation of the feeding technique of the copepods under the binocular, the reason for this pattern of cell destruction became clear. Figure 3, established from short video sequences during observation, schematically shows the feeding behaviour of *T. longicornis* grazing on the large diatom cell. After the capture of the cell, the copepod was able to fix the position of the diatom relative to itself with considerable accuracy (Fig. 3B). When biting off a piece from the frustule, the protoplasma, i.e. the nutritious content of the diatom, concentrated on the opposite side of the cell, as shown when breaking the frustules with a needle (Fig. 1). During feeding, this happened as soon as the frustule was broken by the mandibles of the copepod (Fig. 3C). *T. longicornis* then turned the cell with its feeding appendages so that the concentrated protoplasma was directly adjacent to the mouth opening (Fig. 3D). The copepod bit again a piece out of the frustule and could then reach and ingest the content (Fig. 3E). The empty silica frustule was rejected, since it contains no digestible material for the copepod. The rejected empty frustules showed a characteristic pattern with mostly two (up to five) parts of the frustules missing (Fig. 2, Fig 3F).

While feeding on *C. wailesii*, *T. longicornis* used its swimming feet to fix the position of the large prey item. The copepod was therefore sinking slowly to the bottom of the beaker while handling the prey. When the copepod was disturbed (for instance by moving the beaker) the copepod stopped handling the cell and rejected it in order to escape with a quick jump.

It is not clear whether or not *T. longicornis* is grazing on *C. wailesii* in the presence of alternative food types and further investigation is needed.

Faecal pellet content

Analysis of gut- or faecal pellet content can give a qualitative insight in what have been grazed by a copepod, but can rarely lead to quantitative results (but see Wexels Riser et al. 2003, Jansen et al. 2006). In cases where copepods do not ingest whole cells, interpretation of stomach or faecal pellet content may be difficult or even impossible as only few recognizable pieces of evidence of the diatom-prey may be found. Indeed, faecal pellets of *T. longicornis* produced when grazing on *C. wailesii* look brownish and only very few pieces of the diatom frustule can be identified under the light microscope (Fig. 4). The greenish or brownish content within the observed pellets made up for the majority of the content. There are studies describing more than 90% brownish or greenish content within the guts or faecal pellets of copepods in the field and this was interpreted as the copepods have been grazing on detritus or just described as unidentified matter (Pasternak & Schnack-Schiel 2001). However, the present study has shown that this general conclusion may not be true for all cases. The importance of species specific observations and the need of new methods to measure grazing were clearly demonstrated, since traditional methods like gut- or faecal pellet content analysis come to their limits in many cases. Ideally, it should be possible to directly quantify *in situ* ingestion of all prey of a single zooplankton by analysis of a freshly caught individual or its feces, without any prior interference with the food environment (Nejstgaard et al. 2003). The genetic approach, introduced by Nejstgaard and co-workers (2003) seems to be most promising, but is still in its infancy.

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Figure captions:

Figure 1:

Micrographs of *Coscinodiscus wailesii*, the largest diatom commonly found in the North Sea (mean diameter at the time of this study: 380 µm). Micrograph (A) shows an intact cell, while in (B) the cell was destroyed with a needle. When crushing the frustules of the cell, the protoplasma concentrates on the opposite side of the destructed point.

Figure 2:

Empty *Coscinodiscus wailesii* cells after *Temora longicornis* grazing on these cells. Most of the cells show destruction on either opposed sides of the frustule. Scale bar is given.

Figure 3:

A-F: Schematic diagram of the feeding behaviour of *Temora longicornis*, grazing on *Coscinodiscus wailesii*. See text for further descriptions

Figure 4:

Light microscope micrograph of a faecal pellet produced by *Temora longicornis*, grazing on a monoculture of *Coscinodiscus wailesii*. The faecal pellet content consists mainly of brownish indefinable matter.

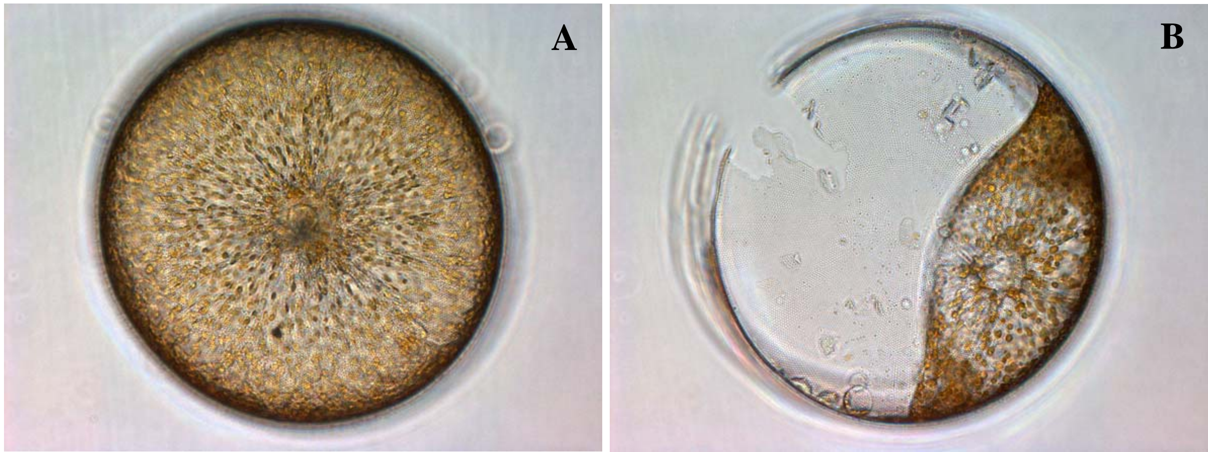

Figure 1

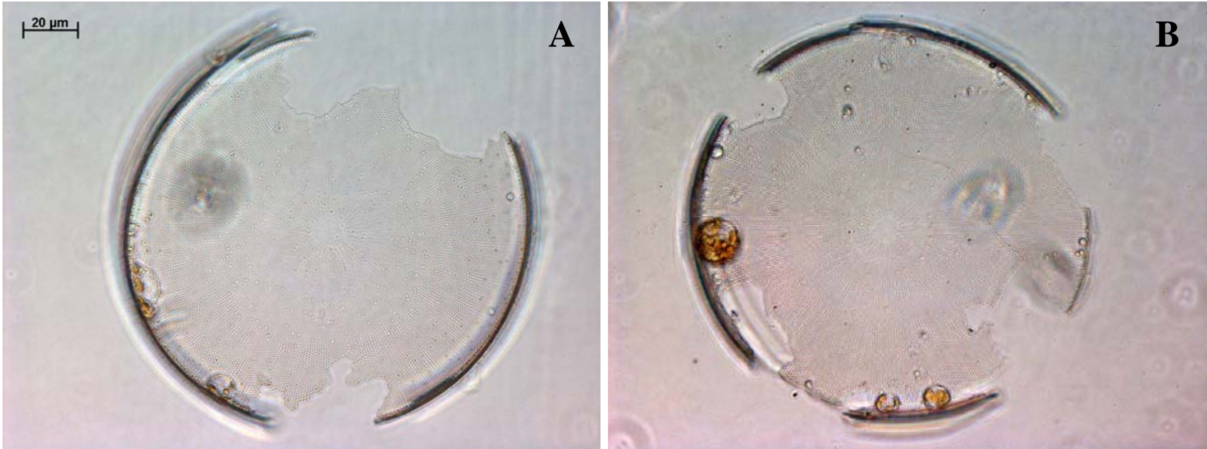

Figure 2

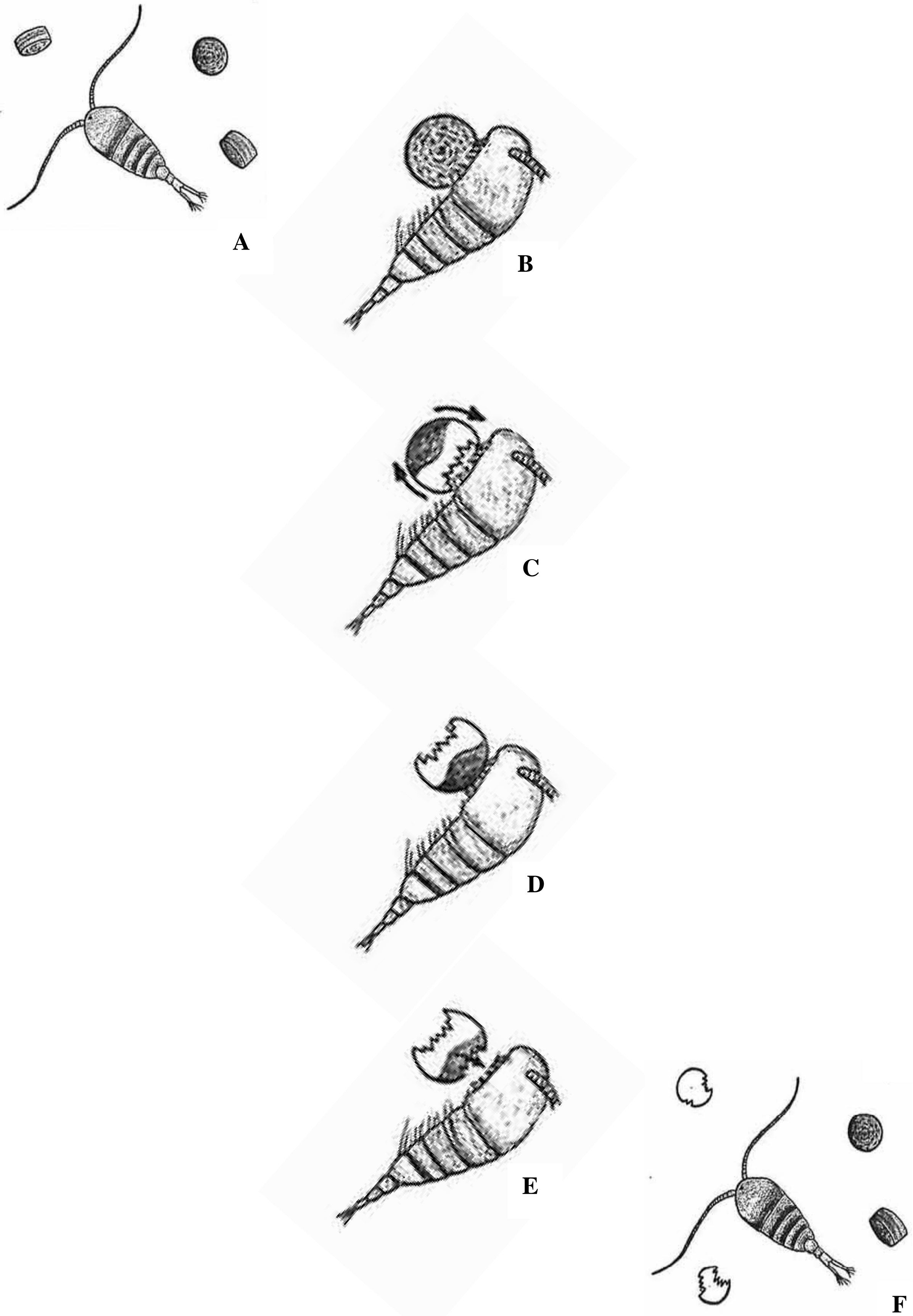

Figure 3

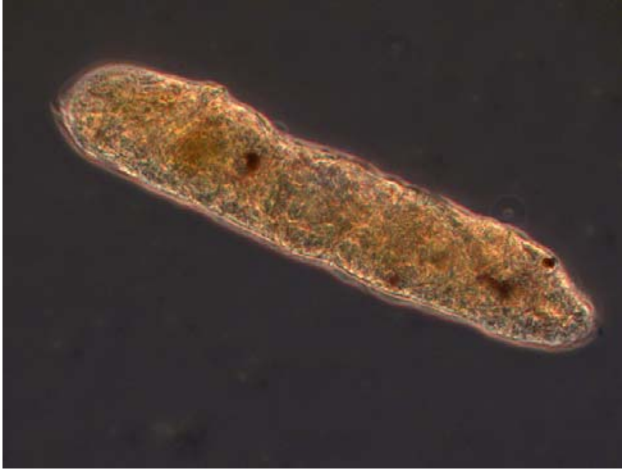

Figure 4

MANUSCRIPT VI

Copepods grazing on Phytoplankton –

First steps of identification using molecular tools

Kerstin Töbe, Sandra Jansen, Uwe John

Draft

Copepods grazing on Phytoplankton – First steps of identification using molecular tools

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Abstract

The development and evaluation of a molecular approach for the identification of phytoplankton in copepod guts was in focus of the present study. Species specific primers were designed for *Coscinodiscus* sp. and four different strains of *Alexandrium* spp. Two of the analysed strains were known to produce PSP toxins, while the two others were non-toxic. Grazing experiments were performed under laboratory conditions with the North Sea copepods *Temora longicornis* and *Calanus helgolandicus* feeding on *Coscinodiscus wailesii* in a first approach and on a mixture of four strains of *Alexandrium* spp. in a second experiment. Copepod gut contents were analysed using real time quantitative PCR (RT-qPCR). Preliminary, but promising results indicated that *C. helgolandicus* was able to feed selectively on the non-toxic *Alexandrium* spp. strains. Problems arose analysing the gut content of *T. longicornis*, independent on the prey grazed upon.

