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The role of copepods and heterotrophic dinoflagellates in the production of dissolved organic matter and inorganic nutrients

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**THE ROLE OF COPEPODS AND HETEROTROPHIC DINOFLAGELLATES
IN THE PRODUCTION OF DISSOLVED ORGANIC MATTER
AND INORGANIC NUTRIENTS**

A Dissertation

Presented to

The Faculty of the School of Marine Science

The College of William and Mary in Virginia

In Partial Fulfillment

of the Requirements for the Degree of

Doctor of Philosophy


by

Grace Kathleen Saba

2009

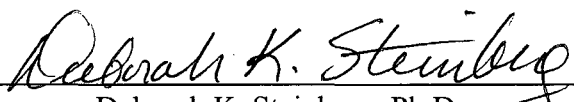
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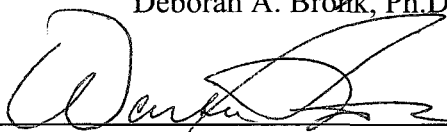
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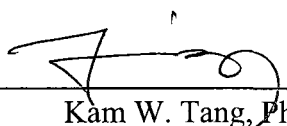
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DEDICATION

I dedicate this dissertation to my husband, Vince, for his remarkable patience and unwavering love and support over the course of my research, and also for the countless days of daddy daycare to our beautiful son, Dean, during my final months of writing.

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ABSTRACT

Zooplankton play a key role in the cycling of dissolved organic matter (DOM) and inorganic nutrients. The factors that affect these processes, however, are not fully understood. I measured the effects of various diets on DOM and inorganic nutrient production by the copepod *Acartia tonsa* and the heterotrophic dinoflagellate *Oxyrrhis marina*, and explored the mechanisms of nutrient release from copepods. Copepods feeding on a mixed diet, the preferred diet of most copepods, had significantly lower dissolved organic carbon (DOC), ammonium (NH_4^+), and total dissolved nitrogen (TDN) release rates compared to feeding on a carnivorous or herbivorous diet. Thus, copepod feeding strategy can control the magnitude and composition of regenerated nutrients supplied to bacteria and phytoplankton. Secondly, I determined the effects of non-bloom and bloom concentrations of non-toxic and toxic cultures of harmful algal bloom (HAB) species *Prorocentrum minimum* and *Karlodinium veneficum* on grazing and production of DOM and inorganic nutrients by *A. tonsa* and *O. marina*. All algal diets deterred grazing, which likely resulted in starvation and subsequent catabolism of grazer body tissue. Additionally, DOM was typically a higher proportion of total dissolved nutrients released by zooplankton while feeding on the toxic algal culture, suggesting algal nutrient quality or direct toxic effects played a role in the differential nutrient release. Low ingestion rates coupled with high nutrient release rates could lead to feedback mechanisms that could intensify HABs. Finally, the various mechanisms of *A. tonsa* nutrient release, including sloppy feeding, excretion, and fecal pellet leaching, were isolated. Excretion and sloppy feeding were the dominant modes of DOC and NH_4^+ release, while sloppy feeding and fecal pellet leaching were dominant modes of urea release. A large proportion of ingested PON was lost as dissolved NH_4^+ and urea from copepods via all release mechanisms. These results have implications for the rapidity and location at which the regenerated nutrients are recycled in the water column. My dissertation results emphasize the importance of diet and release mechanism on the production of nutrients, particularly DOM, by zooplankton, which are important in understanding the recycling and transfer of nutrients and organic matter in marine food webs.

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AUTHOR'S NOTE

The primary research chapters of this dissertation were written in the format of the respective journal under which each is either published, in review, or to be submitted. Therefore, these chapters were written in the third person to represent my co-authors. The citations for the chapters are as follows:

Chapter 2

Saba, G.K., Steinberg, D.K., Bronk, D.A., 2009. Effects of diet on release of dissolved organic and inorganic nutrients by the copepod *Acartia tonsa*. ***Marine Ecology Progress Series*** 386, 147-161.

Chapter 3

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Chapter 4

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*additional coauthors to be determined

**THE ROLE OF COPEPODS AND HETEROTROPHIC DINOFLAGELLATES
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CHAPTER 1

Introduction

Understanding the mechanisms that influence carbon and nutrient cycling in estuarine and coastal systems is of growing importance, particularly if we are to fully understand anthropogenic influences such as global climate change and eutrophication on ecosystem functioning. Zooplankton play a key role in the cycling of dissolved organic and inorganic material and the flux of particulate organic matter from the surface to deeper waters (Steinberg et al. 2000, 2002, Carlson 2002, Schnetzer & Steinberg 2002); thus, dissolved organic matter (DOM) and inorganic nutrient release by zooplankton are important in understanding the recycling and transfer of nutrients and organic matter in marine food webs. Research on plankton dynamics and nutrient cycling has largely focused on cycling of inorganic nitrogen and phosphorus and flux of particulate organic carbon and nitrogen. However, dissolved organic carbon (DOC), dissolved organic nitrogen (DON), and dissolved organic phosphorus (DOP), make up the bulk C, N, and P, respectively, in marine systems (Nagata & Kirchman 1992). Additionally, reactivity in one pool may alter other dissolved pools, ultimately affecting nutrient recycling in the water column. Only a handful of studies, however, have investigated the importance of mesozooplankton (copepods) and microzooplankton (protozoan ciliates and flagellates) in the cycling of DOM.

Zooplankton nutrient cycling

Mesozooplankton grazing and metabolic processes release DOM and inorganic nutrients into the surrounding water (Lampert 1978, Møller 2007), which fuels the microbial loop (Azam et al. 1983; Møller & Nielson 2001). The subsequent bacterial

activity can convert DOM into bioavailable inorganic nutrients (NH_4^+ , PO_4^{3-}) and CO_2 , which can be taken up by phytoplankton. These processes are not exclusive to the euphotic zone, as DOM actively transported via excretion by diel vertically migrating zooplankton could be utilized by deep-sea microbial communities (Steinberg et al. 2000, 2002, 2008).

The rate of DOM release by zooplankton likely exceeds that by phytoplankton (Jumars et al. 1989, Strom et al. 1997). For example, zooplankton grazers released 16-37% of algal cell total C content as DOC, compared to only 3-7% DOC release as direct exudation from the algal cell (Strom et al. 1997). While crustacean zooplankton are considered to be primarily ammonotelic, releasing ammonium (NH_4^+) as a metabolic byproduct (Bidigare 1983), DON can also be a significant proportion of the total N released by both crustacean (Miller & Glibert 1998, Miller & Roman 2008) and gelatinous (Condon et al. in press) zooplankton. This excreted DON is also more biologically available than previously thought, and may be taken up and utilized by bacteria and phytoplankton, including toxic species, during both N-limited and eutrophic conditions (Granéli et al. 1999; Glibert et al 1991; Mulholland & Capone 1999). Miller & Glibert (1998) found *Acartia tonsa* copepod DON excretion (urea and dissolved primary amines, DPA) was between 62 and 89% of total N excreted in mesocosm experiments. While only a handful of studies have focused on DOC or DON production from grazing processes, even less is known about DOP production. An early study showed that up to 74% of the total P released was labile DOP (Hargrave & Geen 1968). More recently the importance of copepod feeding activity on the release of bioavailable DOP (as deoxyribonucleic acid, DNA) has been demonstrated (Titelman et al. 2008).

Nutrient release rates, and the chemical composition of the nutrients produced, may be affected by a number of factors, including ingestion rates (Corner et al. 1976, Kiørboe et al. 1985) as well as the type and quality of the food source (Caron & Goldman 1990, Gismervik 1997, Strom et al. 1997, Elser & Urabe 1999, Besiktepe & Dam 2002, Frost et al. 2004, Mitra & Flynn 2007, Miller & Roman 2008, Saba et al. 2009, Ch. 3). Many crustacean zooplankton are omnivorous or carnivorous, and in many conditions, such as low phytoplankton biomass or poor algal quality, microzooplankton contribute a significant portion of their diet (Stoecker & Capuzzo 1990, Fessenden & Cowles 1994, Merrell & Stoecker 1998, Broglio et al. 2004). Additionally, recent studies show that some zooplankton can graze on harmful algal species and therefore may suppress the formation of harmful algal blooms (HABs) (Mallin et al. 1995; Hamasaki et al. 2003, Dam & Colin 2005, Roman et al. 2006, Breier & Buskey 2007, Colin & Dam 2007). Other studies show that zooplankton either avoid or are adversely affected by feeding on toxic phytoplankton (Huntley et al. 1986, Carlsson et al. 1995, Colin and Dam 2003, Kozlowsky-Suzuki et al. 2003, Vaqué et al. 2006, Cohen et al. 2007). These complex interactions between zooplankton and HAB species can affect zooplankton grazing and reproduction (Sunda et al. 2006), and may ultimately affect zooplankton nutrient regeneration. Little is known, however, about how diet or the influence of HAB species affect zooplankton metabolic processes, including the release of dissolved inorganic nutrients and DOM.

Microzooplankton (e.g., ciliates and flagellates), which graze on bacteria and phytoplankton, also play a significant role in DOM production due to their high rates of growth, feeding, and excretion, and overall high nutrient turnover rates (Andersson et al.

1985, Caron et al. 1985, Goldman et al. 1985, Andersen et al. 1986, Nagata & Kirchman 1992, Gaul et al. 1999, Nagata 2000, Strom 2000). For example, Taylor et al. (1985) found that microzooplankton released 3-88% of ingested carbon as DOC. Using a sensitive ^{15}N -label technique, Hasegawa et al. (2000) found micrograzer DON release accounted for 59% of NH_4^+ regeneration rates. Furthermore, microzooplankton grazers excreted 15-70% of total phosphorus as DOP (Andersen et al. 1986). However, we still know comparatively little about DOM and inorganic nutrient release from microzooplankton, and measurements on the biochemical composition of marine protozoans are limited.

Mechanisms of zooplankton DOM and inorganic nutrient release

Crustacean zooplankton release DOM and inorganic nutrients via sloppy feeding, excretion, and fecal pellet leaching (Lampert 1978, Møller 2007). Most previous zooplankton feeding and nutrient release studies do not differentiate between nutrient release processes (Miller & Glibert 1998; Isla et al. 2004; Saba et al. 2009), and the few that have, measured only sloppy feeding or fecal pellet leaching. Furthermore, a majority of these studies measured release of DOC (Lampert 1978; Møller & Nielson 2001, Møller et al. 2003, Møller 2007), while only few have measured DON release (Roy et al. 1989; Vincent et al. 2007). Nonetheless, these studies show that sloppy feeding and fecal pellet leaching can generate significant amounts of DOM.

The amount of organic and inorganic material transported from the surface to deep waters is dependent upon the mechanism of release. Products of sloppy feeding and

excretion will likely be recycled quickly in the euphotic zone, while sinking fecal pellets have the potential to transfer particulate and dissolved organic material below the euphotic zone. Separating DOM and inorganic nutrient production by different zooplankton-mediated release mechanisms is important in accurately determining the DOM supply to bacteria and the microbial loop in surface and deep waters, and the amount of nutrition available for transfer to higher trophic levels.

Significance of zooplankton-mediated nutrient cycling

Now that evidence leads us to believe a principal pathway of DOM and inorganic nutrients from phytoplankton to bacteria is via by-products of zooplankton feeding and metabolism (Lampert 1978; Jumars et al 1989, Carlson 2002), it is pertinent to understand the role of zooplankton nutrition on the conditions and magnitude of this release, as well as the various mechanisms of release, because changes in the sources and sinks of released nutrients may significantly influence other nutrient pools. Additionally, determining the stoichiometry of released C, N, and P is vital to understand how these pools are coupled.

Structure of dissertation

This dissertation is separated into three main chapters (2-4) and presents results from laboratory experiments examining release rates and composition of nutrient release from copepods and heterotrophic dinoflagellates. Chapters 2 and 3 explore the effects of various diets on zooplankton dissolved organic matter and inorganic nutrient production, and Chapter 4 explores the mechanisms of nutrient release from copepods.

In Chapter 2, I discuss the effects of an exclusively carnivorous diet, and exclusively herbivorous diet, and a mixed omnivorous diet on the release of dissolved organic and inorganic nutrients by the copepod *Acartia tonsa*. I also discuss the impacts of different release rates and different release ratios of dissolved carbon, nitrogen, and phosphorus.

In Chapter 3, I discuss the effects of harmful algal species and food concentration on release of dissolved organic matter and inorganic nutrients by two grazers, the copepod *Acartia tonsa* and the heterotrophic dinoflagellate *Oxyrrhis marina*. The impacts of grazer deterrence, starvation, algal nutrient quality, and direct toxic effects on grazer nutrient production are discussed.

In Chapter 4, the various nutrient release mechanisms of *Acartia tonsa* copepods, including sloppy feeding, excretion, and fecal pellet leaching, are examined. The relative importance of these modes to DOC, NH_4^+ , and urea release are estimated, and the impacts these various modes have on nutrient cycling and transfer to higher trophic levels are discussed.

Finally, in Chapter 5, I conclude with a summary of my results and how they contribute to our knowledge of nutrient cycling in estuarine and coastal systems. I propose directions for future research to further increase our understanding of the central role zooplankton play in consuming lower trophic levels, providing nutrition to higher trophic levels, and regenerating nutrients available for bacteria and phytoplankton.

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CHAPTER 2

Effects of Diet on Release of Dissolved Organic and Inorganic Nutrients by the Copepod *Acartia tonsa*

ABSTRACT

Acartia tonsa copepods are not limited to herbivory and can derive up to half their daily ration from predation on heterotrophic ciliates and dinoflagellates. The effects of an omnivorous diet on nutrient regeneration, however, remain unknown. In this study, we fed *A. tonsa* an exclusively carnivorous diet of either (1a) heterotrophic dinoflagellate *Oxyrrhis marina* or (1b) *Gyrodinium dominans*, (2) an exclusively herbivorous diet of *Thalassiosira weissflogii* diatoms, or (3) a mixed omnivorous diet. We measured the release rate, composition, and stoichiometry of dissolved organic carbon (DOC), dissolved organic phosphorus (DOP), and nitrogen (urea) in addition to the inorganic nutrients ammonium (NH_4^+) and phosphate (PO_4^{3-}). Despite similar ingestion rates among treatments, as well as similar C:N ratios of food items, *A. tonsa* release rates of DOC and NH_4^+ were highest while feeding on a carnivorous diet and lowest while feeding omnivorously. In contrast, urea, on average, was a higher portion of total nitrogen released in the mixed diet treatment (32 to 59%). DOP release rates were only detectable in diets containing microzooplankton prey. Our results suggest that copepod diet plays an important role in determining the quantity and composition of regenerated C, N, and P available to phytoplankton and bacteria. Additionally, the uncoupling of ingestion and nutrient release rates and the variability in released ratios of dissolved C:N:P in our study suggests that stoichiometric models based solely on predator and prey C:N and N:P ratios may not be adequate in determining stoichiometry of total nutrient release.

INTRODUCTION

Consumption of different food resources by zooplankton not only affects their growth and reproduction, but also helps structure planktonic communities and potentially controls biogeochemical cycling of various elements. It is well known now that many planktonic crustacean species are not limited to herbivory and will also consume other zooplankton or detritus (reviewed in Steinberg & Saba 2008). Mesozooplankton typically have higher clearance rates for heterotrophic protozoans compared to phytoplankton (Stoecker & Capuzzo 1990, Fessenden & Cowles 1994, Merrell & Stoecker 1998, Broglio et al. 2004). For instance, the copepod *Acartia tonsa* was found to derive 3 to 52% of its daily ration from predation on ciliates and dinoflagellates >10 μm in a subtropical estuary (Gifford & Dagg 1988, Stoecker & Capuzzo 1990), and some copepods feed solely on microzooplankton during periods of relatively low phytoplankton biomass (Fessenden & Cowles 1994). Protozoan diets may enhance growth and survival of predators and also increase egg production most likely due to their typically lower carbon:nitrogen (C:N) ratios and higher levels of essential nutrients such as polyunsaturated fatty acids (PUFAs including eicosapentaenoic acid [EPA] and docosahexaenoic acid [DHA]), sterols, and amino acids compared to phytoplankton (Stoecker & Egloff 1987, Stoecker & Capuzzo 1990, Gifford 1991). Some microzooplankton species, such as the heterotrophic dinoflagellates *Oxyrrhis marina* and *Gyrodinium dominans*, are important for trophic upgrading, possessing the ability to synthesize EPA, DHA, and sterols from low quality algae and thus enhancing the transfer of essential nutrients through the microbial food web from phytoplankton to

mesozooplankton (Klein Breteler et al. 1999, Tang & Taal 2005). While we now know the importance of protozoans in copepod diets, little is known about how carnivorous or omnivorous diets affect metabolic processes, including the release of dissolved inorganic nutrients and dissolved organic matter (DOM) that support phytoplankton and bacterial growth and fuel the microbial loop.

Mesozooplankton contribute to nutrient release via sloppy feeding (the physical breaking of the food source), excretion, egestion, and subsequent fecal pellet leaching (Møller 2007). In our study we did not differentiate between these modes of nutrient production; thus, our reported copepod 'release rates' incorporate nutrient production from all of these modes. While crustacean zooplankton are considered to be primarily ammonotelic, releasing ammonium (NH_4^+) as a metabolic byproduct (Bidigare 1983), organic N can also be a significant proportion of the total N released by zooplankton. For example, organic N excretion (urea and dissolved primary amines, DPA) by *Acartia tonsa* copepods was between 62 and 89% of total N excreted in mesocosm experiments (Miller & Glibert 1998). Additionally, the rate of DOM release by zooplankton likely exceeds that directly released by phytoplankton (Jumars et al. 1989). Strom et al. (1997) found that zooplankton grazers release 16 to 37% of an algal cell's total C content as dissolved organic carbon (DOC) compared to only 3 to 7% DOC release as direct exudation from algal cells. Studies measuring phosphorus (P) release by zooplankton are scarce and few report dissolved organic phosphorus (DOP) release, which can be readily available to phytoplankton and bacteria (Hargrave & Geen 1968, Titelman et al. 2008). A recent study demonstrated the importance of copepod feeding activity on the release of bioavailable DOP (as deoxyribonucleic acid, DNA) (Titelman et al. 2008). In another

study, up to 74% of total P released was DOP (as opposed to inorganic phosphate, PO_4^{3-}) and was readily available to bacteria (Hargrave & Geen 1968).

Nutrient release rates, and the chemical composition of the nutrients produced, may be affected by a number of factors. In many studies, higher ingestion rates are correlated with higher zooplankton excretion rates (Corner et al. 1976, Kiørboe et al. 1985). Additionally, copepods have variable functional responses to different prey items (Besiktepe & Dam 2002, Mitra & Flynn 2007), potentially causing differential release of byproducts. Zooplankton elemental composition regulates the elemental ratio of nutrients released; thus, a change in the zooplankton taxa or food source may cause a change in the excreted nutrient quantity and composition (Caron & Goldman 1990, Gismervik 1997a, Strom et al. 1997, Elser & Urabe 1999). For example, a consumer with low N and high P body content feeding on prey with high N and low P content will retain the necessary P and excrete more N. Conversely, a consumer feeding on N-limited food would retain the needed N and excrete more P (Sterner 1990, Touratier et al. 2001). Additionally, the composition of N and P released can be indirectly affected by feeding strategy. For example, Corner et al. (1976) showed that NH_4^+ was a higher portion of the total N released while copepods were feeding carnivorously. In contrast, Bidigare (1983) suggested that herbivores may be expected to excrete more urea than carnivores, as the conservation of arginine (a precursor of urea) is higher in marine phytoplankton than in zooplankton. However, this has not been supported by laboratory experiments, as *Acartia tonsa* urea excretion rates were higher when feeding on ciliates compared to diatoms, and these excretion rates increased with decreasing food C:N (Miller & Roman 2008).

Nearly all copepod feeding experiments that measure nutrient excretion have been conducted with phytoplankton as food. Only 2 studies (Strom et al. 1997, Miller & Roman 2008) have investigated DOM release by copepods feeding carnivorously on microzooplankton. Strom et al. (1997) measured DOC production, and Miller & Roman (2008) measured the forms of N released. With the exception of 1 study using the freshwater grazer *Daphnia* (Frost et al. 2004), no studies have measured simultaneous C, N, and P release from marine zooplankton, nor how release of dissolved organic (DOC, DON, DOP) and inorganic nutrients are related. Additionally, no previous nutrient-release studies have included an omnivorous diet, the feeding strategy of most copepods. Thus, we know little about the effects of microzooplankton or mixed diets on the stoichiometry of regenerated nutrient pools. In the present study, we determined the effects of herbivorous, omnivorous, and carnivorous feeding by *Acartia tonsa* copepods on the release rate of dissolved organic C, N, and P and inorganic nutrients, ammonium and phosphate. We also explored the stoichiometry of excretion, as well as the composition of the excreted N and P.

Understanding the role of zooplankton nutrition on the conditions and magnitude of DOM release is pertinent, because changes in the sources and sinks of marine DOM may significantly influence other nutrient pools. Additionally, determining the stoichiometry of released C, N, and P is vital to understand how these pools are coupled.

MATERIAL AND METHODS

Collection and culture of organisms. *Acartia tonsa*, a common coastal omnivorous calanoid copepod, were collected from the York River, USA, a tributary of Chesapeake Bay, by near-surface net tows (0.5 m diameter net, 200 μ m mesh, non-filtering cod end). Copepods for the 2 experiments were collected 5 d apart, but from the same location and during the same tidal cycle. Upon collection, healthy, active *A. tonsa* were placed in 0.2 μ m filtered seawater for 1 to 2 h until the start of the acclimation period (see below). The mean size of adult *A. tonsa* was determined from 50 randomly selected individuals from the tow for which we measured cephalothorax width and total body length (from the top of the head to the base of the caudal rami) under an Olympus SZX12 dissecting scope at 230x magnification.

Two common estuarine heterotrophic dinoflagellates were used as prey items for *Acartia tonsa*: *Oxyrrhis marina* and *Gyrodinium dominans* (both isolated from Narragansett Bay). Both microzooplankton species are readily ingested by *A. tonsa* copepods (Tang & Taal 2005). Dinoflagellate cultures were maintained in F/2 medium (20‰ salinity) prepared with the 0.2 μ m filtered seawater (FSW) used in the experiment. The FSW consisted of a 1:1 ratio of deep Santa Barbara Channel seawater (SBSW) to artificial seawater (ASW) made with sodium chloride combusted at 500°C for 2 h to remove organics. ASW was used in order to start the experiments with a low background of DOM (Protocols for the Joint Global Ocean Flux Study [JGOFS] Core Measurements 1994), and it was combined with low DOM, deep SBSW, to prevent the copepods in the experiments from becoming lethargic, as has been noted for 100% ASW (Strom et al.

1997). The final seawater mixture had DOC and total dissolved nitrogen (TDN) concentrations 23 and 2 $\mu\text{mol l}^{-1}$, respectively. The cultures were incubated at 20°C in the dark. Both *O. marina* and *G. dominans* were maintained on a diet of the chlorophyte *Dunaliella tertiolecta* (CCMP 1320). The experiments were conducted once the dinoflagellate cultures reached the early stationary phase, when protozoan cell abundance was maximum and algal food was minimum (Tang & Taal 2005). The diatom prey, *Thalassiosira weissflogii* (CCMP 1336), was chosen as the food alga in our experiments due to its similar size to *O. marina* and *G. dominans*. These cultures were grown on F/2 + Si medium made with 20‰ FSW, incubated at 20°C on a 12 h light:12 h dark regime, and maintained in exponential phase by diluting with medium every 3 to 4 d. The length and width of the food items were measured after the experiment on a Nikon DIAPHOT-TMD inverted microscope at 600x magnification (fixed in 2% Lugol's solution). Cell volumes were calculated according to geometric cell shapes (*T. weissflogii*, cylinder; heterotrophic dinoflagellates, prolate ellipsoid). Cell volumes were corrected for fixative shrinkage after Montagnes et al. (1994) for diatoms, and using athecate dinoflagellate shrinkage estimates for *O. marina* and *G. dominans* from Menden-Deuer et al. (2001).

Experimental procedure. To examine the impact of diet on *Acartia tonsa* ingestion and nutrient release, 3 food categories were used: (1) exclusively microzooplankton/carnivorous diet (μZ), (2) exclusively diatom/herbivorous diet (DIATOM), and (3) mixed omnivorous diet (MIX) in microzooplankton and diatoms each contributed 50% to the food carbon. Food C contents were estimated from volume measurements made prior to the start of the experiments using cell C to volume conversions from Menden-Deuer & Lessard (2000) for heterotrophic dinoflagellates

Oxyrrhis marina and *Gyrodinium dominans* and from Dam & Lopes (2003) for *Thalassiosira weissflogii* diatoms. Two experiments were conducted using heterotrophic dinoflagellates as microzooplankton prey items, Expt A (*O. marina*) and Expt B (*G. dominans*). Both experiments used the diatom *T. weissflogii*.

Twenty-four hours prior to experimental incubations, freshly collected adult copepods were individually transferred from beakers into 3 separate 3.5 l bottles, each with FSW and the appropriate food items for the μ Z, DIATOM, and MIX food categories, to a final concentration of 60 copepods l^{-1} , which is near the maximum concentration that occurs in Chesapeake Bay (CBP 2000) and the lowest concentration for which we could detect nutrient release in preliminary trials with varying copepod densities and incubation times. Food items were standardized to 300 μ g C l^{-1} , a food density at which *Acartia tonsa* shows maximum ingestion rates on *Thalassiosira weissflogii* and *Oxyrrhis marina* (Besiktepe & Dam 2002), using the size to C conversion factors noted above. Food C was never depleted to <30% of the initial food concentration in any of the experiments. All bottles were topped off with FSW, covered with parafilm to remove bubbles, capped, and placed on a rotating wheel in the dark at 1 rpm for 24 h, similar to acclimation times used in other copepod feeding studies (Merrell & Stoecker 1998, Tang et al. 2001).

