Interactions between harmful algae and calanoid copepods in the Baltic Sea

SANNA SOPANEN

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### CONTRIBUTIONS

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AR = Alexander Rühl, CL = Catherine Legrand, CS = Camilla Svensen, EG = Edna Granéli, FATE = the working group of the EU project “Transfer and Fate of Harmful Algal Bloom (HAB) Toxins in European Marine Waters”, KE = Katrin Erler, MK = Marja Koski, OS = Outi Setälä, PK = Pirjo Kuuppo, PU = Pauliina Uronen, RA = Riitta Autio, SL = Sirpa Lehtinen, SS = Sanna Sopanen, TT = Timo Tamminen.
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Zooplankton have a central role in marine food webs as mediators of energy to higher trophic levels. Eutrophication favours harmful algal blooms and may induce changes in the phytoplankton community. These changes, in turn, may affect grazers, because the quality of food, such as the content of toxic substances, may have effects on feeding, growth and reproduction, and may influence both the species composition and the production of copepods. This will affect the food availability of planktivorous animals, such as Baltic herring. The aim of the studies reported in this thesis was to examine the feeding interactions between calanoid copepods and toxic algae in the Baltic Sea. The central questions in this research concerned the feeding, survival and egg production of copepods exposed to toxic algae. Furthermore, the importance of copepods as vectors in toxin transfer was examined.

These questions were examined experimentally using various harmful algal species occurring in the Baltic Sea. The haptophyte *Prymnesium parvum*, which produces extracellular toxins, was the only studied species that directly harmed copepods. Beside this, it had allelopathic effects (cell lysis) on non-toxic *Rhodomonas salina*. Copepods that were exposed to *P. parvum* filtrates died or became severely impaired, although filtrates were not haemolytic (indicative of toxicity in this study). Monospecific *Prymnesium* cell suspensions, in turn, were haemolytic and copepods in these treatments became inactive, although no clear effect on mortality was detected. These results suggest that haemolytic activity may not be a good proxy of the harmful effects of *P. parvum*, and this algal species may produce other chemical compounds that have not been identified, but are harmful to grazers. In addition, *P. parvum* deterred feeding, and low egestion and suppressed egg production were consequently observed in monospecific suspensions of *Prymnesium*. Similarly, ingestion and faecal pellet production rates were suppressed in high concentration *P. parvum* filtrates and in mixtures of *P. parvum* and *R. salina*. These results indicate that the allelopathic effects of *P. parvum* on other algal species together with lowered viability as well as suppressed production of copepods may contribute to bloom formation and persistence. Furthermore, the availability of food for planktivorous animals may be affected due to reduced copepod productivity.

A plankton community that contained the cyanobacterium *Nodularia spumigena* was size-fractionated in order to follow nodularin transfer from different size fractions to the copepod *Eurytemora affinis*. Nodularin produced by *N. spumigena* was transferred to copepods via grazing on filaments of small *N. spumigena* and by direct uptake from the dissolved pool. Copepods also acquired nodularin in fractions where *N. spumigena*
filaments were absent. Thus, the importance of microbial food webs in nodularin transfer should be considered. However, the mechanism behind nodularin accumulation in these fractions remained unclear. Copepods were able to remove particulate nodularin from the system, but at the same time a large proportion of the nodularin disappeared. This indicates that copepods may possess effective mechanisms to remove toxins from their tissues. The importance of microorganisms, such as bacteria, in the degradation of cyanobacterial toxins could also be substantial.

Our results were the first reports of the accumulation of diarrhetic shellfish toxins (DSTs) in copepods. The PTX2 content in copepods after feeding experiments corresponded to the ingestion of <100 *Dinophysis* spp. cells. However, no DSTs were recorded from field-collected copepods. Neither of the copepod species selected the dinoflagellate *Dinophysis* spp. and consumption remained low (up to 226 *Dinophysis* spp. cells ind$^{-1}$ d$^{-1}$). Instead, the dinoflagellate *Heterocapsa triquetra* and ciliates were selected, irrespective of their abundance. Although not selected, *Dinophysis* spp. formed an important part of the copepod diet when the food concentration was low. It seems likely that copepods are an unimportant link in the transfer of DSTs in the northern Baltic Sea. However, at times when other food sources are scarce, or if copepods encounter a *Dinophysis* spp. bloom with high cell densities typical for the Baltic Sea, these dinoflagellates may form a significant part of the copepod diet.

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1. INTRODUCTION

1.1. Harmful algal blooms (HABs) and reasons for their magnitude

Phytoplankton blooms usually refer to mass developments of microscopic algae and are natural events in all marine food webs. However, during past decades, the occurrence of “exceptional”, often toxic or otherwise harmful algal blooms has increased globally (GEOHAB 2001). The definition “bloom” is not unambiguous. For non-toxic species, biomass is the most often used parameter to define the status of a bloom. However, harmful effects on co-occurring marine biota as well as human health may accompany very different levels of cellular abundance. Thus, not only the abundance of a particular species but also whether its occurrence has harmful consequences is of importance. Generally, the presence of harmful species or measurable toxin levels is increasingly defined as a bloom occurrence (Smayda 1997). For example, the high-abundance, low-biomass bloom of the haptophyte *Chrysochromulina polylepis* in Scandinavian coastal waters during 1988 had deleterious effects on large parts of the heterotrophic components of the plankton community (Nielsen et al. 1990). Another example is the high-biomass blooms of diazotrophic cyanobacteria. These filamentous, nitrogen fixing algae dominated by toxic *Nodularia spumigena* and potentially toxic *Aphanizomenon flos-aquae* and *Anabaena* sp. are commonly present in the Baltic Sea plankton community, but regularly form late-summer blooms in the Baltic Sea (Kahru et al. 1994, Kononen et al. 1996). At the other extreme are blooms with a low abundance and biomass. Filter feeders, such as oysters, can accumulate diarrhetic shellfish toxins, which has led to human poisoning despite the low density of the dinoflagellate *Dinophysis* spp. in the water (Belin 1993). In some cases, the ingestion of small quantities of toxic algae can be lethal to grazers. For example, the ingestion of only a few cells of the dinoflagellate *Alexandrium tamarense* has found to be lethal to first-feeding larvae of capelin (*Mallotus villosus*) and herring (*Clupea harengus harengus*) (Gosselin et al. 1989). For this reason, the International Council for the Exploration of the Sea (ICES) has implemented an annually updated list of potentially harmful algae in the Baltic Sea. In 2007, the list contained about 60 species with effects connected to toxicity, mechanical disturbance, anoxia or hypoxia and bloom formation (ICES 2007).

Anthropogenic nutrient loading to coastal waters has led to eutrophication in the Baltic Sea (HELCOM 2009). Nutrient loading has caused a corresponding increase in nutrient concentrations and changes in the ratios of dissolved inorganic nutrients, nitrogen and phosphorus (Kangro et al. 2007, Tamminen & Andersen 2007, HELCOM 2009). It has been suggested that the eutrophication of the Baltic Sea has increased phytoplankton primary production and consequently led to higher biomasses, caused changes in phytoplankton community structure and increased the incidence of harmful algal blooms (Cloern 2001, HELCOM 2009). Consequently, water transparency has become lower and higher sedimentation rates increased. 

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1 Harmful algal bloom (HAB): A mass occurrence of algae that can cause fouling, oxygen deficiency, shading, clogging of fish gills, poisoning of various organisms and other harmful effects.

2 Eutrophication arises when excessive amounts of nutrients, mainly nitrogen (N) and phosphorus (P) but also organic matter, build up in aquatic ecosystems and cause accelerated growth of algae and plants, often resulting in undesirable effects.
have caused bottom water anoxia, resulting in kills of bottom-dwelling fish and invertebrates (Cloern 2001, HELCOM 2009). The Baltic Sea, like other low saline estuaries, is considered an area at high risk of species invasions (Carlton & Geller 1993, Leppäkoski & Olenin 2000). Species that have resistant cysts or resting eggs in their life-cycle have a high invasive potential (e.g. Cohen & Carlton 1998, Panov & Cáceres 2007, Sopanen 2008), and the ship-mediated introduction of potentially harmful phytoplankton species or other aquatic organisms to new areas can disturb the whole ecosystem. For example, the potentially toxic dinoflagellate Prorocentrum minimum spread to the Baltic Sea in the early 1980s (Hajdu et al. 2000). Since then, it has been reported to bloom in many eutrophied coastal areas of the Baltic (Hajdu et al. 2005). HABs have become a recurrent phenomenon in the Baltic Sea and coastal regions globally (ICES 2007). Therefore, it is essential to examine how these blooms are controlled and, on the other hand, what effects harmful algae can have on grazing zooplankton such as copepods.

### 1.2. Role of copepods in planktonic food webs

Calanoid copepods are abundant planktonic crustaceans that have an important role in the transfer of energy to higher trophic levels in the planktonic food web. In general, copepods obtain energy through two main food pathways (Fig. 1). In the classical food web (Steele 1974), copepods are the main consumers of large phytoplankton and, in turn, are eaten by planktivorous fish (Fig. 1, upper part). In the microbial food web, carbon is transferred through several trophic steps (Azam et al. 1983). Bacteria take up dissolved organic matter that originates from phytoplankton and other planktonic organisms (Azam et al. 1983, Sherr & Sherr 1988, Sherr & Sherr 2000). On the next level, heterotrophic flagellates consume picoplankton (bacteria, picocyanobacteria, picoeucaryotes) (González et al. 1990). Ciliates, in turn, are considered to be an important functional group in the microbial food web as grazers of pico- and nanoplankton (e.g. Sherr & Sherr 1987, Rassoulzadegan et al. 1988). They are themselves controlled by heterotrophic flagellates, which in turn are controlled by bacteria.

**Fig. 1.** Schematic representation of the pelagial food web (Steele 1974, Azam et al. 1983, Sherr & Sherr 1988), emphasizing the routes of toxin transfer to copepods. Symbols: (light grey arrows) pathways of dissolved organic matter, (dark grey arrows) particulate carbon flows, (I) bacteria, picocyanobacteria, HNF, PNF, (II) microzooplankton and mixotrophic organisms, (III) phytoplankton, (IV) mesozooplankton. Potential pathways of nodularin transfer to copepods: (1) through direct grazing on Nodularia spumigena filaments, (2) direct uptake from the dissolved pool, (3) through grazing on organisms in the microbial food web.
by metazooplankton\(^3\), thus connecting the microbial with the classical food web (e.g. Stoecker & Capuzzo 1990, Lenz 2000) (Fig. 1, lower part). Both food webs co-exist, but their relative significance varies regionally and temporally.

In the northern Baltic Sea, the classical food web prevails during the spring bloom (Lignell et al. 1993). Due to thermal stratification, nutrients are locked in deep water and the spring bloom uses up nutrients from the productive layer above the thermocline. Much of the algal biomass settles to the bottom during this season (Lignell et al. 1993). The reason for this is the low abundance of zooplankton because of the long development time of resting eggs as well as the longer generation time of copepods in cold water (Katajisto et al. 1998, Mauchline 1998, Hagström et al. 2001). The microbial food web dominates in energy transfer during the summer, and the summer community is largely driven by the recycling of nutrients (Hagström et al. 2001). The seasonal variation in food resources affects the growth and reproduction rates of copepods, and ultimately the biomass available for the next trophic level varies seasonally (Koski 1999). It has been estimated that in the northern Baltic Sea up to 70 % of the copepod biomass is consumed by planktivorous mysid shrimps and fish, such as Baltic herring (*Clupea harengus*) (Rudstam et al. 1992, 1994). Thus, the importance of copepods may also be substantial in the context of toxin accumulation and transportation through pelagic food webs due to feeding interactions (Granéli & Turner 2006).

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1.3. Algal toxins

Toxic phytoplankton species can either produce intracellular toxins or excrete dissolved toxins into the water (GEOHAB 2001). Intracellular toxins are transferred along the food web through grazers that serve as vectors for toxin transfer to higher trophic levels (Granéli & Turner 2006, Turner 2006). This is indicated by the mortality of sea mammals and birds following the ingestion of algal toxins or organisms that have accumulated toxins (Andersson & White 1992, Scholin et al. 2000). Extracellular toxins have detrimental effects on diverse marine organisms (Richardson 1997), affecting coastal marine ecosystems and causing considerable economic losses for commercial aquaculture.