Introduction

Several methods have been developed to determine the grazing impact of copepods (Båmstedt et al. 2000), while most of them were applied some decades ago (Frost 1972, Mackas & Bohrer 1976). The need for new methods is increasingly discussed since all available ones have their specific limitations (reviewed by Båmstedt et al. 2000). The microscopic analysis of gut or faecal pellet content from freshly caught copepods may provide a direct and good tool to investigate the copepod grazing *in situ*. However, it can give only qualitative insight in what have been grazed (Turner 1984, Turner 1991, Pasternak & Schnack-Schiel 2001), and can rarely lead to quantitative results (but see: Wexels Riser et al. 2003). Also, prey types with mineral skeletons like diatoms or coccolithophores for example, are much easier to classify then e.g. naked flagellates or protozoan and their importance may therefore be overestimated in such studies. In cases where copepods do not ingest whole cells but suck out their prey (Elbrächter 1973), interpretation of gut and faecal pellet content may lead to wrong conclusions. For example, the large centric diatom *Coscinodicus wailesii* was shown to be sucked out by the copepod *Temora longicornis* which guts and faecal pellets consequently mainly contained “undefined matter” (Jansen, unpublished data). This diatom served as first prey item in our study. As second prey item, we used four different strains of the dinoflagellate *Alexandrium* spp. With traditional methods, it is impossible to distinguish different strains from the same species or morphotypes which exhibit toxic and non-toxic representatives, like known for the dinoflagellate *Alexandrium.* It is an important bloom forming species (Turner & Tester 1997) which causes the so called “red tides” and synthesizes potent neurotoxins which are called paralytic shellfish poisoning (PSP) toxins. Copepods can serve as vector to larger predators since they can accumulate high levels of PSP toxins (Teegarden & Cembella 1996). Therefore, it is of interest to analyse if copepods are able to feed selectively on non-toxic algal species. The outcome of such studies seems to be highly species specific (Turner et al. 1998, da Costa et al. 2005) and an accurate method is missing to determine feeding preferences on the level of different algae strains, just differing in their toxicity.

New methods should be able to directly qualify and quantify *in situ* ingestion of all prey of a single zooplankton by analysis of a freshly caught individual or its feces, without any prior interference with the food environment (Nejstgaard et al. 2003). The use of

molecular tools seemed to be the most promising upcoming approach in the recent years (Nejstgaard et al. 2003) and the polymerase chain reaction (PCR) seemed to be an excellent suited tool to identify microalgae in copepod guts or faecal pellets.

Nowadays PCR is routinely used in various fields, like in medical applications and molecular biology, to amplify DNA. The ribosomal DNA is often used as a target for the PCR amplification, because ribosomal DNA (rDNA) genes are repeated tandemly in high copy numbers and are highly conserved (Woese 1987). These regions of the rDNA molecule can be used to develop oligonucleotide probes of different hierarchical levels to discriminate phytoplankton at various taxonomic levels from classes down to species or even strains by using whole-cell and cell-free formats (Scholin et al. 2003, John et al. 2003, 2005). Traditional PCR applications use Agarose gel detection at the final phase of the PCR reaction. In this end point of the PCR reaction, it is not possible to gain information about the basic raw material in the sample whereas in real time quantitative PCR (RT-qPCR) approaches, data is collected over the whole cycles of the PCR and therewith also directly in the exponential growth phase. In this phase, PCR is precise and without the problem of the amplification plateau, which occurs in traditional PCR applications. The accurate doubling of the product is monitored at every cycle and exact information about the quantity of the amplified product in the raw material is enabled.

There are different possible approaches to use RT-qPCR, among which the SYBR Green approach is the most commonly used. SYBR Green is an intercalating fluorescent dye, with an excitation wavelength of 483 nm and an emission wavelength of 533 nm. This fluorescing dye binds to the minor groove of double stranded DNA and results in an increase of the fluorescent emissions which is proportional to an increase of the double-stranded PCR amplicon formation after each PCR cycle.

Another more specific approach is the use of species specific primers together with a species specific fluorigenic oligonucleotide probe, called a TaqMan®probe or MGB®probe. In these probe-based approaches, the probe is designed to anneal between the forward and reverse primers and is labelled at the 5’ end with a reporter dye such as 6-carboxy-fluorescein (FAM) and at the 3’ end with a quencher dye such as 6-carboxy-tetramethyl-rhodamine (TAMRA). In the TaqMan approach, the 5’ exonuclease activity of the applied DNA polymerase is used to separate the quencher from the reporter dye. After hybridization of the probe, the DNA polymerase binds to the specific primers, and during replication of the

template, the enzyme nicks the labelled probe. The PCR product is detected simultaneously and automatically by the emission of the reporter, which is separated from the quencher (Lee et al. 1993). The use of species specific primers and the oligonucleotides (probes) enables the detection of up to four different original templates, and with it, of up to four different species in one sample. As mentioned above, this leads to the opportunity to address the question of preference feeding in mixed prey assemblages up to microcosm experiments.

The aim of the present study was to develop and evaluate a molecular approach based on the use of RT-qPCR to obtain an analytical tool for the quantification and identification of microalgae in copepod guts. We tested the molecular approach under controlled laboratory conditions using the abundant North Sea copepods *C. helgolandicus* and *T. longicorni*s feeding on the diatom *C. wailesii* in a first experiment and on four different strains of the dinoflagellate *Alexandrium* spp., two of them toxic, in a second approach.

Material and Methods

Phytoplankton

Coscinodiscus wailesii is the largest centric diatom commonly found in the North Sea. Single cells were isolated from a net sample, taken at the permanent station Helgoland Roads (North Sea, 54°11´N, 7°54´E). *C. wailesii* was cultivated in F/2 medium (Guillard and Ryther 1962) at 15°C and a light: dark cycle of 16:8 hours was applied. At the time of the experiments, cells had a diameter of ~380 µm.

Alexandrium minutum and *A. tamarense* strains were isolated from different origins (Table 1). Algae were cultured in enriched seawater media K (Keller et al. 1987) and grown with a light: dark photocycle of 12:12 hours at 15°C and a photon flux density of 100 µmol $m^{-2}s^{-1}$. Generally, the genus *Alexandrium* (Halim) consists of approximately 29 species, nine of which are known to be toxic. Among *Alexandrium* species, the *A. tamarense* species complex and *A. minutum* play the most prominent role in Harmful Algal Blooms (HABs). Genetically analysis of representative from the *A. tamarense* species complex divided these groups into six genetically distinct clades. The North American (NA), temperate Asian (TA), and the Tropical clades consists only of toxic strains, whereas the western European (WE), the Tasmanian, and the Mediterranean clades (ME) are exclusively non-toxic (John et al 2003). Tillmann and John (2002) showed that beside the potential capability to produce PSP

toxins, *Alexandrium* species produce a variety of allelochemicals which might be responsible for effects such as grazing deterrent. In the present study, four different *Alexandrium* species were used. Two of them were non-toxic species while two of the species used were known to produce PSP toxins (Table 1).

Copepod collection and feeding experiments

Copepods were collected at the permanent station Helgoland Roads in the North Sea (54°11´N, 7°54´E). A zooplankton net (160 µm mesh size) was slowly towed from 15 meter depth to surface and the catch was diluted in 10 L of filtered seawater immediately after retrieval. Back on land, healthy females of the abundant copepod species *T. longicornis* and *C. helgolandicus* were isolated under the binocular in a temperature controlled room at 15°C. In the first experiment, copepods were fed on *C. wailesii* at a concentration of ~800 µgC L^{-1}. Ten females of each copepod species were first acclimated for six hours to the algae suspension, before they were transferred into new bottles containing the same algae concentration as before. After twelve hours, animals were carefully rinsed with filtered seawater, transferred to a 200 µm mesh, wrapped in aluminium foil and then immediately frozen to - 80°C.

In the second experiment, *T. longicornis* and *C. helgolandicus* females were fed a mixture of four strains of *Alexandrium* spp, listed in Table 1. Like in the first experiment, a food concentration of 800 µgC L^{-1} was chosen in order to achieve maximum feeding rates (Dutz 1998). After six hours acclimation and twelve hours experimental duration, all copepods were treated as described for the first experiment.

Additionally, *T. longicornis* and *C. helgolandicus* females with empty guts were frozen for control analysis. In order to empty their guts, animals were kept in filtered seawater for 48 hours, before they were transferred to a 200 µm mesh, wrapped in aluminium foil and frozen. All samples were kept at - 80°C until further handling.

DNA preparation

DNA extractions from the microalgae were made from 1 L of culture in logarithmic growth phase and were harvested with filtration through a 3 µm Isopore membrane filter (Millipore, USA) and afterwards washed once with sterile seawater. Cells were washed from the filters with prewarmed (65°C) 400 µL lysis buffer and 4 µL RNase A (Qiagen, Germany).

DNA was extracted using the DNeasy kit (Qiagen, Germany) according to the manufacturer's instructions, with a modification in the lysis procedure, which was performed for 2 h at 65°C for a better lysis of the cells. The DNA concentration was measured spectrophotometrically at 260 nm, and the DNA quality was analysed for integrity with agarose gel electrophoresis.

Copepod preparation for Standard and RT-qPCR Analysis

One copepod in 40 µL molecular biology grade water (Eppendorf, Germany) per tube was transferred in sterile conical microtubes (Neolab, Germany) and incubated at 95°C for 10 minutes, manually treated with sterile sticks (Dstroy-S, Biozym, Germany) to break up the copepods, afterwards thoroughly mixed, centrifuged in a mini centrifuge (Roth, Germany) and incubated in liquid nitrogen for several minutes. The process was repeated once. For standard PCR the residual pieces of one treated copepod was added to the PCR master mix, or up to 8 µL (in steps of 2, 4, 6 and 8 µL) of the crude cell lysate of the copepods were given to the master mix and the appropriate amount of water was equalized**.**

Standard PCR amplification

The small subunit rRNA genes of *C. wailesii* and *A. minutum* were amplified using the universal eukaryotic primers 1F (5´- AAC CTG GTT GAT CCT GCC AGT A-3´) and 1528R (5´-GAT CCT TCT GCA GGT TCA CCT AC-3´) as described by Medlin et al. (1988) without the polylinkers. Each 50 µL PCR reaction contained 10 µL of 10 x HotMaster Taq buffer with 25 mM Mg^{2+} (Eppendorf, Germany) and 0.5-1.5 units of HotMaster-Taq DNA polymerase (Eppendorf, Germany). PCR reactions were prepared including the Taq polymerase and cooled on ice until placed in an Eppendorf Mastercycler Gradient (Eppendorf, Germany), with the block preheated to 94°C. After the initial denaturation (94°C, 5 min), 29 cycles of 94°C for 2 min, annealing at 54°C for 4 min and elongation at 70°C for 2 min were carried out, followed by a final extension at 72°C for 7 min and cooling to 4°C. The variable D1/D2 region of the large subunit rRNA of the different *Alexandrium tamarense* clade members was amplified using the forward primer DIR-F and the reverse primer D2CR (Scholin et al. 1994) with initial denaturation (94°C, 5 min) followed by 35 cycles of 94°C for 20 seconds, annealing at 54°C for 20 seconds, elongation at 70°C for 1 min and a final extension at 70°C for 10 min. Amplification products were cooled to 4°C and

checked for appropriate length and purity by agarose gel electrophoresis. Negative controls with no DNA added were performed in each PCR reaction.

Development and testing of Primer and MGB-probes

The species specific primers and the MGB-probes targeting the small, or large ribosomal subunit of the used microalgae (Table I) were designed by using Primer Express software Version 2.0.0 (Applied Biosystems, USA) and the ARB software packaging and its subfunction 'probe design' (Ludwig et al. 2004) by using an alignment of all available sequences downloaded from GenBank or obtained from our laboratory. Species or strain specific regions on the SSU or LSU of the ribosomal operon were detected using the probe design tools of ARB. With the Primer Express software, primer and probes were modified according the prerequisite of RT-qPCR. The primers and PCR conditions were tested in SYBR green assays (see below) to ensure the specificity of the reaction.

SYBR Green Approach for primer specificity tests

Real-time quantitative PCR SYBR Green assays were performed on an ABI PRISM 7000 SDS (Applied Biosystems, USA) in a final volume of 20 µL containing 10 µL of a 2 x Power SYBR Green PCR Master Mix (including SYBR Green 1 dye, AmpliTaqGold®DNA polymerase, dNTPs, Applied Biosystems, UK) and primers at a final concentration of 300 nM. One microliter of PCR amplificated DNA of the different microalgae was added to 19 µL of the PCR master mixture to generate the standard dilution series for each microalgae species to test the specificity of each newly designed Primer set. Experiments were performed in triplicates for each lysate sample and for each standard curve point. The standard curve was constructed with 1000-fold dilutions of a purified 18S/28S PCR product of the target microalgae. The curve range was a 10 x serial dilution of these PCR products from 10^3 to 10^8. In each RT-qPCR experiment, the standard dilution series for the particular microalgae and a no template control were present. Additionally, the primers were tested on non-target species to confirm their specificity. The cycle conditions for the SYBR Green approach were: Stage 1, Step 1: Hold 95°C for 10 min, followed by 40 cycles of stage 2, Step 1: Hold 95°C for 15 min, Step 2: Hold 59°C for 1 min. At the end of the program a melt curve protocol was added to confirm that only the specific PCR product was present. The fluorescence threshold was set by the analytical software for the ABI PRISM cycle. The PCR cycle during which this

threshold was crossed for each sample was designated the C_T, the reported C_T is the average triplicate of experimental samples, standards or internal controls. Sample C_T can be compared to the C_T of standards with a known cell count or DNA concentration to specify the number of cells present in the sample.