At the end of the food acclimation period for each experiment, 12 incubation bottles (300 ml) each were taken for the carnivorous, herbivorous, and mixed diet. Each set included 6 controls (FSW + food) and 6 treatments (FSW + food + copepod predators). All bottles were set up the same way as the acclimation bottles. For each of the sets, 3 controls and 3 treatments were set aside for initial sample collection.

Remaining bottles were incubated as in the acclimation period. A suite of samples was taken initially and at the end of the 24 h incubation.

Sample analyses. *Bacteria nutrient uptake:* Because bacteria can utilize both DOM and inorganic nutrients, we accounted for their potential uptake during experimental incubations in our copepod release rate calculations. Samples for bacterial enumeration were fixed with formaldehyde (final conc. 2%), stained with 4',6-diamidino-2-phenylindole (DAPI; final conc. 0.005%), filtered onto 0.2 μm black polycarbonate filters with 0.45 μm cellulose backing filters, and slide mounted according to Sherr et al. (1983). For each sample, cells in 10 viewing fields were counted on a Nikon Eclipse 80i epifluorescent microscope at 1000x magnification. Using bacterial abundance data, we calculated an average concentration of bacteria, [C], as defined by Frost (1972). Separate samples were taken for bacterial production measurements using the [^3H]-leucine uptake method (Azam et al. 1983, Kirchman & Ducklow 1993). Assuming a bacterial growth efficiency (BGE) of 50% (Azam et al. 1983), the bacterial C demand (BCD, $\text{ng C l}^{-1} \text{h}^{-1}$) was estimated for each incubation bottle using Eq. (1a). We calculated potential daily bacterial DOC uptake (U, $\text{ng C l}^{-1} \text{d}^{-1}$) during the grazing experiments using Eq. (1b), such that:

$$\text{BCD} = \frac{\text{BP} * 3.1}{\text{BGE}} \quad (1a)$$

$$\text{U} = \text{BCD} * \text{T} \quad (1b)$$

where BP is bacterial production ($\text{pmol leucine l}^{-1} \text{h}^{-1}$), 3.1 is the conversion from picomoles of leucine to nanograms of C, and T is incubation time (24 h d^{-1}).

Additionally, using conservative estimates of bacterial molar C:N (4.5; Goldman & Dennett 1991) and C:P (50; Kirchman 2000), we estimated maximum potential N and P uptake, respectively. Because bacteria can utilize both organic and inorganic N, we assumed 16% of the N uptake source was organic urea (calculated from Table 1 in Andersson et al. 2006) and 84% was inorganic NH_4^+ . Bacteria can utilize DOP under certain conditions (Titelman et al. 2008); however, inorganic PO_4^{3-} is their preferred P substrate (Cotner & Wetzel 1992, Kirchman 2000). Because PO_4^{3-} was available in our incubation bottles, we assumed 100% of the P source was inorganic and did not correct DOP release for bacterial uptake.

Feeding rates: Whole-water samples for algal and protozoan cell counts were preserved with acid Lugol's solution (final conc, 2%). Subsamples for algal cell counts were settled in 1 ml Sedgewick rafters, and 5 replicate frames each of at least 100 cells were counted with a Nikon DIAPHOT-TMD inverted microscope at 600x magnification. Subsamples (2 to 5 ml) for protozoans were settled in 5 ml Utermöhl settling chambers, and entire contents (100 cells or more) were counted under an inverted microscope after at least a 24 h settling period (Utermöhl 1931; Hasle 1978). Clearance and ingestion rates of *Acartia tonsa* on both algae and microzooplankton were calculated according to Frost (1972). The possible ingestion of diatoms by the heterotrophic dinoflagellates in the MIX treatment was examined by monitoring the abundance of diatoms over the incubation time in the control bottles. *Thalassiosira weissflogii* concentration in the MIX controls remained constant over the incubation, similar to *T. weissflogii* in the DIATOM controls. This suggests no significant grazing occurred by heterotrophic dinoflagellates in the MIX treatments.

Nutrient analyses: After bacterial production and all abundance samples were collected, the remaining volume from each bottle was prescreened through a 200 μm sieve (to retain copepods in treatments; controls were treated the same) directly into 2 filter towers and filtered through combusted GF/F filters into acid-cleaned, combusted flasks. One GF/F filter was collected for fluorometric chlorophyll analysis (Parsons et al. 1984). The second filter was collected for particulate carbon (PC) and particulate nitrogen (PN) (carbon-hydrogen-nitrogen elemental analyzer, EA1108). The collected copepods, which were all alive and active after incubation, were filtered onto a combusted GF/F, counted under a dissecting scope (Olympus SZX12), and analyzed for PC and PN content. The remaining filtrate for each replicate was analyzed for organic and inorganic nutrient concentrations: DOC, Shimadzu TOC analyzer 5000A (minimum detection limit [MDL] = 0.5 to 1.0 $\mu\text{mol l}^{-1}$) after acidification and purging of dissolved inorganic carbon (Peltzer et al. 1996); ammonium, phenol/hypochlorite Koroleff method with MDL = 0.05 $\mu\text{mol l}^{-1}$ (Grasshoff et al. 1983); urea, diacetyl monoxime procedure with MDL = 0.05 $\mu\text{mol l}^{-1}$ (adapted from Price & Harrison 1987); DPAs, fluorescent O-phthalaldehyde (OPA) method with MDL = 0.05 $\mu\text{mol l}^{-1}$ (Parsons et al. 1984); nitrate and nitrite (NO_x ; Grasshoff method) (MDL = 0.05 $\mu\text{mol l}^{-1}$), phosphate (PO_4^{3-} ; Koroleff method) (MDL = 0.05 $\mu\text{mol l}^{-1}$), and TDN and TDP (persulfate oxidation; MDL = 1.0 $\mu\text{mol l}^{-1}$), were determined with a QuikChem 8500 AutoAnalyzer (Grasshoff et al. 1983, Bronk et al. 2000, Sharp 2002). Concentrations of bulk DON and DOP were calculated by the difference between TDN and inorganic N ($\text{NO}_x + \text{NH}_4^+$) and TDP and PO_4^{3-} , respectively. Copepod release rates (in $\text{ng ind.}^{-1} \text{h}^{-1}$) were calculated according to Miller & Glibert (1998), but modified to include bacterial uptake, such that:

$$\frac{[(\Delta C_t + U_t) - (\Delta C_c + U_c)] \times V}{(N \times T)} \quad (2)$$

where ΔC_t is the change in nutrient concentrations ($\text{ng l}^{-1} \text{d}^{-1}$) in the treatment bottles and ΔC_c is the average change in nutrient concentrations ($\text{ng l}^{-1} \text{d}^{-1}$) in the control bottles; U_t and U_c are estimated values of bacterial uptake ($\text{ng l}^{-1} \text{d}^{-1}$) in the treatment and control bottles (see Eq. 1b); V is the incubation volume (l), N is the number of copepods in the treatment bottles, and T is incubation time (24 h d^{-1}).

Statistical analysis. Statistical comparisons of the effects of diet on ingestion rates, release rates, and stoichiometry were made by 1-way ANOVA, employing the $p = 0.05$ level of significance.

RESULTS

Predator size and C and N content

Acartia tonsa copepods collected for Expts A and B were of similar sizes and had similar C:N ratios. The total body length of the population of adult *A. tonsa* showed a normal size distribution, with mean values of 1085 μm for Expt A and 1121 μm for Expt B, and coefficients of variance (CV) of 6.47 and 5.84%, respectively (Table 1). Copepod C and N contents ranged from 2.1 to 3.7 $\mu\text{g C}$ and 0.5 to 0.9 $\mu\text{g N}$, respectively, yielding C:N ratios between 3.7 and 4.1 (g g^{-1}). The averages are reported in Table 1.

Food size, C and N content, and initial concentration

The cell volumes of food items *Thalassiosira weissflogii*, *Oxyrrhis marina*, and *Gyrodinium dominans* ranged from 673 to 2875, 1016 to 2228, and 520 to 2228 μm^3 , respectively (averages reported in Table 2), and the CV ranged from 33 to 38%. Equivalent spherical diameter (ESD) was highest in *O. marina* and lowest in *G. dominans* (Table 2), with a combined average CV of 11.4%. Despite being the smallest food item, the heterotrophic dinoflagellate *G. dominans* had the highest cellular C and N content. Cellular C contents of all food items were lower than the estimates derived from Menden-Deuer & Lessard (2000) and Dam & Lopes (2003), which we used to standardize the C in the experimental bottles. Thus, initial food concentrations were about half the targeted 300 $\mu\text{g C l}^{-1}$ (Table 3). However, these food concentrations do not

fall below threshold feeding levels and are at the near-saturating levels determined for *Acartia tonsa* by Besiktepe & Dam (2002). In Expt A, the DIATOM treatment had significantly higher initial food C concentration compared to the MIX treatment ($p < 0.01$). All other initial food concentrations were similar between treatments. Initial C concentration of *G. dominans* in the μ Z treatment (Expt B) was significantly higher than C concentrations in the DIATOM and MIX treatment ($p < 0.01$).

Feeding rates

Ingestion rates of copepods feeding on the μ Z, DIATOM, and MIX diets in Expt A (*Oxyrrhis marina* as the microzooplankton food source, *Thalassiosira weissflogii* as the algal food source) were not statistically different from each other and averaged 1.25, 1.58, and 1.13 $\mu\text{g C ind.}^{-1} \text{d}^{-1}$ or 42, 53, and 38% of copepod body C d^{-1} , respectively (Fig. 1a). Ingestion rates for all treatments in Expt B (*Gyrodinium dominans* as the microzooplankton food source, *T. weissflogii* as the algal food source) were significantly higher than those in Expt A ($p < 0.05$ in μ Z and DIATOM; $p < 0.01$ in MIX), averaging 1.77, 1.83, and 1.93 $\mu\text{g C ind.}^{-1} \text{d}^{-1}$ or 66, 68, and 72% of copepod body C d^{-1} , respectively. The ingestion rates for μ Z, DIATOM, and MIX in Expt B, however, were not significantly different from each other. In the MIX treatments, diatom C accounted for 52% of total C ingested in Expt A and 37% of total C ingested in Expt B. Clearance rates of copepods were similar between treatments in Expt A with averages ranging from 0.63 to 0.75 $\text{ml ind.}^{-1} \text{h}^{-1}$ (Fig. 1b). Clearance rates of copepods in Expt B, however, were significantly different between all treatments, being highest in the MIX treatment, lower

in the DIATOM treatment, and lowest in the μ Z treatment, and averaged 1.14, 0.74, and 0.44 ml ind.⁻¹ h⁻¹, respectively (Fig. 1b).

Bacterial nutrient uptake

Estimated bacterial uptake of C, N, and P was minimal (1.4 to 27, 0.4 to 7.1, and 0.1 to 1.4 ng C, N, and P l⁻¹ d⁻¹, respectively). Uptake was also similar between the controls and copepod treatments for each diet in both experiments (Table 4; $p > 0.05$). This is most likely due to the similar bacterial abundance, [C], between the controls and copepod treatments (Table 4; $p > 0.05$). Thus, there were no significant differences in uncorrected and uptake-corrected nutrient release rates ($p > 0.05$). To test this further, we recalculated bacterial uptake to increase the potential uptake of C, N, and P using further conservative conversion factors including BGE = 10% (del Giorgio & Cole 2000), C:N = 3.8 (Fukuda et al. 1998), and C:P = 8 (Bratbak 1985). These uptake-corrected release rates were not significantly different from the uncorrected release rates either ($p > 0.05$).

Copepod nutrient release

DOC release rates in the μ Z treatment for both experiments were significantly higher than the DOC produced by copepods feeding on an exclusively diatom or on a mixed diet (Fig. 2). DOC release in the MIX treatment was undetectable in Expt A and near zero in Expt B. Average release rates for the μ Z and DIATOM treatments ranged from 34 to 83 ng C ind.⁻¹ h⁻¹ and 4 to 15 ng C ind.⁻¹ h⁻¹ and correspond to 67-116 and 6-

20% of C ingested d^{-1} , respectively. Additionally, DOC release rates were higher for copepods feeding on *Gyrodinium dominans* (Expt B) compared to *Oxyrrhis marina* (Expt A) in the μ Z treatments ($p < 0.05$).

Mean NH_4^+ release rates for each treatment ranged from 1.4 to 17 ng N $ind.^{-1} h^{-1}$ (Fig. 3a). Similarly to DOC, NH_4^+ release rates were significantly higher in the μ Z treatment and lowest in the MIX treatment for both Expts A and B (Fig. 3a; $p < 0.05$). Low release rates of DOC and NH_4^+ in the MIX treatment were unexpected due to the combined diet as well as the similar ingestion rates in the MIX treatment compared to the other treatments. NH_4^+ release rates in the μ Z treatment were also higher in Expt B compared to Expt A ($p < 0.01$).

Bulk DON release rates (calculated by subtracting inorganic N sources, NO_x and NH_4^+ , from TDN) were undetectable due to a high background of NO_x during our experiments (up to 80 μ mol l^{-1}). DPA release rates were also below the detection limit. Thus, the released organic N we have reported in the present study is urea. Contrary to the patterns observed in DOC and NH_4^+ release rates, urea release rates were highest in the MIX treatment and lowest in the μ Z treatments for both experiments and ranged from undetectable to 4.1 ng N $ind.^{-1} h^{-1}$, but these differences were not statistically significant (Fig. 3b). Urea was a higher portion of the total N released in the MIX treatment (reaching up to 59%) compared to in the other treatments (Fig. 3c; $p < 0.05$, Expt B).

Release rates of P were considerably more variable across treatments compared to those of other nutrients measured (Fig. 4). Phosphate release rates were mostly on the order of 1 to 2 ng P $ind.^{-1} h^{-1}$ (Fig. 4a), but did reach as high as 11.5 ng P $ind.^{-1} h^{-1}$ (Fig. 4a). In Expt A, the average PO_4^{3-} release rates were highest in the DIATOM treatment

and lower in the μ Z and MIX treatments ($p < 0.05$). The average PO_4^{3-} release rates in Expt B were highest in the μ Z treatment (average = $10.6 \text{ ng P ind.}^{-1} \text{ h}^{-1}$), lower in the DIATOM treatment (average = $1.65 \text{ ng P ind.}^{-1} \text{ h}^{-1}$), and undetectable in the MIX treatment ($p < 0.05$). Similarly to DOC and NH_4^+ , release rates for PO_4^{3-} were higher for copepods feeding on *Gyrodinium dominans* (Expt B) compared to *Oxyrrhis marina* (Expt A) in the μ Z treatments ($p < 0.01$). When DOP release rates were detectable, they were higher than inorganic P release rates and contributed 54 to 100% of the total P released (Fig. 4b). The detectable DOP release only occurred in treatments that contained microzooplankton prey.

Stoichiometry of nutrients released from copepods was quite variable (Fig. 5). Molar DOC:urea release ratios were highest in the μ Z treatment (averages ranging from 172 to 187), lower in the DIATOM treatment (averages ranging from 13 to 63), and, when data were available (Expt B), lowest in the MIX treatment (9.0; Fig. 5a; $p < 0.05$). These release ratios were also well above the Redfield ratio for C:N of 6.6, with averages ranging from 9 to 187 (mol mol^{-1}). DOC:TDN and TDN:TDP release ratios, on the other hand, were all below the Redfield ratio of 6.6 and 16, respectively. Released DOC:TDN ratios were highest in the μ Z treatment (averages ranging from 3.0 to 5.7) and lower in the DIATOM (from 0.4 to 1.8) and MIX treatments (average for Expt B = 1.3; Fig. 5b), but these differences were not significant. TDN:TDP release ratios, however, were highest in the DIATOM treatment (average = 12.5) and lower in the treatments containing microzooplankton prey items (3.6 to 6.6 for μ Z, 1.3 to 8.3 for MIX; Fig. 5c; $p < 0.01$ for Expt B).

DISCUSSION

Diet has been the focus of studies examining copepod feeding and reproduction (Stoecker & Egloff 1987, Stoecker & Capuzzo 1990, Kleppel & Burkart 1995, Bonnet & Carlotti 2001, Broglio et al. 2003). The central theme in these published studies is the importance of protozoans in the copepod diet. The effect of a mixed diet (phytoplankton + protozoans), as opposed to mono-diets, on copepod metabolic processes has not been previously examined. Our study demonstrates for the first time that copepod diet affects relative organic and inorganic nutrient release rates as well as release stoichiometry.

Feeding and nutrient release rates

Average copepod C ingestion rates (1.13 to $1.58 \mu\text{g C ind.}^{-1} \text{d}^{-1}$) were similar to those reported for *Acartia tonsa* in Miller & Roman (2008; 0.05 to $2.96 \mu\text{g C ind.}^{-1} \text{d}^{-1}$), but lower than those measured in Besiktepe & Dam (2002; ca. 6 and $3.5 \mu\text{g C ind.}^{-1} \text{d}^{-1}$ for copepods feeding on *Thalassiosira weissflogii* and *Oxyrrhis marina*, respectively, at prey concentrations similar to those in our study).

Despite similar ingestion rates among treatments, as well as similar C:N ratios of food items, *Acartia tonsa* release rates of DOC, urea, DOP, NH_4^+ , and PO_4^{3-} were extremely variable between diet treatments. Because our release rates represent not only excretion, but also sloppy feeding and egestion/fecal pellet leaching, the hypothesis of ingestion-independent rates of excretion (Miller & Landry 1984) can neither be supported nor rejected. *A. tonsa* DOC release rates (as percentage of food C ingested) in our study

are higher than those shown by Strom et al. (1997) for *Calanus pacificus* copepods feeding on *Oxyrrhis marina* (67 to 100% vs. ca. 16 to 28%) and *Thalassiosira weissflogii* (5.8 to 20% vs. undetectable). Differences in the method, as well as conversion factors, used to correct for bacterial uptake could be one source of variation. While we measured bacterial production using the [³H]-leucine uptake method, Strom et al. (1997) calculated potential bacterial DOC uptake using measured change in bacterial abundance, an estimated 40 fg C bacterial cell⁻¹, and an estimated bacterial growth efficiency of 50%. Using these conversions, bacteria utilized between 9 and 80% of the DOC produced according to Strom et al. (1997), while the proportion of DOC utilized by bacteria was negligible in our study.

Variation in DOC release rates between our study and that of Strom et al. (1997) are also likely due to the different sizes of copepods used for the experiments and the subsequent differences in DOC release by sloppy feeding. When copepod-to-prey ESD ratios are below the threshold of 55 as defined by Møller (2005), DOC release via sloppy feeding can occur. The copepod *Calanus pacificus*, used by Strom et al. (1997), is much larger (ESD = 1060 μm; Møller 2005) than *Acartia tonsa* (ESD = 432 μm; present study). Thus, the calculated copepod-to-prey ESD ratios for *C. pacificus* feeding on prey items *Thalassiosira weissflogii*, *Oxyrrhis marina*, and *Gyrodinium dominans* are always above the threshold for sloppy feeding (76.6, 71.2, and 81.5, respectively) compared to those calculated for *A. tonsa* (31.2, 29.0, and 33.2, respectively). Thus, sloppy feeding could be the source of the higher DOC release in our study compared to that by Strom et al. (1997). Using our copepod-to-prey ESD ratios in the equation of Møller (2005), we predicted the fraction of C removed from suspension and lost as DOC via sloppy feeding

by *A. tonsa* feeding on *T. weissflogii*, *O. marina*, and *G. dominans* to be 30.8, 33.7, and 28.2%, respectively. These estimates are even lower when we use the more conservative sloppy feeding DOC release equation of Møller (2007). The actual DOC release (as the fraction of C removed from suspension) measured in our study for *A. tonsa* grazing on *T. weissflogii* (8.7% in Expt A, 29% in Expt B) was within the range of release predicted by Møller (2007); however, it is higher and above the ranges of sloppy feeding release predicted by Møller (2005, 2007) for *O. marina* (67.1%) and *G. dominans* (116%). This suggests that excretion, and possibly, fecal pellet leaching, were also important sources of DOC release in treatments with microzooplankton prey. However, no studies to date have attempted to separate the modes of DOC release (sloppy feeding vs. excretion vs. fecal pellet leaching), so the relative importance of each mode of release in the present study is not known.

Ammonium release rates (1.4 to 17 ng N ind.⁻¹ h⁻¹) were similar to those reported for *Acartia tonsa* by Miller & Glibert (1998; undetectable to 28 ng N ind.⁻¹ h⁻¹) and Ikeda et al. (2001; 6.0 ng N ind.⁻¹ h⁻¹) but slightly higher than those reported by Miller & Roman (2008; 1.4 to 7.0 ng N ind.⁻¹ h⁻¹) for a range of food qualities. Additionally, DOC and NH₄⁺ release rates were higher for copepods feeding on *Gyrodinium dominans* (Expt B) compared to on *Oxyrrhis marina* (Expt A) in the μZ treatments, most likely due to the higher ingestion rates on *G. dominans* (Fig. 1) as well as the relatively higher food concentration in this treatment (Table 3) and higher cellular C and N of *G. dominans* (Table 2). *A. tonsa* urea release rates (0 to 4.1 ng N ind.⁻¹ h⁻¹) were lower compared to those measured by Miller & Glibert (1998; 0 to 38 ng N ind.⁻¹ h⁻¹). However, the portion of total N release as urea (0.6 to 6.6% in μZ, 13 to 16% in DIATOM, and 32 to 59% in

MIX; Fig. 3c) is similar to that measured by Miller & Glibert (1998; 30 to 54%) and higher than that for copepod *Pleuromamma xiphias* (Steinberg et al. 2002; 21%). These results reiterate the importance of organic N in nutrient remineralization.

Although P release rates for copepods are scarce in the literature, we did find similar PO_4^{3-} release rates (mostly 1 to 2 but reaching $11.5 \text{ ng P ind.}^{-1} \text{ h}^{-1}$) compared to those for the similar-sized copepod *Acartia australis* (Ikeda et al. 2001; $1.3 \text{ ng P ind.}^{-1} \text{ h}^{-1}$), but higher release rates than those measured for the smaller cyclopoid copepod *Oithona nana* (Atienza et al. 2006; 0.34 to $0.37 \text{ ng P ind.}^{-1} \text{ h}^{-1}$). When DOP release rates were detectable, they were higher than inorganic P release rates and contributed 54 to 100% to the total P released (Fig. 4b), which was similar to the adult *A. tonsa* DOP release determined by Hargrave & Geen (1968; 74%). Zooplankton nutrient release experiments, specifically in marine environments, typically ignore P. Our results emphasize the importance of including zooplankton-mediated P release into nutrient budgets, especially in P-limited environments that depend on remineralization processes as the primary source of P.

Potential diatom nutrient uptake

Nutrient uptake by diatoms likely occurred during incubations, as evidenced by declines in NH_4^+ and urea concentrations from T_0 to $T_{24\text{h}}$ in the DIATOM controls. Although this uptake was not directly measured in our experiments using labeled isotope techniques, the calculation for copepod nutrient release rate (Eq. 2) does incorporate these nutrient declines in the controls (uptake) in the term ΔC_c .

Effect of diet on release rates

The highest copepod DOC, NH_4^+ , and TDN release rates occurred while feeding carnivorously. The lowest release rates occurred while feeding omnivorously, perhaps due to higher copepod C and N gross growth efficiencies (GGE) in the mixed diet. GGE is defined as the portion of nutrients from the ingested food delegated to growth and reproduction. A higher GGE for C and N would result in higher copepod egg production rates (EPR), increased biosynthesis (retention) of nutrients, and thus lower metabolic excretion of dissolved C and N. We did not measure EPR in the present study; however, previous studies support the idea that a mixed diet comprised of phytoplankton and microzooplankton results in higher EPR. *Acartia tonsa* copepods exhibited highest EPR and egg hatching success in treatments that included a mixed diet of *Oxyrrhis marina* and the alga *Isochrysis galbana* (Kleppel & Burkart 1995). Stoecker & Egloff (1987) reported 25% higher EPR for *A. tonsa*, and Bonnet & Carlotti (2001) reported a 3- to 7-fold higher EPR and survival rates for *Centropages typicus*, when ciliates were mixed with a phytoplankton diet compared to an exclusively algal diet. Additionally, *A. tonsa* convert ingested food to eggs more efficiently in mixed diets, compared to exclusively algal and exclusively microzooplankton diets (Kleppel et al. 1998). These results were not confirmed by Ederington et al. (1995) or by Dam & Lopes (2003). This is likely due to their use of the bacterivorous ciliates, *Pleuronema* sp. and *Uronema* sp., respectively, as this microzooplankton food source for copepods may either lack, or contain insufficient, fatty acids, including EPA and DHA (Ederington et al. 1995, Dam & Lopes 2003). The heterotrophic dinoflagellates *Oxyrrhis marina* and *Gyrodinium dominans*

(maintained on an algal diet of *Dunaliella tertiolecta*) used in our experiments, however, have previously been shown to be nutritionally beneficial to copepod growth, egg production, and egg hatching success (Klein Breteler et al. 1999, Tang & Taal 2005) due to their high EPA and DHA contents.