The reason for algal toxin production is not clear. It has been argued that the production of these toxins evolved to deter grazers (Turner 2006). However, many of these toxins harm upper-level consumers, such as marine mammals or humans, but are rather harmless to primary grazers, zooplankton and bivalves (Turner et al. 1998). Therefore, it is possible that some toxins are secondary metabolites associated with other processes such as nitrogen storage, nucleic acid biosynthesis, chromosomal structural organization, bioluminescence or bacterial endosymbiosis rather than serving as grazing deterrents (Cembella 1998, 2003). Some toxins may also be involved in allelopathy\(^4\), which primarily causes deleterious effects on other phytoplankton (Fistarol et al. 2003, Legrand et al. 2003, Fistarol 2004).

Powerful algal toxins have been found from various aquatic environments. These

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\(^{3}\) A category that includes multicellular phagotrophic organisms, but is not based on size. In the Baltic Sea, copepods, cladocerans, rotifers and meroplankton are included in this category.

\(^{4}\) The chemical inhibition of one plant (or other organism) by another, due to the release into the environment of substances acting as growth inhibitors.
toxins cause poisonings to humans who consume intoxicated seafood. The best known examples are paralytic shellfish poisoning (PSP), diarrhetic shellfish poisoning (DSP), amnesic shellfish poisoning (ASP) and ciguatera fish poisoning (CFP) (FAO 2004, Granéli & Turner 2006). The harmful consequences of different toxic algae and species that are responsible for the effects are summarized in Zingone & Enevoldsen (2000). The paralytic shellfish toxins (PSTs) are mainly produced by dinoflagellates belonging to the genus *Alexandrium*. During the last 20 years, there seems to have been an increase in PST intoxications (FAO 2004), and at least 21 PSTs have been recorded. These toxins are potential neurotoxins that can cause symptoms such as cramps, signs of paralysis and inhibition of respiration by blocking the excitatory current in nerve and muscle cells (reviewed by Luckas et al. 2005). The diarrhetic shellfish toxins (DSTs) are mainly produced by the genus *Dinophysis* and *Prorocentrum*. These toxins can be divided into three groups according to their chemical structure: (i) acidic toxins, including okadaic acid (OA) and its derivatives, dinophysis toxins (DTXs); (ii) neutral toxins, which consist of polyether-lactones of the pectenotoxin group (PTXs); and (iii) sulphated polyether and its derivative, yessotoxin (YTX) (FAO 2004). The OA group causes diarrhoea, while pectenotoxins are hepatotoxic and may damage the liver, and yessotoxin affects cardiac muscle cells (van Egmond et al. 1993). Because of health problems to humans, these toxins are routinely monitored in water worldwide and especially in shellfish meat in countries with a commercial seafood farming industry (FAO 2003).

Cyanobacterial toxins, which are mainly found from fresh- or brackish water environments, are broadly classified as causative of either neurotoxic or hepatotoxic symptoms (Carmichael et al. 1990). The most important hepatotoxins are cyclic pentapeptides, nodularin and microcystin (e.g. Sivonen et al. 1992, Sivonen & Jones 1999, Vaitomaa 2006), which are produced, for instance, by *Nodularia spumigena* and species of the genus *Microcystis* and *Anabaena*. These toxins inhibit the eukaryotic serine-threonine protein phosphatases PP1 and PP2A (Yoshizawa et al. 1990) and are potent tumor promoters (Nishiwaki-Matsushima et al. 1992). Cyanobacterial neurotoxins, such as saxitoxins and anatoxins, are produced by the genus *Anabaena* and *Microcystis* (e.g. Vaitomaa 2006). Cyanobacterial toxins are regularly found in drinking water supplies, and standardized monitoring methods as well as safety limits are thus provided by the WHO (WHO 2003).

### 1.4. Feeding and food selection

Selective feeding is an important factor in the relationships between grazers and their food. It is of particular interest in the formation of harmful algal blooms, because selection might enable harmful algae to gain a competitive advantage (Huntley et al. 1986, Uye & Takamatsu 1990, Solé et al. 2006).

Early studies suggested that suspension-feeding copepods were non-selective feeders with size-dependent particle selection based on the morphology of the filtering mesh of the second maxillae (e.g. Frost 1972, Boyd 1976). However, subsequent experimental and behavioural studies have demonstrated that copepods possess a complex suite of behavioural responses to different stimuli. These include the ability to generate and alter the near-field flow patterns of feeding-

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5 Specific prey items are consumed disproportionately to their numerical abundance in the food medium.
currents around the appendages to entrain and transport food items into the copepod’s capture area (e.g. Koehl & Stickler 1981, Greene 1988, Jiang et al. 1999, Malkiel et al. 2003), to capture and manipulate different shapes of food items, such as algal chains (e.g. Paffenhofer et al. 1982), to detect dissolved compounds in the water (Cowles et al. 1988), and to actively select or reject food particles (e.g. Paffenhofer et al. 1982, Schultz & Kiørboe 2009).

Several criteria may be involved in food selection. Prey size defines the upper and lower boundaries within which other criteria are also involved. Copepods can exploit prey over a wide range of sizes (approximately 5 to 200 μm), depending on the size of the copepod itself (Hansen et al. 1994, Gasparini & Castel 1997). They are also able to retain the largest particles more efficiently than the smallest ones (Frost 1972). Among other criteria involved in selection are manageability (e.g. prey morphology), quality (e.g. nutritional value, toxicity, physiological condition) or visual stimuli such as motility (e.g. Frost 1972, Bergreen et al. 1988, Cowles et al. 1988, Hansen et al. 1994, Kiørboe et al. 1996, Schmidt & Jónasdóttir 1997).

Numerous studies have demonstrated the importance of food quality in selection. For example, Poulet and Marsot (1978) reported that marine copepods are able to select microcapsules enriched with phytoplankton homogenate over untreated capsules. It has also been shown that copepods can ingest live cells in preference to dead cells, and that the presence of senescent algae may inhibit overall feeding rates (e.g. Cowles et al. 1988). Selection has also been shown to occur under natural conditions. Meyer-Harms et al. (1999) demonstrated by analysing gut pigments that copepods are able to utilise cyanobacteria along with eukaryotic phytoplankton. Thus, food selection in copepods seems to be complicated and may have effects on the prey species composition in the plankton community, as well as on the copepods themselves if, through selective feeding, they can obtain food with a higher quality.

1.5. Interactions between harmful algae and copepods

1.5.1. Grazing interactions are highly variable

The grazing responses of copepods to toxic algae seem to vary considerably. Most studies addressing selection against harmful algae have been carried out in the laboratory with high cell concentrations and only a few food species, and may not therefore give an ecologically relevant picture of the feeding and food selection of grazers (e.g. Turner et al. 1998). An increasing number of zooplankton grazing studies have been performed with natural plankton assemblages during blooms of harmful algae (e.g. Turner et al. 1998, Teegarden et al. 2001, Koski et al. 2005, Koizlowksy-Suzuki et al. 2006). Although also these studies have yielded variable results, it seems that under natural conditions both micro- and mesozooplankton6 grazers unselectively ingest toxic species together with other phytoplankton. This might dilute the potential adverse effects that toxins have on grazers, i.e. those effects that are very often seen in laboratory experiments (reviewed in Turner 2006).

The effects of different harmful algae on copepods in the Baltic Sea and adjacent areas are summarised in Table 1. Toxicity is evi-

6 Micro & mesozooplankton: Heterotrophic planktonic organisms that belong to the size group of 20-200 μm (micro) and 0.2-20 mm (meso) (Sieburth et al. 1978).
Table 1. Summary of the effects of various harmful algae on copepods in the Baltic Sea and adjacent areas. Studies have been carried out experimentally on cell cultures (CULT), mesocosm communities (MC) or on natural phytoplankton communities (NC). Abbreviations: EPR = egg production rate, IR = ingestion rate, nd = no data, not detected = no negative effects found. Species: *A. cla* (*Acartia clausi*), *A. bif* (*Acartia bifilosa*), *A. ton* (*Acartia tonsa*), *Cal. fin* (*Calanus finmarchicus*), *Cal. hel* (*Calanus helgolandicus*), *C. ham* (*Centropages hamatus*), *C. typ* (*Centropages typicus*), *Cent. sp.* (*Centropages sp.*), *E. aff* (*Eurytemora affinis*), *T. lon* (*Temora longicornis*).

<table>
<thead>
<tr>
<th>Species</th>
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<th>Data source</th>
<th>Copepod species</th>
<th>Negative effects</th>
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<td>nd</td>
<td>NC</td>
<td><em>Acartia sp.</em>, <em>T. lon</em> &amp; <em>C. ham</em></td>
<td>EPR, survival</td>
<td>Nielsen et al. 1990</td>
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<td><em>Prymnesium patelliferum</em> (cf. <em>parvum</em>) &amp; <em>Emiliania huxleyi</em></td>
<td>LDH (lactate dehydrogenase leaking)</td>
<td>MC</td>
<td><em>A. cla</em></td>
<td>IR</td>
<td>Nejstgaard et al. 1995</td>
</tr>
<tr>
<td><em>P. patelliferum</em> (cf. <em>parvum</em>)</td>
<td>nd</td>
<td>MC</td>
<td><em>E. aff</em></td>
<td>EPR</td>
<td>Nejstgaard &amp; Solberg 1996</td>
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<td>HA (haemolytic activity)</td>
<td>CULT</td>
<td><em>E. aff</em> &amp; <em>A. bif</em></td>
<td>IR, EPR</td>
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<td>NC</td>
<td><em>Cal. hel</em></td>
<td>not detected</td>
<td>Wexels Riser et al. 2003</td>
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<td><em>D. norvegica</em> &amp; <em>Dinophysis</em> spp.</td>
<td>PTX2, PTX2SA</td>
<td>NC</td>
<td><em>Cal. hel</em>, <em>Cent. sp.</em>, <em>T. lon</em> &amp; <em>Acartia sp.</em></td>
<td>IR (mixed species-specific responses)</td>
<td>Jansen et al. 2006</td>
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<td>CULT</td>
<td><em>E. aff</em> &amp; <em>A. bif</em></td>
<td>IR</td>
<td>Kozlowsky-Suzuki et al. 2006</td>
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<td>CULT</td>
<td><em>A. bif</em>, <em>T. lon</em>, <em>C. typ</em></td>
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<td>nd</td>
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<td><em>E. aff</em> &amp; <em>A. bif</em></td>
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enced if grazers die by direct intoxication after ingesting toxic algae or when exposed to exudates (Turner et al. 1998). More often, however, the effects of toxins on grazers may be sublethal, such as changes in behaviour or lowered ingestion, fecundity or egg hatching rates (Turner 2006). For example, copepods may reduce their filtering rates or refuse to ingest toxic phytoplankton, which may lead to low egg production and elevated mortality due to starvation (e.g. Huntley et al. 1986, Uye & Takamatsu 1990, Buskey & Stockwell 1993, Dutz 1998, Koski et al. 1999b, Maneiro et al. 2000, Colin & Dam 2002, Guisande et al. 2002, Barreiro et al. 2006). This, in turn, may lead to reductions in the biomass that is available to the next trophic level. Other adverse effects have also been demonstrated, such as regurgitation and loss of motor control, which are generally related to the physical condition of the copepods (e.g. Ives 1985, Sykes & Huntley 1987).