MGB probe approach

Real-time quantitative PCR assays were performed on an ABI PRISM 7000 SDS (Applied Biosystems, USA) in a final volume of 20 µL containing 10 µL of a 2 X TaqMan®Universal PCR Master Mix (contains AmpliTa Gold DNA polymerase and dNTPs, Applied Biosystems, manufactured by Roche, USA) and primers at a final concentration of 300 nM. One microliter of PCR amplificated DNA of the different microalgae was added to 19 µL of the PCR master mixture to generate the standard dilution series for each microalgae species, either from traditional PCR amplified DNA from microalgae or from crude cell lysates from copepods. Additionally, experiments were performed in which the residual pieces of one copepod (preparation see above) were added to the PCR Master Mix, or up to 8 µL (in steps of 2, 4, 6 and 8 µL) of the crude cell lysate of the copepods. For the single cell PCR of *C. wailesii,* one, two and three cells were given to the PCR Master Mix, respectively. Experiments were performed in triplicates for each lysate sample and for each standard curve point. The applied MGB probes were labelled with the fluorescence dyes Vic (excitation wavelength 523 nm, emission wavelength 568 nm) or FAM (excitation wavelength 483 nm, emission wavelength 533 nm). In each RT-qPCR experiment the standard dilution series for the particular microalgae and a no template control were present. Additionally, in order to confirm specificity of primers and probes, the probes and primers were tested on non-target species and on copepods fed with non-target species or which were not fed at all. The cycle conditions for the MGB approach were: Stage 1, Step 1: Hold 50 °C for 2 min, Stage 2, Step 2: Hold 95°C for 10 min, followed by 40 cycles of stage 3, Step 1: Hold 95°C for 15 min, Step 2: Hold 59°C for 1 min.

Results and Discussion

Copepods constitute up to 80% of the mesozooplankton biomass of the ocean (Verity & Smetacek 1996). With phytoplankton as main food source, they present an important link

from the primary producers to higher trophic levels such as birds and mammals but also for commercially important fish (Turner & Tester 1997). Algal blooms are therefore advantageous for the marine environment and the aquaculture in general. Problems arise when the bloom species are toxic and so called harmful algal blooms (HABs) can cause great financial losses for industry and tourisms.

In the present study we concentrated on the development of a PCR based detection method of prey in copepod guts. In a first approach we chose *C. wailesii* as a large food item and in a second one, we chose four different strains of *Alexandrium* spp., similar in size and morphology but different in its toxicity in terms of PSP toxins (Table 1). We chose these exemplary phytoplankton species as the specialized and possible selective feeding on these species by copepods can't be fully examined with traditional methods.

In the first step, primer and probe sets were developed which are species or even strain specific for the different prey species under investigation. Afterwards we had to proof if the PCR worked in single cell PCR in order to get the lower detection limit, and then we had to investigate how reproducible we can detect the prey in copepod guts. In the following we show and discuss the first results.

SYBR Green Approach and MGB-approach for primer and probe specificity tests

The newly designed species specific primer and probes targeting the 18S / 28S rDNA (Table II) are tested on DNA of target and non-target microalgae species. The primer and probes show a high species specificity with no cross-reaction with DNA from other closely related non-target species, which make them adequate for the specific identification and quantification of the content of copepod guts by using RT-qPCR as an analytical molecular tool. The species specific primer established here in this study can be further used in studies where single species approaches, single cell approaches or only single heterotrophic dinoflagellates or ciliates are used. In such set ups the background genomic DNA is low enough that interfering problem of intercalating SYBR Green should be excluded.

MGB probe approach for detecting Coscinodiscus wailesii fed to copepods

Figures 1-5A show the PCR amplification course of the standard dilution series from 10^3-10^8 of a standard PCR product of the respective microalgae species and demonstrate where the threshold for the C_T values have been set. The resulting C_T values were used to

calculate the standard curves. Figures 1-5B show the standard curves of the respective standard dilution series which are important for the evaluation of the PCR efficiency. The latter should be close to 100% in order to be suitable for quantification and for comparison reasons. The optimal PCR efficiency of 100% corresponds to a slope of -3.32 of the standard curve. In our study, we got for the *Coscinodiscus* primer and probe set a slope of -3.19 with a probability of 0.99 (Fig. 1B), a slope of -3.47 for *A. tamarense* WE31/4, with a probability of 0.99 (Fig.2B), a slope of -3.75 for *A. tamarense* SZN1 with a probability of 0.99 (Fig. 3B), a slope of -3.73 for *A. tamarense* NA with a probability of 0.99 (Fig. 4B), and finally a slope of -3.22 for *A. minutum* AMP4 with a probability of 0.96 (Fig. 5B).

With RT-qPCR (Fig. 1A-D) amplificated Standard PCR products, we could neither clearly detect *C. wailesii* fed to *C. helgolandicus* nor detect the diatom in *T. longicornis.* However, we found putative positive signals with the *Coscinodiscus* probe generated from crude cell lysates from *C. wailesii* and *C. helgolandicus* (Fig. 1C), which were not present in the negative results. However, we could not generate a positive amplicon in every replicate. The standard PCR amplificated 18S DNA products from crude cell lysates from *C. wailesii* and *C. helgolandicus* were only weakly visible in agarose gel electrophoresis and we assume that only small amounts of the diatoms were ingested by the copepods. Another possible explanation for the weak reaction may be that their digestion rate is too fast in relation to the ingestion speed. It is important to point out that we are able to amplify DNA of single *C. wailesii* cells with RT-qPCR (Fig. 1D). Hence if enough DNA material would have been left in the copepod, we should have detected it. Previous experiments showed that *C. helgolandicus* as well as *T. longicornis* were able to graze on *C. wailesii*. In further experiments we plan to combine the genetically approach with direct observations of the copepods and calculations of the ingestion rates with traditional methods for calibration.

MGB probe approach for detecting Alexandrium fed to copepods

With RT-qPCR amplificated Standard PCR products, we detected the non-toxic *A. tamarense* WE31/4 belonging to the Western European clade and *A. tamarense* SZN1, belonging to the Mediterranean clade, in crude cell lysates from single *C. helgolandicus* (Figs. 2C & 3C), which were fed with the four different clades of *Alexandrium* spp. Furthermore, we did not detect the toxic *A. tamarense* of the North American clade and *Alexandrium minutum*

AMP4 with RT-qPCR in these animals (Fig. 3C & 4C). More precisely, the two non-toxic species *A. tamarense* of the Western European clade and of the Mediterranean clade were ingested by the copepod whereas the toxic forms seemed to be avoided. This leads to the assumption that *C. helgolandicus* is able to distinguish between toxic and non-toxic clades of the same algal species, feeding selectively on the non-toxic strains. However, further investigations are needed for definitive conclusions as we could only show preliminary, but promising results. In contrast to *C. helgolandicus*, the gut content of *T. longicornis* females showed again no clear results. It is unclear whether the copepods did not feed in the present study, or if there is another existing inhibitor, which does not allow a successful amplification via PCR of the target species DNA fragments in this copepod.
In upcoming experiments, we plan to feed monocultures to the copepods to be sure about the traceability of all algae species. The ability to quantify the ingestion rates of copepods with the presented molecular tools will be compared with classical incubation methods in the laboratory in the future.

Reproducability and sensitivity in amplifying the potential target species in copepods with RT-qPCR, can be increased by an additional PCR step. The whole copepod extract is used for 18S or 28S rDNA amplification and this amplicon was used for species discrimination in RT-qPCR. The major disadvantage of this procedure is the biased amplification of potential target DNA. As a consequence of that the resulting distribution might not reflect the original copepod prey. Therefore it is desirable to avoid this additional step for quantatitative analysis. However, this step could prove useful for qualitative analysis, e.g. mesocosm and field studies, because the potential prey composition is more complex and could be unknown. The PCR amplicons from the mediated step can then be screened with many different primer/probe sets for a high variety of species.

Real time quantitative PCR is also an excellent analytical tool for the detection and quantification of target DNA in field samples (Dyhrmann et al. 2006, Coyne et al. 2006). However, field samples require preservation, which could influence RT-qPCR results or even inhibit the reaction. Hosoi-Tanabe and Sako (2005) investigated the influence of fixatives on RT-qPCR and found that the sensitivity of RT-qPCR was considerably lower with preservation using formalin and glutaraldehyde than with no preservation. However, it was possible to detect and quantify target cells from three year old field samples preserved by freezing (−20°C) and ethanol treatment.

In this study, we demonstrated that DNA from microalgae ingested by *C. helgolandicus* can be detected by RT-qPCR. However, further tests under defined laboratory conditions are necessary to calibrate this method before it can be applied to field samples. This will provide new insight into the *in situ* feeding behaviour of copepods.

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Table 1:

Phytoplankton organisms used in this study. Strain code, geographic origin and preferred culture medium is given, as well as information on the toxicity.

Organisms	*Strain Code*	*Geographic origin*	*PSP*	*Medium*
Coscinodiscus wailesii		Helgoland, Germany	-	F/2
Alexandrium minutum	AMP4	Galicia, Spain	toxic	K
Alexandrium tamarense Western European clade	WE 31/4	Cork Harbour, Ireland	non-toxic	K
Alexandrium tamarense, Northern American clade	NA	Orkney Islands, UK	toxic	K
Alexandrium tamarense, Mediterranean clade	SZN1	Gulf of Naples, Italy	non-toxic	K

Table 2:

Species specific primer and MGB-probes used in this study

MGB probe [5'-3']	**Primer forward [5'-3']**	**Primer reverse [5'-3']**	**Specific for:**
6-FAM-ccagccctaaccatc-MGB	tgg atg tgg tag ccg ttt ctc	att ggc cta cca tgg ctt taa c	*Coscinodiscus* sp., (18 S)
6-FAM-catccaaacctgacttc-MGB	aca tgg ata act gtg gta att cta tag cta a	gtt ggt tct gta act aat gac cac aac	*Alexandrium minutum*, (18S)
VIC-caacctcaaacacatgga-MGB	ttg tgt gtg tca ggg ctt gta ag	gaa tgt gtc tgg tgt atc tgt ttt tgt	*Alexandrium tamarense*, Western European clade, (28S)
6-FAM-aacactcccaccaagca-MGB	cca cag ccc aaa gct ctt gt	cca tga ggg aaa tat gaa aag ga	*Alexandrium tamarense*, Northern American clade, (28 S)
VIC-agcacaacaacctcacca-MGB	gca tgc caa gaa aga atc att gta	tgt att tgc tga aca aaa agt aaa cag act	*Alexandrium tamarense*, Mediterranean clade, (28 S)

Figure captions:

Figure 1:

(A): Amplification plot for standard curve from 10^3 to 10^8 generated from dilutions of a 18S PCR amplified DNA from *Coscinodiscus wailesii*. The cycle number is plotted versus the Delta Rn. The Delta Rn represents the Rn minus the baseline signal established in the early PCR cycles.

(B): Calibration curve plotting log starting copy number (C_0) versus C_t. Slope, -3.19 correlation coefficient (R^2), 0.99.

(C) Amplification plot generated of *Calanus helgolandicus* extracts fed with *Coscinodiscus wailesii*.

(D) Amplification plot from *Coscinodiscus wailesii* single cells. The cycle number is plotted versus the Delta Rn. The Delta Rn represents the Rn minus the baseline signal established in the early PCR cycles.

Figure 2:

(A): Amplification plot for standard curve from 10^3 to 10^8 generated from dilutions of a 28S PCR amplified DNA from *Alexandrium tamarense* strain WE 31/4. The cycle number is plotted versus the Delta Rn. The Delta Rn represents the Rn minus the baseline signal established in the early PCR cycles.

(B): Calibration curve plotting log starting copy number (C_0) versus C_t. Slope, -3.47 correlation coefficient (R^2), 0.99.

(C): Amplification plot generated from 10^3-10^5 dilutions of a 28S PCR amplified DNA from *Alexandrium tamarense* strain WE 31/4 fed to *Calanus helgolandicus*.

Figure 3:

(A): Amplification plot for standard curve from 10^3 to 10^8 generated from dilutions of a 28S PCR amplified DNA from *Alexandrium tamarense* strain SZN1. The cycle number is plotted versus the Delta Rn. The Delta Rn represents the Rn minus the baseline signal established in the early PCR cycles.

(B): Calibration curve plotting log starting copy number (C_0) versus C_t. Slope, -3.75 correlation coefficient (R^2), 0.99.
(C): Amplification plot generated from 10^3-10^5 dilutions of a 28S PCR amplified DNA from *Alexandrium tamarense* strain SZN1 fed to *Calanus helgolandicus*.