The idea of higher GGE and higher egg production in the mix diet also suggests that this diet may be more balanced than either of the mono-diets, as a higher consumer-resource composition imbalance results in a lower consumer GGE (Sterner & Elser 2002). Additionally, imbalances in diet could create differential assimilation patterns in order for the copepod to regulate synthesis of nutrients to match its needs, thus resulting in differential catabolism and eventual release of C, N, and P (Sterner & Elser 2002).

Although gut transit time, egestion rate, and assimilation efficiency (AE) were not measured in our study, variability in these processes may have occurred in copepods feeding on the different diets. For instance, *Acartia clausi* copepods exhibited longer gut transit times, and *Temora stylifera* had lower egestion rates, while feeding on dinoflagellates compared to diatoms, the latter of which typically have lower molecular complexity (Ianora et al. 1995, Tirelli & Mayzaud 2005). These studies suggest that copepods feeding on a more complex diet (i.e. more proteins, carbohydrates, lipids, etc.) may need a longer time to digest their food. This may have caused lower copepod nutrient release rates in the MIX treatment compared to the mono-diet treatments. However, if gut transit times or AE were solely a function of food molecular complexity, then nutrient release rates by copepods feeding on dinoflagellates in the μ Z treatment would also be higher than those in the DIATOM treatment, and this did not occur in our study.

The differences in P release rates between treatments may be a result of variable food P composition. TDP release rates were highest in the microzooplankton diet, followed by the mixed diet, and lowest in the diatom diet, and DOP was only detectable in treatments containing microzooplankton prey. We did not measure particulate P contents in our food items and there are no published data on P content in microzooplankton. Compared to algae, however, dinoflagellates have a larger genome (Raven 1994) and much higher amounts of DNA in their nucleus (Rizzo 1987). Because DNA is rich in P (Sterner & Elser 2002), the higher release rates of P in our microzooplankton prey treatments could be a result of higher DNA contents in these heterotrophic dinoflagellates compared to *Thalassiosira weissflogii* diatoms.

Possible behavioral effects on release rates

Variations in nutrient release rates could also be due to copepods exerting different feeding behaviors on the 3 diets. Omnivorous copepods quickly hop and seize microzooplankton prey in 'ambush mode', generate continuous feeding currents in the more passive 'suspension mode' for non-motile phytoplankton food including diatoms, and exhibit prey-switching behavior when feeding on a mixed diet (Saiz & Kiørboe 1995, Kiørboe et al. 1996). Although the energetic costs of each feeding mode have not been directly determined, the copepod *Metridia pacifica* displays slower swimming speeds and fewer high-speed bursts when feeding on an exclusively phytoplankton diet compared to a more active feeding mode with frequent high-speed bursts when feeding on a carnivorous diet of *Artemia* sp. nauplii (Wong 1988). If more energy is expended by

copepods feeding in the ambush mode compared to suspension mode, then nutrient release rates would also be higher in the ambush mode. This hypothesis is supported by our results: highest copepod DOC and TDN release rates while feeding on microzooplankton and lower release rates while feeding on diatoms (when copepods are likely feeding mainly in suspension mode) and on the mixed diet (where the energetic cost of ambush feeding is potentially cut by 50%), as well as the release of DOP in the only treatments containing microzooplankton. Future research is needed in order to determine the energetic costs of feeding behaviors and their potential effects on copepod nutrient release.

Microzooplankton and nutrient release

The nutrient release directly from the heterotrophic dinoflagellate prey in the μ Z treatment was investigated by calculating the change in nutrients in these control bottles during incubation (using the term ΔC_c in Eq. 2). The only detectable positive release calculated in any control was PO_4^{3-} release by *Oxyrrhis marina* in Expt A. The PO_4^{3-} release by *O. marina* was significantly lower than that released by the copepods ($p < 0.05$); however, it most likely contributed to the lower calculated PO_4^{3-} release (Eq. 2) by copepods feeding on *O. marina* (Expt A) compared to those feeding on *Gyrodinium dominans* (Expt B). Due to the negligible contribution of DOC, NH_4^+ , and, in Expt B, PO_4^{3-} from the heterotrophic dinoflagellates in the present study, we infer that the elevated release of these nutrients in the μ Z treatments came directly from the copepods.

Inorganic versus organic N release

Relative to inorganic N release, urea release rates were higher and accounted for a higher proportion of TDN released while copepods fed on a mixed diet. This could be due to the preferential metabolism of nucleic acids (RNA, DNA) via the uricogenesis/ureogenesis pathways of which urea is the primary byproduct (Regnault 1987). Ammonia formation, on the other hand, is the major byproduct of the catabolism of amino acids (Regnault 1987). Reasons for preferential catabolism of certain molecules over others, as related to zooplankton diet are, however, unclear and have not been reported. As discussed above, it is possible that the mixed diet is more balanced and allows higher efficiency in metabolizing nucleic acids as opposed to the other 2 mono-diets. Variability in the types of N released could also be due to differences in release processes. Both urea and NH_4^+ can be released from the copepod body via simple diffusion across membrane surfaces (Pandian 1975, Bidigare 1983). However, while NH_4^+ is rapidly released to avoid its toxic properties, urea has a slower diffusive property compared to NH_4^+ , and thus disperses more slowly through the membranes (Pandian 1975). Thus, if copepods feeding on the mixed diet are efficiently retaining N for growth and reproduction, then a higher portion of the N that is being released may be the passive leakage of urea. Conversely, if copepods feeding on the mono-diets are not efficiently retaining N, then more NH_4^+ may be actively released. Diffusion of NH_4^+ and urea are most likely short-term processes and may not be reflected in release rates during the 24-h incubation.

Stoichiometry of nutrient release

Copepod molar DOC:urea nitrogen release ratios were well above the classic Redfield C:N ratio (6.6); however, when all forms of released N and P were accounted for, molar DOC:TDN and TDN:TDP release ratios were either lower than or close to Redfield ratios (6.6 and 16, respectively). Non-Redfield remineralization has been shown for a variety of diel-migrating zooplankton taxa in the Sargasso Sea: DOC:DON (range from 5.1 to 14.9), DIC:DIN (6.1 to 12.6), and DIN:DIP (6.1 to 15.7) (Steinberg et al. 2002), as well as for Barents Sea zooplankton, which exhibit wide ranges of ratios of respiration and inorganic excretion: DIC:DIN (range 4 to 44) and DIN:DIP (2 to 45) (calculated from Table 3 in Ikeda & Skjoldal 1989).

Released C:N and N:P ratios were also variable between treatments. High molar DOC:urea release ratios in the μ Z treatment were a result of the low proportion of urea release (as the total percentage of N), which ranged from 0.6 to 6.6%. DOC:urea release ratios, as well as the proportion of urea release in the DIATOM and MIX treatments (5.1 to 14.9 and 21 to 40%, respectively) more closely resembled those found by Steinberg et al. (2002). The higher TDN:TDP ratio of the released products in the DIATOM treatment was most likely due to lower P contents in the diatoms relative to microzooplankton prey items, similar to those found for *Daphnia* feeding on P-limited prey items (Frost et al. 2004). Additionally, we cannot discuss stoichiometric imbalances without considering predator (copepod) P content, which, if variable between treatments, could potentially explain the different TDN:TDP release ratios. We did not measure copepod P content in our experiments; however, Walve & Larsson (1999) found that

while *Acartia* sp. C and N contents were stable, their P content (and N:P) varied greatly. These variations were seasonal, as were those for *A. clausii* C:P and N:P according to Gismervik (1997b), and thus may also be a function of copepod diet composition as well as differences in growth rate (changes in P-rich DNA and RNA). Additional research is required to attain a more complete understanding of how predator and prey P content affects copepod P release rates and organic and inorganic N:P release ratios.

Stoichiometric theoretical models that have been implemented to further understand consumer-driven nutrient recycling processes all agree that the stoichiometry of nutrients released from zooplankton is mainly a function of both prey and grazer elemental composition (Sterner 1990, Elser & Urabe 1999, Touratier et al. 2001). However, our results show the uncoupling of copepod ingestion and nutrient release rates, as well as variable release rates of DOC, and dissolved organic and inorganic N and P, on different food types (phytoplankton vs. microzooplankton vs. mix) but with similar prey C:N. This is most likely because these aforementioned models are limited to excretion, and do not incorporate sloppy feeding and egestion/fecal pellet leaching. Thus, stoichiometric models based exclusively on predator and prey C:N and N:P ratios may not be adequate in determining stoichiometry of total release, especially when considering variability in diet.

Finally, differences in the stoichiometry may also be explained by other aspects of food composition (i.e. relative amounts of complex lipids vs. simple protein or amino acid contents, differential nucleic acid content), which may have affected the rate at which C, N, and P were individually metabolically processed, digested, and released creating differential C:N and N:P release ratios. Extended models, which incorporate

dietary constituents such as essential fatty acids (Anderson & Pond 2000), prey selectivity (Mitra & Flynn 2006), and digestion efficiency/gut transit time (Mitra & Flynn 2007), may be more appropriate when copepods feed on a diversity of prey items.

CONCLUSION

We have shown that copepod nutrient release rates, composition, and stoichiometry are significantly affected by feeding strategy. In particular, we have revealed key nutrient release differences in copepods feeding omnivorously compared to those feeding on mono-diets of either phytoplankton or microzooplankton. While we could not directly distinguish the source(s) of variable nutrient release, we provide a black box view of zooplankton nutrient release as a function of diet and discuss multiple factors that may drive nutrient release variability. Including mixed diets of phytoplankton and microzooplankton should be an important component of future studies examining copepod metabolism and digestion, growth efficiency, and inorganic and organic nutrient release. Differences in these processes with diet, as well as the proportion of time copepods spend feeding herbivorously, carnivorously, and omnivorously, need to be accounted for in order to estimate the quantity, quality, and stoichiometry of regenerated nutrients available for the growth and metabolism of phytoplankton and heterotrophic bacteria, and to better model the role of zooplankton in ocean nutrient and C budgets.

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Table 1. *Acartia tonsa*. Size (length and equivalent spherical diameter, ESD), carbon (C) and nitrogen (N) contents of the calanoid copepod predator *A. tonsa* for Expts A and B. Values are mean \pm SD with n=50 (length and ESD) and n=5-8 (C and N contents) for each experiment

Expt	Length (μm)	ESD (μm)	C ($\mu\text{g copepod}^{-1}$)	N ($\mu\text{g copepod}^{-1}$)	C:N (g g^{-1})
A	1085 \pm 70	418 \pm 59	3.1 \pm 0.3	0.8 \pm 0.1	3.9 \pm 0.2
B	1121 \pm 65	446 \pm 51	2.6 \pm 0.1	0.7 \pm 0.1	4.0 \pm 0.1

Table 2. *Thalassiosira weissflogii*, *Oxyrrhis marina*, *Gyrodinium dominans*. Food size (length, width, volume, and equivalent spherical diameter, ESD), carbon (C) and nitrogen (N) contents. Values are mean \pm SD with n=50 (size parameters), n=6 (C and N contents) for each experiment. Food size and C and N contents were measured at the start of the experiments, cell volumes were corrected for Lugol's-derived shrinkage (see 'Materials and methods')

Food	Length (μm)	Width (μm)	Volume (μm^3)	ESD (μm)	C (pg cell^{-1})	N (pg cell^{-1})	C:N (g g^{-1})
Expt A							
<i>T. weissflogii</i>	12 \pm 2.2	9.1 \pm 1.4	1511 \pm 577	14 \pm 1.7	53 \pm 6.6	10 \pm 1.9	5.6 \pm 0.6
<i>O. marina</i>	23 \pm 2.4	12 \pm 1.5	1802 \pm 624	15 \pm 1.6	268 \pm 63	52 \pm 7.6	5.1 \pm 0.9
Expt B							
<i>T. weissflogii</i>	12 \pm 2.0	8.8 \pm 1.1	1396 \pm 463	14 \pm 1.5	73 \pm 10	13 \pm 2.0	5.5 \pm 0.3
<i>G. dominans</i>	20 \pm 2.0	10 \pm 1.5	1209 \pm 420	13 \pm 1.5	329 \pm 54	65 \pm 9.2	5.1 \pm 0.3

Table 3. *Oxyrrhis marina*, *Thalassiosira weissflogii*, *Gyrodinium dominans*.

Average initial food conditions. Values are mean \pm SD, n=3 for each treatment

Initial food concentration ($\mu\text{g C l}^{-1}$)	
Expt A	
<i>Oxyrrhis marina</i>	147 \pm 30
<i>Thalassiosira weissflogii</i>	162 \pm 18
<i>O. marina</i> / <i>T. weissflogii</i> MIX	
<i>O. marina</i>	63 \pm 15
<i>T. weissflogii</i>	58 \pm 5.0
Total	121 \pm 16
Expt B	
<i>Gyrodinium dominans</i>	233 \pm 19
<i>Thalassiosira weissflogii</i>	160 \pm 3.0
<i>G. dominans</i> / <i>T. weissflogii</i> MIX	
<i>G. dominans</i>	80 \pm 17
<i>T. weissflogii</i>	56 \pm 6.0
Total	136 \pm 18

Table 4. Mean bacterial abundance, [C], and mean estimated daily bacterial C, N, and P demands used for uptake corrections on release rates in Expts A and B. The μ Z prey in Expt A was *Oxyrrhis marina*, and in Expt B was *Gyrodinium dominans*. The diatom food for both experiments was *Thalassiosira weissflogii*, and the mixed diet is a combination of the μ Z and diatom prey items. Nutrient demands (total C, N, and P) were calculated using $^3\text{[H]}$ -leucine bacterial production data, a bacterial growth efficiency estimate of 50%, and estimates of bacterial molar C:N (4.5) and C:P (50) (see 'Materials and methods' for details); n=3 for [C] and C, N, and P daily nutritional demands

	[C] (cells ml ⁻¹ x 10 ⁵)	Daily Nutritional Demand (ng l ⁻¹ day ⁻¹ x 10 ⁻¹)		
		C	N	P
Expt A				
μ Z Control	1.8 ± 0.1	41 ± 0.5	11 ± 0.1	2.2 ± 0.03
μ Z + Copes	2.3 ± 0.2	53 ± 4.4	14 ± 1.1	2.8 ± 0.03
DIATOM Control	0.7 ± 0.1	14 ± 0.5	3.8 ± 0.1	0.6 ± 0.03
DIATOM + Copes	1.3 ± 0.1	28 ± 2.8	7.0 ± 0.7	1.5 ± 0.15
MIX Control	2.2 ± 0.1	48 ± 1.2	12 ± 0.3	2.5 ± 0.06
MIX + Copes	1.9 ± 0.1	42 ± 2.0	11 ± 0.6	2.2 ± 0.12
Expt B				
μ Z Control	11 ± 0.1	270 ± 3.7	70 ± 1.0	14 ± 0.19
μ Z + Copes	10 ± 0.4	256 ± 10	66 ± 2.7	13 ± 0.53
DIATOM Control	5.6 ± 0.5	115 ± 11	29 ± 2.8	5.9 ± 0.56
DIATOM + Copes	5.6 ± 0.2	116 ± 4.9	29 ± 1.3	5.9 ± 0.25
MIX Control	2.4 ± 0.1	55 ± 1.2	14 ± 0.3	2.8 ± 0.06
MIX + Copes	3.2 ± 0.3	76 ± 5.8	20 ± 1.5	4.0 ± 0.31

Fig. 1. *Acartia tonsa*. Feeding rates on a carnivorous microzooplankton diet (μ Z), a herbivorous diatom diet (DIATOM), and an omnivorous mixed diet (MIX) in Expts A and B. The μ Z prey in Expt A was *Oxyrrhis marina*, and in Expt B was *Gyrodinium dominans*. The diatom food for both experiments was *Thalassiosira weissflogii*, and the mixed diet was a combination of the μ Z and diatom prey items. All values are mean \pm SD (n=3). (a) Ingestion rate (I); rates were converted from cells per individual per day using average measured C contents of food items shown in Table 2. Ingestion rates with different letters were significantly different from each other (y>z; 1-way ANOVA, p < 0.05 in μ Z and DIATOM, p < 0.01 in MIX). (b) Clearance rates (C) in ml per individual per hour; for the MIX treatments, rates for microzooplankton food and diatom food were calculated separately and then combined. Clearance rates with different letters were significantly different from each other (x>y>z; 1-way ANOVA, p < 0.05)

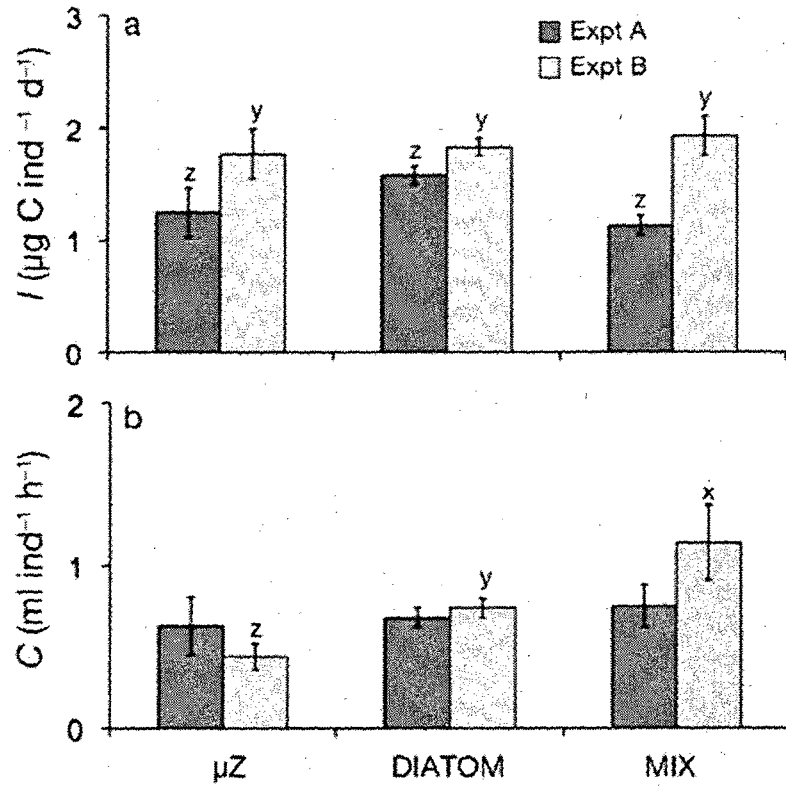


Fig. 2. *Acartia tonsa*. Dissolved organic carbon (DOC) release rates in nanograms C per individual per hour while feeding on a carnivorous microzooplankton diet (μ Z), a herbivorous diatom diet (DIATOM), and an omnivorous mixed diet (MIX) in Expts A and B. The μ Z prey in Expt A was *Oxyrrhis marina*, and in Expt B was *Gyrodinium dominans*. The diatom food for both experiments was *Thalassiosira weissflogii*, and the mixed diet was a combination of the μ Z and diatom food items. DOC release rates with different letters were significantly different from each other ($x>y>z$; 1-way ANOVA, $p < 0.05$). Values are mean \pm SD (n=3). nd: DOC release not detected

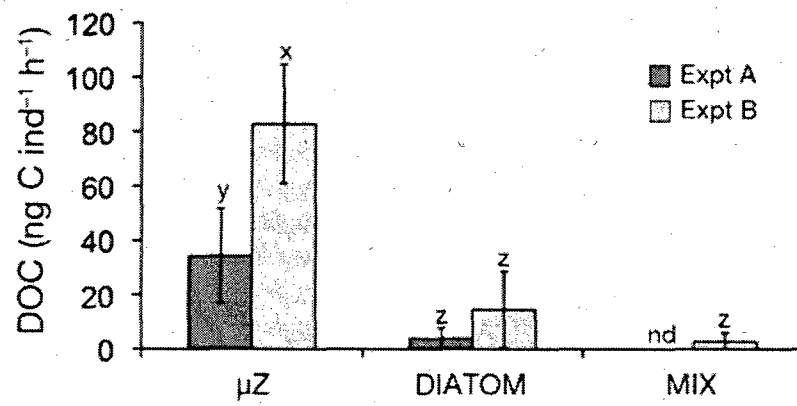


Fig. 3. *Acartia tonsa*. N release while feeding on a carnivorous microzooplankton diet (μ Z), a herbivorous diatom diet (DIATOM), and an omnivorous mixed diet (MIX) in Expts A and B. The μ Z prey in Expt A was *Oxyrrhis marina*, and in Expt B was *Gyrodinium dominans*. The diatom food for both experiments was *Thalassiosira weissflogii*, and the mixed diet was a combination of the μ Z and diatom food items. All values are mean \pm SD (n=3). Release rates with different letters were significantly different from each other (v>w>x>y>z; 1-way ANOVA, p < 0.05). (a) Inorganic N (NH_4^+) release rates in nanograms N per individual per hour. (b) Urea release rates in nanograms N per individual per hour. (c) Proportion of urea (organic N) release as percentage of TDN (total dissolved nitrogen, NH_4^+ + urea) release

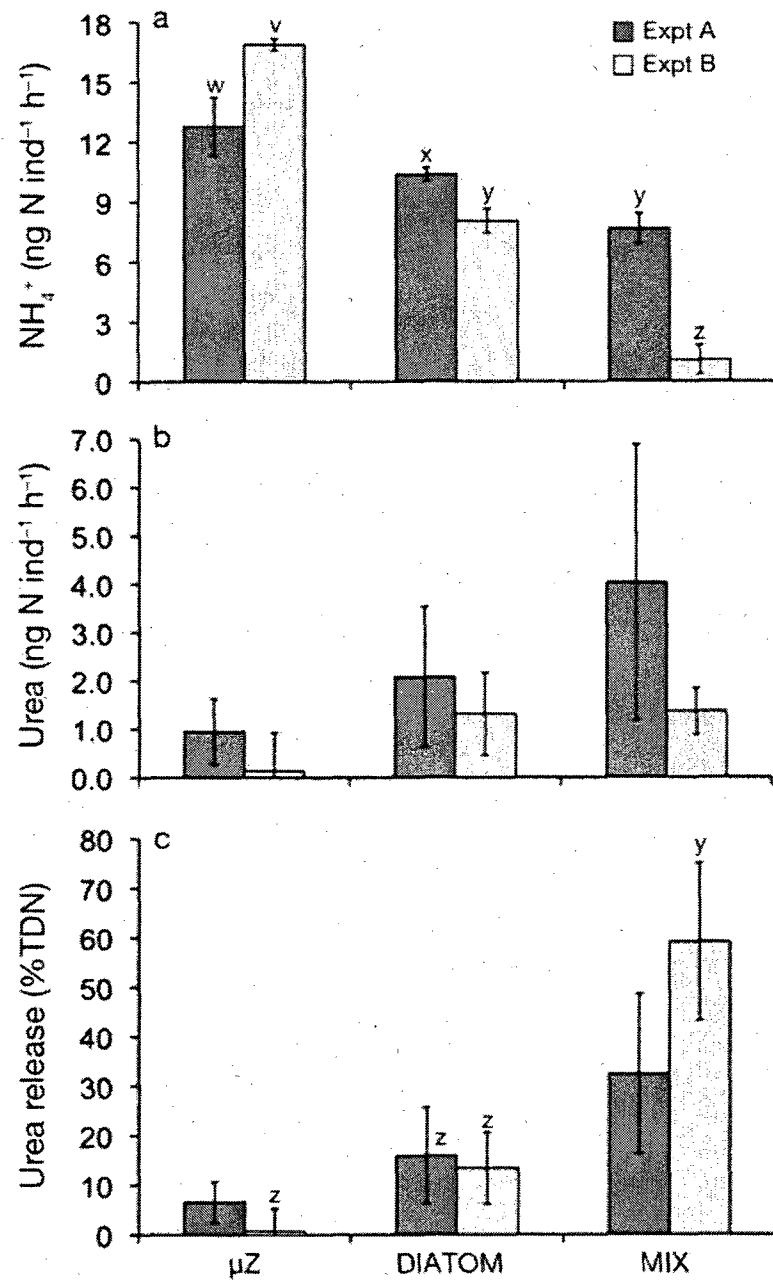


Fig. 4. *Acartia tonsa*. P release rates in nanograms P per individual per hour while feeding on a carnivorous microzooplankton diet (μ Z), a herbivorous diatom diet (DIATOM), and an omnivorous mixed diet (MIX) in Expts A and B. The μ Z prey in Expt A was *Oxyrrhis marina*, and in Expt B was *Gyrodinium dominans*. The diatom food for both experiments was *Thalassiosira weissflogii*, and the mixed diet was a combination of the μ Z and diatom food items. Values are mean \pm SD (n=3). nd = P release not detected. (a) Inorganic P (PO_4^{3-}) release. Release rates with different letters were significantly different from each other ($x > y > z$; 1-way ANOVA, $p < 0.05$). (b) Dissolved organic phosphorus (DOP) release