Contradictory results with no apparent negative effects have also been reported (reviewed by Turner 2006). This variability is probably related to the dose of particular toxins (Turner & Tester 1997), because the toxin content in algae may vary within a single strain and also be modified by the culture age and nutrient conditions (e.g. Taroncher-Olendenburg et al. 1997, Béchemin et al. 1999, Johansson & Granéli 1999, Hansen et al. 2003, Maclean et al. 2003, Frangópulos et al. 2004, Uronen et al. 2005). Generally, diets with higher toxin concentrations should cause greater effects. However, grazers probably also have species- and site-specific responses or adaptations to different toxins. It has been demonstrated that the ecological relationships between zooplankton grazers and HABs are closely related to their common history (Colin & Dam 2002, 2005, 2007, Hairston et al. 2002). For instance, Colin and Dam (2002) found that copepods from areas where they are constantly exposed to blooms of toxic dinoflagellate *Alexandrium* spp. were less affected by ingesting toxic cells than copepods of the same species from areas where they were not naturally exposed to these toxins. This was explained by the evolutionary adaptations and selection of grazers for resistance to the toxic effects of these algae. However, interactions between grazers and algae are not unidirectional and phytoplankton can also respond to the presence of grazers, which suggests that the hypothesis of grazing deterrence is not entirely incorrect. For instance, Selander et al. (2006) demonstrated that copepods induced an increase in the PST content of *Alexandrium minutum*, and that toxin production correlated with an increased resistance to grazing. Another example of chemical interaction is a study by Tang (2003), which showed that grazing, and specifically grazing-related chemical signals, induced an increase in colony size of the prymnesiophyte *Phaeocystis globosa*. Therefore, aspects such as genetic selection and physiological adaptations should be taken account when examining interactions between grazers and toxic algae.

### 1.5.2 The impact of zooplankton grazing on HABs

Harmful algal blooms and phytoplankton in general are controlled by bottom-up regulation, which is related to factors that control the availability of nutrients and nutrient uptake by the phytoplankton (summarised in Uronen 2007), and by top-down control, which refers to grazing and predation by organisms at higher trophic levels.

Regulation by grazing is determined by the balance between algal growth (μ) and grazing rates (g) (e.g. Smayda 2008). This
relationship has rarely been demonstrated in natural conditions. A good example of this relationship is a high-density low-biomass brown tide bloom of the pelagophyte *Aureococcus anophagefferens*, which persisted for 5 months in Narraganset Bay, USA. During this bloom, the authors were able to separate several stages of grazer influence, characterized by three grazer-breakdown episodes (I-III, Fig. 2) and three grazer-restoration stages (V-VII) (Smayda 2008). As this example demonstrates, the contribution of grazing by the zooplankton community (micro- and mesozooplankton) can be significant during bloom initiation and termination. However, during its peak, the bloom escapes grazing control due to the high biomass and population growth rate, although individual growth rates usually slow down (Turner 2006). Recent studies indicate that the grazing impact of copepods on HABs is variable, but in most situations quite low (Campbell et al. 2005, Jansen et al. 2006, Kozlowsky-Suzuki et al. 2006). In contrast, the grazing impact of microzooplankton on blooms appears to exceed that of mesozooplankton. For example, Calbet et al. (2003) found that microzooplankton grazing in the Mediterranean was similar to or exceeded that of *Alexandrium minutum* growth rates. Although the copepods *Acartia granii* and *Oithona* sp. were able to graze on *Alexandrium*, the daily consumption rate was only 0.003-0.007 % of the *A. minutum* standing stock due to the low standing stocks of copepods. Such comparisons between the grazing impact of mesozooplankton and microzooplankton have not been carried out in the Baltic Sea.

**Fig. 2.** Schematic representation of the cascading effects during an *Aureococcus anophagefferens* bloom in Narraganset Bay, USA, illustrating the grazing pressure by ciliates and subsequent zooplankton collapse during bloom initiation (I and II). The reduction in the zooplankton grazing pressure was responsible for the continued increase in *A. anophagefferens* abundance ($\mu > g$). The greatest breakdown in grazer control was linked to cessation of benthic grazing by filter feeders, especially *Mytilus edulis*. The combined reduction in grazing regulation by micro- and mesozooplankton and benthic grazers led to a huge excess of *A. anophagefferens* net population growth ($\mu >> g$). It is possible that viral infection and ingestion of *A. anophagefferens* by phagotrophic flagellates contributed to the decline of the bloom (IV) (Milligan & Cosper 1994, Smayda 2008). During bloom senescence, grazers recovered over time and were able to take control of the declining *A. anophagefferens* population (redrawn after Smayda 2008).
2. OBJECTIVES OF THE STUDY

Harmful algal blooms, especially if they produce toxic substances, can have widespread negative effects on aquatic food webs. These effects include fish kills, kills of marine mammals and birds as well as lowered production and viability of grazers exposed to toxic algae. Copepods have a central role in the food web, because they transfer energy to higher trophic levels. The toxicity of food species may affect their feeding, viability and reproduction, reducing the biomass that is available to the next trophic level. Additionally, if copepods accumulate toxins, they may transfer them to zooplanktivores. The aim of the studies reported in this thesis was to advance our understanding of the feeding interactions between copepods and toxic algae.

Feeding interactions between copepods and toxic algae are probably the main mechanisms that determine what kind of negative effects algae may have on copepods or whether toxins accumulate in grazers. Papers I and II examine the outcomes of the interaction between copepods and toxic algae. The main objective in papers III and IV, however, were to investigate feeding and food selection and their role in toxin transfer to copepods. This links the separate studies in this thesis together. The experimental design and objectives of all the studies are summarized in Table 2.

In papers I and II the feeding-responses, reproduction and behavioural changes in copepods feeding on toxic *Prymnesium parvum* were examined (potential negative effects / responses and treatments are presented in Fig. 3). The aim was to investigate what kind of feeding-related responses copepods could have when they are exposed to *P. parvum*. Could there be changes in feeding rates, and are these reflected in reproduction or viability, which may alter the population growth rates and decrease the grazer biomass available to higher trophic levels? Are there changes in the behaviour of copepods due to *P. parvum*, such as lowered swimming activity, and are these changes linked to toxicity or just an outcome of lowered feeding?

The importance of the microbial food web in the transfer of nodularin has rarely been considered, although the microbial community associated with the bloom-forming cyanobacteria is presumably a valuable food source for copepods (Sellner 1997, Engström-Öst et al. 2002, see Uronen 2007). In experiments with *Nodularia spumigena* (paper III), the transfer of nodularin to *Eurytemora affinis* was followed by testing three potential pathways: (1) a direct pathway by grazing on *N. spumigena* filaments; (2) a direct pathway from the dissolved nodularin pool; and (3) through grazing on organisms in the microbial food web (see Fig. 1).

Paper IV deals with the feeding-interactions between *Dinophysis* spp. and copepods. The aim was to test whether copepods are able to feed on *Dinophysis* spp., and whether the relative abundance of *Dinophysis* spp. compared to other species in the community could influence feeding and selection. The importance of copepods as vectors in the transfer of toxins derived from *Dinophysis* spp. to higher trophic levels was also considered.
Table 2. Objectives and experimental design in the studies of this thesis. Grazing studies were carried out with either *Eurytemora affinis* or *Acartia bifilosa*. *Prymnesium parvum* was grown in nitrogen-limited (-N), phosphorus-limited (-P), and in nutrient-balanced (+NP) conditions. Different treatments were prepared from these cultures (see also Fig. 3). Non-toxic *Rhodomonas salina* and filtered seawater (FW) were used as controls (papers I & II). Copepods were incubated in different size fractions to estimate how much nodularin was transferred from these fractions (paper III). Feeding behaviour and toxin transfer were assessed in plankton communities that contained *Dinophysis* spp. (paper IV).

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<th>Objectives</th>
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<td><em>P. parvum</em> (+NP, -N, -P), <em>R. salina</em> (+NP)</td>
<td>Monospecific <em>Prymnesium</em> (5 cell concentrations) <em>Rhodomonas</em> control (5 cell concentrations) FW control</td>
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<td>I</td>
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<td>See above</td>
<td>See above</td>
<td><em>E. affinis</em></td>
<td>See above</td>
<td>Low concentration filtrate (Filt 1-2: 2 concentrations &amp; <em>Rhodomonas</em>) High concentration filtrate (Filt 3-4: 2 concentrations &amp; <em>Rhodomonas</em>) Mixture 1 &amp; 2 (1:1, 2 cell concentrations) <em>Rhodomonas</em> control</td>
<td>6 / 4</td>
<td>II</td>
</tr>
<tr>
<td>Nodularin transfer to copepods through the microbial food web. Grazing, food selection, faecal pellets, survival</td>
<td>Bottle incubation 1 × 24 h; 2 experiments (Expt 1, Expt 2)</td>
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<td>4 / 3 (130 ml units) 15 / 3 (30 ml units)</td>
<td>IV</td>
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Fig. 3. Experimental set-up in studies with *Prymnesium parvum* (papers I and II). Symbols: (P) *P. parvum* cells, (R) *Rhodomonas salina* cells, (-N, -P, +NP) N-deficient, P-deficient and NP-balanced cultures that were used for the preparation of different treatments. The range of cell concentrations in different treatments is presented and the potential effects of *P. parvum* are summarised.
3. INTRODUCTION TO HARMFUL ALGAL SPECIES IN THIS STUDY

3.1. Prymnesium parvum Carter

The haptophyte *Prymnesium parvum*, which is a common member of coastal plankton communities worldwide, excretes toxins into the surrounding water. It forms blooms with deleterious effects on diverse marine organisms, such as fish kills, affecting coastal marine ecosystems and causing considerable economic losses to commercial aquaculture (Richardson 1997). *P. parvum* occurs in many parts of the Baltic Sea (Hällfors 2004), but has formed few blooms associated with high fish mortality in Finnish coastal waters (Lindholm & Virtanen 1992, Lindholm et al. 1999). The toxicity of *P. parvum* blooms in the Åland archipelago has been related, for instance, to reduced water exchange and phosphorus deficiency (Lindholm et al. 1999). Because fish farms in Finland are usually located in sheltered bays, they are vulnerable to noxious *P. parvum* blooms. Natural concentrations of *P. parvum* during blooms usually vary between 50 × 10^3 and 100 × 10^3 cells ml^−1, but extreme cell concentrations of up to 800-1600 × 10^3 cells ml^−1, have been observed (Edvardsen & Paasche 1998). The highest *P. parvum* concentrations in our experiments were approximately 350 × 10^3 cells ml^−1, thus representing a normal bloom event.

Several compounds have been described as causing the haemolytic effect\(^7\) of *P. parvum*, such as proteolipids, glycolipids (haemolysins) and polyethers (Prymnesin 1 and 2) (Shilo & Rosenberger 1960, Igarashi et al. 1996, Morohashi et al. 2001, Edvardsen & Imai 2006). *P. parvum* has also been reported to cause ichtyotoxic and cytotoxic\(^8\) effects (Shilo 1971, Igarashi et al. 1998). Both nitrogen and phosphorus limitation has been found to enhance the toxicity of *P. parvum* (Shilo 1971, Johansson & Granéli 1999, Uronen et al. 2005). Therefore, we used both nutrient-limited and nutrient-balanced cultures in our experiments (Fig. 3).

Several studies have indicated that *P. parvum* has allelopathic effects on other algae (e.g. Legrand et al. 2003) and is able to feed on other algae and bacteria (Nygaard & Tobiessén 1993, Tillmann 1998, Legrand et al. 2001). Mixotrophy\(^9\) is stimulated by nutrient limitation (Skovgaard et al. 2003). In addition, noxious effects on ciliates (Fistarol et al. 2003, Granéli & Johansson 2003), rotifers (Barreiro et al. 2005) and copepods (Nejstgaard & Solberg 1996, Koski et al. 1999b, Sopanen et al. 2006, Sopanen et al. 2008) have been reported. Thus, a bloom of this species is likely to have adverse effects on large parts of the plankton community. Since these chemicals are rapidly degraded in water (e.g. Fistarol et al. 2003), the possibility of deleterious effects is most pronounced during the bloom.

3.2. Nodularia spumigena Mertens ex Bornet & Flathault 1886

Blooms of diazotrophic cyanobacteria\(^10\) naturally occur in the Baltic Sea (Bianchi et al. 2000). However, the frequency and

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\(^7\) Haemolytic effect, haemolytic activity (HA): the potential to induce rupture or destruction of red blood cells. Describes the toxicity of *Prymnesium parvum* in this study.