Figure 4:
(A): Amplification plot for standard curve from 10^3 to 10^8 generated from dilutions of a 28S PCR amplified DNA from *Alexandrium tamarense* strain NA. The cycle number is plotted versus the Delta Rn. The Delta Rn represents the Rn minus the baseline signal established in the early PCR cycles.
(B): Calibration curve plotting log starting copy number (C_0) versus C_t. Slope, 3.73 correlation coefficient (R^2), 0.99.
(C): Amplification plot generated from 10^3-10^5 dilutions of a 28S PCR amplified DNA from *Alexandrium tamarense* strain NA fed to *Calanus helgolandicus*.

Figure 5:
(A): Amplification plot for standard curve from 10^3 to 10^8 generated from dilutions of a 18S PCR amplified DNA from *Alexandrium minutum* strain AMP4. The cycle number is plotted versus the Delta Rn. The Delta Rn represents the Rn minus the baseline signal established in the early PCR cycles.
(B): Calibration curve plotting log starting copy number (C_0) versus C_t. Slope, -3.22 correlation coefficient (R^2), 0.96.
(C): Amplification plot generated from 10^3-10^5 dilutions of a 18S PCR amplified DNA from *Alexandrium minutum* strain AMP4.fed to *Calanus helgolandicus*.

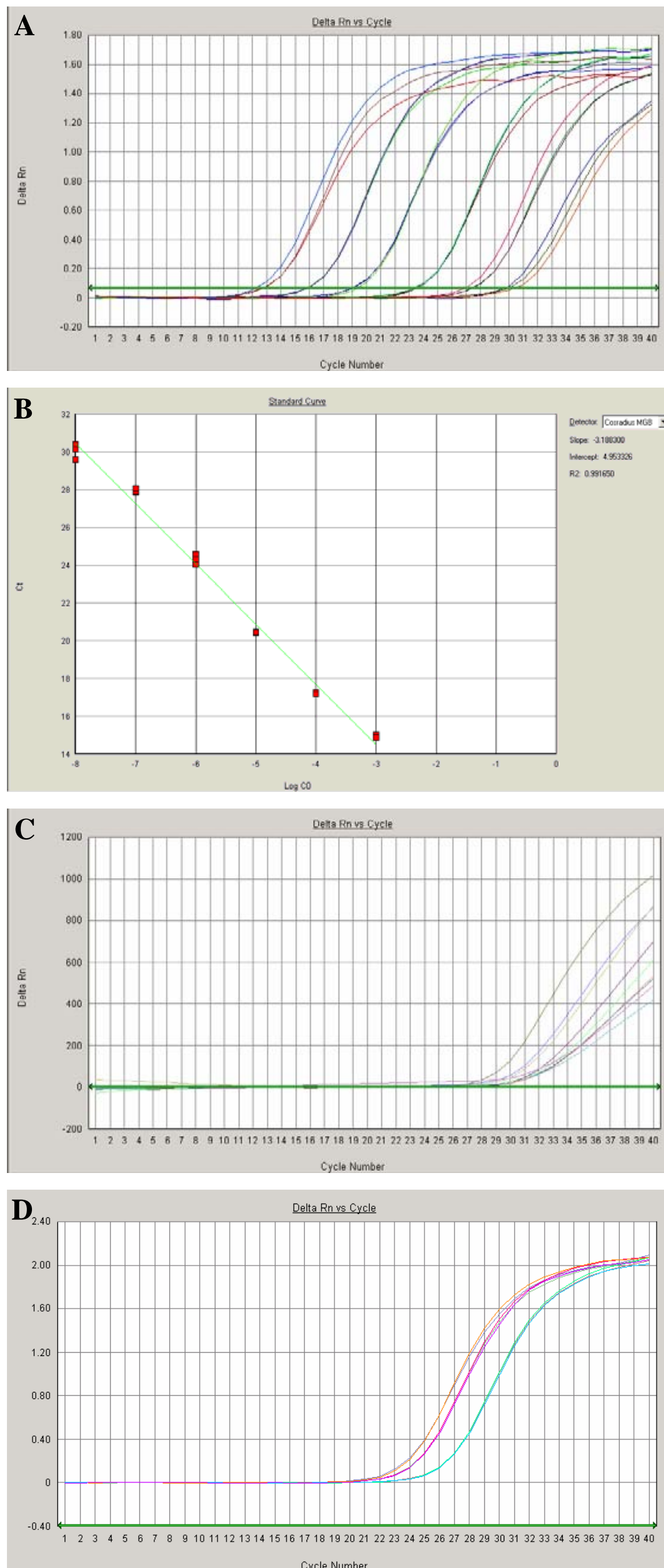

Figure 1

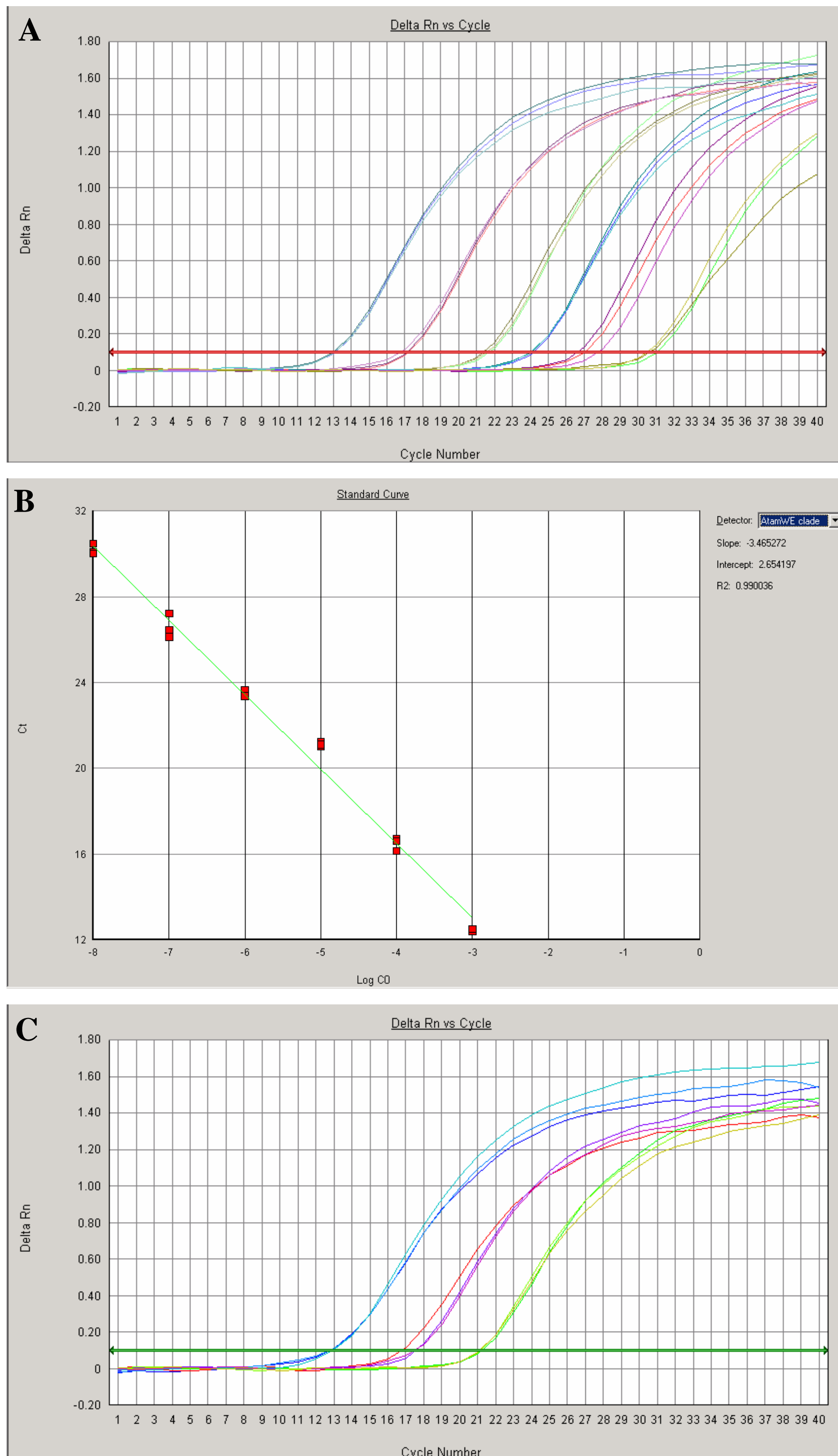

Figure 2

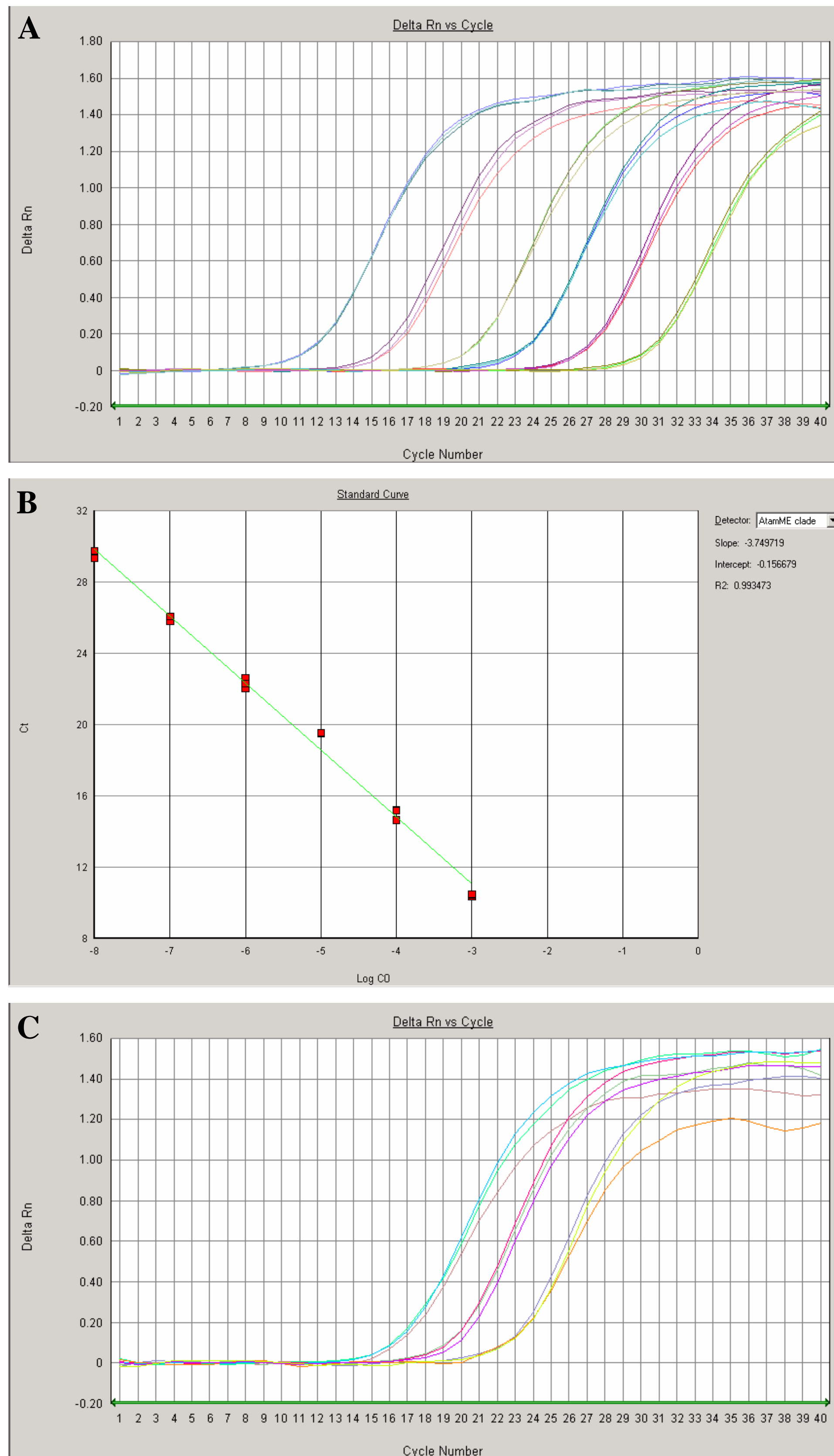

Figure 3

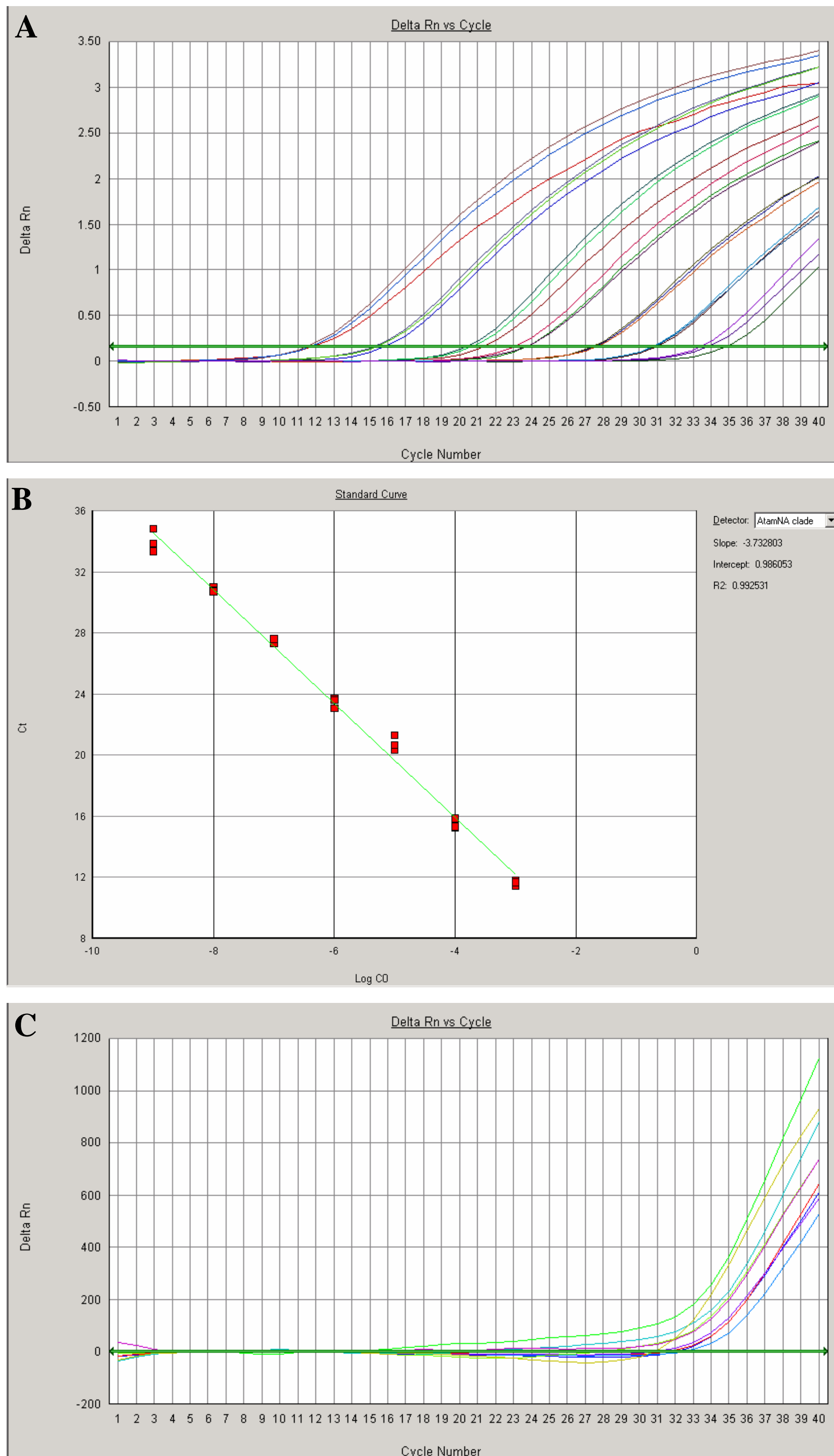

Figure 4

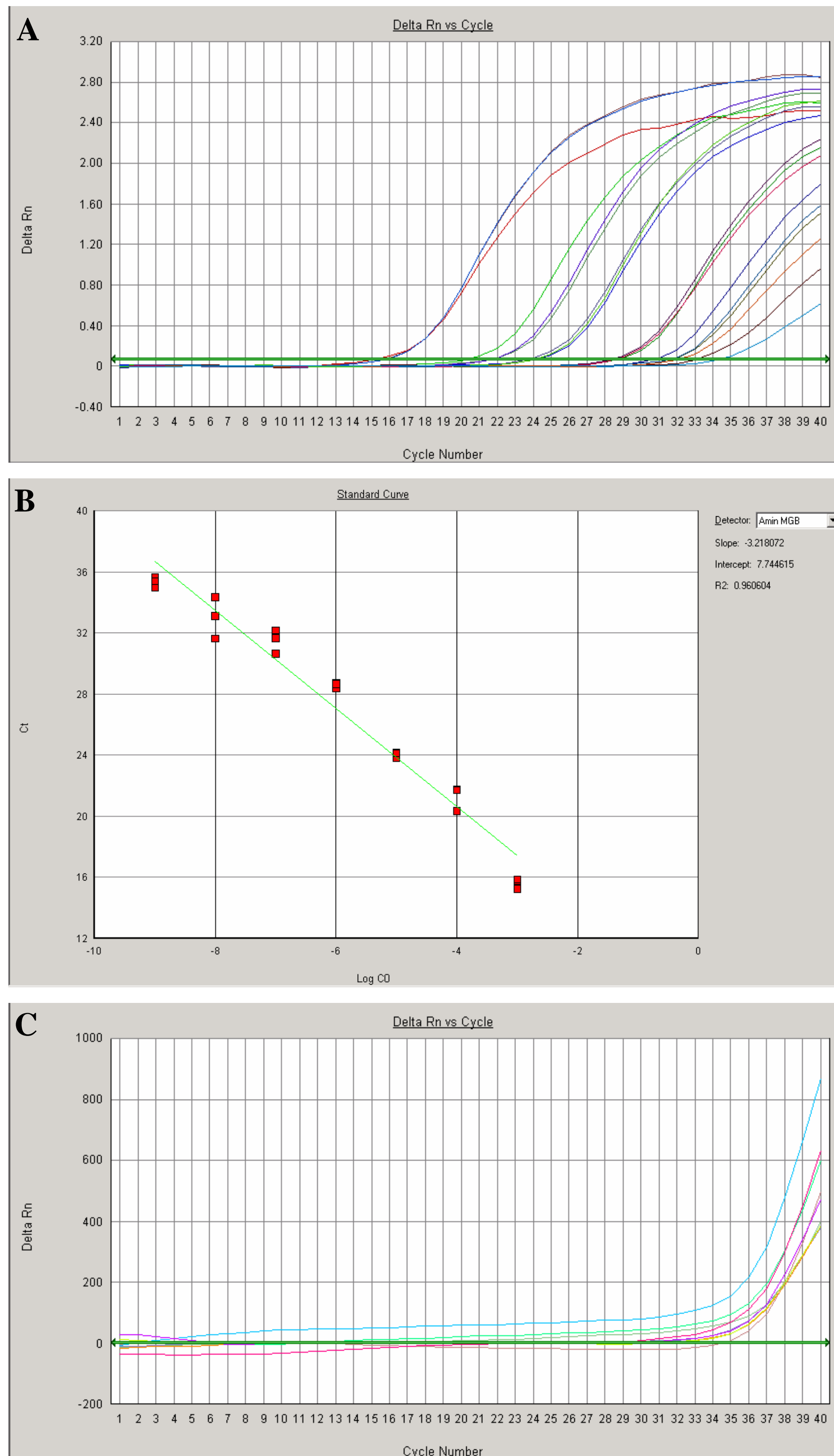

Figure 5

Manuscript VII

Reproductive response of the copepod *Rhincalanus gigas* to an iron-induced phytoplankton bloom in the Southern Ocean

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ORIGINAL PAPER

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Reproductive response of the copepod *Rhincalanus gigas* to an iron-induced phytoplankton bloom in the Southern Ocean

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Abstract The reproductive response of *Rhincalanus gigas* to the build up of a phytoplankton bloom in the Southern Ocean was studied during the European iron fertilization experiment (EIFEX). Egg production experiments were conducted over a period of approximately 5 weeks during development of a diatom dominated bloom. *R. gigas* showed a clear response to increasing chlorophyll *a* concentrations and the total egg production of the *R. gigas* population was highest just after the peak of the bloom at day 29 after fertilization. The average peak production was 50 eggs female^{-1} day^{-1}. The percentage of egg producing females increased from about 0 to 90% during the course of the experiment. Accordingly, the maturation of the gonads reflected the positive response towards enhanced chlorophyll *a* concentrations. The fast reproductive response indicate that *R. gigas* was food limited during the period of this study in the Antarctic Polar Front region (APF).

Introduction

The Southern Ocean is known as a high nutrient—low chlorophyll (HNLC) ecosystem where production is generally low, even though macronutrients like nitrate and phosphate are available all year round (Martin et al. 1990). In recent years, it has been shown that primary production in the Southern Ocean is limited by iron availability (Martin et al. 1990). Large scale artificial iron fertilization experiments of HNLC waters have induced phytoplankton blooms (e.g. Boyd et al. 2000; Gervais et al. 2002; Coale et al. 2004). Copepods dominate the zooplankton communities of the Southern Ocean in terms of numbers and biomass (Voronina 1998; Pakhomov et al. 2000), but our understanding of copepod population dynamics in this area is still poor. As a consequence of the short growth season and low primary production, zooplankton growth and reproduction in the Southern Ocean may be limited by food availability. A productive area within the Southern Ocean is associated with the Antarctic Polar Front (APF) where blooms are frequently reported (Laubscher et al. 1993). In the Atlantic sector of the APF *Rhincalanus gigas* is one of the most abundant large copepod species (e.g. Ommanney 1936; Atkinson 1991; Pakhomov et al. 2000), and is known to have a protracted period of recruitment through the summer into the autumn (Ommaney 1936). In recent years it has become more and more apparent, that growth and development of populations of large calanoid copepods within the Southern Ocean depend on the availability of food (Ward and Shreeve 1999; Shreeve et al. 2002). However, most of the reported studies give only snapshot results where temporal development is not taken into consideration.