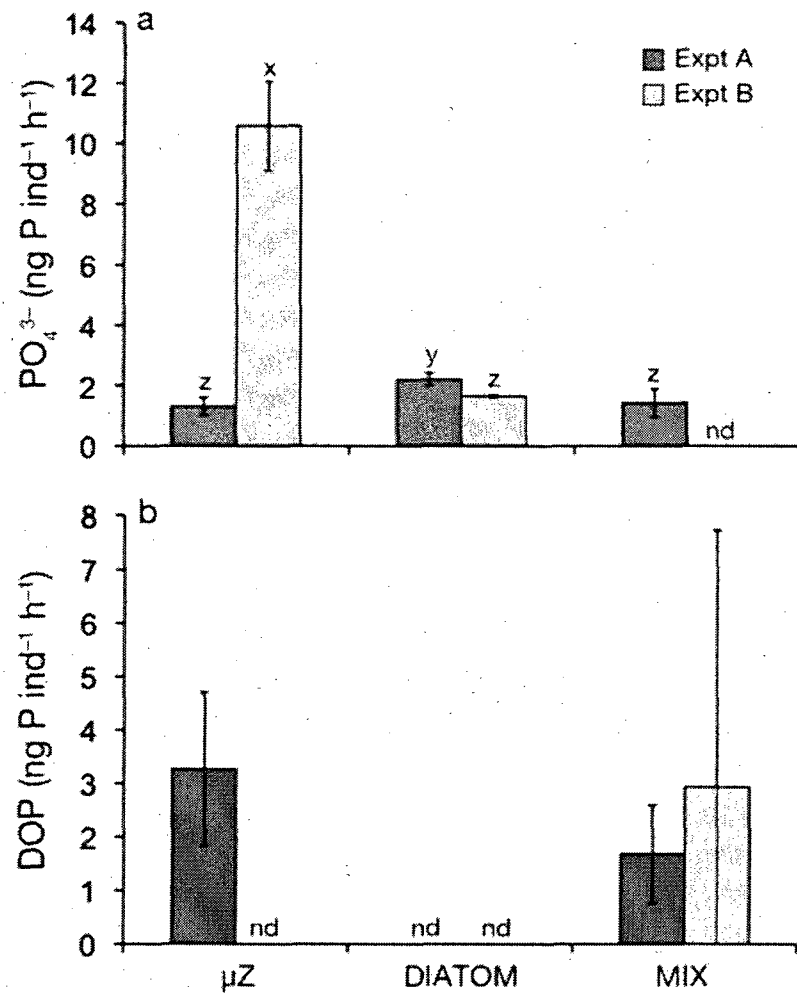
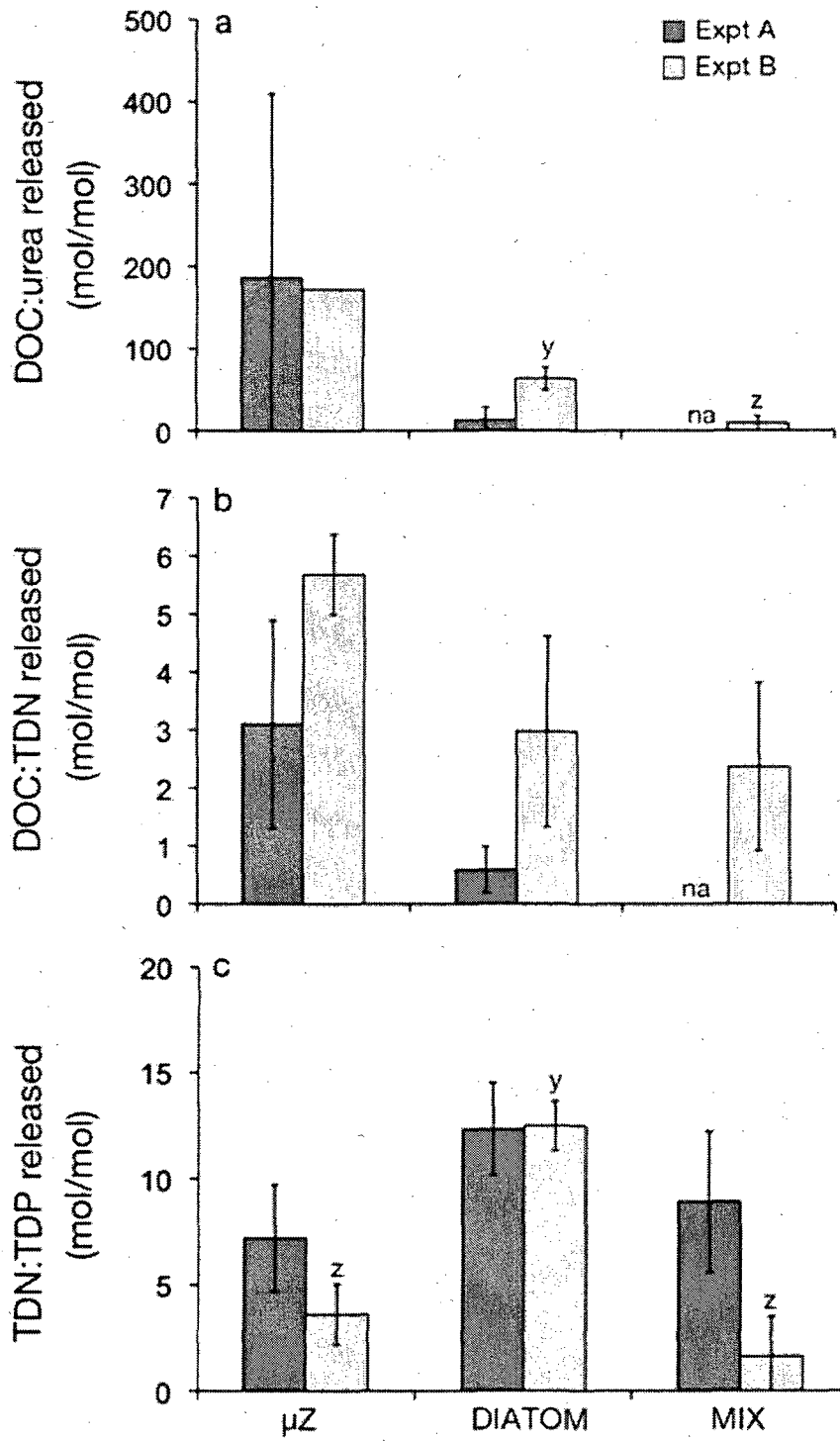


Fig. 5. *Acartia tonsa*. Stoichiometry of nutrient release while feeding on a carnivorous microzooplankton diet (μ Z), a herbivorous diatom diet (DIATOM), and an omnivorous mixed diet (MIX) in Expts A and B. The μ Z prey in Expt A was *Oxyrrhis marina*, and in Expt B was *Gyrodinium dominans*. The diatom food for both experiments was *Thalassiosira weissflogii*, and the mixed diet was a combination of the μ Z and diatom food items. All values are mean \pm SD (n=3). Release ratios with different letters were significantly different from each other (y>z; 1-way ANOVA, p < 0.05). na = data not available. (a) DOC:urea release. (b) DOC:TDN release, and (c) TDN:TDP release. Ratios calculated with TDN (total dissolved nitrogen) and TDP (total dissolved phosphorus) represent combined dissolved inorganic + organic forms



CHAPTER 3

The Effects of Harmful Algal Species and Food Concentration on Zooplankton Grazer Production of Dissolved Organic Matter and Inorganic Nutrients

Abstract

Harmful algal blooms (HABs), including toxic species, have been increasing in frequency, range, and duration over the past several decades. The effect of a harmful or toxic algal diet on zooplankton nutrient regeneration, however, has not been previously examined. In this study, we determined the effects of non-bloom and bloom concentrations of non-toxic and toxic cultures of HAB species *Prorocentrum minimum* and *Karlodinium veneficum* on grazing and production of dissolved organic carbon (DOC), nitrogen (DON), and phosphorus (DOP) and inorganic nutrients, ammonium (NH_4^+) and phosphate (PO_4^{3-}), by the copepod *Acartia tonsa* and the heterotrophic dinoflagellate *Oxyrrhis marina*. Ingestion rates of grazers were significantly higher while feeding on bloom algal concentrations compared to non-bloom algal concentrations, but were always below 1% body C d^{-1} for *A. tonsa* (ingestion rate range of 0.5–31 ng C individual $^{-1}$ d^{-1}) and below 2% body C d^{-1} for *O. marina* (range of 0.1–8.8 pg C individual $^{-1}$ d^{-1}). However, rates of inorganic nutrient and dissolved organic matter (DOM) release, when detected, were always > 100% of C, N, and P ingested. Additionally, the quantity and forms (organic vs. inorganic) of nutrients released by zooplankton were significantly different between non-toxic and toxic algal treatments, and typically higher grazer dissolved organic matter (DOM) release occurred while feeding on the toxic algal strain. DOM was the only detected form of nutrients released from *O. marina*, and DON and DOP were significant portions of total dissolved N and P released for *A. tonsa* feeding on toxic *K. veneficum* (69–84% and 73%, respectively). All algal diets used in our study, regardless of cell concentration, deterred grazing, which

likely resulted in starvation and subsequent catabolism of grazer body tissue. The potential for other factors affecting variable grazer nutrient release between toxic and non-toxic algal treatments, including algal nutrient quality and direct toxic effects, are discussed. Our results suggest these grazers may not be capable of controlling bloom formation of these HAB species, and that nutrient cycling dynamics in the coastal ocean are likely to change with increases in the presence of harmful and toxic algal blooms.

1. Introduction

Estuarine and coastal marine systems are sensitive to eutrophication, which can lead to subsequent shifts in phytoplankton composition and favor the chronic occurrence of harmful and toxic phytoplankton (Uye et al. 1999, Anderson et al. 2002, Beaugrand and Reid 2003). Harmful algal blooms (HABs), including toxic species, have been increasing in frequency, duration, and range since the 1970s (Sellner et al. 2003). Chesapeake Bay, in particular, has experienced an increase in the number of potential toxin-producing algal species over the past several decades (Marshall et al. 2005). Bloom-forming dinoflagellates *Prorocentrum minimum* and *Karlodinium veneficum* (syn. *Karlodinium micrum*) are common and wide spread in Chesapeake Bay (Johnson et al. 2003) and both have been associated with fish and shellfish mortality (Luckenbach et al. 1993, Deeds et al. 2002, Kempton et al. 2002, Heil et al. 2005, Tango et al. 2005). HABs also have complex interactions with zooplankton, which can affect zooplankton grazing and reproduction (Sunda et al. 2006), and may ultimately affect zooplankton nutrient regeneration.

Zooplankton grazers may be adversely affected by anti-grazing properties developed by some HAB-forming species, including toxin production, which can lead to grazer deterrence and starvation, decreased growth rates, increased mortality, regurgitation, and decreased egg production, which will likely promote HAB bloom formation (Huntley et al. 1986, Carlsson et al. 1995, Colin and Dam 2003, Kozlowsky-Suzuki et al. 2003, Vaqué et al. 2006, Cohen et al. 2007). For example, *Acartia tonsa* copepods avoided feeding and thus starved when exposed to the toxic dinoflagellate

Karenia brevis (Cohen et al. 2007). The copepods *A. margalefi* and *A. tonsa* both had lower ingestion rates, and *A. margalefi* had decreased viability of eggs, while feeding on a toxic strain of *Karlodinium* compared to non-toxic strains of *Gymnodinium* and *Karlodinium*, respectively (Vaqué et al. 2006; Waggett et al. 2008). Similarly, the heterotrophic dinoflagellate *Oxyrrhis marina* had low grazing and growth rates on toxic strains of *Karlodinium* (Vaqué et al. 2006, Adolf et al. 2007), and the haemolytic lipophilic toxins, called karlotoxins, produced by some *Karlodinium* species, caused cell lysis and decreased grazing in *O. marina* (Deeds & Place 2006; Adolf et al. 2007; 2008).

While much emphasis has been placed on grazer-mediated control of HABs (feeding and growth rates, egg production, and mortality), the effects of harmful or toxic algae on zooplankton nutrient regeneration and the potential for feedback into the bloom cycle is unknown. Previous studies show that zooplankton nutrient release can be affected by diet (Sterner & Smith 1993, Strom et al. 1997, Frost et al. 2004, Miller & Roman 2008, Saba et al. 2009). These studies, however, focused on how nutrient release varies with food type (various algal or zooplankton prey) or prey quality (food carbon:nitrogen, C:N, ratios) and did not specifically examine diets with HAB species. Sunda et al. (2006) suggest that unpalatable HAB species reduce zooplankton grazing rates and thereby decrease the regeneration of nutrients by those grazers. This will accelerate bloom development for HAB species that are adapted to nutrient-limited environments. Feedback mechanisms caused by zooplankton nutrient regeneration such as this are vital to our understanding of HAB dynamics. Thus, in this study, we determined the effects of toxic and non-toxic harmful algae and food concentration on copepod and microzooplankton grazer production of dissolved organic C, N, and

phosphorus (P), and of inorganic nutrients, ammonium (NH_4^+) and phosphate (PO_4^{3-}). Because current climate change models project worldwide increases in eutrophication and consequential increases in the frequency of HABs, it is critical to understand how zooplankton-mediated nutrient release will change with varying phytoplankton community composition, including harmful and toxic algal species.

2. Methods

To examine the impact of harmful algal food on ingestion and nutrient release of zooplankton grazers, we conducted one experiment in which *Acartia tonsa* copepods and the heterotrophic dinoflagellate *Oxyrrhis marina* were fed non-toxic and toxic cultured strains of *Prorocentrum minimum*, and another experiment in which *A. tonsa* copepods were fed non-toxic and toxic *Karlodinium veneficum*. Both experiments included non-bloom and bloom algal food concentrations.

2.1. Culture and collection of organisms

The algal food item used in the first experiment was *Prorocentrum minimum* (JA-98-01). Previous studies using *P. minimum* as a food source have demonstrated lethal and sub-lethal effects on shellfish (Luckenbach et al. 1993, Wikfors & Smolowitz 1993, Wikfors & Smolowitz 1995, Hégaret & Wikfors 2005). Although certain clones of *P. minimum* can produce toxins (Grzebyk et al. 1997, Denardou-Queneherve et al. 1999, Wikfors 2005), a specific toxin compound isolate has not yet been characterized. However, stationary-phase *P. minimum* is toxic to scallops, causing mortality within 24 hours, while log-phase *P. minimum* is less toxic (Hégaret & Wikfors 2005; Wikfors 2005). Thus, we cultured *P. minimum* in two separate batches, a non-toxic batch in which the culture was kept in exponential growth phase with fresh additions of nutrient-replete L1 media every 2-3 days, and a toxic batch where the culture was grown in low-nutrient media (L1/20) to late stationary phase before being transferred into new L1/20

media. Daily cell counts were used to monitor culture growth phases. We tested for relative toxicity between the two growth phases by conducting an oyster exposure experiment, the results of which indicated that late stationary phase *P. minimum* was toxic to oysters and log phase *P. minimum* was not (see below). Thus, the non-toxic and toxic cultures used for the experiment (and referred to as such below) were *P. minimum* at log phase and *P. minimum* at late stationary phase, respectively.

The non-toxic and toxic algal food cultures selected in the second experiment (Expt. 2) were *Karlodinium veneficum* (CCMP 1609), which produces little to no karlotoxin (data presented here) and *K. veneficum* (CCMP 2778), which produces KmTx 2 karlotoxin (Place et al. 2010). CCMP 1609 was isolated by A. Lewitus in 1991 from the Choptank River, MD and deposited in 1993. Based on the ITS sequence (see Bachvaroff et al. 2009) and pigment profile, CCMP 1609 is a *K. veneficum* strain. Both cultures were maintained in exponential growth phase with fresh additions of L1 media every 2-3 days. All cultures were incubated at 20°C on a 12:12 h light:dark regime.

The heterotrophic dinoflagellate grazer used in the *P. minimum* experiment, *Oxyrrhis marina* (isolated from Narragansett Bay), was maintained on a diet of the chlorophyte *Dunaliella teriolecta* (CCMP 1320) in f/2 media and incubated at 20°C in the dark. The experiments were conducted once the dinoflagellate culture reached early stationary phase, when protozoan cell abundance was maximal and algal food was minimal (Saba et al. 2009).

Acartia tonsa copepods were collected from the York River, U.S.A., a tributary of Chesapeake Bay, by near-surface net tows using a 0.5 m diameter net with 200 µm mesh and a non-filtering cod end. Copepod collections for the two experiments in this study

were conducted two weeks apart, but were from the same location and during the same tidal cycle. Upon collection, actively swimming *A. tonsa* were placed in 0.2 μm filtered seawater for 1-2 hours until the start of the acclimation period (see below).

The 0.2 μm filtered seawater (FSW) used for the experiment acclimation periods and incubations, and used to make the L1, L1/20, and F/2 nutrient medias for the cultures, had a salinity of 20 psu and a low background of DOM, consisting of a 1:1 ratio of deep Santa Barbara Channel seawater (SBSW) to artificial seawater (ASW) made with sodium chloride combusted at 500°C for 2 hours (Saba et al. 2009).

2.2. Experimental Procedures

Feeding and Nutrient Release Experiments. Two experiments were conducted, each with a non-toxic and toxic algal treatment at non-bloom and bloom concentrations. In the first experiment, copepods (*Acartia tonsa*) and heterotrophic dinoflagellates (*Oxyrrhis marina*) were fed *Prorocentrum minimum* in the following growth phase/treatments: (1) log (non-toxic) non-bloom, (2) log bloom, (3) late stationary (toxic) non-bloom, and (4) late stationary bloom. Blooms of *P. minimum* are defined as cell concentrations $>3000 \text{ cells ml}^{-1}$; localized blooms can reach concentrations up to $\sim 10^5 \text{ cells ml}^{-1}$ in Chesapeake Bay (Tango et al. 2005). In our study, the non-bloom and bloom concentrations of *P. minimum* were 1500 and 15,000 cells ml^{-1} , which corresponded to ~ 3 and $35 \mu\text{g chl } a \text{ l}^{-1}$, respectively. In the second experiment, *A. tonsa* copepods were fed non-bloom and bloom concentrations of non-toxic and toxic strains of *Karlodinium veneficum* in the same four combinations as listed above for *P. minimum*. Low cell

concentrations *in situ* range from 100 to 1000 ml⁻¹, but *K. veneficum* can reach bloom concentrations ranging from 10⁴ to 10⁵ cells ml⁻¹ (Adolf et al. 2007). In our study, the non-bloom and bloom concentrations of *K. veneficum* were 1000 and 15,000 cells ml⁻¹, which corresponded to ~1 and 35 µg chl *a* l⁻¹, respectively. Only the copepod grazer (*A. tonsa*) was used in the second experiment because in preliminary experiments, *O. marina* did not consume *K. veneficum* (CCMP 1974, data not shown), and in another study, *K. veneficum* was lethal to *O. marina* (Deeds & Place 2006).

Each experiment had a 48-hour acclimation period prior to experimental incubations. In the *P. minimum* experiment, freshly collected adult copepods were individually transferred from beakers into four separate 3.5 l bottles using wide-bore Pasteur pipettes, each with FSW and the appropriate algal food culture and concentration, to a final concentration of 60 copepods l⁻¹ (Saba et al. 2009). Additionally, *P. minimum* food (in the appropriate four treatment conditions) were added to four separate 500 ml bottles of heterotrophic dinoflagellate *O. marina* culture. All bottles were topped off with FSW, covered with parafilm to remove bubbles, capped, and placed on a rotating wheel (1 rpm), and incubated on a 12:12 h light:dark regime at 20°C for 48 hours, similar to acclimation times used in previous copepod feeding studies (Besiktepe & Dam 2002).

At the end of the acclimation period for the *P. minimum* experiment, 72 incubation bottles (500 ml, combusted borosilicate glass) were split into four sets of 18 bottles, each set representing a food condition: log non-bloom, log bloom, late stationary non-bloom, and late stationary bloom. Each set included six controls (FSW + algal food), six copepod treatments (FSW + algal food + copepod grazers), and six *O. marina* treatments (FSW + algal food + *O. marina* grazers). The bottles were filled with FSW

and the appropriate algal food culture and concentration. Zooplankton grazers acclimated to the food conditions were then added to designated treatment bottles. Copepods were added to a final concentration of 60 copepods l^{-1} , and *O. marina* grazers were gently added with silicon tubing affixed to a syringe to a final concentration of 100 cells ml^{-1} , a density common in Chesapeake Bay (Johnson et al. 2003, Vaqué et al. 2006). A suite of samples was taken initially and at the end of the 24-hour incubation: three controls, three copepod treatment bottles, and three *O. marina* treatment bottles for each of the sets were sacrificed for initial sample collection, and the other three control and six zooplankton grazer treatment bottles for each of the sets were incubated similar to those in the acclimation period.

In the second experiment, the acclimation and experimental incubations were set up as in the *P. minimum* experiment, but without the heterotrophic dinoflagellate *O. marina* treatment. The four prey conditions were: (1) non-toxic *Karlodinium veneficum* non-bloom, (2) non-toxic *K. veneficum* bloom, (3) toxic *K. veneficum* non-bloom, and (4) toxic *K. veneficum* bloom.

Oyster Exposure Experiment. To determine the potential toxicity of *Prorocentrum minimum*, we conducted a simultaneous exposure experiment using triploid spat of the eastern oyster, *Crassostrea virginica*, and the Asian oyster, *C. ariakensis*, using methods modified from those used for oyster embryos and larvae in Stoecker et al. (2008). Oyster spat of similar size (shell length 0.8 – 1 mm) were obtained from the oyster hatchery at the Virginia Institute of Marine Science. Oysters were spawned in filtered natural seawater at a salinity of 20-22 psu and a temperature of ~20°C. Upon collection, oysters were incubated in gently aerated FSW for 48 h to purge

any consumed algae and to acclimate to the experimental conditions (20°C, 12:12 h light:dark regime). Oysters were then inspected for good health by checking feeding activity, mantle movement when tapped with a dissecting needle, and valve closure ability, using a dissecting microscope (Olympus SZX12) (Shumway et al. 2006). Oysters that did not pass these criteria were discarded. Individual oysters were then randomly transferred into BD Falcon 6-well culture plates (1 oyster well⁻¹). The treatments used for this experiment are listed in Table 1. Aliquots of 15ml of either FSW, or FSW + algal food at the appropriate cell density, were added to each well. The cell densities of *P. minimum* cultures used for oyster exposure were equivalent to those used in the grazer feeding and nutrient release experiment (non-bloom = 1500 cells ml⁻¹, bloom = 15000 cells ml⁻¹). *Dunaliella tertiolecta* (CCMP 1320) was used as a non-toxic control alga and was supplied to oysters at the same non-bloom and bloom concentrations. To prevent food and oxygen depletion during the exposure, water in each well was emptied and replenished daily with fresh FSW and algae. As oysters were exposed to the experimental algal conditions, they were assessed daily for mortality over 3 days using the criteria described above. Mortality was defined as lack of feeding activity, absence of response to mantle stimulation, and inability of the oyster to maintain valve closure ability (Shumway et al. 2006).

2.3. Sample analyses

Karlotoxin analyses. In the *Karlodinium veneficum* experiment, samples were collected at the beginning and end of the incubation to determine karlotoxin (KmTX2)

concentration. In the non-bloom and bloom treatments, 25-50 ml and 5-10 ml, respectively, of water was filtered through 13 mm PTFE syringe filters (Whatman, 0.2 μm). KmTX2 concentrations (ng ml^{-1}) were measured by liquid chromatography-mass spectrometry (LC-MS) after methanol elution of filters as described in Backvarroff et al. (2008). Under normal nutrient replete conditions CCMP 1609 has no detectable karlotoxin production. Based on our lower detection threshold for karlotoxin, we estimate the toxin level was <0.01 pg/cell.

Bacterial nutrient uptake. Because bacteria can utilize both DOM and inorganic nutrients, we accounted for their potential uptake during experimental incubations in our copepod release rate calculations. Samples for bacterial enumeration were fixed with formaldehyde (final concentration 2%) and frozen (-80°C) until quantified using flow cytometry according to Bouvier et al. (2007). Briefly, fixed cells were stained with SYTO-13 (Invitron Molecular Probes, S7575), and abundance was enumerated on a Cytopeia Influx cell sorter/analyzer (488 nm argon laser) calibrated with 1.1 μm microsphere bead stock using the protocol described by Bouvier et al. (2007). Each sample's cytogram of side scatter (SSC) versus green fluorescence (FL1) was manually gated and analyzed with FloJo 8.8.6 software to determine total bacteria abundance (Bouvier et al. 2007). We calculated specific growth rate, μ (d^{-1}), for each incubation bottle using the following equation (1):

$$\mu = \frac{\ln(B_F/B_0)}{T} \quad (1)$$

where T is the incubation time (1 d), and B_0 and B_F are the initial and final estimates of bacterial biomass in ng C l^{-1} , which were calculated by converting bacterial cell concentration to C biomass by assuming a bacterial cellular C content of $20 \text{ fg C cell}^{-1}$ (Lee & Fuhrman 1987) and then dividing by 10^6 to convert biomass from fg C to ng C .

The potential daily bacterial uptake of DOC during the grazing experiments, (U , $\text{ng C l}^{-1} \text{ d}^{-1}$) was estimated for each incubation bottle assuming a bacterial growth efficiency (BGE) of 50% (Azam et al. 1983) using the following equation (2):

$$U = \frac{\mu * B_0}{\text{BGE}} \quad (2)$$

In treatments with negative μ values, we assumed the nutrient demand, U , was 0. Additionally, using conservative estimates of bacterial molar C:N (4.5; Goldman & Dennett 1991) and C:P (50; Kirchman 2000), we estimated maximum potential N and P uptake, respectively, as described in Saba et al. (2009).

Feeding rates. Whole water samples for algal and protozoa cell counts were preserved with acid Lugol's solution (final concentration 2%). Subsamples for algal cell counts were settled in 1 ml Sedgewick rafter, and five replicate frames of at least 100 cells were counted with a Nikon DIAPHOT-TMD inverted microscope at 600X magnification. Subsamples (2-5 ml) for protozoans were settled in 5 ml Utermöhl settling chambers, and entire contents (100 cells or more) were counted under an inverted microscope after at least 24 hours (Utermöhl 1931; Hasle 1978). Clearance and ingestion rates of *Acartia tonsa* and *Oxyrrhis marina* on algal prey were calculated according to the equations of Frost (1972). Due to changes in *O. marina* abundances during the

incubation, we further adjusted clearance rate calculations according to those described in Båmstedt et al. (2000). Specific growth rates of the heterotrophic dinoflagellates were calculated as in Vaqué et al. (2006).