\(^8\) Toxic to fish and cells, respectively.

\(^9\) Mixotrophy: organisms that are able to combine phototrophy (i.e. assimilate dissolved CO\(_2\) from the water) and heterotrophy (i.e. use only carbon from organic sources for biosynthesis).

\(^10\) Cyanobacterial species that have an ability to fix dissolved atmospheric nitrogen gas.
intensity of these blooms, dominated by *Nodularia spumigena* and *Aphanizomenon flos-aquae*, have increased during recent years (Kahru et al. 1994, Finni et al. 2001, Poutanen & Nikkilä 2001), and have been related to eutrophication in the Baltic Sea (Vahtera 2007). The average bloom biomass of *N. spumigena* in the Gulf of Finland, Baltic Sea, usually ranges between 0.08 and 0.28 mg WW l\(^{-1}\), but values of up to 2.91 mg WW l\(^{-1}\) have been recorded (Kanoshina et al. 2003). The biomasses during our grazing experiments (0.25 to 0.99 WW l\(^{-1}\)) represented normal bloom values.

Increased toxin production by *N. spumigena* has been related to a high biomass as well as an increase in production and growth (Lehtimäki et al. 1997). Nodularin is mainly intracellular during optimal growth, but is released into the water when the bloom is decaying (e.g. Codd et al. 1989, Lehtimäki et al. 1997). *N. spumigena* has caused fatal poisonings of livestock, dogs and ducks (reviewed in Karjalainen 2005). Because nodularin is a stable molecule, it can be transferred through the food web. Food-chain experiments have demonstrated that zooplankton can act as vectors for nodularin transfer to consumers at higher trophic levels (Engström-Öst et al. 2002). In the Baltic Sea, nodularin has also been found to accumulate, for example, in mysids (Engström-Öst et al. 2002), fish (Sipiä et al. 2001) and eiders (Sipiä et al. 2003).

Copepods are able to graze on *Nodularia spumigena* and accumulate toxins in their body tissues and faecal pellets (Sellner et al. 1994, Koski et al. 1999a, Lehtiniemi et al. 2002, Kozlowsky-Suzuki et al. 2003). In general, however, cyanobacteria are considered to be ‘poor’ food for zooplankton (reviewed by Lampert 1987) due to the low manageability of large filaments or colonies, or nutritional inadequacy and toxicity (e.g. Lampert 1987, Ahlgren et al. 1990, Schmidt & Jónasdóttir 1997, Kozlowsky-Suzuki et al. 2003). Meyer-Harms et al. (1999) demonstrated that copepods are also able to ingest cyanobacteria under natural conditions, and they considered that a cyanobacterial bloom in the late phase may actually provide nutritious food for copepods because of the accompanying diverse fauna of microzooplankton. Therefore, it is essential to evaluate the importance of the different routes by which nodularin is transferred to grazers.

### 3.3. *Dinophysis* spp.

An increasing trend in dinoflagellate stocks has been observed in the Baltic Sea (Wasmund & Uhlig 2003), and incidences of massive late-summer blooms of potentially toxic species, such as *Alexandrium ostenfeldii*, have been reported (Hajdu et al. 2006, Kremp et al. 2009). If the frequency and intensity of potentially toxic dinoflagellate blooms increases, grazers will encounter toxic dinoflagellate blooms more frequently. Three toxic *Dinophysis* species – *D. acuminata*, *D. norvegica* and *D. rotundata* – commonly occur in the northern Baltic Sea. The abundances of *Dinophysis* spp. in the Baltic Sea are typically quite low (about 1-5 cells ml\(^{-1}\)), but high concentrations of up to 150 cells ml\(^{-1}\) have also been found (Carpenter et al. 1995, Kuuppo et al. 2006). The concentrations of *Dinophysis* spp. during our study (40-77 cells ml\(^{-1}\)) were thus higher than is typically seen in non-bloom conditions.

*Dinophysis*-derived toxins, such as pectenotoxins (PTXs) and dinophysistoxins (DTXs), have been detected in the northern Baltic phytoplankton communities (Goto et al. 2000, Kozlowsky-Suzuki et al. 2006, Kuuppo et al. 2006). Dinoflagellate-derived
toxins accumulate in consumers, such as shellfish, and are vectored to higher trophic levels through trophic interactions, causing diseases to humans though the consumption of seafood (reviewed in Hallegraeff 1993). In the Baltic Sea, these problems are not entirely relevant, since brackish water mussels are not commercially exploited. However, animals that feed on mussels are exposed to toxins. For example, OA has been detected in blue mussels (Mytilus edulis), and in the common flounder (Platichthys flesus) in the northern Baltic Sea (Pimiä et al. 1998, Sipiä et al. 2000). Therefore, it is important to investigate the dinoflagellate-grazer interactions and toxin transfer between species naturally occurring in the Baltic Sea.

4. MATERIAL AND METHODS

4.1. Experiments

4.1.1. Experiments with Prymnesium parvum (papers I & II)

We were interested in determining whether toxic Prymnesium parvum is able to harm the copepods Eurytemora affinis and Acartia bifilosa (Fig. 3), which are important grazers in the coastal areas of the northern Baltic Sea (Kivi et al. 1996, Uitto 1997, Koski et al. 1999a, 1999b, Engström et al. 2000) and valuable prey for zooplanktivores such as mysids and fish. Adult females were collected from the Tvärminne sea area on the SW coast of Finland (59° 49’ N, 23° 17’ E). For a detailed description of the area, see Niemi (1975).

To illustrate the effects of P. parvum, the survival, feeding (clearance- and weight-specific ingestion rates), faecal pellet production rate and egg production rate were examined in copepods exposed to P. parvum. Copepods were incubated with: (1) cell suspensions of Rhodomonas salina, which represented a non-toxic control treatment (Rhodomonas control); (2) filtered seawater (FW, as starvation control); and (3) cell suspensions of P. parvum (monospecific Prymnesium) (paper I). P. parvum excretes toxic substances into the water. These exudates could have direct effects on copepods, and also allelopathic effects on other algae. In order to examine these effects, copepods were exposed to: (1) cell-free P. parvum filtrates in the presence of a saturated concentration of non-toxic R. salina cells (high and low concentration filtrates); (2) 1:1 cell mixtures of P. parvum and R. salina (mixture 1 & 2); and (3) R. salina in a saturated food concentration (Rhodomonas control) (paper II). P. parvum cell concentrations that were used during our studies were selected to represent normal bloom conditions. The study design, treatments, the range of cell concentrations and responses are presented in Figure 3. The study aims, experimental set-ups and methods are presented in Tables 2 and 3. Because the haemolytic activity of P. parvum, which represented toxicity in this study, has been found to increase in nutrient-limited conditions (e.g. Johansson & Granéli 1999), all cell suspensions and cell-free P. parvum filtrates were prepared from P. parvum cultures, grown under NP-balanced conditions or under nitrogen (-N) or phosphate (-P) deficiency (for detailed culture methods and P. parvum growth and toxicity, see Uronen et al. 2005). Non-toxic control food, Rhodomonas salina, was grown under NP-balanced conditions.

11 Filtrates were prepared by gently GF/C filtering the cell suspensions taken from P. parvum cultures and diluting them with 0.2 μm filtered seawater to obtain appropriate concentrations.
4.1.2. Experiments with Nodularia spumigena and Dinophysis spp. (papers III & IV)

The aim of these studies was to examine selective feeding and the role of copepods in toxin transfer (see Table 2). Nodularia transfer experiments were conducted with cultured females of *Eurytemora affinis* to ensure that the copepods were free of nodularin. The phytoplankton community, collected from the Baltic proper and grown in mesocosms, was gently fractionated into 5 size fractions12 in order to create different plankton communities, and animals were allowed to feed on the size-fractionated plankton community to follow nodularin transfer to copepods via these fractions (see Table 2 and Fig. 1). Studies were carried out in two different situations regarding *N. spumigena* biomass and the phase of the bloom. The first experiment (Expt 1) was performed during the initial growth phase of an *N. spumigena* bloom with low chl-a (1.3 μg C l⁻¹), and the second one (Expt 2) during stationary growth conditions with high chl-a (9.4 μg C l⁻¹) and decaying filaments of *N. spumigena*. Since the initial *N. spumigena* concentration was low in the mesocosm community in Expt 1, cultured *Nodularia spumigena* were inoculated to the mesocosms. This resulted in the presence of two types of filaments in Expt 1: long-chained filaments, with a cell size of 3.5-6 × 10⁻¹³ μm and a short morphotype13, with a cell size of 2-4 × 4-5 μm. These filaments are referred to in this text as “large” and “small” *N. spumigena*. In the second experiments (Expt 2), only long-chained filaments were present.

In the experiments with *Dinophysis* spp. (paper IV), selective feeding of the copepods *Acartia bifilosa* and *Eurytemora affinis* was evaluated in plankton communities that differed regarding the proportion of *Dinophysis* spp. in phytoplankton mixtures. The aim was to examine whether the copepods fed on *Dinophysis* spp. and may thus accumulate toxins (Table 2). Experiments were conducted with a natural plankton community that was collected below the thermocline from Tvärminne Storfjärd, SW coast of Finland. Experimental water was filtered through a 76 μm mesh net to remove zooplankton and larger phytoplankton cells. During the procedure, some of the largest *Dinophysis* spp. cells could have been removed. The proportion of *Dinophysis* spp. in the food suspensions was adjusted by inverse filtration through a 20 μm net. Due to filtration, food communities differed regarding the total cell concentration and the proportion of different algae or algal groups. Because of variation in total food concentrations, experiments I – VI are referred to as “high food experiments” and experiments VII – X as “low food experiments”14. The initial cell

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12 <150 μm size fractions represented the total community in Expt 1 and 2. These fractions were further divided into <45, <20, <10, <3 and <0.2 μm fractions.

13 Our aim was to remove *Nodularia spumigena* filaments in all but the largest size fractions. However, in Expt 1, small filaments of *N. spumigena* passed filtration all the way into <45, <20 and <10 μm size fractions (see Table 5), while in Expt 2, only few small pieces of large *N. spumigena* were found in the <45 μm fraction.

14 Expts I – VI consisted almost entirely of the dinoflagellate *Heterocapsa triquetra*, which often forms blooms during late summer, and the total cell concentrations in the plankton communities were relatively high (up to 1300 cells ml⁻¹). In Expts VII – X, the total cell concentrations were up to 20 fold lower and the community had shifted towards *Dinophysis* spp. dominance (the initial proportions of different species/species groups are presented in the respective papers).
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<td>Carbon conversions for plankton</td>
<td>Biovolumes were calculated from cell dimensions with species-specific formulas and calculated further to carbon</td>
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<td>III, IV</td>
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<td>Absorbance at 540 nm was read on a Fluorstar 403 microplate reader</td>
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<td>HPLC-MS with electrospray ionization (LC–ESI–MS) (PE series 200 autosampler and pump coupled to an Applied Biosystems API 165 mass spectrometer), which was operated in selective ion monitoring mode using positive ionization of nodularin</td>
<td>III</td>
<td>Dahlmann et al. 2003 (Dissolved nodularin sample preparation: ISO 20179:2005, water quality. Determination of microcystins: Method using solid phase extraction [SPE] and HPLC with UV detection)</td>
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<td>DSP toxins</td>
<td>The copepod toxin content was measured and expressed as pg individual(^{-1})</td>
<td>HPLC system equipped with a PE series 200 autosampler and pump coupled to an Applied Biosystems API 165 SCIEX mass spectrometer. The electrospray ionization operated in positive mode</td>
<td>IV</td>
<td>McNabb et al. 2005</td>
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concentrations were in the range of natural concentrations of nano- and microplankton reported in the study area (Kononen 1988, Setälä & Kivi 2003, Suikkanen et al. 2007).