The European iron fertilization experiment (EIFEX) provided an unique opportunity to follow the reproductive response of *R. gigas* during the entire development of a diatom dominated phytoplankton bloom. This Lagrangian type experiment made it possible to study the same zooplankton assemblage for 38 consecutive days, which has so far not been achieved for the Southern Ocean.

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Material and methods

Study location

The iron fertilization experiment EIFEX was carried out during austral autumn (21 Jan 2004–25 Mar 2004) in the Atlantic sector of the Southern Ocean on *RV* Polarstern. We choose a cyclonic eddy (extending over

60 × 100 km) for our experiment, embedded in a meander of the APF and centred at approximately 49.5°S and 02°E (Strass et al. 2005). On the 10th of February the centre of this eddy was marked with a drifting buoy and an area of 150 km^2 around the buoy was fertilized with seven tons of iron sulphate solution ($FeSO_4$). The first indication of algal response to iron enrichment was a small but detectable increase in the photosynthetic efficiency (F_v/F_m) after about 24 h (Röttgers et al. 2005). On board measurements of in situ phytoplankton F_v/F_m and later measurements of the partial pressure of CO_2 (pCO_2) served as markers of the fertilized patch. Sampling was carried out within the eddy, inside and outside the fertilized patch throughout the duration of the experiment (38 days).

Egg production experiments

Net samples from the upper 50 m of the water column were taken with Bongo nets (100 μm mesh size) around dusk. Net catches were diluted in 20 l of surface water. Sub-samples were transferred to a Petri dish and sorted under a binocular. For the egg production experiments, healthy *R. gigas* females were handpicked individually and transferred to 100 ml beakers filled with filtered seawater. The females were incubated individually at in situ temperature in the dark. Depending on how many females were present in the net haul, 12–36 parallel incubations were set up (Table 1). After 24 h, the females were removed from the beakers and eggs in the beakers were counted immediately under the binocular. For each station the average egg production rate was derived using data from all incubations, including those where females did not spawn.

Carbon mass (CM) analyses

Rhincalanus gigas females for carbon analysis were handpicked from additional Bongo net catches (100 μm mesh size; 50–0 m). Females were kept in filtered seawater for 24 h before the carapace length was measured under a binocular. Subsequently, animals were transferred individually onto pre-combusted GFF-filters and stored at −80°C. Back on land, samples were dried overnight at 60°C and analysed for carbon and nitrogen on a C/N-Analyser (Carlo Erba NA-1500).

Maturation of the gonads

Zooplankton was sampled using a Mulitnet (100 μm mesh size) at five depth intervals: 400–160, 160–100, 100–50, 50–25 and 25–0 m, respectively. The samples were preserved in buffered formaldehyde (4% final concentration) and stored at 4°C until further analysis. Between 40 and 60 *R. gigas* females from selected stations were examined to determine the stage of the ovarian development. Stations were selected to give good temporal coverage, irrespective of the time of the day at capture. Gonad maturation stages can be established from whole preserved specimens without staining (Niehoff and Runge 2003) in copepods with transparent carapaces. This is the case for female *R. gigas*. Therefore, gonads of whole, unstained animals were studied under a dissecting microscope and five developmental stages (GS) were distinguished (Table 2; modified after Niehoff and Hirche 1996; Niehoff and Runge 2003).