Predator C, N, and P content. Subsamples of the copepod *Acartia tonsa* (for both *Prorocentrum minimum* and *Karlodinium veneficum* experiments) and the heterotrophic dinoflagellate *Oxyrrhis marina* were collected and analyzed for particulate C (PC), particulate N (PN), and particulate P (PP) content (see analytical methods below) before the start of the acclimation period and after the experimental incubation.

Copepods were filtered onto combusted GF/F filters (n = 30 copepod per filter; n = 3 filters for each food condition), and we filtered 50 ml of *O. marina* culture onto each combusted GF/F filter (n = 3 for each food condition). Replicate blanks (n=3) for PC/PN and PP analysis were prepared by filtering 50 ml or 100ml (depending on volume filtered for other PC/PN samples) of 0.2 µm FSW through combusted GF/F filters. All filters were frozen until analysis.

Nutrient analyses. After bacteria, algal food, and *Oxyrrhis marina* grazer abundance samples were collected, the remaining volume from each bottle was prescreened through a 200 µm sieve (to retain copepods in treatments; controls were treated the same) directly into three filter towers (100 ml each) and filtered through combusted GF/F filters into acid-cleaned, combusted flasks. One GF/F filter was collected for fluorometric chlorophyll analysis (Parsons et al. 1984), a second filter was collected for PC and PN (Carbon-Hydrogen-Nitrogen elemental analyzer, EA1108), and a third for PP was muffled and extracted in hydrochloric acid (Aspila et al. 1976). The remaining filtrate for each replicate was analyzed for organic and inorganic nutrient

concentrations. Concentration of DOC was measured with a Shimadzu TOC analyzer 5000A (minimum detection limit, MDL = 0.5-1.0 $\mu\text{mol l}^{-1}$; coefficient of variance, CV = 2-6%) after acidification and purging of dissolved inorganic carbon (Peltzer et al. 1996; Sharp et al. 2002). Ammonium was measured with the phenol/hypochlorite Koroleff method with MDL = 0.05 $\mu\text{mol l}^{-1}$ and CV = 2.5% (Grasshoff et al. 1983; Parsons et al. 1984), urea was measured with the diacetyl monoxime procedure with MDL = 0.05 $\mu\text{mol l}^{-1}$ and CV = 2% (adapted from Price & Harrison 1987), and DPAs were analyzed using the fluorescent O-phthalaldehyde (OPA) method (MDL = 0.05 $\mu\text{mol l}^{-1}$; CV = 2-4%) (Parsons et al. 1984). Concentrations of nitrate and nitrite (NO_x ; Grasshoff method) and PO_4^{3-} (Koroleff method) (MDL = 0.05 $\mu\text{mol l}^{-1}$; CV = 2-3%), as well as TDN and TDP following persulfate oxidation (MDL = 1.0 $\mu\text{mol l}^{-1}$; CV = 2-3%), were determined with a QuikChem 8500 AutoAnalyzer (Grasshoff et al. 1983, Bronk et al. 2000, Sharp 2002). Concentrations of bulk DON and DOP were calculated by the difference between TDN and inorganic N ($\text{NO}_x + \text{NH}_4^+$) and TDP and PO_4^{3-} , respectively. Copepod release rates (in $\text{ng individual}^{-1} \text{hour}^{-1}$) were calculated according to Saba et al. (2009):

$$\frac{[(\Delta C_t + U_t) - (\Delta C_c + U_c)] \times V}{(N \times T)} \quad (3)$$

where ΔC_t is the change in nutrient concentrations ($\text{ng l}^{-1} \text{day}^{-1}$) in the treatment bottles and ΔC_c is the average change in nutrient concentrations ($\text{ng l}^{-1} \text{day}^{-1}$) in the control bottles; U_t and U_c are estimated values of bacterial uptake ($\text{ng l}^{-1} \text{day}^{-1}$) in the treatment and control bottles (see equation 1b); V is the incubation volume (l), N is the number of grazers in the treatment bottles, and T is incubation time ($24 \text{ hours day}^{-1}$). Nutrient

uptake by algae likely occurred during the incubations, and this nutrient decline is incorporated in this equation in the controls as ΔC_c .

Statistical analysis. Statistical comparisons of the effects of diet on ingestion and release rates, were made by one-way ANOVA, employing the $p = 0.05$ level of significance, using Minitab 15.

3. Results

3.1. Oyster exposure experiment

All *Crassostrea virginica* and *C. ariakensis* oyster spat survived and actively fed by the end of day 3 in the FSW treatment (no food) and in the treatments with non-bloom and bloom concentrations of *Dunaliella tertiolecta* and log phase *Prorocentrum minimum* (Table 1). However, both oyster species experienced mortality after being exposed to late stationary phase *P. minimum* at non-bloom and bloom concentrations. All mortalities occurred between days 2 and 3 of exposure. The percent survival of *C. ariakensis* feeding on non-bloom and bloom densities, and of *C. virginica* feeding on bloom densities, were 87, 80, and 93%, respectively (Table 1). The oysters that lost valve closure ability (considered dead) also appeared to have little internal tissue compared to healthy oysters.

3.2. Karlotoxin concentration

Concentrations of KmTx 2 in *Karlodinium veneficum* (CCMP 1609) and *K. veneficum* (CCMP 2778) at the start of the experimental incubation are shown in Fig. 1. The average initial KmTx 2 concentration in the bloom treatment of *K. veneficum* CCMP 2778 (105 ng ml^{-1}) was significantly higher than in the non-bloom treatment (4 ng ml^{-1} ; Fig. 1). Average cellular concentrations were 9 and 6 pg cell^{-1} for this strain in the bloom

and non-bloom treatments, respectively. *K. veneficum* CCMP 1609, however, had no measureable quantity of KmTx 2 (Fig. 1).

3.3. Algal C, N and P content

Log and late stationary phase *Prorocentrum minimum* cells had similar C contents, averaging 290 and 313 pg C individual⁻¹, respectively (Table 2). However, while log phase cell molar C:N and C:P (7.6 and 93.9, respectively) were near the Redfield molar ratio (6.6 and 106, respectively), late stationary cells were deficient in both N and P, yielding significantly higher average molar C:N and C:P ratios of 13.5 and 277, respectively ($p < 0.05$).

Toxic *Karlodinium veneficum* had slightly higher cellular C, N, and P contents compared to those of non-toxic *K. veneficum* (Table 2), however these differences were not significant. Toxic and non-toxic *K. veneficum* had similar C:N and C:P ratios that were close to the Redfield ratio.

3.4. Grazer C, N, and P content

The *Acartia tonsa* copepod grazers used in the *Prorocentrum minimum* experiment (Expt. 1) had slightly higher initial C, N, and P contents compared to *A. tonsa* used in Expt. 2 (Table 2), but these differences were not significant. While the C:N ratios of *A. tonsa* in both experiments were lower than the Redfield ratio, C:P atomic molar ratios were well above Redfield (235 in Expt.1, 199 in Expt. 2), suggesting that copepods

may have been P deficient at the start of the acclimation period. After 3 days of exposure to experimental algal diets (acclimation + experimental incubation), decreases in copepod body C ranged from 30-38% in Expt. 1 and 19-30% in Expt. 2 (Fig. 2). Losses in copepod body N and P were also significant in both experiments, ranging from 8-51% and 2-37%, respectively (data not shown).

The cellular C, N, and P contents of *Oxyrrhis marina*, the heterotrophic dinoflagellate grazer used in the *P. minimum* experiment, yielded average molar C:N and C:P ratios of 5.7 and 57.8, respectively (Table 2). We were unable to measure elemental contents of heterotrophic dinoflagellate *Oxyrrhis marina* at the end of the experiment due to interference of algal cells remaining on the filters.

3.5. Feeding rates

Algal food C was never grazed below 50% of the initial algal concentration during any experiment. Ingestion rates of all grazers in both experiments were significantly higher in the algal bloom treatments, compared to algal non-bloom treatments (Fig. 3; $p < 0.05$). Ingestion rates of *Acartia tonsa* copepods feeding on non-bloom densities of non-toxic and toxic *Prorocentrum minimum* were not statistically different from each other and averaged 0.8 and 0.5 ng C ind⁻¹ day⁻¹, respectively (Fig. 3a). Ingestion rates of copepods in the toxic *P. minimum* bloom treatment were slightly lower than those in the non-toxic bloom treatment, averaging 25 and 31 ng C individual⁻¹ day⁻¹ or 0.6 and 0.7% of copepod body C day⁻¹, respectively, but these differences were also not significant (Fig. 3a). The heterotrophic dinoflagellate *Oxyrrhis marina* had low

growth rates (0.3 d^{-1}) while feeding on both log and late stationary phases of *P. minimum* bloom treatments, but exhibited mortality in the non-bloom *P. minimum* treatments (mean mortalities = $32 \pm 19\%$ feeding on log phase and $21 \pm 13\%$ feeding on late stationary phase). Ingestion rates of *O. marina* on non-bloom densities of *P. minimum* were significantly lower while feeding on the non-toxic culture compared to the toxic culture ($p < 0.05$), averaging 0.1 and $0.6 \text{ pg C individual}^{-1} \text{ day}^{-1}$, respectively (Fig. 3b). Ingestion rates of *O. marina* feeding on bloom densities of non-toxic and toxic *P. minimum* were not statistically different (mean = 8.2 and $8.8 \text{ pg C individual}^{-1} \text{ day}^{-1}$ or 1.6 and 1.8% of body C day^{-1} , respectively) (Fig. 3b). Ingestion rates of *A. tonsa* copepods feeding on toxic *Karlodinium veneficum* (mean non-bloom vs. bloom = 1.4 vs. $16.2 \text{ ng C ind}^{-1} \text{ day}^{-1}$) were slightly higher compared to those for non-toxic *K. veneficum* (mean non-bloom vs. bloom = 1.1 vs. $11.7 \text{ ng C ind}^{-1} \text{ day}^{-1}$), but these differences were not significant (Fig. 3c).

3.6. Bacteria nutrient uptake

Initial bacterial abundance was higher in the bloom treatments compared to non-bloom treatments for all algal strains used in our study (Table 3). In Expt. 2, bacterial abundances were significantly higher in the toxic *Karlodinium veneficum* non-bloom and bloom treatments compared to their non-toxic *K. veneficum* counterparts. However, in Expt. 1 bacterial abundances were similar in *Prorocentrum minimum* non-toxic log vs. toxic late stationary phases. In the *P. minimum* experiment (Expt. 1), bacterial specific growth rate, μ , was less than 0.2 d^{-1} in all treatments (Table 3). The highest bacterial

abundance and specific growth rates were in non-bloom treatments with the grazer *Oxyrrhis marina*. Bacterial growth was higher in non-toxic *K. veneficum* treatments in Expt. 2, compared to negligible growth in all treatments containing the toxic *K. veneficum*, and there were no differences in μ between non-bloom and bloom treatments. Due to variability in bacterial specific growth rate in the two experiments, estimated average daily bacterial demand of C, N, and P ranged from 0-52.2, 0-13.5, and 0-2.7 $\mu\text{g C, N, and P l}^{-1}\text{ day}^{-1}$, respectively (Table 3). The highest nutritional demands were in treatments with higher initial bacterial biomass and μ (see equation 2).

3.7. Grazer nutrient release

Acartia tonsa DOC release rates were significantly higher in the toxic vs. non-toxic treatments for both non-bloom (11 ng C ind⁻¹ h⁻¹) and bloom (42 ng C ind⁻¹ h⁻¹) *Prorocentrum minimum* densities in Expt. 1 (Fig. 4a). *Oxyrrhis marina* DOC release was detectable only in the toxic *P. minimum* bloom treatment, ranging from 2-20 pg C ind⁻¹ h⁻¹ (Fig. 4b). Similarly, DOC release by *A. tonsa* copepods in Expt. 2 occurred only when feeding on toxic *Karlodinium veneficum*, with average DOC release rates in non-bloom and bloom treatments of 14 and 28 ng C ind⁻¹ h⁻¹, respectively (Fig. 4c).

In contrast to DOC release, copepods in Expt. 1 had significantly higher inorganic N (NH₄⁺) release rates while feeding on non-toxic *P. minimum* at non-bloom and bloom algal densities compared to those densities of toxic *P. minimum* (Fig. 5a). Release rates of NH₄⁺ were also significantly higher in copepods feeding on non-bloom densities of the non-toxic culture of *P. minimum*, averaging 2.4 ng N ind⁻¹ h⁻¹. There was no detectable

NH_4^+ release by the grazer *O. marina* in any *P. minimum* treatment. Similar to Expt. 1, NH_4^+ release rates by copepods in Expt. 2 were significantly higher when feeding on non-toxic *K. veneficum* compared to toxic *K. veneficum* (Fig. 5b). Copepod NH_4^+ release rates were also higher in Expt. 2 than those in Expt. 1 ($p < 0.05$).

When grazer DON release was detected, it was typically a significant portion of total N released (Fig. 6). Although variable, release rates of DON by copepods feeding on *P. minimum* were only detected in the non-toxic bloom treatment, averaging $12.3 \text{ ng N ind}^{-1} \text{ h}^{-1}$ and accounting for 94% of the total N released (Fig. 6a). DON was the only form of released N detected for grazer *O. marina* (Fig. 6b). DON release rates were significantly higher while feeding on the toxic *P. minimum* at bloom concentrations (mean = $10.3 \text{ pg N ind}^{-1} \text{ h}^{-1}$) compared to those feeding in any other treatment. In contrast to NH_4^+ release, copepods in Expt. 2 had significantly higher DON release rates while feeding on toxic *K. veneficum* compared to non-toxic *K. veneficum* (Fig 6c). Additionally, unlike DOC release in Expt. 2, DON release was higher in non-bloom compared to bloom densities ($p < 0.05$, toxic).

Inorganic P (PO_4^{3-}) release rates by *A. tonsa* in Expt. 1 occurred only when feeding on non-toxic *P. minimum* at bloom densities, and averaged $1.7 \text{ ng P ind}^{-1} \text{ h}^{-1}$ (Fig. 7a). There was no detectable PO_4^{3-} release by the grazer *O. marina* in any *P. minimum* treatment, as noted above for NH_4^+ . Release rates of PO_4^{3-} were only detectable for copepods feeding on toxic *K. veneficum* at bloom densities, and averaged $0.4 \text{ ng P ind}^{-1} \text{ h}^{-1}$ (Fig. 7b).

Similar to DON release, when DOP release was detected, it was typically a significant portion of total P released (Fig. 8). Release rates of DOP by *A. tonsa*

copepods and *O. marina* (Expt. 1) were only detectable in treatments with the non-toxic *P. minimum* culture (Fig. 8a and 8b). Additionally, DOP release rates were higher when grazers fed on non-bloom vs. bloom *P. minimum* densities. In contrast to Expt. 1, detectable DOP release by copepods in Expt. 2 occurred only in the toxic bloom *K. veneficum* treatment, and averaged $1.2 \text{ ng P ind}^{-1} \text{ h}^{-1}$ (Fig. 8c).

4. Discussion

Prorocentrum minimum and *Karlodinium veneficum* form widely distributed annual blooms in Chesapeake Bay (Johnson et al. 2003). In our study, we report low feeding rates of copepod *Acartia tonsa* and heterotrophic dinoflagellate *Oxyrrhis marina* on all algal species, regardless of food concentration or toxicity, which suggests these grazers may not be capable of controlling formation of these blooms. Compared to these low feeding rates, however, inorganic nutrient and DOM release rates were often higher and, in addition to nutrient composition, were quite variable with food concentration and between toxic and non-toxic treatments.

4.1. Toxicity of algal cultures

The *Prorocentrum minimum* in late stationary growth phase in our experiment appeared to be toxic (although a toxin was not directly isolated and quantified), as mortality occurred in oyster spat of *Crassostrea virginica* and *C. ariakensis* only when exposed to *P. minimum* in this growth phase. The Asian oyster, *C. ariakensis*, may have been more sensitive to this algae, as it had 80% survival compared to 93% survival of the native oyster, *C. virginica* after 3 days of exposure to bloom concentrations. In comparison, juvenile *C. virginica* oysters had a mean survival of 53% after 11 days of exposure to bloom concentrations of *P. minimum* (Luckenbach et al. 1993). No mortality occurred in either oyster species when exposed to non-bloom and bloom concentrations of *Dunaliella tertiolecta*, log phase *P. minimum*, or when starved, indicating that late

stationary phase *P. minimum* caused a specific adverse reaction in oysters that was unrelated to food concentration.

Karlotoxin (KmTx2) was undetectable in *Karlodinium veneficum* (CCMP 1609) but was present at non-bloom and bloom concentrations of *K. veneficum*, reaching levels shown to be acutely toxic to fish (100 ng ml⁻¹; Deeds et al. 2006).

There were no lethal toxic effects from the algae on the zooplankton grazers used in the experiments. All copepods in both experiments were alive and active after incubation. The heterotrophic dinoflagellate *Oxyrrhis marina* had low mean specific growth rates of 0.3 d⁻¹ in the log and late stationary phases of *P. minimum* bloom treatments, and an overall mean mortality of *O. marina* of 26% occurred in non-bloom treatments with both *P. minimum* growth phases. These results suggest that growth phase, and thus toxicity, of *P. minimum* had no direct effect on grazer *O. marina* growth, but that instead *O. marina* may have been food limited at all algal concentrations (see below).

4.2. Low grazer ingestion rates

Low growth and ingestion rates of *Oxyrrhis marina* could be caused by factors unrelated to food quality. *O. marina* can ingest up to 560% body C d⁻¹ of certain HAB species (Jeong et al. 2001, 2003a; calculated using our estimated 500 pg C cell⁻¹ for *O. marina*), but in our study *O. marina* ingestion rates were below 2% body C d⁻¹. Threshold prey concentrations of *O. marina* range from 80 cells ml⁻¹ (Jeong et al. 2003a) to 10⁵ cells ml⁻¹ (Goldman et al. 1989) depending on the algal food offered. Initial

Prorocentrum minimum concentrations in our study were 1500 and 15,000 cells ml⁻¹, and may have been below *O. marina* feeding threshold levels. Additionally, because algal densities were possibly below *O. marina* threshold feeding levels causing food limitation during the acclimation period, *O. marina* could have started the experimental incubation in stationary or early senescence, phases when low growth and ingestion rates may occur (Goldman et al. 1989).

In contrast, algal concentrations offered in our study were well above known threshold ingestion levels for *Acartia tonsa* copepods (Houde & Roman 1987, Besiktepe & Dam 2002). While copepod grazing rates on *P. minimum* in our study (0.5-31 ng C ind⁻¹ d⁻¹) were similar to those found for *A. tonsa* on non-toxic *P. minimum* in Cohen et al. (2007; 5-21 ng C copepod⁻¹ d⁻¹), they were low ($\leq 2\%$ body C d⁻¹) compared to ingestion rates reported in other studies (Besiktepe & Dam 2002, Colin & Dam 2002, Miller & Roman 2008, Waggett et al. 2008, Saba et al. 2009). For example, *A. tonsa* can ingest >100% of its body C d⁻¹ (Kiorb e et al. 1985, Durbin & Durbin 1992, Besiktepe & Dam 2002), and *A. tonsa* reached its maximum ingestion rate of 10 μ g C ind⁻¹ d⁻¹ (~200% body C) while feeding on *P. minimum* at concentrations of 300-800 μ g C l⁻¹ (Besiktepe & Dam 2002). Additionally, the critical N ingestion rate, the minimum ingestion rate required to balance endogenous metabolism (Gardner & Scavia 1981), of *A. tonsa* was 10% body N copepod⁻¹ d⁻¹ (Miller & Roman 2008). Our ingestion rates were below this critical ingestion rate ($\leq 2\%$ body N d⁻¹), suggesting the copepods in our study were feeding well below potential despite being offered sufficient food concentrations during experimental incubations.

4.3. Similarity in ingestion rates between non-toxic and toxic strains

Grazer ingestion rates in our study were also generally similar between non-toxic and toxic strains at both non-bloom and bloom algal densities. Dam and Colin (2005) also reported no difference in ingestion rates of *Acartia tonsa* feeding on actively growing *Prorocentrum minimum* cells versus cells in the stationary growth phase. In contrast, lower ingestion rates of *A. tonsa* feeding on toxic *Karlodinium* strains compared to a non-toxic strain of *K. veneficum* (CSIC1; Waggett et al. 2008) or *Gymnodinium* sp1 (Vaqué et al. 2006) have also been reported. The source of reduced grazing rates on non-toxic strains as well as toxic strains is unclear, but there may have been other factors that made the non-toxic strains equally unpalatable to grazers. The non-toxic strain of *K. veneficum* used in our study (CCMP 1609) did not produce karlotoxins, but it did have lower C, N, and P contents and slightly higher C:P ratios compared to toxic *K. veneficum* (CCMP 2778), which could have decreased its nutritional sufficiency and palatability to the copepods, resulting in similar grazing rates on both toxic and non-toxic forms. Additionally, the strain of *P. minimum* used in our study (JA-98-01), regardless of growth phase or nutrient content, could have contained unidentified feeding deterrents or toxic compounds that caused ingestion rates to be low in all treatments. Rosetta & McManus (2003) reported that ciliates feeding on *P. minimum* (clone Exuv) had high growth rates compared to those feeding on *P. minimum* (JA-98-01). The toxins associated with French strains of *P. minimum* act by blocking sodium channels (Denardou-Queneherve et al. 1999), which would act to decrease ingestion rates (Colin & Dam 2003, Dam & Colin 2005); however, toxins were not identified in our study or in Rosetta & McManus (2003),

so the exact cause of low feeding rates is unknown. Despite low feeding rates, inorganic nutrient and DOM release rates, when detected, were always > 100% of C, N, and P ingested and were quite variable with food concentration and between toxic and non-toxic treatments.

4.4. Potential causes of variability in nutrient release rates

Signal:noise ratio. Nutrient release rates were highly variable both within individual treatments and between algal food treatments. Variability within treatments could be a result of a low signal:noise ratio. For example, the signal:noise ratio for DOC release was 1-8, the lower end of which is near the MDL. The large error associated with DOC release rates (see error bars, Fig. 4) led to difficulty in closing the C budget. Thus, even after accounting for C ingestion, the observed C lost as DOC is at least two times higher than the decrease in copepod body C content.

Copepod sex ratios. Variability within treatments could also be caused by the presence of both male and female adult copepods in the grazer treatment bottles. Most studies measuring feeding or nutrient release from copepod use only females. However, the male:female ratio of *Acartia tonsa* copepods *in situ* is about 1:1 (Kjørboe 2006). From a subsample of 100 adult copepods used in our study, 44% were male, yielding a male:female ratio of 0.8. Although male copepods typically eat ~50% less than females (Conover 1956; Saage et al. 2009), females may have lower nutrient release rates due to their higher energy requirements for egg production. Thus, variation in the ratio of male:female copepods between replicate experimental bottles in our study may have

caused variations in nutrient release rates within treatments. However, our average release rates are likely closer to those of natural field assemblages than previous studies using females only.

4.5 Effects of starvation on grazer nutrient release

All algal diets used in our study, regardless of toxicity or cell density, deterred grazing by *A. tonsa* and *Oxyrrhis marina*, which likely resulted in starvation and affected the amount and type of nutrients released. When zooplankton are starved, they typically reduce respiration and excretion (Mayzaud 1973, Mayzaud 1976, Fenchel 1982, Kiørboe et al. 1985). In our study, however, when release rates were measurable, they were nearly always greater than what was ingested, and in some cases were three orders of magnitude higher. Because of these variable high nutrient release rates during a time when grazers were not meeting critical C, N, and P requirements, grazers were likely catabolizing body tissue for survival (Mayzaud 1973, Mayzaud 1976, Miller & Roman 2008), specifically proteins (DOC, DON, and NH_4^+ release), amino acids (P release via gluconeogenesis), and possibly RNA (P release). For example, starvation in heterotrophic microflagellates leads to digestion of mitochondria and RNA (Fenchel 1982), which could potentially result in released C, N, and P byproducts. Catabolism of body tissues in our study was also indicated by the loss of copepod body C, N, and P. Differences in reserves within individual copepods likely affected their degree of starvation and subsequent catabolism of material, contributing to variation within treatments.

If nutrient release rates were solely based on catabolism of body tissue due to starvation, however, we might expect to see less variable nutrient release because ingestion rates were low ($\leq 2\%$ body C d⁻¹) and copepod C, N, and P composition was similar in all treatments regardless of toxicity. However, nutrient release rates and the forms of nutrients released (organic vs. inorganic) in our study were significantly different between non-toxic and toxic treatments. This suggests that, in addition to starvation, other factors, including algal food quality and the presence of toxins, may have caused the differences in grazer nutrient release between treatments.