4.2. Analytical and statistical methods

Descriptions of the incubations, sampling procedures and methods are presented in Tables 2 and 3. The species abundances in all studies were converted to carbon by using volume-based carbon conversion factors (Table 3). Both phytoplankton and protists were grouped in the further analyses of grazing and food preference (papers III & IV). Grazing (clearance and ingestion rates) was calculated according to Frost (1972) from the disappearance of food cells from the experimental bottles containing copepods in comparison with the control bottles without copepods (papers I–IV). The Chesson selectivity index \( \alpha \) was estimated as the ratio of the grazed units to the availability of particular food in the incubation bottles (Chesson 1983) (papers III & IV). The faecal pellet production rate (PPR) quantifies the part of the food that has not been absorbed in the digestive tract of the animal and can be used as an estimate of ingestion. In order to estimate faecal pellet production in different studies, conversion factors that were based on volume were used (Gonzales & Smetacek 1994) (papers I, II & III). To estimate reproduction, the egg production rate (EPR) for the egg-carrying copepod E. affinis was measured, taking into account the temperature-dependent development time of the eggs (Andersen & Nielsen 1997), whereas the eggs of A. bifilosa were counted after each incubation (papers I & II). Gross growth efficiency \( \text{GGE, } \% \text{ day}^{-1} \) in each treatment was calculated from the weight-specific egg production rate divided by the average weight-specific ingestion.

The toxicity of P. parvum, measured by haemolytic activity, was analysed daily from the P. parvum cultures and cell-free P. parvum filtrates, and on the first experimental day from monospecific Prymnesium treatments (papers I & II, see Table 3). The allelopathic effect of P. parvum on R. salina was estimated by comparing the control treatments without copepods in the filtrate and mixture treatments with the Rhodomonas controls (paper II). Nodularin in particulate and dissolved forms was analyzed before and after the experiments from the size fractions and after the incubations in copepods (paper III, Table 3). The toxin content (DSP toxins) in copepods was measured after the incubations (paper IV, Table 3).

Differences in grazing, egestion, egg production and survival were tested with 2-way ANOVA \( \text{GGE, } \% \text{ day}^{-1} \) in each treatment was calculated from the weight-specific egg production rate divided by the average weight-specific ingestion.

**Selectivity indices are used to estimate food selection. In the Chesson selectivity index, a particular food type is neither selected nor avoided (i.e. each food type is grazed proportionally to its availability) if \( \alpha = m^{-1} \) (m = number of food types available). When \( \alpha > m^{-1} \) the food type is selected, and when \( \alpha < m^{-1} \) the selection is negative.**

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15 Selectivity indices are used to estimate food selection. In the Chesson selectivity index, a particular food type is neither selected nor avoided (i.e. each food type is grazed proportionally to its availability) if \( \alpha = m^{-1} \) (m = number of food types available). When \( \alpha > m^{-1} \) the food type is selected, and when \( \alpha < m^{-1} \) the selection is negative.

16 Gross growth efficiency expresses the quantitative relationship between growth and ingestion.

17 Dissolved nodularin was measured from the <0.2 and <3 \( \mu \text{m} \) fractions in Expt 1 and <0.2, <3 and <150 fractions in Expt 2 after the incubations. Initial dissolved nodularin was measured from the mesocosms. Detailed descriptions of the toxin analyses and preparation of samples are provided in the respective papers.

18 In paper 1 (experiments with E. affinis, grazing, survival and PPR), cell concentration and algal treatment (R. salina and P. parvum -N, -P, +NP) were used as independent variables, while in experiments with A. bifilosa examining grazing, PPR and EPR, the day and algal treatment were used as inde-
among treatments in the EPR of *E. affinis* were tested with 1-way ANOVA (papers I & II). To identify which groups were grazed on by the copepods, the calculated clearance or weight-specific ingestion rates were compared using 1-way ANOVA (papers III & IV). Pairwise multiple comparisons were made if significant differences between treatments were found. After calculating the Chesson selection index $\alpha$, Dunnet’s method or a non-parametric Mann-Whitney rank sum test was used to distinguish those food groups that were significantly preferred in the copepod’s diet (Papers III & IV). The relative importance of the microbial food web in nodularin transfer to copepods was estimated by comparing the average amount of nodularin in copepods from the dissolved size fraction with those from other size fractions using 1-way ANOVA. Linear regression was performed to examine which variables (ingestion of different plankton groups) could explain the variance in the nodularin content of the copepods (paper III)\(^\text{19}\), and to examine the dependence of clearance and ingestion rates of the copepods on the initial prey cell concentration in different experiments (paper IV). All analyses were performed with the statistical software Sigma Stat for Windows 3.0.1. (SPSS).

5. RESULTS AND DISCUSSION

5.1. Instantaneous toxic effects of ichtyotoxic *Prymnesium parvum*

The feeding, egestion, reproduction and mortality of copepods exposed to toxic *Prymnesium parvum* cells, filtrates that were prepared from *P. parvum* cultures or mixtures containing *P. parvum* and non-toxic *Rhodomonas salina* were examined and responses compared to *R. salina* and filtered water (FW) controls. These studies were carried out because it has been found that *P. parvum* has deleterious effects on many organisms including phytoplankton, microzooplankton, mesozooplankton and fish. Thus, it is crucial to assess what effects *P. parvum* may have on copepods, which are important as grazers in Baltic Sea food webs and as prey for zooplanktivorous animals.

5.1.1. Haemolytic activity cannot be used as a predictor of *P. parvum* toxicity to copepods

*Prymnesium parvum* negatively affected the feeding, survival, behaviour and reproduction of the calanoid copepods *Eurytemora affinis* and *Acartia bifilosa*. The cell concentrations\(^\text{20}\) used in this study represented the normal bloom levels of *P. parvum* (Edvardsen & Paasche 1998). All the observed responses are summarized in Figure 3 and Table 4. The *P. parvum* cultures were in a steady state and all were haemolytic during the experiments (papers I & II) (Uronen et al. 2005). Thus, *P. parvum*...
has the potential to cause adverse effects on grazers, irrespective of the nutrient conditions.

Substances causing the HA appeared to be quite labile. In this study, HA decreased by up to 70 % during the 24 h incubations in all Prymnesium treatments (paper I), and was below detection limits in the filtrates (paper II). The survival of copepods was related to the presence of P. parvum or compounds released by P. parvum, but not necessarily to HA (papers I and II). This was indicated by the finding that filtrates were toxic to copepods, although they were not haemolytic. Survival rates were inversely related to the initial concentration of filtrates. All animals died during the first incubation period in the highest filtrate concentration (Filt 4, -P and +NP) (Fig. 4). Exposure to high concentration filtrates resulted in significantly lower survival compared to low concentration filtrates and the Rhodomonas control (Fig. 4). Despite the absence of HA, the negative effect on survival was stronger in the filtrates in comparison to monospecific Prymnesium suspensions or mixtures. Copepods survived well in the mixtures (>90 %, paper II), and the differences between monospecific Prymnesium, the Rhodomonas control and FW were not significant (paper I). Survival percentages in the Rhodomonas control and FW (43-86 %) were only slightly better compared to the monospecific Prymnesium suspension (26-66 %), and varied irrespective of the cell concentration or nutrient treatment (paper I). We observed relatively high mortality once in the first incubation period in the Rhodomonas control due to an unknown reason, which could explain the lack of significant differences. The visually observed condition of copepods in the monospecific Prymnesium suspension was, however, noticeably worse than the actual survival rates indicated. The copepods were swimming actively in the Rhodomonas control and FW, whereas copepods incubated in the monospecific Prymnesium suspension or in high concentration filtrates were inactive and showed reduced pipette avoidance. This observation was consistent with the video observations of Koski et al. (1999b), according to which copepods feeding solely on Prymnesium patelliferum were inactive. Acartia bifilosa showed relatively high survival in the monospecific Prymnesium suspension and differences among treatments (monospecific Prymnesium 71-84 %; FW 89 %; the Rhodomonas control 43-86 %).

**Fig. 4.** Survival (%) of Eurytemora affinis in Rhodomonas (Rho) control and in high (Filt 3-4) and low (Filt 1-2) concentration Prymnesium parvum filtrates on day 3 (average ± S.E.). Different symbols indicate P. parvum nutrient treatments (re-drawn from paper II).

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21 Filt 3-4: corresponding to 50-354 cells × 10³ cells ml⁻¹.

22 Filt 1–2: corresponding to 5 and 10 × 10³ cells ml⁻¹.

23 In this study means decreased swimming activity (i.e. copepods were completely motionless). The expression is based on visual observation and is thus only qualitative.
Rhodomonas control 100 %) were not significant (paper I). The different survival rates of these copepods suggest that responses to Prymnesium are species-specific and could also reflect the life history of the animals, such as age, feeding history, condition or physiology. For example, Valkanov (1964), who examined the toxic effects of P. parvum, reported that copepods (Cyclops sp. and Calanipeda sp.) were unaffected even after a 3-day exposure to high cell densities (1.1 × 10^5 cells ml^-1). Similarly, Calanus finmarchicus and Acartia clausi were unaffected after a 2-day exposure to P. patelliferum (10^6 cells

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ml⁻¹) in a study by Nejstgaard et al. (1995). In contrast, Koski et al. (1999b) observed the mortality of *E. affinis* to increase when copepods were fed with *P. patelliferum* at a cell density of 13 to 29 × 10³ cells ml⁻¹. Additionally, *P. parvum* strains in distinct growth stages or nutrient conditions may simply vary in their toxicity. Therefore, the results obtained with different *Prymnesium* strains may not be comparable.

What could have caused the different responses in survival that were particularly apparent between filtrates and cell suspensions (mixtures and monospecific *Prymnesium*)? Since filtrates were not haemolytic, they may have contained other chemical compounds that have not been recognized with the available methods. So far, only qualitative indirect measurements of toxicity such as haemolytic activity, or lethal effects on *Artemia salina* nauplii are available (Meldahl et al. 1994). These may not be as accurate as direct measurements of toxicity, and the ultimate factor that caused the observed responses thus remains unclear. In addition, the mechanisms underlying the activation or promotion of toxicity have remained obscure (Edvardsen & Imai 2006). Therefore, HA is not always a good proxy for the toxicity of *P. parvum*.

Other factors could also explain the acute toxicity of *P. parvum*. One possible explanation is linked to a general failure of cell membrane function (changes in permeability). For example, Meldahl and Fonnum (1995) showed that substances extracted from *P. patelliferum* increase the permeability to Na⁺, Ca²⁺ and K⁺ in the synaptosomal membranes as well as causing membrane depolarization and inhibition of the net uptake of L-glutamate. This type of general effect on cell membranes may cause symptoms that were apparent in monospecific *Prymnesium* treatments as well as in filtrates. In addition, many ichthyotoxic phytoplankton species, including *P. parvum*, produce superoxides, also known as reactive oxygen species, which are strong oxidants (Marshall et al. 2005). Reactive agents such as superoxide (O₂⁻) and hydrogen peroxide (H₂O₂) are well-known toxigenic products in biological systems, and have been linked to fish gill tissue injury, alteration of chloride cells and physiological responses such as hypoxia, among other effects (Marshall et al. 2005).

It was interesting that copepods in the mixtures were active and survived well (paper II). The cell concentrations of *P. parvum* in mixtures were comparable to concentrations of the *P. parvum* suspensions from which the filtrates and monospecific *Prymnesium* treatments were prepared, and in which adverse effects (swimming behaviour, survival) were apparent. This indicates that the presence of *R. salina* in mixtures somehow buffered the harmful effects of *P. parvum* on copepods. Exudates released by *P. parvum* have a tendency to bind to particles such as other phytoplankton cells (discussed in Uronen et al. 2005). The total cell numbers in the mixtures were higher than in the filtrates, which mean that if exudates were bound to other particles, this may have diluted the negative effects (paper II). Furthermore, *P. parvum* is mixotrophic and able to ingest other organisms, such as bacteria and other phytoplankton (Legrand et al. 2001, Skovgaard et al. 2003). Mixotrophy may release *P. parvum* from cellular stress, reducing its toxicity (Legrand et al. 2001, Uronen et al. 2007). Our results revealed a strong allelopathic effect in the mixtures (see Fig. 5), which may have promoted mixotrophy and consequently led to lower toxicity (paper II). In addition, the presence of copepods may reduce toxicity. Mechanisms such as sloppy

24 approx. 15 and 50 × 10³ cells ml⁻¹ in mixture 1 and 2, respectively.
feeding, excretion by copepods or leakage of nutrients from dead copepods release nutrients and could relieve *P. parvum* from nutrient deficiency. Responses under natural conditions could be expected to resemble those found in the mixtures. It is, however, possible that copepods may become inactive and stop feeding during a mass occurrence of *P. parvum*, especially in situations with minimal water turbulence. This, together with allelopathic effects, may contribute to bloom formation and persistence.