Results

Before fertilization near-surface (8 m depth) chlorophyll *a* distribution in the eddy was patchy with concentrations ranging from 0.5 up to 1.2 μg chlorophyll *a* l^{-1}. During the course of the experiment samples outside the fertilized patch showed high variability with chlorophyll *a* values ranging from 0.3 μg l^{-1} up to about 1 μg l^{-1}. Inside the fertilized patch, chlorophyll *a* concentrations increased and maximum integrated values (0–100 m depth) were found around day 30. Because of the patchy chlorophyll *a* distribution within the eddy, clear differences between in-patch and out-patch values were not observed until about day 10 after fertilization when in-patch chlorophyll *a* concentrations exceeded those found outside the fertilized patch. Throughout the experiment the phytoplankton assemblage was dominated by chain-forming (*Chaetoceros* spp., *Fragilariopsis kerguelensis*, *Pseudo-nitzschia* spp.) and large single

Table 1 Mean (± SE) egg production rates (EPR) of *Rhincalanus gigas* inside and outside the fertilized patch (out). Station numbers are given with corresponding average chlorophyll *a* concentrations over the upper 60 m of the water column (mg m^{-3}). *n* number of incubated females

Station	*t* (day after fertilization)	Chl *a*	*n*	Mean EPR ± SE
424	0	0.76	20	0
508	10	1.7	36	23.7 ± 3.6
513	15	1.97	12	28 ± 8.1
514 out	17	0.61	12	0
544	23	2.45	20	22 ± 5.4
560	29	2.7	18	49.8 ± 10
561 out	29	1.02	20	0.3 ± 0.3
579	32	1.65	20	15.4 ± 4.3
593	36	2.05	15	8 ± 4.8

Table 2 Classification of the gonad developmental stage (GS) based on macroscopic criteria, modified for *Rhincalanus gigas* after Niehoff and Hirche (1996) and Niehoff and Runge (2003)

GS 1	Oocytes present in the ovary; oviduct empty or only with single, transparent, small oocytes
GS 2	Transparent oocytes in the oviduct in one or maximal two layers
GS 3	Transparent oocytes in the oviduct in several layers; all oocytes similar in size; no nucleus visible
GS 3.5	Oocytes in the oviduct in several layers, all similar in size; ventral row with visible nucleus, but still transparent
GS 4	Several rows of oocytes in the oviduct; oocytes increase in size in ventral direction, ventral row is larger, darker and with visible nucleus

celled diatoms (*Thalassiothrix antarctica*, *Corethron inerme*, *Proboscia* spp., *Rhizosolenia* spp.; P. Assmy, personal communication).

Temperature profiles taken inside and outside the fertilized patch over the duration of the experiment indicated that the mixed layer often extended down to 100 m, temperatures between 3.5 and 4.5°C were stable within the nearly closed eddy circulation (V. Strass, personal communication).

Egg production and CM analyses

Figure 1 shows the egg production rates of *R. gigas* over the course of the experiment. First measurements were conducted at the time of iron release and represent conditions before fertilization. *R. gigas* did not produce eggs at this time. Egg production and the proportion of egg producing females increased significantly inside the fertilized patch with increasing chlorophyll *a* concentrations (Table 1). At day 29 after fertilization an average of 50 eggs female^{-1} day^{-1} were produced, with about 90% of the incubated females producing eggs. The highest individual egg production rate (153 eggs female^{-1} day^{-1}) was also observed at day 29 after fertilization. Outside the fertilized patch, the number of egg producing females as well as egg production rates remained close to zero (Fig. 1, Table 1).

Carbon content of *R. gigas* varied considerably from 220 to 1,780 µg C female^{-1} (Table 3). The mean carbon content of the females did not change significantly over the course of the fertilization experiment (ANOVA: $F = 6.22$, $P = 0.067$), although a slight increase in carbon content was observed.

Maturation of the gonads

The proportion of females in the different gonad developmental stages (GS 1–GS 4) is shown in Fig. 2. The corresponding integrated chlorophyll *a* values for the upper 100 m of the water column are indicated. Until day 12 after fertilization, females in all GS were present in changing proportions with no significant difference between in- and out-patch stations. From day 16 on, about 90% of the *R. gigas* females were in GS 4 at all in-patch stations. In the out-patch stations the relative contribution of GS 4 females remained low (about 10%).

Discussion

Several studies have examined the relationship between reproduction of *R. gigas* and chlorophyll *a* as an indicator of food availability. Published results show high variability in the correlation between egg production rates and surface chlorophyll levels (Ward and Shreeve 1995; Ward et al. 1996; Shreeve et al. 2002). However, although chlorophyll *a* concentration may represent actual food availability it does not give information on past feeding conditions or food quality. Hence, interpretation of the correlation found in previous studies can be problematic. The EIFEX cruise presented an unique opportunity to study the complete build up of a phytoplankton bloom with the corresponding reproductive response of the copepods investigated.

Egg production in relation to food

Although maturing females require food supply for oocyte maturation and egg production, some studies have shown that temperature rather than food controls egg production rates for different species (e.g. Kiørboe et al. 1988; White and Roman 1992). Our survey of the reproductive response of copepods within an iron fertilized patch and non-fertilized water in a nearly closed eddy allows us to separate the temperature effect from that of food supply. Temperature changes were only minor during the experiment (± 1°C) and the temperature field was the same within the eddy. During the present study, females started to produce eggs within the fertilized patch and egg production rate increased concurrent with increasing chlorophyll *a* concentrations (Fig. 1a). In contrast, at the two out-patch stations where female *R. gigas* could be obtained for egg production experiments, chlorophyll *a* values and egg production remained low (Table 1, Fig. 1a). The average egg production of *R. gigas* during the course of the experiment showed a significant positive relationship with chlorophyll *a* concentrations ($r^2 = 0.714$; $P < 0.005$; Fig. 1b). Although the present study was a Lagrangian experiment, some of the *R. gigas* sampled may have just recently turned into adult females or immigrated into the patch (S. Krägefsky, unpublished data). Both factors could affect the correlation between egg production rates and chlorophyll *a*, and may lead to the relatively high variability observed between and within each station (Fig. 1b).

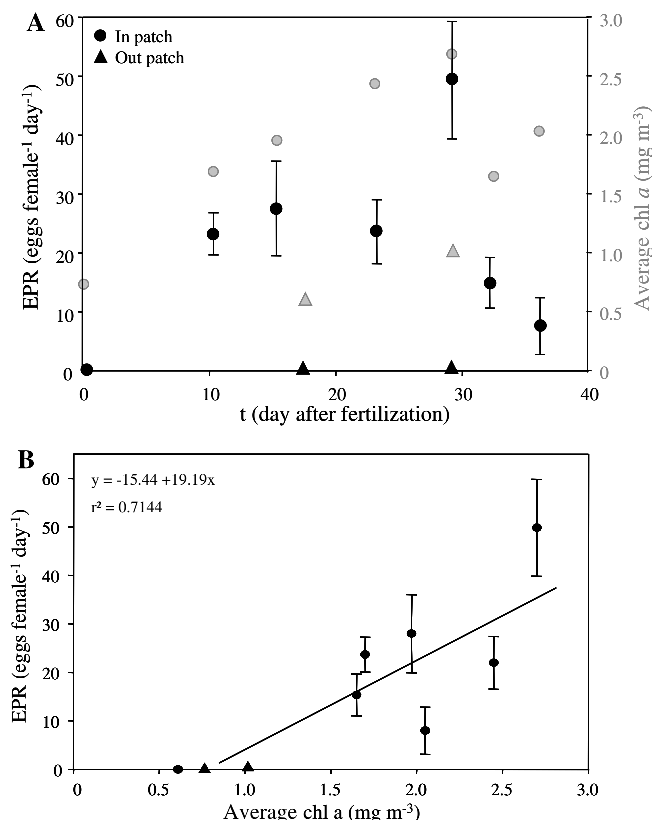


Fig. 1 Mean (± SE) egg production rates (EPR) of *Rhincalanus gigas* after iron fertilization, **a** inside the fertilized patch (*black circles*) and outside the fertilized patch (*black triangles*), with corresponding average chlorophyll *a* concentrations over the upper 60 m of the water column (*grey symbols*). **b** Mean (± SE) egg production rates (EPR) of *Rhincalanus gigas* in relation to the average chlorophyll *a* concentration (0–60 m). Regression line fitted ($P < 0.005$)

In this study we found no saturation level for egg production rate as a function of chlorophyll *a*. It is therefore possible that with even higher chlorophyll *a* the egg production rate may exceed the maximum of ~50 eggs female^{-1} day^{-1}. Indeed, the highest daily egg production rate by a single female was 153 eggs female^{-1} day^{-1}, exceeding the number of ripe eggs counted in preserved females with full oviducts. Consequently, under conditions of abundant food supply, the spawning cycle may take even less than 24 h. This has also been observed for another Antarctic copepod species, *Calanoides acutus*, during a study in Gerlache Strait (Lopez et al. 1993). Assuming a carbon content of 420 ng C per egg (Ward and Shreeve 1999) and an egg production rate of 153 eggs female^{-1} day^{-1}, this corresponds to a daily rate of 5–11% of measured body carbon for day 29 after fertilization. The high variability in the carbon content of adult females found in this study is within the range given by Shreeve et al. (2002; 288–1,791 µg C female^{-1}). Given the high variability of results and the small amount of samples analysed, no significant changes in body carbon could be found during this study.

Gonad maturation with increasing chlorophyll *a* concentration

Different gonad stages for *R. gigas* were already described and illustrated by Ommanney (1936). Similar to the increase in egg production rates, gonad maturation took place concurrent with increasing chlorophyll *a* concentrations (Fig. 2). Females in GS 4 were also found at the beginning of the fertilization experiment and at the out-patch stations which may indicate favourable past feeding conditions for these animals. Consequently, no significant differences between the stages of gonad development between in- and out-patch were found until day 12 (Fig. 2). In contrast, the out-patch station at day 17 after fertilization and all following days show significant differences in *R. gigas* gonad development compared to the in-patch stations where almost all females were found in GS 4. The fact that all other GS stages were more or less absent in the in-patch stations after day 17, show that it took at most 1 week for *R. gigas* to use the increasing food availability during the present study for the completion of the gonad maturation.

The reproductive flexibility of *R. gigas* depending on food conditions, as shown in this study, can be seen as a behavioural response to the episodic and patchy food supply in the Southern Ocean environment.

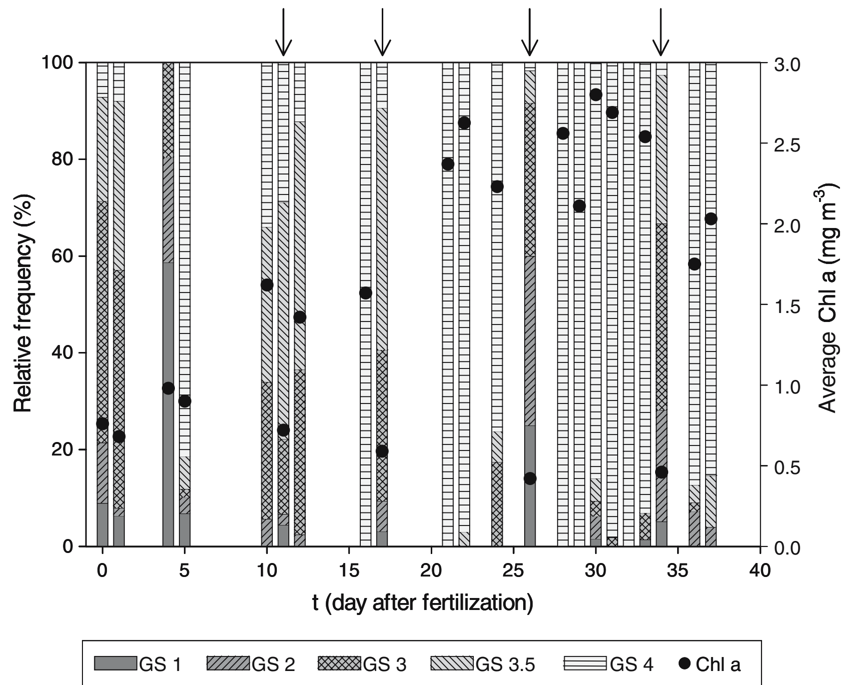
Table 3 Carbon and nitrogen analyses of *Rhincalanus gigas* females during the European iron fertilization experiment (EIFEX)

Station	*t*	Carapax length (µm)	CM (µg)	C:N	*n*
424	0	7,140 (6,720–7,600)	245 (220–267)	3.97 (3.71–4.16)	4
508	10	7,965 (6,743–10,600)	1,034 (636–1,656)	4.89 (2.67–6.79)	23
513	16	7,400 (6,560–8,080)	790 (352–1,780)	6.43 (4.2–9.46)	45
514 out	17	7,300 (6,975–7,518)	760 (519–1,083)	4.38 (3.17–5.95)	5
544	22	6,975 (6,433–7,363)	847 (657–906)	4.82 (4.25–5.73)	6
560	29	7,380 (7,200–7,500)	877 (576–1,218)	4.77 (3.62–5.62)	5
579	33	7,392 (6,975–7,750)	1,102 (899–1,399)	5.13 (4.56–5.73)	8

Mean values of the carapace length, carbon mass (CM) and carbon to nitrogen mass ratio (C:N) are shown, with their range in parentheses

n number of analysed individuals, *t* number of days after the first iron addition

Fig. 2 Percent composition of *Rhincalanus gigas* female population gonad development stages (GS) from multinet samples with corresponding average chlorophyll *a* concentrations over the upper 100 m of the water column (*black dots*). *Arrows* mark the out-patch stations