4.6. *Effects of algal growth phase and quality on grazer nutrient release*

Because zooplankton maintain a relatively stable body nutrient content, changes in the quality of their food source (as regulated by body C:N and C:P ratios - poorest quality having highest C:N and C:P), will generate changes in the relative amounts of DOC, N (NH₄⁺, DON), and P (PO₄³⁻, DOP) excreted (Caron and Goldman 1990; Elser and Urabe 1999). In our study, *Prorocentrum minimum* had lower N and P contents, and thus higher C:N and C:P ratios, in the late stationary growth phase compared to the log phase. These differences in algal food chemical composition could have contributed to the differential release of nutrients from grazers by increasing limitation of N and P in the late stationary *P. minimum* treatment. This is supported by the lower release rates (conservation) of NH₄⁺ and DON by copepods, and P by copepods and heterotrophic dinoflagellates, while feeding on late stationary *P. minimum*. However, the opposite occurred with *Oxyrrhis marina* DON release, which was higher in the late stationary

compared to the log *P. minimum* treatment. Thus, interactions of starvation and algal quality most likely had varying effects on the catabolism of the copepod and heterotrophic dinoflagellate grazers. Additionally, because late stationary *P. minimum* caused mortality in oysters in our exposure study, we cannot rule out possible toxic effects on the zooplankton grazers. However, because a toxin was not isolated and characterized from this algae in our study, we can not establish if and how a toxin could impact the behavior, neurology, or biochemical composition of the grazers.

Grazer-mediated sloppy feeding could also have caused nutrient release. Møller (2005) shows that copepod DOC release via sloppy feeding is enhanced when the copepod-to-prey equivalent spherical diameter (ESD) ratio is < 55 . In our study, *Acartia tonsa*/*P. minimum* and *A. tonsa*/*Karlodinium veneficum* ESD ratios averaged 32 and 35, respectively. Additionally, there was typically higher C, N, and P release (Figs. 4-8) when zooplankton fed on algae with higher C, N, and P contents (Table 2), suggesting that differential release from copepods feeding on non-toxic and toxic *P. minimum* and *K. veneficum* with variable elemental compositions could be due to sloppy feeding.

4.7. Effects of karlotoxin on copepod nutrient release

Karlotoxins are lipid-soluble toxins that can negatively interact with membrane sterols, including cholesterol, causing cell lysis in *Oxyrrhis marina* (Deeds & Place 2006), damage to fish gill epithelia (Deeds et al. 2002, Deeds et al. 2006), and increases in ionic permeability of vertebrate membranes (Deeds 2003). Although these interactions have not been examined specifically in copepods, karlotoxin interaction with copepod

cholesterol likely causes similar responses. Thus, upon exposure and ingestion of toxic *Karlodinium veneficum*, the copepods in our study perhaps became “leaky” and suffered increased membrane permeability and subsequent loss of organic material from the cytosol. This is supported by higher *Acartia tonsa* DOC, DON, PO_4^{3-} , and DOP release rates in the toxic *K. veneficum* treatment compared to the non-toxic *K. veneficum* treatment. Furthermore, DOC, PO_4^{3-} , and DOP release was highest when exposed to the highest KmTx 2 concentration (*K. veneficum* toxic bloom treatment).

5. Conclusion

Many HAB species produce toxins or are unpalatable to grazers. All cultures of *Prorocentrum minimum* and *Karlodinium veneficum* used in our study caused grazer deterrence and illustrate the inability of *Acartia tonsa* and *Oxyrrhis marina* to control blooms of these algal species, regardless of toxicity. However, starvation and subsequent catabolism of body tissue caused grazer nutrient release, at rates higher than they ingested. Furthermore, the forms of nutrients released (organic vs. inorganic) varied between non-toxic and toxic strains, with typically higher grazer DOM release occurring in the toxic treatments. Our results are contrary to the previous hypothesis that grazer deterrence caused by HABs will decrease grazer-mediated nutrient recycling (Sunda et al. 2006), the consequences of which are important in understanding nutrient feedback interactions in HABs. Low ingestion rates coupled with high nutrient release rates by the grazers could intensify HABs, especially HABs with high uptake affinities for organic forms of nutrients, or alternatively lead to feedback mechanisms by which non-harmful algae are able to outcompete HABs. Additionally, toxin production by certain HAB species, which may increase under nutrient limitation or in the presence of DOM (Anderson et al. 2002), may be affected by changes in zooplankton grazer nutrient regeneration. In any case, ingestion of HAB species and subsequent nutrient release by zooplankton may drastically affect nutrient cycling dynamics in estuarine and coastal areas with localized, dense HABs. Nutrient release rates will likely vary dependent upon whether zooplankton can ingest alternative, preferred prey during harmful algal blooms; therefore, future studies examining grazer-HAB nutrient dynamics should include mixed diets containing HAB and non-HAB species, or mixed toxic and non-toxic forms of the

same species. Understanding the mechanisms that influence HABs and marine coastal nutrient cycling is of increasing importance, particularly if we are to understand and predict the effects of climate change or enhanced eutrophication on plankton dynamics.

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Table 1. *Crassostrea ariakensis* and *C. virginica* oyster spat exposure experiment treatments with varying algal food and food density and their percentage (%) survival after a 3-day incubation. Non-bloom and bloom food densities are described in the methods. n=30 oysters for each treatment listed.

Oyster spat	Algal Food	Algal Density	Survival (%)
<i>C. ariakensis</i>	None		100
<i>C. virginica</i>	None		100
<i>C. ariakensis</i>	<i>D. tertiolecta</i>	Non-Bloom	100
<i>C. ariakensis</i>	<i>D. tertiolecta</i>	Bloom	100
<i>C. virginica</i>	<i>D. tertiolecta</i>	Bloom	100
<i>C. ariakensis</i>	<i>P. minimum</i> (log)	Non-Bloom	100
<i>C. ariakensis</i>	<i>P. minimum</i> (log)	Bloom	100
<i>C. virginica</i>	<i>P. minimum</i> (log)	Bloom	100
<i>C. ariakensis</i>	<i>P. minimum</i> (late stationary)	Non-Bloom	87
<i>C. ariakensis</i>	<i>P. minimum</i> (late stationary)	Bloom	80
<i>C. virginica</i>	<i>P. minimum</i> (late stationary)	Bloom	93

Table 2. Chemical composition (individual C, N, and P content, C:N, and C:P) of algae, heterotrophic dinoflagellates, and calanoid copepods used in this study. n=8-18 for *Prorocentrum minimum* cultures, n=5-12 for *Karlodinium veneficum* cultures, n=3 for *Oxyrrhis marina* and *Acartia tonsa* grazers. C:N, C:P ratios converted from weight (g g^{-1}) to atomic molar (mol mol^{-1}) according to formulas: C:N (molar) = (C:N - weight) x (14/12); C:P (molar) = (C:P - weight) x (31/12).

		C	N	P	C:N	C:P
		(pg individual ⁻¹)			(molar)	
Expt. 1						
<i>P. minimum</i> (log)	Algae	290 ± 38	46 ± 7	7 ± 1	7.6 ± 1.8	93.9 ± 20.6
<i>P. minimum</i> (late stationary)	Algae	313 ± 61	27 ± 5	3 ± 0.4	13.5 ± 2.0	277 ± 48.3
<i>O. marina</i>	Grazer	500 ± 34	103 ± 13	19 ± 6	5.7 ± 0.8	57.8 ± 6.6
Expt. 2						
<i>K. veneficum</i> (CCMP 1609)	Algae	207 ± 22	35 ± 2	6 ± 0.3	7.0 ± 0.9	77.1 ± 10.2
<i>K. veneficum</i> (CCMP 2778)	Algae	254 ± 27	47 ± 5	7 ± 0.4	6.3 ± 0.9	80.6 ± 12.0
Calanoid copepod		C	N	P	C:N	C:P
		(μg individual ⁻¹)			(molar)	
<i>A. tonsa</i> (Expt. 1)	Grazer	4.2 ± 0.5	1.2 ± 0.3	0.045 ± 0.007	4.1 ± 0.6	235 ± 21.3
<i>A. tonsa</i> (Expt. 2)	Grazer	3.4 ± 0.6	0.8 ± 0.2	0.038 ± 0.006	4.8 ± 0.4	199 ± 26.1

Table 3. Initial bacterial abundance (BA), mean specific growth rate (μ), and mean estimated daily bacterial C, N, and P demands used for uptake corrections on release rates in experiment (Expt) 1 and 2. Nutrient demands (total C, N, and P) were calculated using a bacterial growth efficiency (BGE) estimate of 50%, and estimates of bacterial molar C:N (4.5) and C:P (50) (see methods section for details). $n=3$ for BA, μ , and C, N, and P daily nutritional demands.

	BA (cells ml ⁻¹ x 10 ⁶)	μ (d ⁻¹)	Daily nutritional demand		
			C	N (ng l ⁻¹ d ⁻¹ x 10 ³)	P
Expt 1					
<i>P. minimum</i> (log)					
Non-bloom Control	1.59 ± 0.01	0.03 ± 0.01	1.7 ± 0.9	0.4 ± 0.2	0.09 ± 0.04
Non-bloom + <i>A. tonsa</i>	1.55 ± 0.05	0.07 ± 0.04	4.6 ± 2.2	1.2 ± 0.6	0.24 ± 0.11
Non-bloom + <i>O. marina</i>	1.67 ± 0.04	0.19 ± 0.07	12.9 ± 5.0	3.4 ± 1.3	0.67 ± 0.26
Bloom Control	9.35 ± 0.82	-0.05 ± 0.03	0	0	0
Bloom + <i>A. tonsa</i>	8.34 ± 0.09	0.02 ± 0.01	6.4 ± 4.9	1.7 ± 1.3	0.33 ± 0.25
Bloom + <i>O. marina</i>	8.36 ± 0.11	-0.01 ± 0.04	0	0	0
<i>P. minimum</i> (late stationary)					
Non-bloom Control	1.92 ± 0.09	0.07 ± 0.02	5.2 ± 7.3	1.3 ± 1.9	0.27 ± 0.38
Non-bloom + <i>A. tonsa</i>	1.82 ± 0.09	0.07 ± 0.05	5.2 ± 3.6	1.3 ± 0.9	0.27 ± 0.18
Non-bloom + <i>O. marina</i>	1.91 ± 0.02	0.18 ± 0.05	13.4 ± 3.8	3.5 ± 1.0	0.69 ± 0.20
Bloom Control	8.93 ± 0.16	0.09 ± 0.09	33.0 ± 33.1	8.6 ± 8.6	1.70 ± 1.71
Bloom + <i>A. tonsa</i>	8.91 ± 0.09	0.03 ± 0.03	11.1 ± 12.2	2.9 ± 3.2	0.57 ± 0.63
Bloom + <i>O. marina</i>	9.60 ± 0.64	-0.05 ± 0.07	0	0	0
Expt 2					
<i>K. veneficum</i> (CCMP 1609)					
Non-bloom Control	0.05 ± 0.01	1.03 ± 0.12	2.0 ± 0.2	0.5 ± 0.1	0.10 ± 0.01
Non-bloom + <i>A. tonsa</i>	0.05 ± 0.01	2.34 ± 0.13	4.4 ± 0.3	1.1 ± 0.1	0.23 ± 0.01
Bloom Control	0.64 ± 0.02	0.90 ± 0.04	22.8 ± 1.0	5.9 ± 0.3	1.18 ± 0.05
Bloom + <i>A. tonsa</i>	0.72 ± 0.17	1.81 ± 0.03	52.2 ± 0.8	13.5 ± 0.2	2.69 ± 0.04
<i>K. veneficum</i> (CCMP 2778)					
Non-bloom Control	2.45 ± 0.03	-0.04 ± 0.08	0	0	0
Non-bloom + <i>A. tonsa</i>	2.48 ± 0.03	0.06 ± 0.02	6.0 ± 2.2	1.5 ± 0.6	0.31 ± 0.11
Bloom Control	17.8 ± 0.97	-0.01 ± 0.04	0	0	0
Bloom + <i>A. tonsa</i>	18.9 ± 0.64	0.00 ± 0.02	6.5 ± 6.3	1.7 ± 1.6	0.34 ± 0.32

Fig. 1. Initial karlotoxin (KmTx 2) concentrations of non-toxic and toxic *Karlodinium veneficum* used in this study. x and y denote significance ($x > y$, one-way ANOVA, $p < 0.05$). Mean of $n=3$, error bars = 1 standard deviation.

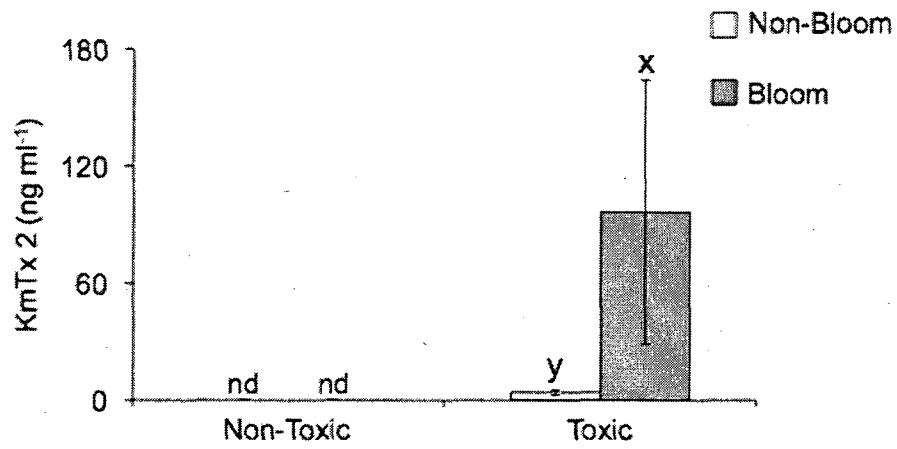


Fig. 2. *Acartia tonsa* C contents in a) experiment 1 with *Prorocentrum minimum* and b) experiment 2 with *Karlodinium veneficum* before the start of the acclimation period (T_0) and after 3 days of exposure to non-toxic and toxic algal cultures of each algal species at non-bloom and bloom algal densities. x and y denote significance ($x > y$, one-way ANOVA, $p < 0.05$). Mean of $n=3$, error bars = 1 standard deviation.

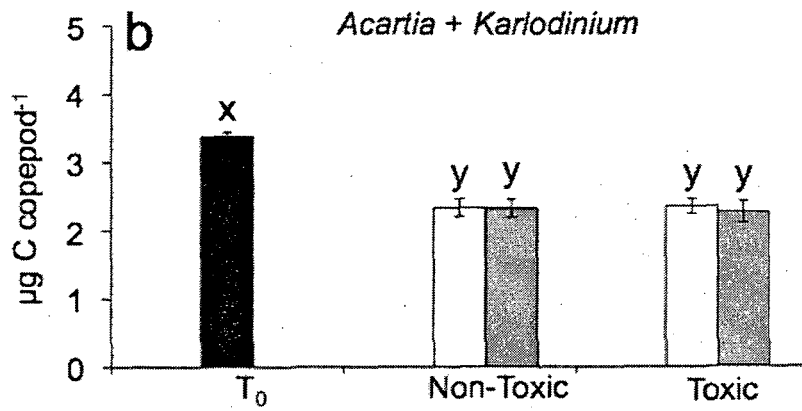
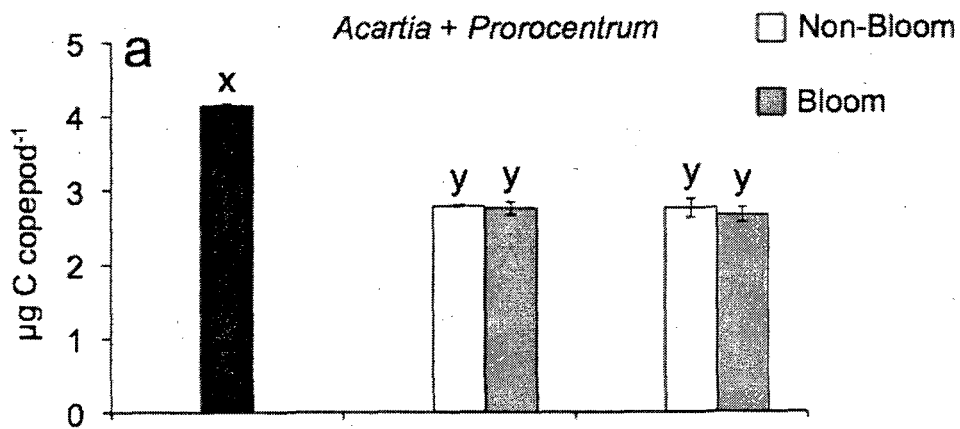


Fig. 3. Ingestion rates (I) of a) copepod *Acartia tonsa* and b) heterotrophic dinoflagellate *Oxyrrhis marina* fed non-toxic (log phase) and toxic (late stationary phase) *Prorocentrum minimum* (Expt. 1), and c) *A. tonsa* fed non-toxic (CCMP 1609) and toxic (CCMP 2778) *Karlodinium veneficum* (Expt. 2) at non-bloom and bloom algal densities. Ingestion rates were converted from cells individual⁻¹ day⁻¹ using average C content of food items shown in Table 2. x and y denote significance (x>y, one-way ANOVA, p < 0.05). Mean of n=3, error bars = 1 standard deviation.

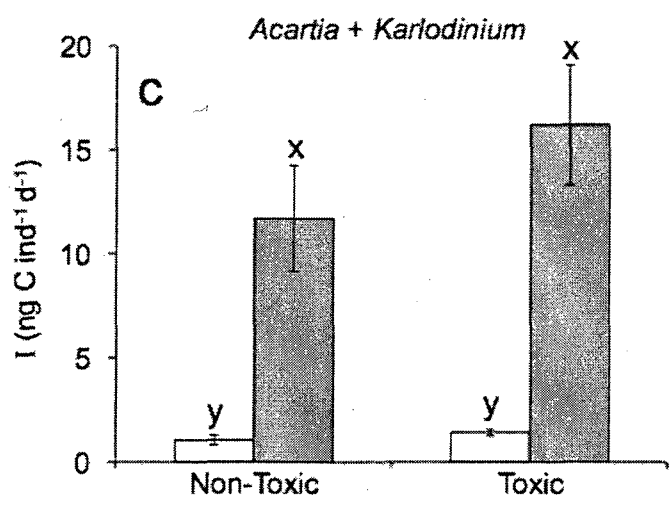
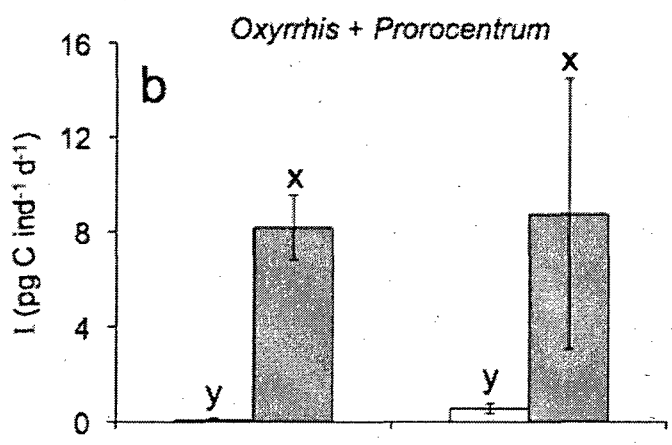
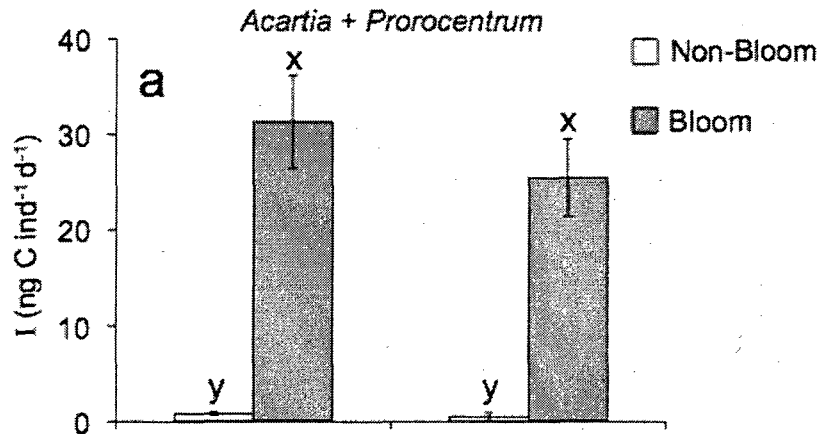


Fig. 4. DOC release rates of a) copepod *Acartia tonsa* and b) heterotrophic dinoflagellate *Oxyrrhis marina* fed non-toxic (log phase) and toxic (late stationary phase) *Prorocentrum minimum* (Expt. 1), and c) *A. tonsa* fed non-toxic (CCMP 1609) and toxic (CCMP 2778) *Karlodinium veneficum* (Expt. 2) at non-bloom and bloom algal densities. x and y denote significance ($x > y$, one-way ANOVA, $p < 0.05$). Mean of $n=3$, error bars = 1 standard deviation. nd = DOC release not detected.

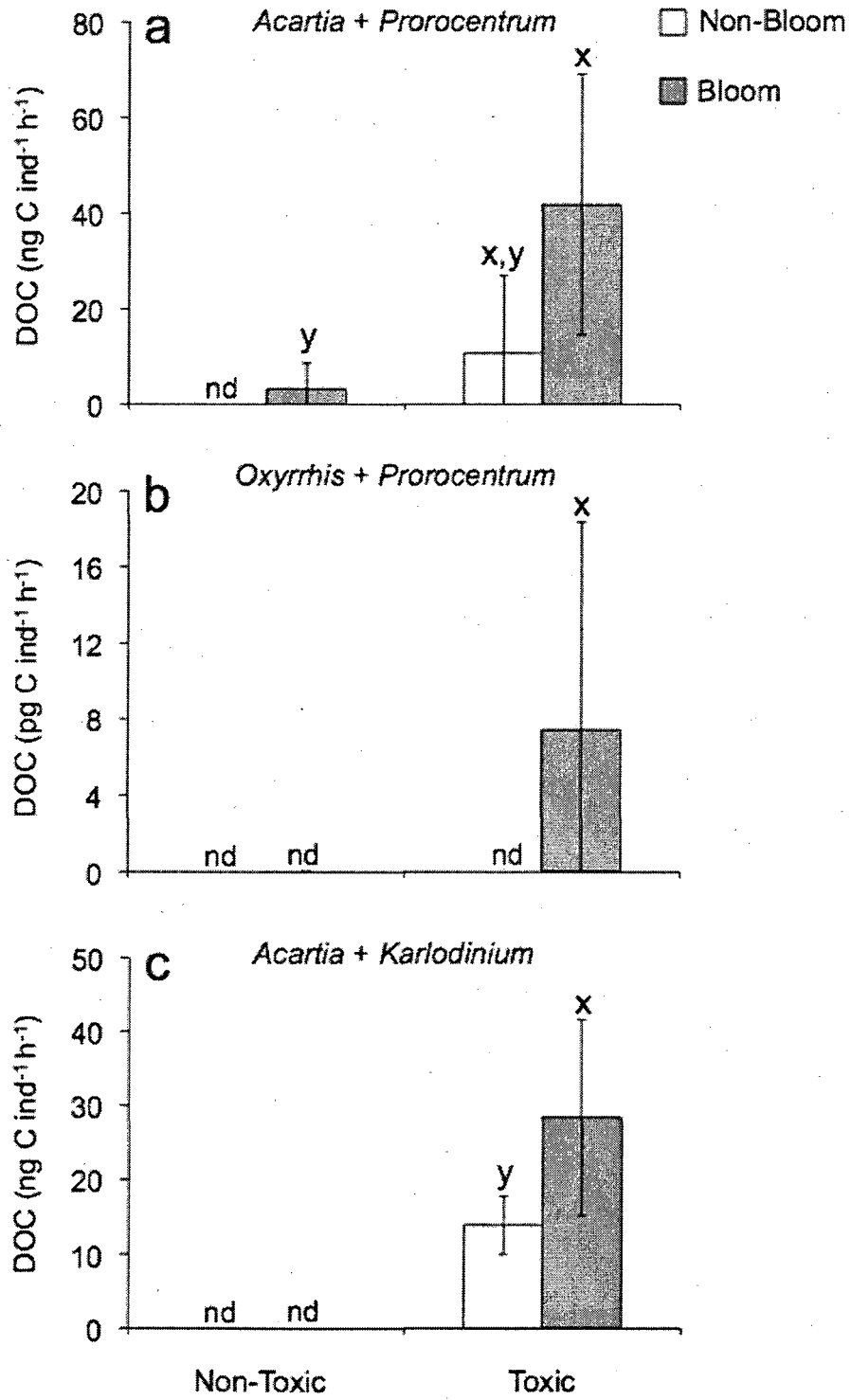


Fig. 5. Inorganic N (NH_4^+) release rates of a) copepod *Acartia tonsa* fed non-toxic (log phase) and toxic (late stationary phase) *Prorocentrum minimum* (Expt. 1), and b) *A. tonsa* fed non-toxic (CCMP 1609) and toxic (CCMP 2778) *Karlodinium veneficum* (Expt. 2) at non-bloom and bloom algal densities. x, y, and z denote significance ($x > y > z$, one-way ANOVA, $p < 0.05$). Mean of $n=3$, error bars = 1 standard deviation. nd = NH_4^+ release not detected.