5.1.2. *P. parvum* impairs the feeding of copepods

The experiments revealed that both *Eurytemora affinis* and *Acartia bifilosa* avoided feeding on *Prymnesium*, but readily consumed non-toxic *Rhodomonas* in monospecific treatments (Fig. 6 A & B, paper I). However, clearance rates at the lowest

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25 Sloppy feeding refers to inefficient ingestion of large cells by zooplankton, which can produce small ungrazed particles.

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26 Two-way ANOVA results are presented in Table 2 in article 2.

*Prymnesium* concentration indicated that *E. affinis* is able to consume *P. parvum* cells when the cell abundance is low. Filtrates clearly interfered with feeding. This was especially apparent in the high concentration filtrates (Filt 3 and 4), in which reduced ingestion rates were recorded, while the clearance and ingestion rates in the low concentration filtrates (Filt 1 and 2) were comparable to the *Rhodomonas* control. Results from other studies have been controversial. Higher ingestion compared to this study or low grazing rates have been observed in grazing experiments with *Prymnesium patelliferum* (Nejstgaard et al. 1995, Koski et al. 1999b). The lack of similarity in feeding responses may thus be linked to the species, sites and common history of grazers and prey.

The faecal pellet production rate (PPR) supported the observed ingestion rates. The average PPR of *E. affinis* and *A. bifilosa* reflected feeding, being high in the *Rhodomonas* controls in comparison with the FW and monospecific *Prymnesium* treat-
Fig. 6. Average (± S.E.) weight-specific ingestion of (A) *Eurytemora affinis* and (B) feeding on *Rhodomonas salina* (Rho) or *Prymnesium parvum* (Pry -N, -P, +NP) in monospecific cell suspensions (redrawn from paper I).

Fig. 7. Average (± S.E.) weight-specific pellet production of *Eurytemora affinis* in filtered seawater (FW) and at different concentrations of *Rhodomonas salina* (Rho) and *Prymnesium parvum* (Pry –N, -P, +NP) in monospecific cell suspensions (redrawn from paper I).

![Graph](image)

Fig. 7. Average (± S.E.) weight-specific pellet production of *Eurytemora affinis* in filtered seawater (FW) and at different concentrations of *Rhodomonas salina* (Rho) and *Prymnesium parvum* (Pry –N, -P, +NP) in monospecific cell suspensions (redrawn from paper I).

![Graph](image)

Both ingestion and PPR revealed that *E. affinis* fed in filtrates and mixtures. However, in the high concentration filtrates, exudates produced by *P. parvum* interfered with *E. affinis* feeding. Interestingly, the PPR in Mixture 2 suggested that feeding was reduced. Thus, it is possible that *P. parvum* exudates negatively affected the overall activity of the copepods, as in the filtrates. However, since copepods were active in the mixtures, other explanations should also be considered. If an alga produces allelochemicals that harm other algae, it may reduce the quantity of

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27 Two-way ANOVA results for filtrates and mixtures are presented in Table 3 in article 2.
competitive algae, which may in turn lead to a reduced availability of alternative food for grazers. *P. parvum* showed strong allelopathic effects on *Rhodomonas salina* (Table 4, Fig. 5). In the mixtures, the cell density decreased approximately 30 % during the incubations, both in controls without copepods and in replicates with copepods (paper II). The effect was weaker in the filtrates, in which only 5 to 10 % of the cells disappeared. The stronger allelopathic effect in the mixtures compared to the filtrates may be due to the continuous release of allelopathic chemicals by *P. parvum* cells. Uronen et al. (2007) observed that practically all *R. salina* cells lysed within a short time-span with *P. parvum* densities (64 × 10^3 cells ml^-1) corresponding to bloom conditions. However, the allelopathic effect during our study was not as strong and the copepods were probably not food-limited. Thus, it is possible that compounds released by *P. parvum* had sublethal effects on copepods that were seen in lowered grazing.

5.1.3. Low ingestion is linked to suppressed egg production

Our results demonstrated a reduced fecundity of both *E. affinis* and *A. bifilosa* on a diet of *Prymnesium*. The EPRs of *E. affinis* and *A. bifilosa* in monospecific *Prymnesium* suspensions were lower compared to the *Rhodomonas* control (paper I). Additionally, the EPR of *A. bifilosa* increased over time when copepods fed on *Rhodomonas*, but remained low or even decreased in the monospecific *Prymnesium* treatment and in FW (Fig. 8). In the filtrates and mixtures, variation in EPR was unrelated to the treatment and was at the same level as in the *Rhodomonas* control (paper II). Gross growth efficiencies (GGEs) were generally higher in the *Rhodomonas* controls (mean ± S.D. = 15 ± 7 % d^-1) compared to monospecific *Prymnesium* or filtrates (5 ± 6 % d^-1). In accordance with this, other studies have also shown a suppressed EPR due to the avoidance of Prymnesiophytes *P. patelliferum* (Nejstgaard & Solberg 1996) or *Chrysochromulina polylepis* (Nielsen et al. 1990). Thus, the reduced EPR was most probably caused by the low feeding rates together with the inactivation of copepods exposed to *Prymnesium*. In the filtrates and mixtures, copepods were able to feed on non-toxic *R. salina*, which diminished the effect. This finding is relevant, because monospecific blooms could be rather rare in nature. Thus, the toxic effects may be diluted if grazers ingest small amounts of harmful taxa along with other non-toxic phytoplankton (Turner 2006). This type of selection, with a wide food-spectrum, was apparent in studies with *Nodularia spumigena* (paper III) and *Dinophysis* spp. (paper IV), in which the phytoplankton community was
diverse. Furthermore, no negative effects, such as increased mortality, were observed in these experiments. Unfortunately, the EPR was not estimated in these studies.

Reproduction is linked to factors that influence the quality or quantity of the diet. Thus, if copepods avoid feeding on algae because of taste, toxicity (Nielsen et al. 1990, Nejstgaard & Solberg 1996, Dutz 1998), shape/size (Infante & Abella 1985, Berggreen et al. 1988) or low nutritional value (Jónasdóttir 1994, Schmidt & Jónasdóttir 1997), reproduction may fail. During bloom events, the proportion of unsuitable food compared to other prey species could be high. In such situations, energy reserves may be used for maintenance rather than allocated to reproduction.

5.2. Accumulating toxins of Nodularia and dinoflagellates

Nodularin as well as Dinophysis-derived toxins are among those toxins that can be vectored in the food web. The aim of these studies was to examine selective feeding and the role of copepods in toxin transfer. In experiments with Nodularia spumigena we were especially interested in the pathways of nodularin transfer. Do copepods gain nodularin by direct ingestion of filaments, can they take up dissolved nodularin, or could nodularin perhaps be transferred via the microbial food web? These two experiments aimed to address these questions.

5.2.1. Transfer of nodularin to copepods through feeding interactions

In the Baltic Sea, copepods mainly obtain nodularin via the ingestion of toxic filaments (e.g. Engström-Öst et al. 2002, Karjalainen et al. 2006), but also directly in the dissolved form (Karjalainen et al. 2006). However, very little is known about the importance of the microbial food web in nodularin transfer, and we thus aimed to focus especially on the importance of this pathway. Potential nodularin sources were estimated by calculating the ingestion and food selection of copepods incubated in different size fractions. Pre-filtration produced relatively different planktonic communities in the size fractions in terms of both total biomass and species composition. Dinoflagellates and ciliates comprised an important group in this study in terms of biomass. The contribution of nano- and pico-sized organisms (flagellates, picocyanobacteria, and bacteria) varied from moderate to high28.

The initial biomass of N. spumigena (27 μg C l⁻¹, 0.25 mg WW l⁻¹) during Expt 1 was in the range of average bloom biomasses in the Gulf of Finland, whereas the biomass during Expt 2 (109 μg Cl⁻¹, 0.99 mg WW l⁻¹) fitted within the range of maximum values29. The initial nodularin concentrations in our experiments (Table 5) were in the range of values naturally observed in the Baltic Sea (80-18 700 ng l⁻¹) (Kononen et al. 1993). The initial concentration of dissolved nodularin (Table 5) in both experiments corresponded to approximately one-fifth of the total nodularin in the <150 μm fraction. Nodularin was found in the copepods incubated in all the plankton size fractions in both experiments, indicating that all pathways presented in Figure 1 are possible (Table 5). The im-

28 Composition of the planktonic community, see Table 2 in article III.
29 The average bloom biomass in the Gulf of Finland ranges between 0.08-0.28 mg WW l⁻¹, while maximum values are in the range of 0.23-2.91 mg WW l⁻¹ (Kanoshina et al. 2003).
Table 5. Initial *Nodularia spumigena* biomass and nodularin concentration in particulate and dissolved forms. Nodularin (pg ind⁻¹) in *Eurytemora affinis* after 24-h incubations, proportion of initial nodularin channelled to copepod total biomass (n = 5 replicate bottles with 35 copepods in each; mean ± S.D.). * Initial dissolved nodularin concentrations were measured from mesocosms.

<table>
<thead>
<tr>
<th></th>
<th>Particulate pool</th>
<th>Dissolved pool</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>&lt;150 μm &lt;45μm &lt;20 μm &lt;10 μm &lt;3 μm &lt;0.2 μm</td>
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<tr>
<td>Expt 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Initial <em>N. spumigena</em> biomass (μg C l⁻¹)</td>
<td>27   13   20   5   0   0</td>
<td></td>
</tr>
<tr>
<td>Initial nodularin (ng l⁻¹)</td>
<td>387   257   85   50   111* 111*</td>
<td></td>
</tr>
<tr>
<td>Nodularin in copepods (pg ind⁻¹)</td>
<td>14.3 ± 11.6  5.7 ±1.3  3.8 ± 2.6  4.3 ± 2.6  1.3 ± 2.8  1.7 ± 3.8</td>
<td></td>
</tr>
<tr>
<td>% copepod biomass</td>
<td>0.1 ± 0.1  0.08 ± 0.02  0.2 ± 0.1  0.3 ± 0.2  0.04 ± 0.09  0.05 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>Expt 2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Initial <em>N. spumigena</em> biomass</td>
<td>109   2   -   0   0   0</td>
<td></td>
</tr>
<tr>
<td>Initial nodularin</td>
<td>199   3   -   2   42* 42*</td>
<td></td>
</tr>
<tr>
<td>Nodularin in copepods</td>
<td>6.6 ± 0.7  3.5 ± 3.3  -   4.2 ± 4.7  3.7 ± 1.6  1.6 ± 3.5</td>
<td></td>
</tr>
<tr>
<td>% copepod biomass</td>
<td>0.1 ± 0.01  4.1 ± 3.4  -   6.5 ± 7.3  0.3 ± 0.3  0.1 ± 0.3</td>
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</table>

The importance of possible pathways will next be discussed by presenting questions and evaluating whether our data are able to support these assumptions.

Do copepods obtain nodularin directly by grazing on toxic filaments? We found that some of the nodularin was obtained through direct grazing on small *N. spumigena* filaments in the <150, <45, <20, and <10 μm fractions in Expt 1 (Fig. 1, pathway 1, Fig. 9 A-D). These filaments were grazed on in the proportion they were available, without significant preference or avoidance (Dunnett’s test p > 0.05 compared to α), while large filaments were avoided in both experiments. Grazing on the small filaments correlated with the amount of nodularin found in the copepods in Expt 1. However, the correlation was not strong enough (r² = 0.39, p < 0.05) to exclude other possible pathways for nodularin transport to copepods. In the smaller fractions (<45 to <0.2 μm), the nodularin content of the copepods was up to 52% lower compared to copepods incubated in the <150 μm community, indicating that a relatively large part of the nodularin was obtained through grazing on filaments.