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CONCLUSIONS

ECOLOGICAL IMPORTANCE OF COPEPOD FEEDING

Since surface waters are generally super-saturated with respect to calcite, theoretically no CALCITE DISSOLUTION should occur in the euphotic zone of the oceans (Thierstein & Young 2004). However, a number of observations suggest that a considerable proportion of biogenic calcite, to great extent produced by coccolithophores, dissolves above the chemical lysocline as result of biological mediation (Milliman et al. 1999). Milliman et al. (1999) suggested the dissolution within the gut or faeces of grazers as a possible mechanism since they may provide an under-saturated microenvironment. Literature dealing with this topic is scarce and results are inconsistent. Faecal pellets studied by means of scanning electron microscopic (SEM) analysis contained well preserved coccoliths which showed no signs of dissolution (Honjo 1976, Honjo & Roman 1978, Bathmann et al. 1987). These findings are supported by *in vivo* pH measurements, showing that the copepod gut is not acidic (Pond et al. 1995). In contrast, significant calcite dissolution (up to 73%) was observed in feeding experiments with the copepods *Pseudocalanus elongates* and *Calanus helgolandicus* (Harris 1994). Using a new experimental approach based on SEM analysis of *Calcidiscus leptoporus* coccoliths we could reinforce earlier SEM observations suggesting insignificant calcite dissolution in copepod guts. In particular, the detachment of the proximal and distal shield of the coccoliths could be defined as an early stage of dissolution occurring already at 8% weight loss of the coccolithophores. Within the faecal pellets of *Temora longicornis* and *C. helgolandicus* all coccoliths comprised of both, proximal and distal shield. We therefore concluded that coccolith dissolution during copepod gut passage is minor and cannot contribute significantly to the observed calcite dissolution in the upper ocean (**manuscript I**). A modelling study by Jansen & Wolf-Gladrow (2001) suggested that reingestion of coccolith-containing faecal pellets may enhance copepod mediated coccolith dissolution. Since this notion is still awaiting experimental testing, the possible impact of faecal pellet reingestion on the calcium and carbon cycle remains speculative. The question where and how the calcite

dissolves above the chemical lysocline of the oceans remains an open question, which deserves further investigation. Copepods as one of the main predators of coccolithophores in the oceans may play an indirect role for this considerable calcite loss. During bloom conditions the transfer of coccoliths from copepod guts to higher trophic levels such as fish may be an important link.

Such vectorial transport through the classical food chain from algae to herbivorous zooplankton and zooplanktivorous fish has so far received most interest due to possible TOXIN TRANSFER (White 1981, Teegarden & Cembella 1996, Tester et al. 2000). Generally, algal blooms are advantageous for marine organisms and their environment but problems arise when the bloom species have harmful impacts on the ecosystem. So called harmful algal blooms (HABs) can cause great financial losses for industry and tourisms. Copepods can serve as vector for toxins to higher trophic levels since they can accumulate these toxins in their somatic tissues (White 1981, Turriff et al. 1995, Teegarden & Cembella 1996, Turner & Tester 1997, Tester et al. 2000, Lehtiniemi et al. 2002), already few hours after being exposed to toxic algae (White 1981, Lehtiniemi et al. 2002). Thus, copepods might transmit toxins to their predators, like commercial important fish. The importance of toxin transfer via copepod faecal pellets is more uncertain, since toxin concentrations measured are rather low (Lehtiniemi et al. 2002). Lehtiniemi et al. (2002) concluded therefore, that it is unlikely that copepod faecal pellets act as an important vector to coprophagous animals in the pelagic or benthic zone. Whereas the egestion of completely intact toxic phytoplankton cells like *Dinophysis* spp may be an alternative important pathway of toxins through the food web which must be considered (**manuscript II & III**). During a *D. norvegica* bloom in the North Sea, faecal pellets produced by *C. helgolandicus* contained almost exclusively intact *D. norvegica* cells (**manuscript II**), while nothing can be said regarding the viability of the cells or the toxicity of the faecal pellets. *Dinophysis* spp is the main phytoplankton genus producing diarrhetic shellfish poisoning (DSP) toxins (Yasumoto 1990, Turner & Tester 1997) and due to difficulties in culturing this species (Sampayo 1993) there is a lack of knowledge about interactions between zooplankton and DSP toxin producing phytoplankton. However, the general ability to ingest harmful algae species like *D. norvegica* and *Ceratium furca* was shown for *C. helgolandicus* during the mentioned field study in the North Sea (**manuscript III**). *C. helgolandicus* ingestion rates on *D. norvegica* and *C. furca* were

measured to be up to 47 and 16 cells female^{-1} hour^{-1}, respectively. Due to low *C. helgolandicus* abundances in the field, total grazing impact on *Dinophysis* spp and *Ceratium* spp at the time of the experiment was assumed to be minor despite high individual grazing rates. In general, the grazing impact of the zooplankton community on the development and termination of HABs is very variable and situation specific (Turner et al. 1998). Selective feeding on non-toxic phytoplankton species may enhance the probability of HABs. Weather or not copepods select non-toxic over toxic algae has been a main question of several studies dealing with HAB species and will be discussed later.

Beside coccolithophores and dinoflagellates, diatoms are the main phytoplankton group in the oceans. They are usually dominating the phytoplankton assemblage in temperate and high latitude areas (Lalli & Parson 1997). For the predominantly herbivorous zooplankton living at high latitudes the problem of long periods with shortage of food arises. Copepods have adapted to this highly seasonal food availability by storing lipid reserves (Albers et al. 1996, Lee et al. 2006). Adult females are able to mobilize these reserves for gonad development and egg production (e.g. Hagen & Schnack 1996, Lee et al. 2006), making REPRODUCTION for some species more or less independent of ambient food concentrations (Smith 1990, Hagen & Schnack 1996, Mauchline 1998). However, it is still questionable, which species are spawning on lipid reserves and which species are able to react directly to enhanced food concentrations (Hagen & Schnack 1996). The European iron fertilization experiment (EIFEX) provided the possibility to analyse the direct reproductive response of *Rhincalanus gigas* during the build up of a diatom dominated phytoplankton bloom in the region of the Antarctic Polar Front (APF). *R. gigas* females clearly reacted on the enhanced food concentration with maturation of their gonads and a significant increase in their egg production rate, compared to the out-patch stations characterised by low phytoplankton concentrations (**manuscript VII**). It is suggested, that *R. gigas* females are dependent on phytoplankton blooms for the maturation of their gonads, not spawning on lipid reserves. In their review, Hagen and Auel (2001) write that *R. gigas* “has been described as an expatriate in high-Antarctic waters (Voronina 1970, Atkinson 1991) and the moderate wax ester levels and the deficiency in long-chain monounsaturated fatty acids and alcohols indicate that this species is not fully adapted to life in high-Antarctic waters (Kattner et al. 1994)”. This is supported by our findings.

POSSIBLE DEFENCE STRATEGIES OF PHYTOPLANKTON

Phytoplankton evolution is ruled by protection (Verity & Smetacek 1996, Smetacek 2001) and a wide range of defence systems have been described, ranging from mechanical (e.g. Hamm et al. 2003) to various types of chemical strategies (e.g. Wolfe 2000, Ianora et al. 2004). Generally, the ecological success of a phytoplankton species depends on its ability to optimise the balance of growth and loss processes. The development of morphological and/or chemical defence mechanisms involves production costs for the phytoplankton which can only be developed to the disadvantage of e.g. nutrient uptake capability, light harvesting efficiency or growth rates. Some of the phytoplankton attributes, commonly assumed as defence strategies against zooplankton grazing will be discussed below.

The fundamental question, why some phytoplankton species produce TOXINS is yet difficult to answer. Beside the common assumption that they act as grazing deterrents, other explanations for toxin production include precursors for sub-cellular organelles, cell wall degradation products, nitrogen storage, nucleic acid synthesis, inhibition of competing co-occurring phytoplankton species or the function as pheromones (Turner & Tester 1997, Wyatt & Jenkinson 1997). Turner and Tester (1997) and Turner et al. (1998) have reviewed the literature dealing with interactions between toxic phytoplankton and their grazers. They conclude that an overall synthesis is not possible today. HABs and grazer interactions are highly situation- as well as species specific and available results are still too disparate. Further, they state that different grazers exhibit different responses to different toxic phytoplankton species or to different clones or blooms of the same species. Generalisations are not possible and further work is needed to complete the mosaic. The same is true for selective feeding in marine copepods, exposed to toxic algae and available results are contradictory, ranging from no observed selectivity (White 1981, Turner & Anderson 1983, Teegarden & Cembella 1996) to lower feeding rates, selective feeding or even regurgitation of toxic cells (Sykes & Huntley 1987, Turriff et al. 1995, Turner et al. 1998, Teegarden 1999). Most studies of grazing on toxic phytoplankton were carried out with monocultures or with mixtures of two different phytoplankton species, while studies on feeding preferences on the level of different algae strains, just differing in their toxicity, are scarce (but see Teegarden 1999). First experimental results obtained in this thesis indicated that *C. helgolandicus* can distinguish between toxic and non-toxic strains of *Alexandrium* spp, selectively feeding on the

non-toxic strains (**manuscript VI**). However, extrapolation of the results must be done with care, since the method applied is not fully established and control experiments are missing.
As a result of studies conducted in the recent years, diatoms are now known to produce chemicals which are thought to impair the reproductive success of copepods (Ianora et al. 2004). Those chemicals, belonging to the class of aldehydes are cleaved from a fatty acid precursor, activated by enzymes within seconds after crushing of the cell (Pohnert 2000). The harmful effect of aldehydes on the hatching success of different copepod species has been shown in several laboratory studies (Ban et al. 1997, Ceballos & Ianora 2003, Ianora et al. 2004), and in the field (Miralto et al. 1999, Halsband-Lenk et al. 2005), while other researchers found no negative relationship between the occurrence of diatoms and the copepod hatching success *in situ* (Irigoien et al. 2002). Copepods seem to differ in their sensitivity to diatom aldehydes (Ceballos & Ianora 2003) and some copepods may be able to reduce or avoid the negative effects of diatom toxins through the production of enzymes such as aldehyde dehydrogenases or glutathion reductases (Ceballos & Ianora 2003). Additionally, differences in the biological activity of aldehydes were detected (Pohnert 2002). However, as stated for other phytoplankton toxins (Turner & Tester 1997, Turner et al. 1998), effects may again be species specific and some copepods may cope with some toxins classes, while others do not.
Irrespective of the aldehyd production, diatoms have evolved silica frustules with special morphology and architecture, providing a maximal mechanical protection with minimal investment in material (Hamm et al. 2003). Hamm et al. (2003) measured the forces necessary to break single, living cells of different diatom species and found their STABILITY to be extremely high. Forces up to 730 µN are necessary to break the frustules of *Fragilariopsis kerguelensis.* The frustules of diatoms have evolved to withstand external pressure and only large copepods or euphausiids have evolved feeding appendages to be able to break such frustules (Hamm et al. 2003). Feeding experiments conducted under laboratory conditions revealed that adult females of the North Sea copepods *C. helgolandicus* as well as the smaller species *T. longicornis* were able to feed on a monoculture of *F. kerguelensis* (**manuscript IV**). The silica edged mandibular gnathobases of the copepods (General introduction, Fig. 2) are assumed to have co-evolved with the arming of the diatoms (Hamm et al. 2003). Some of the grazed *F. kerguelensis* cells survived gut passage of both copepod species and SEM

observations of the produced faecal pellets revealed, that most of the surviving cells were "members" of chains(**manuscript IV**). This leads to the assumption that *F. kerguelensis* in addition to its stable frustules may benefit from CHAIN FORMATION, increasing its chance of survival when being grazed.

In contrast, the chain forming, spiny diatom *Proboscia alata* did not escape digestion and the long SPINES of *Chaetoceros dichaeta* provided no efficient protection against copepod grazing (**manuscript IV**). Both of these diatoms have much thinner frustules than *F. kerguelensis*. It is tempting to assume that the combined effect of chain formation and strong frustules may reduce the risk of destruction, allowing individual cells of *F. kerguelensis* to remain viable after gut passage. A laboratory study conducted by Gifford (1981) revealed that filtration rate of *C. finmarchicus* was 1.7 times higher on cells with spines than on the same species without spines (Gifford et al. 1981). All these findings can at first glance not support the hypothesis that the spines of diatoms serve as a defence against grazing. But such a hypothesis may be true for other species of suspension feeding predators and since the copepods were grazing on monocultures in the presented study, it may be possible however, that they avoid these algae with special morphology in the field.

With *C. wailesii* as the largest diatom regularly occurring in the North Sea plankton, the defence mechanism of large cell SIZE was investigated. In the present study it was observed that those cells were too large to be grazed by *Acartia clausi*. However, *T. longicornis* showed a very skilful handling of individual *C. wailesii* cells, which allowed the copepod to reach the nutritional content of the cell (**manuscript V**). The advantage for copepods grazing on larger prey items is obvious, since they need fewer items to fuel their energy requirements for growth and metabolism. When feeding on food particles of different sizes, copepods are known to feed first on the larger particles (Paffenhöfer & Knowles 1978, Richman et al. 1980, Price & Paffenhöfer 1986), but feeding efficiency may decrease with increasing cell size. When the prey is large, relative to the copepod, some of the grazed material is lost as dissolved organic carbon (DOC) due to sloppy feeding (Lampert 1978, Møller & Nielsen 2001, Møller 2005). If *C. wailesii* is grazed by *T. longicornis* in nature and to what extent, cannot be evaluated within this laboratory study. This is a general problem when species specific interactions are studied in the laboratory. Results cannot be applied directly to the field situation. However, studies of specialised skills of the copepods are important to analyse

for later interpretation of the animals' behaviour in the field and interesting questions emerge: Do copepods avoid feeding on certain prey species, because they simply cannot graze them, or because they just prefer to feed on something else? In the case of *A. clausi* it would be the lack of ability while we now know that *T. longicornis* can feed on *C. wailesii.* Experiments offering a mixture of different food items will be the next step to extend the knowledge of copepod feeding behaviour when grazing on large food items.