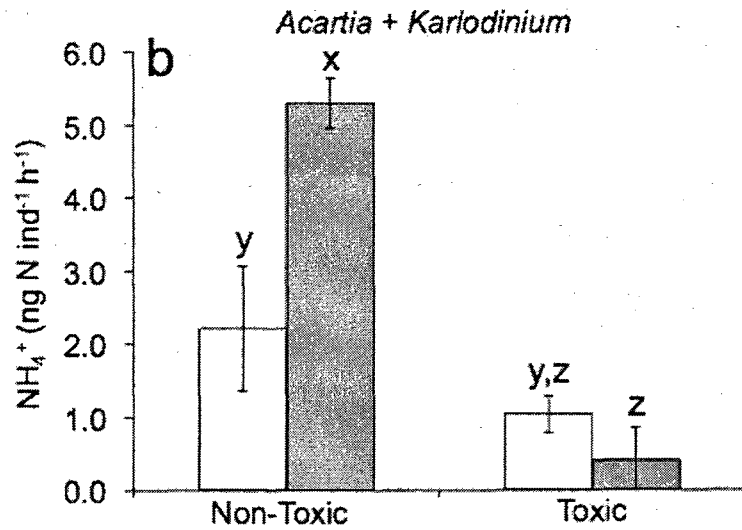
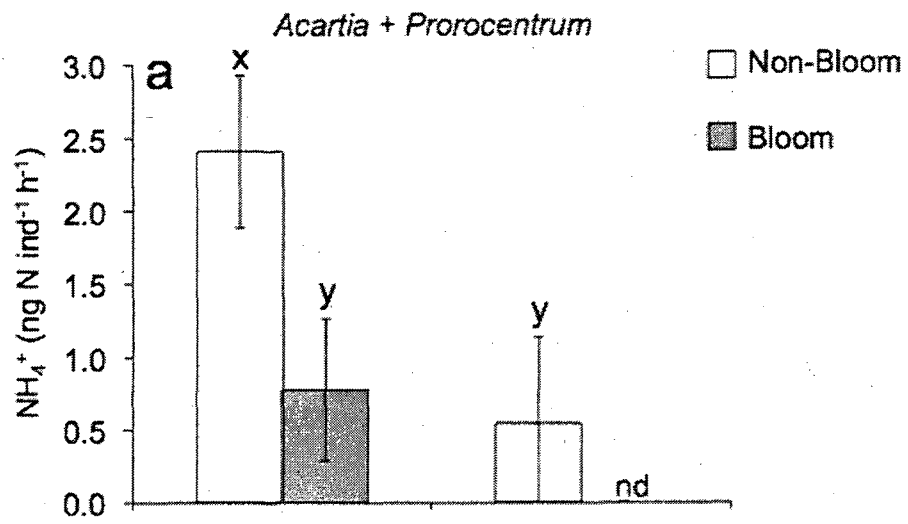


Fig. 6. DON release rates of a) copepod *Acartia tonsa* and b) heterotrophic dinoflagellate *Oxyrrhis marina* fed non-toxic (log phase) and toxic (late stationary phase) *Prorocentrum minimum* (Expt. 1), and c) *A. tonsa* fed non-toxic (CCMP 1609) and toxic (CCMP 2778) *Karlodinium veneficum* (Expt. 2) at non-bloom and bloom algal densities. x and y denote significance ($x > y$, one-way ANOVA, $p < 0.05$). Mean of $n=3$, error bars = 1 standard deviation. nd = DON release not detected. Numbers in parentheses are the average proportion of DON release as % of total dissolved nitrogen ($\text{NH}_4^+ + \text{DON}$) release.

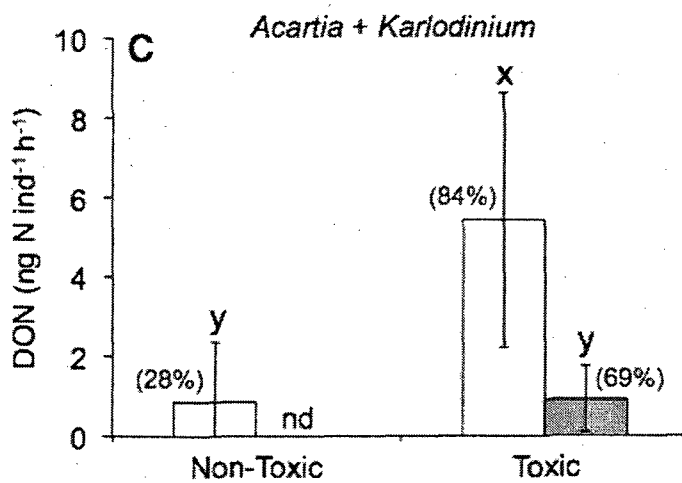
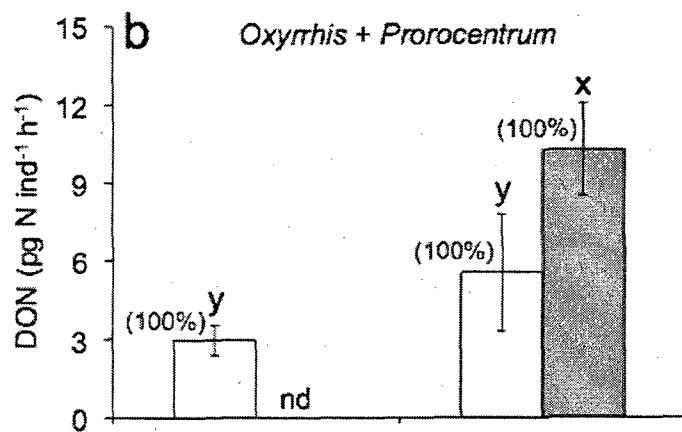
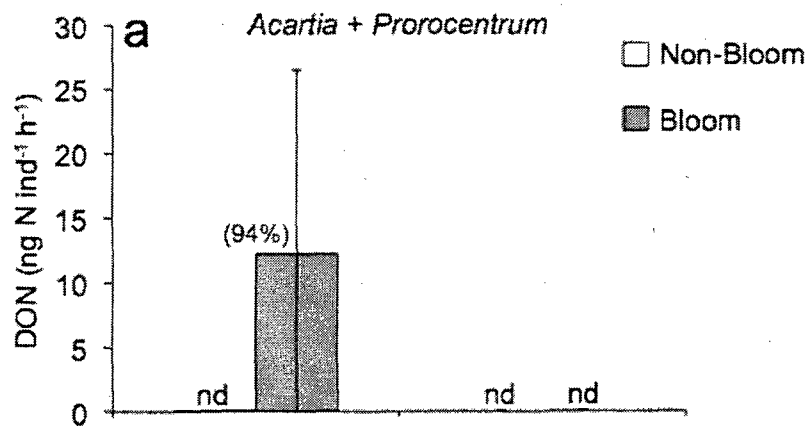


Fig. 7. Inorganic P (PO_4^{3-}) release rates of a) copepod *Acartia tonsa* fed non-toxic (log phase) and toxic (late stationary phase) *Prorocentrum minimum* (Expt. 1), and b) *A. tonsa* fed non-toxic (CCMP 1609) and toxic (CCMP 2778) *Karlodinium veneficum* (Expt. 2) at non-bloom and bloom algal densities. Mean of n=3, error bars = 1 standard deviation. nd = PO_4^{3-} release not detected.

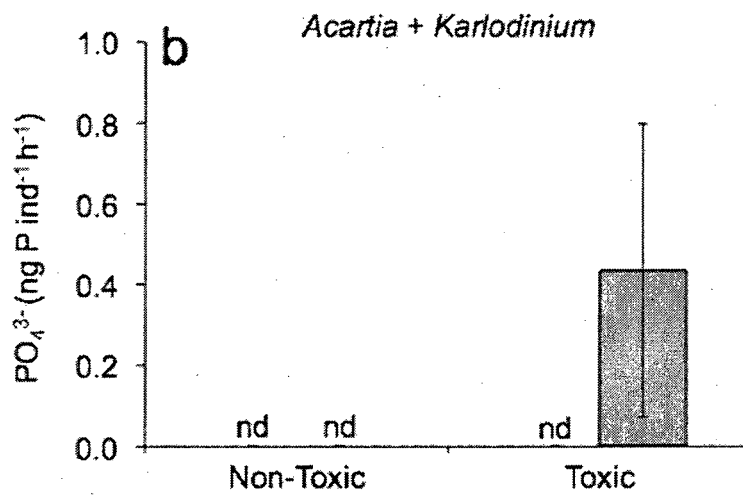
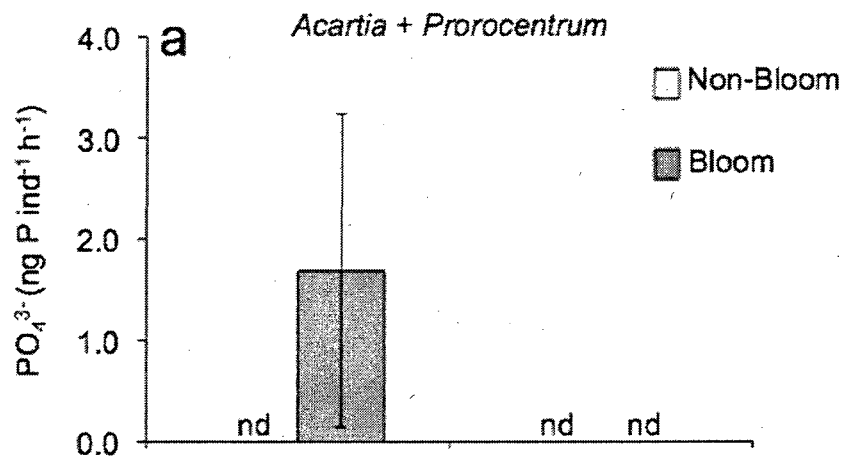
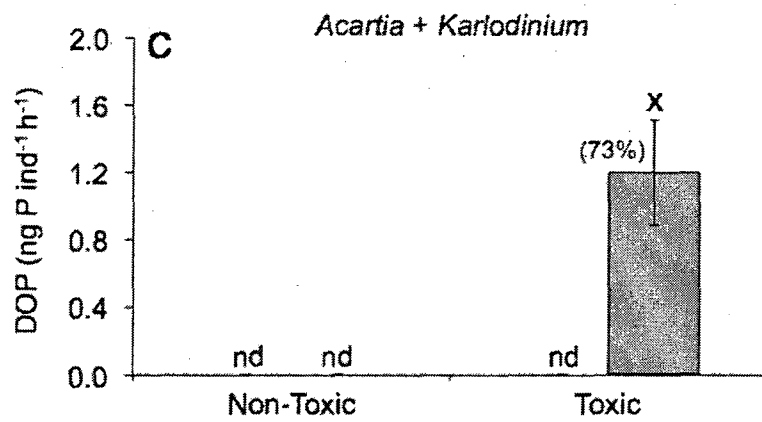
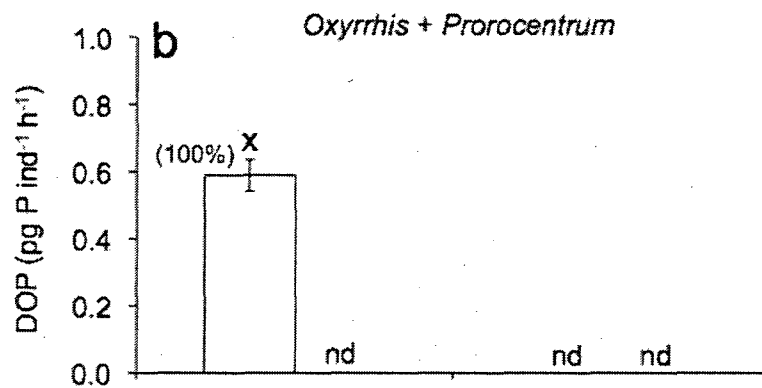
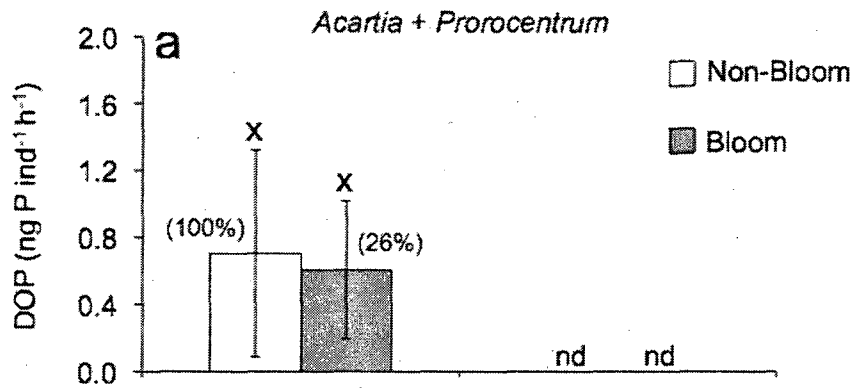


Fig. 8. DOP release rates of a) copepod *Acartia tonsa* and b) heterotrophic dinoflagellate *Oxyrrhis marina* fed non-toxic (log phase) and toxic (late stationary phase) *Prorocentrum minimum* (Expt. 1), and c) *A. tonsa* fed non-toxic (CCMP 1609) and toxic (CCMP 2778) *Karlodinium veneficum* (Expt. 2) at non-bloom and bloom algal densities. x denotes significance (one-way ANOVA, $p < 0.05$). Mean of $n=3$, error bars = 1 standard deviation. nd = DOP release not detected. Numbers in parentheses are the average proportion of DOP release as % of total dissolved phosphorus ($\text{PO}_4^{3-} + \text{DOP}$) release.



CHAPTER 4

The Relative Importance of Sloppy Feeding, Excretion, and Fecal Pellet Leaching in the Release of Dissolved Carbon and Nitrogen by *Acartia tonsa* Copepods

Abstract

Crustacean zooplankton produce dissolved organic matter (DOM) and inorganic nutrients via sloppy feeding, excretion, and fecal pellet leaching. These different release mechanisms of metabolic products, however, have never been individually isolated. Our study was designed to determine the relative importance of these different modes on release of dissolved organic carbon (DOC), ammonium (NH_4^+), and urea from *Acartia tonsa* copepods feeding on the diatom *Thalassiosira weissflogii*. Excretion and sloppy feeding were the dominant modes of DOC production (11 and 5% of particulate organic C ingested, respectively) and NH_4^+ release (34 and 8% of particulate organic nitrogen, PON, ingested, respectively). Urea, however, was predominately produced via sloppy feeding and fecal pellet leaching (10% and 6% of PON ingested, respectively). Urea release via sloppy feeding accounted for 54% of total measured nitrogen (TMN; NH_4^+ + urea) release. TMN release was $> 100\%$ of copepod body N d^{-1} , resulting in low DOC:TMN release ratios (2.2 for sloppy feeding, 2.1 for cumulative release of sloppy feeding, excretion, and fecal pellet leaching). Our results suggest that the mechanism of release plays an important role in the amount of different forms of DOM, NH_4^+ , and urea available to bacteria and phytoplankton.

Introduction

Zooplankton play a key role in the cycling and transfer of nutrients and organic matter in marine food webs (Miller & Landry 1984, Steinberg et al. 2000, 2002, Carlson 2002, Schnetzer & Steinberg 2002, Steinberg & Saba 2008). The products of zooplankton grazing and metabolism are either recycled and available for uptake by bacteria and phytoplankton or transferred to higher trophic levels (Azam et al. 1983, Cushing 1989, Møller & Nielson 2001). Crustacean zooplankton release dissolved organic matter (DOM) and inorganic nutrients via sloppy feeding, excretion, and leaching from egested fecal pellets (Lampert et al. 1978, Møller 2007). Few studies have attempted to tease apart these different mechanisms of nutrient production by zooplankton. These studies focused only on sloppy feeding (Roy et al. 1989, Møller & Nielson 2001, Møller 2005, Møller 2007) or fecal pellet leaching (Roy & Poulet 1990, Urban-Rich et al. 1998, Urban-Rich 1999; Thor et al. 2003), but demonstrate that these processes can generate significant amounts of DOM. Most measured release of DOC (Lampert 1978; Møller & Nielson 2001, Møller et al. 2003, Møller 2007), while only few measured dissolved organic nitrogen (DON) release (Roy et al. 1989; Roy & Poulet 1990, Vincent et al. 2007). Excretion has been extensively reported in the literature (reviewed in Steinberg & Saba 2008). The term “excretion” or “release” used in these studies, however, is typically inclusive of all the release processes cumulatively (Miller & Glibert 1998; Isla et al. 2004; Saba et al. 2009, Ch 2). Our study was designed to isolate the three mechanisms of release: sloppy feeding, fecal pellet leaching, and excretion, to determine their relative importance in C and N release at different time scales.

Release of DOM via sloppy feeding, the physical breakage of the food source, is greatest when cells are too large to be ingested whole and lowest when small phytoplankton cells are ingested whole (Lampert 1978, Møller & Nielson 2001, Møller 2005, Møller 2007). For example, DOM release occurred when the copepod *Calanus hyperboreus* fed on *Thalassiosira fluviatilis* but not when *C. hyperboreus* fed on a smaller prey item, *T. weissflogii* (Strom et al. 1997). DOC released via sloppy feeding (as a fraction of food POC removed from suspension) was 54–69% for *Acartia tonsa* copepods feeding on diatom *Ditylum brightwelli* and dinoflagellate *Ceratium lineatum* (Møller & Nielson 2001), 49% for *Calanus* spp. feeding on natural plankton assemblages (Møller et al. 2003), and 7–36% for three species of copepods feeding on differently sized phytoplankton (Møller 2007). DON release via sloppy feeding was about 28% of total DON release of *Calanus helgolandicus* copepods feeding on *Thalassiosira weissflogii* diatoms (Vincent et al. 2007). Sloppy feeding causes losses of uningested particulate material; thus, determining loss of DOM and inorganic nutrients to sloppy feeding is important in preventing overestimations of ingestion and assimilation (Dagg 1974, Roy et al. 1989).

DOM loss from egested fecal pellets has been argued to be on time scales of minutes for pellets with permeable membranes (Jumars et al. 1989) to hours or even days for pellets with incompletely permeable membranes (Strom et al. 1997; Urban-Rich 1999). Urban-Rich (1999) and Thor et al. (2003) found that *Calanus* spp. and *Acartia tonsa* fecal pellets, respectively, can leach between 34 and 50% of the total C content of the fecal pellet as DOC within the first 48 hours of egestion. Similarly, Roy & Poulet (1990) found a rapid decrease in copepod fecal pellet total dissolved free amino acid

(DFAA) concentration within the first 3 to 5 days. The amount of DOC leaching from fecal pellets can be a function of food concentration and type. Leaching of DOC from fecal pellets is likely higher when copepods are fed high food concentrations (Jumars et al. 1989, Møller et al. 2003) due to higher egestion rates and decreased assimilation efficiencies (Landry et al. 1984; Besiktepe & Dam 2002), higher C:volume ratios of pellets (Urban-Rich et al. 1998), and faster gut-passage times, yielding more dissolved solutes in pellets (Jumars et al. 1989). Additionally, copepod fecal pellets leached more DOC when copepods fed upon dinoflagellates compared to diatoms (Thor et al. 2003), and on heterotrophs compared to phytoplankton (Urban-Rich et al. 1998). Copepods may also enhance DOC release from fecal pellets by ingesting the outer membrane of the fecal pellets (coprophagy), fragmenting pellets via swimming activity (coprorhexy), and morphologically loosening pellets (coprochaly), which leaves the pellet vulnerable to physical and microbial degradation (Lampitt et al. 1990; Noji et al. 1991, Iversen & Poulsen 2007). In addition to passive leaching, DOC and DFAA can be rapidly released from fecal pellets due to bacterial degradation processes and hydrolysis of proteins (Roy & Poulet 1990; Urban-Rich 1999).

The amount of C and N transported from the surface to deep waters is dependent upon the mechanism of release. Products of sloppy feeding and excretion will likely be recycled quickly in the euphotic zone, with the exception of diel vertically migrating zooplankton, which also actively transport dissolved inorganic and organic C and N below the euphotic zone (Steinberg et al. 2000, 2002). Sinking fecal pellets will not only export POC and PON, but also will leach interstitial DOC and DON below the euphotic zone. Separating DOM and inorganic nutrient production by different zooplankton-

mediated release mechanisms is thus important in accurately determining ingestion and assimilation, DOM supply to bacteria in surface and deep waters, stoichiometry of recycled DOM, and will ultimately help us to understand the dynamics of DOM fluxes and standing stocks.

Methods

In order to isolate the three different zooplankton-mediated mechanisms of dissolved C and nutrient release, we conducted this study in two parts. The first experiment was designed to measure sloppy feeding (SF) and total release (TR). Sloppy feeding was assumed to be the only form of nutrient release during a 20 minute incubation with previously starved *Acartia tonsa* copepods feeding on *Thalassiosira weissflogii* diatoms, as 20 minutes is short enough to avoid release via defecation or excretion (Maar et al. 2002, Møller et al. 2003). Total release in this experiment included nutrient release from sloppy feeding, excretion, and fecal pellet leaching after a 3-hour incubation with *A. tonsa* feeding on *T. weissflogii*. In the second experiment, we measured nutrient release directly from fecal pellets (FP) produced by *A. tonsa* copepods, fed *T. weissflogii*, in short (20-minute) and longer (3-hour) incubations. Excretion was then calculated as the difference between total release and the combined sum of sloppy feeding and fecal pellet release, normalized to the 3-hour incubation.

Collection and culture of organisms — *Acartia tonsa*, a common, coastal, omnivorous calanoid copepod, was collected from the York River estuary, U.S.A., a tributary of Chesapeake Bay, via near-surface net tows using a 0.5 m-diameter net with 500 μm mesh and a non-filtering cod end. Healthy, active *A. tonsa* were placed in acid-cleaned buckets with gently aerated 0.2 μm filtered seawater (FSW). Copepods were then fed a diet of *Thalassiosira weissflogii* diatoms for 2-3 days before the start of the experiments.

Thalassiosira weissflogii (CCMP 1336) was cultured using f/2 + Si medium made with the same FSW used in the experiments (salinity = 20 psu). The cultures were

incubated at 20°C on a 12:12 h light:dark regime and maintained in exponential phase by transfers every 3-4 days into fresh media. The FSW used in experiments and nutrient media had a low background of DOM, consisting of a 1:1 ratio of deep Santa Barbara Channel seawater (SBSW) to artificial seawater (ASW) made with sodium chloride combusted at 500°C for 2 hours (Saba et al. 2009).

Experimental Procedure: Sloppy Feeding and Total Release (SF/TR) — The experiment conducted to measure SF and TR contained controls with 450 ml of *Thalassiosira weissflogii* diatoms standardized to 300 $\mu\text{g C l}^{-1}$, and treatments with 36 adult *Acartia tonsa* copepods added to 450 ml of the food described in the control (final concentration of 60 copepods l^{-1}). The polycarbonate incubation bottles were each fitted with a 100 μm mesh screen insert near the bottom to keep copepods separated from their fecal pellets and prevent coprophagy. Copepods were individually placed into FSW and allowed to empty their guts for 3-4 hours prior to being placed into the incubation bottles. Bottles were incubated in the dark at 20°C, and three control and three treatment bottles were sacrificed at each time point [initial (T_0), 20 minutes ($T_{20\text{min}}$; only sloppy feeding release), and 3 hours ($T_{3\text{h}}$; total release)] for analyses. To measure excretion/fecal pellet leaching from non-feeding copepods, an additional triplicate set of treatment bottles with copepods was incubated for 3 hours with *T. weissflogii*, after which the copepods were gently transferred to FSW for another 3-hour incubation. Triplicate bottles filled with FSW served as the controls. All these ‘post-incubation bottles’ were treated the same as those in the feeding experiment.

Fecal pellet production — Fecal pellets became stuck underneath the mesh inserts inside the incubation bottles during the SF/TR experiment; thus we were unable to

accurately enumerate fecal pellets produced during the incubations. Thus, we conducted a separate fecal pellet production experiment immediately following the SF/TR experiment, with feeding bottles (n=6) set up as in the SF/TR experiment. The only difference was instead of the 100 μm mesh disc inserts placed near the bottom, the bottles contained removable 500 μm mesh sieves to retain the copepods and allow fecal pellets to fall through. After the 3-hour incubation, the sieves with copepods were removed, and fecal pellets in each bottle were counted under a dissecting scope. Fecal pellet production was calculated (mean = 2.8 pellets copepod⁻¹ hour⁻¹), and this value was applied to fecal pellet nutrient release rates (below) to estimate the nutrient leaching from fecal pellets in the total release component (T_{3h}) of the SF/TR experiment.

Experimental Procedure: Fecal Pellet Leaching — The experiment conducted to determine nutrient release from fecal pellets (FP) contained controls (190 ml FSW), a ‘biotic’ treatment with 38 *A. tonsa* fecal pellets added to 190 ml FSW, and an ‘abiotic’ treatment of 38 fecal pellets, which were pre-soaked in mercuric chloride (HgCl₂) to kill associated bacteria, added to 190 ml FSW. A suite of samples were taken initially (T₀), after incubating for 20 minutes (T_{20min}), and 3 hours (T_{3h}); three replicate bottles were sacrificed at each time point for controls and treatments. Before the start of the experiment, copepods were fed *Thalassiosira weissflogii* for 12 hours. Then, in small batches, copepods were gently concentrated using 500 μm sieves placed in shallow dishes to retain a small volume of water above the mesh, and individually placed into 20-ml well plates. Using a dissecting scope and an acid-clean, combusted Pasteur pipette, fecal pellets were collected immediately (within 1-2 minutes from evacuation from the copepod) and, for the biotic treatment, placed into appropriate incubation bottles with

FSW. For the abiotic treatment, fecal pellets were gently placed in a concentrated HgCl_2 solution ($20 \text{ g HgCl}_2 \text{ l}^{-1}$; Urban-Rich, pers. comm.) and allowed to soak for 10 minutes before being placed into the incubation bottles containing FSW. Samples for T_0 were taken immediately after each bottle was set up. The $T_{20\text{min}}$ and $T_{3\text{h}}$ bottles were incubated at 20°C in the dark.