Could copepods obtain nodularin directly in the dissolved form? Our data suggest that part of the nodularin was directly obtained from the dissolved pool in the <0.2 μm fraction (Fig. 1, pathway 2; Table 5). This accounted for up to 24% of the *E. affinis* nodularin content compared to the <150 μm fraction. Consistently with this, Karjalainen et al. (2003) found that *E. affinis* was able to incorporate similar amounts of labelled dissolved nodularin (1.8 ± 0.5 pg ind⁻¹), although the initial nodularin concentration was 45 to 120-fold higher than in our study. Thus, the possibility that copepods took up dissolved nodularin has to be considered. It is important to point out, however, that nodularin is a hydrophobic compound, which easily attaches onto surfaces (Hyenstrand et al. 2001). If nodularin attaches to particles, it is thus likely that some of the nodularin was attached to the surface of the copepods.
Fig. 9. Average (± S.D.) clearance rate (ml ind\(^{-1}\) h\(^{-1}\)) of *Eurytemora affinis* on different plankton groups during Expt 1 (upper panel) and Expt 2 (lower panel). The differences between plankton communities in different size fractions are presented schematically. * The few large ciliates (>23 μm diameter) initially found in the <10 μm fraction were grazed to zero during the experiments. These bottles were excluded from the clearance and ingestion rate calculations; thus, the grazing for large ciliates is underestimated (redrawn from paper III).
rather than incorporated in their tissues. In such a case the amount of adsorbed nodularin could be quite constant, irrespective of the initial amount of dissolved nodularin. The toxin would not, however, interfere with the metabolism of the copepods and the degradation of the toxin would not occur in their tissues. The copepods would, nevertheless, act as a vector of adsorbed nodularin to higher trophic levels. To estimate the relevance of adsorption, dead and living animals should be incubated in the dissolved nodularin and their nodularin content compared.

The possibility that nodularin is attached onto particles should also be kept in mind when discussing the role of microbial organisms in nodularin transfer. The <0.2 and <3 μm fractions in our study only contained dissolved nodularin. However, the <0.2 μm fraction contained bacteria and the <3 μm fraction bacteria and nanoflagellates. Thus, part of the nodularin may have been bound to the microbial biomass at low concentrations, below the detection limit of the nodularin analysis. In Expt 2, copepods obtained as much nodularin in the <3 μm fraction as in the <10 μm fraction (Table 5). Hence, it is possible that part of the nodularin was accumulated through grazing on nanoflagellates in this fraction. The production of faecal pellets in the <3 μm fractions support this suggestion, because it was at the same level as in the <10 μm fractions (Fig. 10), although the clearance rates of heterotrophic nanoflagellates (HNF) and photosynthetic nanoflagellates (PNF) were low or negative (Fig. 9, E & I). Low clearance rates could be explained by the high growth potential of nanoflagellates, which probably masked feeding. For example, Uitto (1996) found that copepods are not efficient in removing nano-sized organisms. In her study, copepods were able to clear only 2 to 6 % of the nanoproteoan biomass daily. Therefore, we cannot exclude the possibility that some nodularin was vectored through these organisms to copepods. Thus, based on this study, it is not possible to exclusively conclude whether copepods obtained nodularin directly from the water, via adsorption or via ingestion of bacteria or nanoflagellates.

What could be the role of the microbial food web? Could copepods obtain part of the nodularin through feeding on microbial organisms? The answer is that we do not know. Unfortunately, we were unable to verify this pathway because we failed to remove cyanobacteria filaments from the smaller size fractions in Expt 1. Therefore, results gathered during Expt 2 will be highlighted here, since in this experiment toxic filaments were scarce in the <45 μm fraction and not present in the <10 μm or smaller fractions. To estimate the relative importance of the microbial food web in nodularin transfer, we compared the average amount of nodularin in copepods from the dissolved size fraction (<0.2 μm) with those from the other size fractions. Copepods that were incubated in the <45, <10 and <3 μm fractions obtained roughly 2 to 3 times more nodularin compared to the <0.2 μm fraction in Expt 2 (Table 5), although the differences were not statistically significant (ANOVA, \( p > 0.05 \)). However, these differences indicate that nodularin was probably transferred from other planktonic organisms to the copepods through grazing (Fig. 1, pathways 3 & 4).

Based on this study, dinoflagellates and ciliates could be the most promising candidates for nodularin transfer. The clearance rates on these organisms in the <150, <45, <20 and <10 μm fractions were the highest in both experiments (Fig. 9), and they were an important carbon source for copepods (Expt 1: dinoflagellates 33-64 %, ciliates 27-57 %; Expt 2: dinoflagellates 65-69 %, ciliates approximately 23 %). Grazing on
Dinoflagellates explained 47 % of the variance in the copepod nodularin content ($r^2 = 0.47$, $p = 0.01$) in Expt 1. Recent studies have revealed that increasing numbers of dinoflagellate species are mixotrophic, with the ability to utilise organic matter for their growth (reviewed by Hansen 1998, Ptacnik et al. 2004). Thus, they may be able to take up nodularin, and nodularin transfer via mixotrophic dinoflagellates consuming bacteria and small phytoplankton cannot therefore be excluded. However, further studies on this are required. In Expt 2, copepods selected ciliates (Dunnett’s test, $p < 0.05$ compared with α), and they were an important carbon source for the copepods. These results are important because ciliates graze on organisms at lower trophic levels, but also because they are able to take up nodularin directly in the dissolved form (Karjalainen et al. 2003). On the other hand, as pointed out earlier, nodularin may attach onto the surfaces of particles. This would mean that nodularin could be transferred through overall grazing on any particles.

5.2.2. Processes in the microbial food web: importance in nodularin loss and transfer

We found that copepods were able to remove particulate nodularin from the system. This was not apparent in the <150 μm fraction, where the relative change in the particulate nodularin concentrations was similar between the treatments with copepods and the controls in both experiments (Fig. 11). In the smaller size fractions, the presence of copepods lowered particulate nodularin concentrations compared to the controls (t-test, $p < 0.001$ for <20 and <45 μm fractions, Fig. 11). However, at the end of the experiments, the copepods contained less nodularin than the amount that had been lost. Up to 0.3 % of the initial particulate nodularin in Expt 1 and up to 6.5 % in Expt 2 was found in copepods (Table 5). Of the initial dissolved nodularin in the <3 and <0.2 fractions, up to 0.3 % ended up in copepods. Similarly, Kozlowsky-Suzuki et al. (2003) estimated that only 0.1 % of ingested nodularin was present in copepod tissues.
Part of the ingested nodularin was probably metabolized by the copepods (Karjalainen et al. 2006). It has been found that animals originating from areas with Nodularia spumigena blooms may have the ability to cope with ingested toxins (Kozlowsky-Suzuki et al. 2003). Consistently with this, nodularin had no direct adverse effects on the copepods, as observed in the studies with Prymnesium parvum, and their survival remained above 98%. Toxins with differing chemical composition clearly have variable modes of action in food webs. Some toxic species, such as *P. parvum*, which excretes toxic compounds into water, could harm organisms rather rapidly, using chemical defence to repel grazing (e.g. Granéli & Johansson 2003, papers I and II). On the other hand, if grazers are continuously able to feed on toxic species because they are not negatively affected, they could transfer toxins to other organisms. Copepods may also possess effective mechanisms, such as detoxification enzymes, to remove toxic compounds from their tissues (Sipiä et al. 2002, Kozlowsky-Suzuki et al. 2009). Therefore, the amount of nodularin that was transferred to copepods through ingestion was probably higher than we estimated. Due to the low toxin retention, a smaller proportion was detected in the copepods. However, although the proportion of nodularin in the copepods appears low, they have the potential to act as a link in the transfer of nodularin to planktivorous fish and mysids, because of the high feeding rates of these animals (Viherluoto & Viitasalo 2001, Karjalainen et al. 2005, this study).

Besides direct grazing, other loss factors are also involved in nodularin dynamics. Nodularin concentrations declined more during Expt 2 than during Expt 1 (Fig. 11). The conditions varied between these experiments. During Expt 1, the cyanobacterial community was at initial growths stage, while Expt 2 represented stationary conditions with decaying filaments. This discrepancy could explain the observed variation in nodularin dynamics between these experiments. Interactions in the microbial food web can be important in nodularin dynamics. There could therefore have been changes in the microbial community in our study that promoted degradation during the aging of the cyanobacterial communities.

Cyanobacterial toxins are considered intracellular, but are released into the water during cell decay. Cyclic cyanobacterial toxins, such as nodularin, are relatively stable due to their chemical structure, and are thus not easily degraded by physical factors such as light or temperature (e.g. Twist & Codd 1997, Mazur & Plinski 2001). However, biological factors, including sloppy feeding by copepods and processes in the surface community of the cyanobacterial cells, could lead to toxin release. For example, studies by Teegarden et al. (2003) suggested that regurgitation and sloppy feeding seem to be important mechanisms that release PSP toxins in a dissolved form, which are then available for uptake or degradation. Furthermore, it has been shown that specialized bacteria are responsible for the degradation of cyanobacterial toxins (Mazur & Plinski 2001, Hagström 2006). Bacteria can cause disruption of toxic cyanobacteria (Nakamura et al. 2003), and some bacterial strains are able to metabolize cyanotoxins such as the cyclic pentapeptide microcystin-LR and use it as a carbon and nitrogen source (Bourne et al. 1996). Furthermore, Lahti et al. (1998) isolated several bacterial strains from freshwater sediments and water that are able to degrade microcystins and nodularin.

Although bacteria are presumably essential in nodularin degradation, the role of other members of the microbial food web (e.g. ciliates, mixotrophic organisms, HNF)
in removing and transferring toxins is still largely unknown. In this study, ciliates were largely removed due to grazing by copepods. Bacterial growth rates, on the other hand, were strongly controlled by HNF, as has also been shown in other studies (e.g. Kuuppo-Leinikki 1990). This was indicated by the differences in the specific growth rates of bacteria in the <0.2 μm (no grazers) and <3 μm (small HNF) size fractions (0.5 and 0.3 d⁻¹ in Expt1; 0.5 and 0.2 d⁻¹ in Expt 2). Thus, some of the nodularin could have disappeared as a result of trophic interactions within the microbial food web.

As this study demonstrated, it is relatively easy to show that grazers accumulate toxins, but more challenging to follow the pathways of toxin transfer. According to this study, direct grazing on filaments of small *Nodularia spumigena* was an important pathway.
in nodularin transfer. Copepods also directly acquired nodularin from the dissolved pool, although the mechanism remained obscure. Whether the microbial food web was important in nodularin transfer is questionable. However, this study provided some indications that nodularin could be transferred to copepods via grazing on ciliates, dinoflagellates or HNF, and trophic cascades may actually be important in the transfer of algal toxins in aquatic environments.

5.2.3. Feeding behaviour of Eurytemora affinis and Acartia bifilosa on plankton communities containing Dinophysis spp.

The aim of this study was to test whether copepods feed on Dinophysis spp., and could thereby transport toxins to higher trophic levels in the food web. The rates of ingestion of Dinophysis spp. by E. affinis ranged from no grazing to 226 cells ind\(^{-1}\) d\(^{-1}\) (up to 0.5 \(\mu\)g C ind\(^{-1}\) d\(^{-1}\)). The ingestion rates of A. bifilosa feeding on Dinophysis spp. varied from 43 to 64 cells ind\(^{-1}\) d\(^{-1}\) (up to 0.14 \(\mu\)g C ind\(^{-1}\) d\(^{-1}\)). Although the ingestion rate varied irrespective of the Dinophysis spp. proportion in the planktonic community, Dinophysis formed a relatively large part of the copepod diet in situations where the food concentration was low (Fig. 12 B & C). Total ingestion rates increased with an increase in the food concentration (\(r^2 = 0.62, p = 0.007\)). Thus, in the low food experiments (Exp VII-X), ingestion accounted for only about 8-20% of the copepods’ carbon demand, but in the high food experiments (Exp I-VI) the ingestion rates corresponded to about 25-70% of the body carbon d\(^{-1}\). Heterocapsa triquetra formed the main part of the E. affinis diet at high food concentrations (Fig. 12 A), while at low food concentrations other food groups also contributed to the copepod’s diet (Fig. 12 B & C).