Another possibility for phytoplankton species to defend themselves, neither using toxins nor morphological structures, which may protect them from being eaten, is to avoid being digested. Fowler and Fisher (1983) demonstrated the ability of some phytoplankton species to ESCAPE DIGESTION and found alive cells within the faecal pellets after incubating them in filtered seawater. In **manuscript IV** the new method of fluorescein diacetate (FDA) staining was applied to analyse the ability of phytoplankton to survive the gut passage of copepods. Cell survival after egestion was demonstrated for the dinoflagellate *Scrippsiella trochoida* and for the diatom *F. kerguelensis*. *D. norvegica* cells also showed no sign of dissolution within the faecal pellets collected in the field (**manuscript II & III**), but whether or not cells were viable can only be speculations since FDA staining was not applied in the latter case. Assimilation of food by zooplankton is sometimes incomplete (Marshall & Orr 1955, Conover 1966) and the resistance to digestion is assumed to be common occurrence in the marine environment (Fowler & Fisher 1983).

Kiørboe claims that the recycling of faecal pellets by mesozooplankton might be a strategy to conserve material in the upper mixed layer and consequently a way to prevent the weakening of phytoplankton communities (Kiørboe 1997). In non-coastal and non-upwelling areas, the recycling of nutrients is necessary to provide phytoplankton growth. If large diatom blooms occur in such systems, enhanced vertical flux due to aggregate formation might be the result (Smetacek 1980), leading to an impoverished system with low biomass and available nutrients. With the new knowledge from this study showing that even alive cells can be found in the faecal pellets we can go a step further. The coprophagy, flux feeding and consequently retention of material in the euphotic zone may not just be seen as mechanisms to keep nutrients in the upper water (Kiørboe 1997), but also alive cells to seed the forthcoming phytoplankton community.

APPLICATION OF NEW TECHNIQUES

A number of studies have shown that faecal pellets may contain undigested, intact phytoplankton cells (Fowler & Fisher 1983, Turner 1984, Bathmann & Liebezeit 1986, Turner 1991). Results from the field experiment conducted in the North Sea (**manuscript II & III**) as well as results from **manuscript IV** showed that studies of faecal pellet content in some cases might be a quantitative method describing copepod feeding. Quantitative information can be obtained in cases where the ingested cells remain intact (e.g. because of hard cell wall structures) and are large enough to be enumerated under the microscope. Studies of faecal pellet content can therefore be of great importance to study grazing effects on bloom development and/or bloom termination, since a variable proportion of the phytoplankton cells grazed, may survive passing through the gut of the copepod. Methods that reveal viability of phytoplankton cells within faecal pellets may give us further knowledge, which cannot be obtained by traditional grazing experiments. In **manuscript IV**, the FDA STAINING method was implemented as a first direct attempt to look at this issue. The experiments revealed, that some cells could survive the gut passage and gave new insights on ecosystem functioning.

After several decades of copepod research, a wide variety of different methods exist in literature, to address the problem of quantification and qualification of copepod ingestion (Båmstedt et al. 2000). Like stated in the introduction, each approach has its experimental limitations and new methods are needed to assess *in situ* zooplankton feeding rates and to evaluate their prey selection in the field. The most promising approach was introduced in 2003 and makes use of the trendsetting field of MOLECULAR RESEARCH (Nejstgaard et al. 2003). Within this thesis, we showed an enhancement of this approach with first possibilities of prey quantification in copepod guts (**manuscript VI**). Nevertheless, further development is needed and it still seems to be a long way to go for making this approach one day an easy, fast and accurate method for zooplankton research. But looking through the microscope to learn to know the behaviour and beauty of different species can never be replaced.

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SUMMARY

Observations on the feeding behaviour of calanoid copepods, microscopical analysis of their faecal pellets and the possible ecological importance of copepod grazing will be addressed in this thesis. Laboratory as well as field studies were applied to investigate the responses of different copepod species to different food environments, covering the three main phytoplankton groups - Diatoms, Dinoflagellates and Coccolithophores. Since many of the species belonging to these groups are surrounded by mineral skeletons, qualitative observations can be revealed by the use of scanning electron microscopy (SEM).
The importance of calcifying algae in the marine carbon and calcium cycles is widely known, but the role of copepods for the coccolith calcite dissolution is vague. Dissolution experiments were performed using the calcifying algae *Calcidiscus leptoporus.* SEM observations of the morphological changes of the coccoliths were used for content interpretation of freshly produced faecal pellets in feeding experiments with the common North Sea copepods *Calanus helgolandicus* and *Temora longicornis*. Coccolith calcite dissolution during copepod gut passage was found to be minor and can therefore not contribute significantly to the generally observed calcite dissolution in the upper ocean.
Field experiments conducted during a dinoflagellate bloom in the North Sea clearly showed that some copepod species can feed very efficiently on the dinoflagellate species *Dinophysis norvegica*. SEM observations showed that faecal pellets produced by *C. helgolandicus* were almost exclusively filled with intact cells of this toxic dinoflagellate species. Faecal pellets, containing intact toxic phytoplankton species may be an important vehicle and new pathway transferring toxins within the pelagic and to the benthic community. The viability of *D. norvegica* cells within the faecal pellets was not investigated, but lead to the idea to observe the ability of different phytoplankton species to survive the copepod gut passage. Therefore a new staining method was applied. Viable cells of the dinoflagellate *Scrippsiella trochoida* and the diatom *Fragilariopsis kerguelensis* were detected within the faecal pellets of different copepod species. *F. kerguelensis* may protect itself with its stable frustules and also the formation of chains seemed to be advantageous for the survival of single cells when they are grazed. Whereas the long spines of the diatom *Chaetoceos dichaeta* provided no protection and was reduced to small pieces within the faecal pellets of all copepods investigated. Studies

of species specific interactions among copepods and their prey can greatly improve the current understanding of the benefit of certain phytoplankton cell structures. In an additional experiment conducted with the two copepods *Acartia clausi* and *T. longicornis* the ability to graze on the largest centric diatom commonly found in the North Sea plankton was under investigation. *A. clausi* was not able to graze on *Coscinodiscus wailesii* but *T. longicornis* showed a very skilful handling of individual cells. The copepod did not ingest the whole cells, but bite a piece off the frustules to reach the protoplasma. After ingestion of the cell content, the empty diatom frustules were rejected. Results showed that traditional methods to determine grazing may sometimes be unsuitable and that new methods need to be developed. The new promising approach of genetical gut content analysis to qualify and quantify the grazing impact of copepods was therefore tested in a study and provided preliminary, but promising results.

The general importance of food for copepods can be reflected in their energy requirements for egg production. To observe if and how food concentration is linked to egg production in a common Antarctic copepod species, the reproductive response of *Rhincalanus gigas* was studied during the build up of a diatom dominated phytoplankton bloom in the Southern Ocean. Results from egg production experiments as well as the maturation of their gonads revealed that *R. gigas* was able to react directly to enhanced food concentrations.

ZUSAMMENFASSUNG

Beobachtungen vom Fraßverhalten calanoider Copepoden, mikrospopische Analysen ihrer Kotballen und der mögliche Einfluss ihres Fraßes sind Thema dieser Arbeit. In Laboruntersuchungen und Feldstudien wurden die Reaktionen von verschiedenen Copepodenarten auf unterschiedliche Futtertypen untersucht. Dabei standen die drei wichtigsten und häufigsten Algengruppen Diatomeen, Dinoflagellaten und Coccolithophoriden im Mittelpunkt. Viele Arten dieser Gruppen haben ein mineralisches Skelett, so dass qualitative Untersuchungen mit dem Elektronenmikroskop gut und relativ einfach durchführbar sind.

Die Bedeutung kalzifizierender Algen im marinen Kohlenstoff- und Kalziumkreislauf ist allgemein bekannt, aber die Rolle der Copepoden bei der Lösung des Kalzit von Coccolithophoriden ist nach wie vor unklar. Zur Klärung dieser Frage wurden Lösungsexperimente mit der kalzifizierenden Alge *Calcidiscus leptoporus* durchgeführt und die morphologischen Veränderungen der Coccolithen mit Hilfe des Elektronenmikroskops untersucht. Diese Beobachtungen dienten zur Interpretation von Coccolithen in frisch produzierten Kotballen der Nordseecopepoden *Calanus helgolandicus* und *Temora longicornis*. Die Lösung von Coccolithen Kalzit während der Darmpassage von Copepoden konnte als sehr gering eingestuft werden. Copepoden scheinen somit keinen direkten Einfluss auf die generell festgestellte Kalzitlösung in den oberen Ozeanregionen zu haben. Ein indirekter Einfluss ist aber nicht auszuschließen.

Feldstudien, die während einer Dinoflagellatenblüte in der Nordsee durchgeführt wurden, zeigten, dass manche Copepoden in der Lage sind große Mengen von *Dinophysis norvegica* zu fressen. Anhand von rasterelektronenmikroskopischen Untersuchungen wurde festgestellt, dass die produzierten Kotballen von *C. helgolandicus* fast ausschließlich mit intakten Zellen des toxischen Dinoflagellaten gefüllt waren. Solche Kotballen könnten eine entscheidende Rolle beim Transfer von Toxinen sowohl im pelagischen als auch im benthischen Nahrungsnetz spielen. Während dieser Studie wurde allerdings nicht untersucht, ob die *D. norvegica* Zellen in den Kotballen noch lebten. Mit einer neuen Färbemethode wurde in einer folgenden Studie untersucht, ob und welche Algenarten die Darmpassage von verschiedenen Copepodenarten überleben können. Lebende Zellen des Dinoflagellaten *Scrippsiella*

trochoida und der Diatomee *Fragilariopsis kerguelensis* konnten mit dieser Methode in den Kotballen verschiedener Copepodenarten identifiziert werden. Die Diatomee *F. kerguelensis* schützt sich vermutlich durch ihre stabile Schale vor der Verdauung, und auch das Ausbilden von Ketten könnte ein weiterer Vorteil für die Alge sein, der dazu führt, dass einzelne Zellen der Ketten überleben, wenn sie gefressen werden. Die langen Stacheln von *Chaetoceos dichaeta* konnten dahingegen keinen Schutz vor Copepodenfraß bieten. Alle untersuchten Copepodenarten waren in der Lage *C. dichaeta* zu fressen und die Untersuchung vom Kotballeninhalt zeigten, dass alle Zellen stark zerkleinert wurden. Das Studium der artspezifischen Interaktionen von Copepoden und ihren Nahrungsorganismen kann helfen, den Vorteil verschiedener Zellstrukturen von Algen zu verstehen. In einer weiteren Studie wurde deshalb untersucht, ob die Copepoden *Acartia clausi* und *T. longicornis* in der Lage sind die größte, zentrische Diatomee zu fressen, die regelmäßig in der Nordsee zu finden ist. *A. clausi* war nicht in der Lage *Coscinodiscus wailesii* zu fressen, aber *T. longicornis* zeigte eine sehr geschickte Handhabung von einzelnen Zellen. Der Copepode fraß nicht die ganzen Zellen, sondern biss ein Stück aus der Silikatschale um an das Protoplasma zu gelangen. Nach Ingestion des Zellinhaltes wurden die leeren Schalen zurückgelassen. Diese Ergebnisse zeigen, dass traditionelle Methoden zur Bestimmung des Fraßes von Copepoden oft an ihre Grenzen stoßen, und neue Methoden vor allem gebraucht werden um den *in situ* Fraßeinfluss besser bestimmen zu können. Eine vielversprechende Möglichkeit, um dies zu erreichen sind genetische Analysen des Darminhaltes von Copepoden oder von deren Kotballen. In einer Studie dieser Arbeit wurde eine neue genetische Methode zum Nachweis zweier Algenarten in Fraßexperimenten erfolgreich getestet sowie die Möglichkeit aufgezeigt, mit dieser Methode in Zukunft auch quantitative Ergebnisse zu erzielen.

Die allgemeine Wichtigkeit von Futter für Copepoden spiegelt sich in ihrem Energiebedarf bei ihrer Eiproduktion wider. Ob und wie die Futterkonzentration mit der Eiproduktion bei dem antarktischen Copepoden *Rhincalanus gigas* zusammenhängt, wurde während einer induzierten Algenblüte in Südozean untersucht. Ergebnisse von Eiproduktionsexperimenten sowie die Untersuchung der Gonadenentwicklung zeigte eine deutliche Reaktion dieses Copepoden auf eine erhöhte Futterkonzentration.

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ANLAGE ZUR DISSERTATION

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