Neither of the copepod species selected Dinophysis spp., while H. triquetra and ciliates were the most preferred food in all experiments (Fig. 13). The feeding interactions between copepods and Dinophysis spp. have been shown to vary. Different studies have reported grazing rates that vary from no grazing up to 1 128 cells ind\(^{-1}\) d\(^{-1}\) (Maneiro et al. 2000, Jansen et al. 2006, Kozlowsky-Suzuki et al. 2006). The rates of Dinophysis spp. ingestion in our study (up to 0.5 \(\mu\)g C ind\(^{-1}\) d\(^{-1}\)) were at the lower end of those reported by Kozlowsky-Suzuki et al. (2006) for Acartia bifilosa feeding on D. norvegica (0.3 to 3.6 \(\mu\)g C ind\(^{-1}\) d\(^{-1}\), Baltic Sea). The low feeding rates on Dinophysis spp. at both high and low food concentrations suggest that grazing was not related to the relative concentration of Dinophysis spp. in the plankton community. Several criteria may be involved in food selection. Factors that contribute to selection have been presented earlier in the introduction (chapter 1.4). Both Eurytemora affinis and Acartia bifilosa are relatively small (adults 1.0-1.5 mm) compared to oceanic species such as Calanus helgolandicus, which have been found to feed efficiently on D. norvegica (Jansen et al. 2006). Acartia bifilosa is considered to be more selective compared to E. affinis because it is able to switch between filtering-type and more active ambush-type feeding modes (Kiørboe et al. 1996). However, in this study, the selection patterns of these two copepods were similar (Fig 13).

The dinoflagellate H. triquetra is considered to be high quality food for copepods. It is of a suitable size and known to support high egg production rates in copepods (Uye & Takamatsu 1990). Similarly, ciliates are a nutritionally adequate and suitable sized food for copepods (Stoecker & Egloff 1987, Klein
Breteler et al. 1999). However, it should be mentioned that opposite observations have also been reported. Ciliates cannot synthesize sterols and HUFA (highly unsaturated fatty acids), which are essential for copepods (Klein Breteler et al. 2004). Thus, the nutritional quality of ciliates may vary depending on their diet. Copepods selected *H. triquetra* and ciliates irrespective of their abundance in the community (Fig. 13). This implies that copepods selected food that was of high quality and of a suitable size, and hence easier to manage than the large *Dinophysis* spp. cells. Similarly, Jansen et al. (2006) suggested that *Dinophysis* cells may be simply too large to be grazed on by smaller species. Although *Dinophysis* spp. was not the preferred food for the copepods, the weight-specific ingestion rates were reasonably high in some experiments due to the large cell volume (Olenina et al. 2006).

Diarrhetic shellfish toxin (PTX2) was found from the copepods after incubation in two combined samples (Expt I & II: 142 pg ind⁻¹, Expt III & IV 53 pg ind⁻¹). The ingestion rate in the replicates in these experiments ranged from no grazing to 226 cells ind⁻¹ d⁻¹. Kuuppo et al. (2006) reported that PTX2 concentrations in the Baltic Sea area vary between 1.6 and 19.2 pg cell⁻¹. Based on their results, we calculated that the measured toxin content in the copepods corresponded to the ingestion of less than 100 cells. The detection of PTX2 in copepods suggests that the toxins were not instantaneously metabolized, as they usually are in filter-feeding shellfish such as mussels and scallops (e.g. Draisci et al. 1996, Suzuki et al. 2001). However, feeding on *Dinophysis* does not necessarily indicate digestion or toxin accumulation in the grazer. This assumption is supported by observations in which the ingested *Dinophysis* cells have remained intact in the faecal pellets (Maneiro et al. 2000, 2002, Wexels Riser et al. 2003).

Fig. 12. Average (± S.D.) weight-specific ingestion rates of copepods in (A) high food concentrations (Exp I-VI, *Eurytemora affinis*), (B) low food experiments (Exp VII & IX, *E. affinis*), and (C) low food experiments (Exp VIII & X, *Acartia bifilosa*). Experiments were pooled in order to compress the original number of figures. (Dinoph) *Dinophysis* spp., (Htri) *Heterocapsa triquetra*, (Auto) phototrophic dinoflagellates, (Cil) ciliates, (open bars) total ingestion (redrawn from paper IV).
The water column had settled in the trap material. These low estimates are in agreement with our results of low feeding rates on *Dinophysis* spp. cells by the copepods. Consistently with this, no DSTs were found from animals that were individually collected in a field study from the same area (Setälä, Sopanen, Autio, Erler, unpublished data). The high loss rate implies that toxins could have passed along the food web, decomposed in the sediment or transformed into other toxin derivatives (Kuuppo et al. 2006). In accordance with this, we found PTX-2 seco acid in zooplankton net samples at 9 of 13 locations in the northern Baltic Sea. However, weak positive signals of PTX-2 seco acid probably originated from disrupted *Dinophysis* spp. cells (Setälä, Sopanen, Autio, Erler, submitted manuscript). These observations imply that similar mechanisms to those involved in nodularin degradation (see chapter 5.2.2.) could also apply in the degradation of other toxins, such as those derived from toxic dinoflagellates.

On the basis of this study and the observations of other authors, it seems likely that copepods are an unimportant link in the transfer of DSTs in the northern Baltic Sea. On the other hand, at times when other food sources are scarce, or if copepods encounter a *Dinophysis* spp. bloom with high cell densities typical for the Baltic Sea (Kuosa 1990, Carpenter et al. 1995), these dinoflagellates may form a significant part of the copepod diet.

Since the faecal pellets were not examined in our experiments, it is not clear whether the *Dinophysis* cells were actually digested or simply passed through the copepods. Nevertheless, whether the copepods accumulate toxins or not, they may become toxic after ingesting toxin-containing cells, or produce toxic faecal pellets.

Kuuppo et al. (2006) examined the transfer of DSTs to sediments in the Gulf of Finland. Their sediment trap material contained PTX2 and DTX1. The authors suggested that copepod faecal pellets might be the source of these toxins, since no *Dinophysis* spp. cells or cell remnants were found. They estimated that only 0.01% of the PTX2 and 0.1% of the DTX1 that was found suspended in the water column had settled in the trap material.

These low estimates are in agreement with our results of low feeding rates on *Dinophysis* spp. cells by the copepods. Consistently with this, no DSTs were found from animals that were individually collected in a field study from the same area (Setälä, Sopanen, Autio, Erler, unpublished data). The high loss rate implies that toxins could have passed along the food web, decomposed in the sediment or transformed into other toxin derivatives (Kuuppo et al. 2006). In accordance with this, we found PTX-2 seco acid in zooplankton net samples at 9 of 13 locations in the northern Baltic Sea. However, weak positive signals of PTX-2 seco acid probably originated from disrupted *Dinophysis* spp. cells (Setälä, Sopanen, Autio, Erler, submitted manuscript). These observations imply that similar mechanisms to those involved in nodularin degradation (see chapter 5.2.2.) could also apply in the degradation of other toxins, such as those derived from toxic dinoflagellates.

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30 Tvärminne sea area, SW coast of Finland, Baltic Sea.

31 A metabolic product of PTX2 and produced, for instance, by shellfish that have been exposed to algae containing PTX2 (Suzuki et al. 2001).
6. SUMMARY AND CONCLUDING REMARKS

We found that algal species producing extracellular toxins could have acute negative effects on grazers, such as elevated mortality and suppressed feeding and egg production. In contrast, other algal toxins, especially those that grazers are able to accumulate, may not be potent enough to induce immediate adverse effects. However, they could have sublethal effects that may be seen, for example, as suppressed production after a longer time period due to low food quality. These changes may influence both species composition and production in the long term and affect the food availability of planktivorous organisms, such as fish. Grazers, in turn, could regulate HABs especially during bloom initiation and termination. Microzooplankton grazers could be more effective in this, but no comparative studies have been carried out in the Baltic Sea.

These results revealed that the ichtyotoxic haptophyte *Prymnesium parvum* has negative effects on copepods. Although not haemolytic, *P. parvum* filtrates clearly reduced the viability of copepods. In contrast, even though the monospecific *Prymnesium* treatments were haemolytic during the experiments, the toxicity was not reflected in the mortality of the copepods. The copepods in these treatments were, however, severely impaired and reacted slowly to mechanical disturbance. This effect was not due to starvation, as copepods in the FW control were active. Therefore, high mortality and inactivation might not be dependent on HA alone, but also on other unknown substances that *P. parvum* releases into the water. Thus, HA is not always a good proxy for algal toxicity. The results indicated that *P. parvum* is able to deter grazing. Due to the low feeding rates, suppressed pellet production and egg production rates were observed. Harmful effects on the well-being of potential grazers, together with severe allelopathic effects on other phytoplankton species, may accelerate the formation of *P. parvum* blooms and contribute to bloom persistence.

Nodularin was transferred to copepods through three pathways: by feeding on *Nodularia spumigena* filaments, by direct uptake from the dissolved pool, and via organisms in the microbial food web. According to this study, direct grazing could have been a relatively important pathway in nodularin transfer. However, we were unable to conclude whether the copepods obtained nodularin directly in the dissolved form, via adsorption or via ingestion of bacteria or nanoflagellates in the smallest fractions. Whatever the mechanism, copepods could still act as vectors of nodularin transfer to higher trophic levels. However, the role of the microbial food web remained unclear. While our results indicated that copepods obtained nodularin through grazing on microbial organisms, nodularin accumulation could have been a result of overall grazing on particles if the toxin had been adsorbed onto the particles. Nodularin was not so potent in either a particulate or dissolved form to deter grazers such as *P. parvum*, and no acute effects, such as high mortality, were detected. Thus, algal toxins of this kind have the potential to accumulate in grazers and further along the food web.

We found that copepods were able to remove nodularin from the system. However, only a small proportion of the initial nodularin was channelled into the copepods, while most of the toxin disappeared. It may have partly been metabolized by the copepods, but several other processes might also have been responsible for the degradation of nodularin. This implies that processes in the microbial food web, such as bacterial degradation,
probably contribute to the biodegradation of toxins. Because nodularin is chemically closely related to microcystins, which are produced, for example, by the freshwater species *Microcystis* and *Anabaena*, they probably share common mechanisms of toxin transfer. Thus, trophic cascades could also be important in the transfer of cyanobacterial toxins in freshwater environments.

Neither of the copepod species in our experiments selected the dinoflagellates *Dinophysis* spp., which may simply be too large to be grazed on by these smaller copepod species. Instead, the dinoflagellate *Heterocapsa triqueta* and ciliates were the preferred food in all experiments. This implies that copepods selected food that was of high quality and a suitable size. However, in experiments where the food concentration was low, the large *Dinophysis* spp. cells formed a relatively large component of the copepod diet. PTX2 toxin was found in the animals after the incubations. However, no DSTs have been detected in field-collected animals from the same area. According to these observations, copepods seem to be an unimportant link in the transfer of DSTs in the Baltic Sea.

This study examined the interactions between copepods and toxic algae. Comparisons with various other studies, especially when considering *P. parvum* – copepod interactions, revealed that the tolerance of algal toxins, as well as feeding and reproduction responses, are species-specific. Moreover, dissimilar responses due to different algal toxins, such as *P. parvum*-derived substances and nodularin or DSTs, could shape zooplankton communities in different ways. A *P. parvum* bloom could have detrimental effects on a large part of the community if water turbulence is low. However, nodularin and DSTs could be rather harmless to grazers, although due to their transport along the food web they may harm organisms at higher trophic levels. Despite the variable results, experimental studies provide valuable information on potential interactions between grazers and harmful algal species, leading to better understanding of the importance of grazers in the regulation of harmful algal blooms, and processes that control the accumulation and degradation of algal toxins.
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