Sample analyses

Bacterial nutrient uptake — Because bacteria can utilize both DOM and inorganic nutrients, we accounted for potential nutrient uptake during experimental incubations in our release rate calculations. Samples for bacterial abundance were fixed with formaldehyde (final concentration 2%) and frozen (-80°C) until analysis. Fixed cells were stained with SYTO-13 (Invitron Molecular Probes, S7575), and abundance was enumerated on a Coulter Epics Altra flow cytometer (488 nm argon laser) calibrated with $1.1 \mu\text{m}$ microsphere bead stock using the protocol described by Bouvier et al. (2007).

We calculated bacterial specific growth rate, $\mu \text{ (h}^{-1}\text{)}$, for each incubation bottle using the following equation (1):

$$\mu = \frac{\ln(B_F/B_0)}{T} \quad (1)$$

where T is the incubation time (h), and B_0 and B_F are the initial and final estimates of bacterial biomass in nmol C L^{-1} . Bacterial biomass was calculated by converting bacteria cell concentration to C biomass by assuming a bacterial cellular C content of $20 \text{ fg C cell}^{-1}$ (Lee & Fuhrman 1987), dividing by 10^6 to convert biomass from fg C to ng C , and dividing by 14.01 to convert ng C to nmol C .

The potential hourly bacterial uptake of DOC during the experiments, (U , nmol C L⁻¹) was estimated for each incubation bottle assuming a bacterial growth efficiency (BGE) of 50% (Azam et al. 1983) using the following equation (2):

$$U = \frac{\mu * B_0 * T}{BGE} \quad (2)$$

Additionally, using conservative estimates of bacterial molar C:N (4.5; Goldman & Dennett 1991), and assuming 16% of the N uptake source was organic urea (calculated from Table 1 in Andersson et al. 2006) and 84% was inorganic NH₄⁺, we estimated maximum potential N uptake (Saba et al. 2009).

Feeding rates — Whole water samples for *Thalassiosira weissflogii* diatom cell counts were preserved with acid Lugol's solution (final concentration 2%). Subsamples for algal cell counts were settled in 1 ml Sedgewick rafters, and five replicate frames each of at least 100 cells were counted with a Nikon DIAPHOT-TMD inverted microscope at 600X magnification. Clearance and ingestion rates of *Acartia tonsa* on diatoms were calculated according to the equations of Frost (1972).

Nutrient analyses — Prior to filtering for nutrient analysis, bacteria (FP, SF/TR experiments) and diatom abundance (SF/TR experiment) samples were collected directly from each incubation bottle. In the SF/TR experiment, the remaining volume from each bottle was prescreened through a 200 µm sieve (to retain copepods; controls were treated the same) directly into two filter towers (90 ml each) and filtered through combusted GFF filters into acid cleaned, combusted flasks. One GF/F filter was collected for fluorometric chlorophyll analysis (Parsons et al. 1984), and the second filter was

collected for particulate organic carbon (POC) and particulate organic nitrogen (PON) analysis (CHN elemental analyzer, EA1108). The collected copepods were filtered onto a combusted GF/F, counted under a dissecting microscope (Olympus SZX12), and analyzed for POC and PON content. For each incubation bottle in the FP experiment, the entire content (190 ml) was filtered onto a combusted GF/F filter, and pellets were counted under a dissecting scope and analyzed for PC and PN content. Replicate blanks (n=3) for PC/PN analysis were prepared by filtering 90 ml (SF/TR) or 190 ml (FP) of 0.2 μm FSW through combusted GF/F filters. All POC/PON samples were dried at 55°C and desiccated with 6 N HCl to remove inorganic C prior to measurement (Condon & Steinberg 2008). The remaining filtrate for each replicate was analyzed for organic and inorganic nutrient concentrations. Concentration of DOC was measured with a Shimadzu TOC analyzer 5000A (minimum detection limit, MDL = 0.5-1.0 $\mu\text{mol l}^{-1}$; coefficient of variance, CV = 2-6%) after acidification and purging of dissolved inorganic carbon (Peltzer et al. 1996; Sharp et al. 2002). Ammonium (NH_4^+) was measured with the phenol/hypochlorite method with MDL = 0.05 $\mu\text{mol l}^{-1}$ and CV = 2.5% (Grasshoff et al. 1983; Parsons et al. 1984) and urea was measured with the diacetyl monoxime procedure with MDL = 0.05 $\mu\text{mol l}^{-1}$ and CV = 2% (adapted from Price and Harrison 1987). The sum of NH_4^+ and urea was defined as total measured nitrogen (TMN).

All release rates (sloppy feeding, total release, and fecal pellet leaching; in nmol ind^{-1} or $\text{pellet}^{-1} \text{h}^{-1}$) were calculated according to Saba et al. (2009):

$$\frac{[(\Delta C_t + U_t) - (\Delta C_c + U_c)] \times V}{(N \times T)} \quad (3)$$

where ΔC_t and ΔC_c are changes in nutrient concentrations (nmol L^{-1}) in the treatment (food + copepods or FSW + fecal pellets) and control (food or FSW only) bottles, respectively; U_t and U_c are estimated values of bacterial uptake (nmol L^{-1}) in the treatment and control bottles (see equation 2); V is the incubation volume (L), N is the number of copepods or fecal pellets in the treatment bottles, and T is incubation time (hours). Release rates in the post-incubation bottles were also calculated using this equation, but the controls were bottles with FSW and the treatments were bottles with FSW + copepods. Because these copepods were not feeding, post-incubation release rates incorporate only that from excretion and fecal pellet leaching. In order to compare relative amounts of each mode of release, all rates were converted from nmol ind^{-1} or $\text{pellet}^{-1} \text{ h}^{-1}$ to nmol h^{-1} by multiplying by the number of copepods in the SF/TR or post-incubation experiment bottles (36) or number of fecal pellets in the FP experiment bottles, respectively.

Release due to fecal pellet leaching in the SF/TR experiment, FP_{0-3h} , was estimated using release rates calculated from the FP experiment according to equation 3, and converted from $\text{nmol pellet}^{-1} \text{ h}^{-1}$ to nmol h^{-1} by multiplying by 101, the mean number of pellets 36 copepods produced in 1 hour at our measured fecal pellet production rate of $2.8 \text{ pellets copepod}^{-1} \text{ h}^{-1}$.

In the SF/TR experiment, we made two key assumptions. First, sloppy feeding was the only form of release by copepods between the T_0 and $T_{20\text{min}}$ time points. This incubation time was short enough to avoid production of fecal pellets and excretion of nutrients from previously starved copepods that had just begun feeding. Secondly, based on the linear relationship of sloppy feeding DOC release and C ingestion of *Calanus* spp.

(Møller et al. 2003), we assumed the release of DOC, or of NH_4^+ and urea, from sloppy feeding is proportional to *Acartia tonsa* copepod ingestion rate of C or N, respectively. Thus, reduced feeding rates will yield proportionally lower sloppy feeding release rates. We then estimated sloppy feeding release rates between $T_{20\text{min}}$ and $T_{3\text{h}}$ ($\text{SF}_{20\text{min}-3\text{h}}$, nmol h^{-1}) using the following equation:

$$\text{SF}_{20\text{min}-3\text{h}} = \frac{(\text{SF}_{0-20\text{min}} \times I_{20\text{min}-3\text{h}})}{I_{0-20\text{min}}} \quad (4)$$

where $\text{SF}_{0-20\text{min}}$ is the sloppy feeding release rate (nmol h^{-1}) between T_0 and $T_{20\text{min}}$, and $I_{0-20\text{min}}$ and $I_{20\text{min}-3\text{h}}$ are copepod ingestion rates ($\mu\text{g ind}^{-1} \text{h}^{-1}$) between T_0 and $T_{20\text{min}}$ and between $T_{20\text{min}}$ and $T_{3\text{h}}$, respectively. Finally, we calculated the overall sloppy feeding release rate that occurred in the total 3-hour incubation ($\text{SF}_{0-3\text{h}}$, nmol h^{-1}) using the following equation:

$$\text{SF}_{0-3\text{h}} = \frac{(\text{SF}_{0-20\text{min}} \times 0.333 \text{ h}) + (\text{SF}_{20\text{min}-3\text{h}} \times 2.67 \text{ h})}{3 \text{ h}} \quad (5)$$

where 0.333 and 2.67 h are the amounts of time copepods spend sloppy feeding at release rates of $\text{SF}_{0-20\text{min}}$ and $\text{SF}_{20\text{min}-3\text{h}}$, respectively. The term $\text{SF}_{0-3\text{h}}$ was used to calculate excretion rates (nmol h^{-1}) in the SF/T experiment:

$$\text{Excretion rate} = \text{Total Release}_{0-3\text{h}} - \text{SF}_{0-3\text{h}} - \text{FP}_{0-3\text{h}} \quad (6)$$

Statistical analysis — Statistical comparisons of the effects of diet on ingestion and release rates, were made by one-way ANOVA, employing the $p = 0.05$ level of significance, using Minitab 15.

Results

C and N content of prey, predators, and fecal pellets — The average C and N content of the prey diatom species *Thalassiosira weissflogii* was 68.6 $\mu\text{g C}$ and 11.2 $\mu\text{g N cell}^{-1}$, with an average molar C:N of 7.1 (Table 1). The initial concentration of *T. weissflogii* in the incubation bottles for the sloppy feeding/total release experiment was $249 \pm 41 \mu\text{g C L}^{-1}$. *Acartia tonsa* copepods averaged 5.9 $\mu\text{g C}$ and 1.2 $\mu\text{g N copepod}^{-1}$, yielding a molar C:N ratio of 5.7. Fecal pellets produced by *A. tonsa* contained an average of 17.7 ng C; PON content was below detection level for the number of pellets analyzed.

Feeding rates — Carbon ingestion rates of copepods feeding on *T. weissflogii* in the SF/TR experiment were significantly higher for the first 20 minutes of the incubation ($T_{0-20\text{min}}$) compared to rates from 20 minutes to 3 hours ($T_{20\text{min}-3\text{h}}$) and those calculated over the entire 3-hour incubation ($T_{0-3\text{h}}$; $p < 0.05$; Fig. 1), averaging 24.6, 5.2, and 6.6 $\mu\text{g C ind}^{-1} \text{d}^{-1}$, respectively. Nitrogen (N) ingestion rates, which were calculated using the average molar C:N ratio of *T. weissflogii* (7.1), followed a similar pattern, and averaged 4.0, 0.8, and 1.1 $\mu\text{g N ind}^{-1} \text{d}^{-1}$, respectively (Fig. 1). Average ingestion rates for C and N, calculated over any time period, were all $\geq 100\%$ copepod body C or N d^{-1} .

Release rates — Copepod release rates of DOC and NH_4^+ in the SF/TR experiment were highest between the 20 minute and 3 hour time points, reaching averages of 71 ng C and 30 ng N $\text{ind}^{-1} \text{h}^{-1}$, respectively, lower in the first 20 minutes when sloppy feeding was assumed to be the only form of release, and lowest in the post-incubation during which copepods were not feeding (Fig. 2). However, release rates of urea by *Acartia tonsa* were highest in the first 20 minutes (mean = 20 ng N $\text{ind}^{-1} \text{h}^{-1}$)

compared to all other time periods, but were only significantly higher than the post-incubations (Fig. 2c). Urea accounted for 22% of TMN (NH_4^+ + urea) released from 0-3 hr. and 23% of TMN in the post-incubations. Molar C:N ratios of DOC:urea-N released from 0-3h. were above the Redfield ratio of 6.6 (10.8 ± 5.1), while DOC:TMN ratios were below the Redfield ratio of 6.6 (2.1 ± 0.5).

Copepod sloppy feeding —DOC, NH_4^+ , and urea sloppy feeding release rates were highest in the first 20-minutes, compared to the normalized sloppy feeding release rates (see methods) calculated over other time periods (Fig. 3). Cumulative sloppy feeding release ($\text{SF}_{0-3\text{h}}$) of DOC averaged $13.8 \text{ ng C ind}^{-1} \text{ h}^{-1}$, or 5.0% of C ingested (Fig. 3, Table 2; Fig. 4). Rates of urea release via sloppy feeding were higher than those for NH_4^+ ; however, these differences were not significant (Table 2). Urea averaged 54% of TMN released from sloppy feeding. The molar ratio of released DOC:urea-N and DOC:TMN averaged $5.4 (\pm 3.3)$ and $2.2 (\pm 0.1)$, respectively.

Copepod excretion — Excretion rates of DOC and NH_4^+ were a higher portion of C and N ingested, respectively, compared to release rates of sloppy feeding and fecal pellet leaching (Table 2) but was only significantly higher for NH_4^+ ($p < 0.05$, one-way ANOVA). However, the sum of urea release from sloppy feeding and fecal pellet leaching exceeded total (0-3h) urea release, yielding a slightly negative excretion rate (Eq. 6). Theoretically, excretion rates cannot be negative; thus, we assumed the negative urea excretion rates were 0 (Table 2; Fig. 3).

Fecal pellet release — DOC and NH_4^+ release from fecal pellet leaching in the FP experiment were below detection at all time points (minimum detection limit, MDL = 0.5 to $1 \mu\text{M}$ DOC, $0.05 \mu\text{M}$ NH_4^+). Urea release was below detection in the first 20 minutes

(MDL = 0.05 μM), but averaged 1 ng N pellet⁻¹ h⁻¹, or 6% of N ingested, over the 3-hour incubation in both the biotic and abiotic (HgCl₂-soaked) pellet treatments (Table 2).

Relative importance of various modes of release — Excretion was the greatest proportion of total DOC and NH₄⁺ released (EXC_{0-3h}) in the SF/TR experiment, averaging 68% and 81% of the total, respectively (Fig. 3); this was followed by sloppy feeding (SF_{0-3h}) averaging 32% and 18% of the total, respectively. However, sloppy feeding and fecal pellet leaching were the dominant modes of urea release. Release of DOC, NH₄⁺, and urea during the post-incubation are likely derived mostly from excretion, as fecal pellet production and subsequent leaching will be minimal, and sloppy feeding absent, for non-feeding copepods.

Copepod C and N budgets — The average estimates of ingestion, sloppy feeding, excretion, and fecal pellet egestion and subsequent leaching were used to model C and N budgets of *Acartia tonsa* copepods (Figs. 4 and 5). The relative amount of C allotted to respiration and growth/egg production, and N allotted to growth/egg production, were calculated by subtracting the sum of estimated excretion and egestion from C or N ingestion, respectively. Fecal pellets produced by *A. tonsa* contained an average of 17.7 ng C, which was used to estimate C egestion (Fig. 4). Fecal pellet N content was below detection in this study due to sample size; thus, we assumed a pellet mass C:N ratio of 4.3 determined by Butler & Dam (1994) for *A. tonsa* feeding on exponential phase *Thalassiosira weissflogii* at 2405 cells ml⁻¹ in order to estimate N egestion (Fig. 5). The C budget illustrates that 5% of C removed from suspension was released as DOC via sloppy feeding, 18% POC was egested, and 77% was assimilated (Fig. 4). From the assimilated C, 13% was released via DOC excretion and 87% was allotted to respiration

and growth/egg production. In the N budget, 15% of N removed from suspension was lost as NH_4^+ and urea by sloppy feeding, 23% PON was egested, and 62% was assimilated (28% excreted, 34% allotted to growth/eggs). The assimilation efficiency for C and N, calculated as $(I-E)/I$, where I = ingestion and E = egestion, 82% and 74%, respectively. When we included sloppy feeding, assimilation efficiencies decreased to 81% and 69%, respectively.

Discussion

The present study is the first to tease apart all mechanisms of zooplankton grazer-mediated release (sloppy feeding, excretion, and fecal pellet leaching) simultaneously. Additionally, we compare simultaneous release of DOC, NH_4^+ , and urea. Until now, previous studies have been limited to measuring only one or two release mechanisms at a time or measuring only DOC or DON, and most studies measuring DOM and inorganic nutrient release from zooplankton typically report total release or assume excretion is the main mode of release. We demonstrate that the relative importance of the mechanism of release is different for DOC, NH_4^+ , and urea. Additionally, our study is the first to report urea release via sloppy feeding and fecal pellet leaching.

C and N contents of copepods and diatoms — *Acartia tonsa* copepod C and N contents and C:N molar ratios are within the range of those found for *A. tonsa* copepods in Miller & Roman (2008; 2.5-5.5 $\mu\text{g C}$, 0.50-1.5 $\mu\text{g N}$, and 4.1-5.8 mol:mol, respectively). The average C and N content of the food item, diatom *Thalassiosira weissflogii*, were also within range of those measured by Saba et al. (2009; 46-83 pg C and 8-15 pg N , respectively). The initial concentration of *T. weissflogii* in the incubation bottles for the sloppy feeding/total release experiment, $249 \pm 41 \mu\text{g C L}^{-1}$, was near the prey concentration for which maximum ingestion rates of *A. tonsa* feeding on *T. weissflogii* were found by Besiktepe & Dam (2002).

Fecal pellet production rates and C and N content — The fecal pellet production rate of *A. tonsa* feeding on diatom *T. weissflogii* in the present study, 2.8 pellets $\text{ind}^{-1} \text{h}^{-1}$, is within range of *A. clausi* (syn. *A. hudsonica*) feeding on a variety of prey items (1.0-4.1 pellets $\text{ind}^{-1} \text{h}^{-1}$; Honjo & Roman 1978) and slightly lower than *A. tonsa* feeding on

exponential-phase *T. weissflogii* at 2405 cells ml⁻¹ (3.5 pellets ind⁻¹ h⁻¹; Butler & Dam 1994). POC content of fecal pellets produced by *A. tonsa* in our study (18 ng C pellet⁻¹) were near the minimum of the range of C contents of pellets produced by larger copepods *Calanus hyperboreus* and *C. finmarchicus* (20 to 80 ng C pellet⁻¹) reported in Urban-Rich (1999) but were lower than those measured by Honjo & Roman (1978) for *A. clausi* (syn. *A. hudsonica*) feeding on coccolithophores (133-276 ng C pellet⁻¹) or natural seawater (96-187 ng C pellet⁻¹) and also for *A. tonsa* feeding on *T. weissflogii* (ca. 30-375 ng C pellet⁻¹, Butler & Dam 1994; 121 ng C pellet⁻¹, Hansen et al. 1996). The inclusion of inorganic C in fecal pellet total C estimates by Honjo & Roman (1978) and Butler & Dam (1994) may partially account for the comparatively lower pellet C content in the present study (POC only). Additionally, fecal pellet C estimates by Butler & Dam (1994) and Hansen et al. (1996), which were converted from ng um⁻³ to ng pellet⁻¹, may be artificially high due to their determination of pellet volume using linear two-dimensional measurements (Hansen et al. 1996).

Feeding rates — The average copepod C ingestion rate from 0-20 min (24.6 µg C ind⁻¹ d⁻¹) was higher than any previously reported ingestion rate for *Acartia tonsa* feeding on *Thalassiosira weissflogii* or any food item at concentrations used in our study (250 µg C L⁻¹). This is likely because ingestion rates are typically measured from longer incubation times (hours to days). Copepods in our study were starved for 3 to 4 hours prior to experimental incubations, which likely resulted in the large grazing and sloppy feeding signal observed at the beginning of the incubation (Fig. 1, Fig. 3). The ingestion rate calculated for the remainder of the incubation (20 min– 3 hr) and the overall ingestion rate during the entire incubation (0-3 hr) (3-7 µg C ind⁻¹ d⁻¹) were similar to

rates previously reported (ca. 6-9 $\mu\text{g C ind}^{-1} \text{d}^{-1}$) for *A. tonsa* copepods feeding on *T. weissflogii* at food concentrations similar to those in our study (Besiktepe & Dam 2002).

Relative importance of various modes of DOC and NH_4^+ release — In studies conducted by Møller et al. (2003) and Møller (2007), sloppy feeding played a major role in copepod DOC production. Although sloppy feeding by copepods resulted in release of DOC and NH_4^+ in our study, excretion was the dominant mode of release during the 3-hour incubation (11% C ingested; 10% C removed from suspension). Total DOC release of *Acartia tonsa* feeding on *Thalassiosira weissflogii* in our study (15% of C ingested) was within range of that previously reported (6 to 20% of C ingested) (Saba et al. 2009). NH_4^+ excretion rates in our study (mean = 15.4 $\text{ng ind}^{-1} \text{h}^{-1}$) are within range of those previously reported for *Acartia tonsa* (1.4 to 17 $\text{ng N ind}^{-1} \text{h}^{-1}$, Saba et al. 2009; undetectable to 28 $\text{ng N ind}^{-1} \text{h}^{-1}$, Miller & Glibert 1998).

Sloppy feeding release of DOM is dependent upon the size of the prey relative to the predator (Lampert 1978, Møller & Nielson 2001, Møller 2005, Møller 2007). As such, the ratio of copepod-to-prey equivalent spherical diameter (ESD) has been used as a predictor of release of DOM by sloppy feeding, with significant release occurring below a certain threshold value (Møller 2005, Møller 2007). By applying our calculated copepod-to-prey ESD ratio of 31.8, we predicted the fraction of total C removed from suspension lost as DOC via sloppy feeding by *A. tonsa* feeding on *T. weissflogii* to be 30.1 and 8.2% using the predictive equation by Møller (2005) and Møller (2007), respectively. The actual DOC release by sloppy feeding (as the fraction of total C removed from suspension) measured in our study for *A. tonsa* feeding on *T. weissflogii* was higher for the first 20 min (10%) compared to the entire 3 hr experiment (5%),

resulting from the decline in ingestion rate after the first 20 min. Similarly, high release of DOC via sloppy feeding (as percentage of POC removed from suspension) reported in Møller et al. (2003; 49%) and Møller (2007; 7-36%) was likely due to starving their copepods for 3-6 hours and running their experiments for only 20-30 min. Our normalized rate of sloppy feeding release calculated over the entire 3 hr experiment (SF_{0-3h}), as opposed to the sloppy feeding release rate in the first 20 min of the incubation ($SF_{20min-3h}$), is more likely to occur *in situ* where copepods that are exposed to a relatively constant food supply and are feeding at lower, less variable constant rates. However, rapid rates of sloppy feeding and subsequent nutrient release may occur in the surface waters during nighttime feeding of diel migrating zooplankton after periods of reduced or no feeding activity (and reduced release of metabolic byproducts) during the daytime.

Because we did not use a more sensitive method for detecting DOC release, such as ^{14}C isotope tracer methods (Urban-Rich 1999; Møller et al. 2003; Thor et al. 2003), we likely underestimated the significance of fecal pellet leaching to the total DOC pool. Although DOC release rates from fecal pellets were below lower detection limits, leaching likely occurred. Fecal pellet leaching of urea, which contains organic C and N, was detectable in our study. If we assume 1 mole of DOC (urea-C) is released from fecal pellets with every 2 moles of urea-N, DOC release is < 1% of total C ingested. Likewise, if we apply a maximum pellet DOC release rate of $0.22 \text{ ng DOC pellet}^{-1} \text{ h}^{-1}$ measured in copepods by Urban-Rich (1999) to estimate DOC leached from pellets in our study, DOC release would still be < 1% of total C ingested, a significantly low contribution to the total DOC pool compared to excretion and sloppy feeding (Fig. 4). These low rates of DOC release from pellets may be due to the diatom prey used in this study. Previous

studies have shown that pellets derived from diatoms have slower rates of DOC release and decomposition compared to pellets derived from cryptophytes, nanoflagellates, or dinoflagellates (Hansen et al. 1996; Thor et al. 2003).

Relative importance of various modes of urea release — Urea release did not follow the same patterns as DOC and NH_4^+ release. Instead, sloppy feeding and fecal pellet leaching were the dominant modes of urea release, and the contribution of excretion to total urea release was negligible. In contrast, sloppy feeding accounted for a lower portion of total DON release (21%) compared to excretion (79%) in estimates by Vincent et al. (2007) for *Acartia discaudata* feeding on the diatom *Skeletonema costatum*. However, their “excretion” term includes fecal pellet leaching, as the two were not experimentally separated. We further discuss sloppy feeding release of urea below.

Previous studies have measured significant DFAA content in copepod fecal pellets (Poulet et al. 1986) as well as release of DFAA from leaching of fecal pellets within 3 to 5 days of production (Roy and Poulet 1990). Our study is the first to report urea release from fecal pellets, which was an estimated 60% of the total urea released in the 3-hour incubation. Furthermore, urea release rates from fecal pellets were similar in the biotic and abiotic treatments, suggesting that direct leaching, and not active bacterial degradation, was the mechanism of urea release from fecal pellets in our study. This is likely due to pellet type, as diatom-based pellets are poor substrates for pellet-associated bacteria (Hansen et al. 1996). These results suggest that fecal pellets can be a significant source of urea, and thus sinking pellets may be important in exporting urea below the euphotic zone.

The rate of total urea release (0-3 hr; Fig. 3c) should equal the sum of the release from sloppy feeding, excretion, and fecal pellet leaching in the same time frame. However, the sum of urea release in the latter three terms exceeds the total, suggesting uptake of urea by bacteria during the 3-hour incubation that was unaccounted for. Lampert (1978) also found lower cumulative DOC release in a 3-hour incubation compared to a shorter 15-minute incubation, and also argued that this was due to bacterial uptake as well as reingestion of previously broken cells (causing decreased release via sloppy feeding) in the longer incubation. The estimate we used for bacterial uptake of urea was 16% of total N uptake; however, bacteria could have utilized more urea relative to NH_4^+ in this study. For example, bacterial urea uptake of $\geq 50\%$ total N, or a lower BGE ($\leq 40\%$, compared to the 50% used in uptake calculations), would have accounted for the discrepancy between total urea release and the sum of sloppy feeding, excretion, and fecal pellet leaching.

Sloppy feeding release of NH_4^+ and urea — Sloppy feeding by copepods also resulted in the release of NH_4^+ and urea at surprisingly high proportions of particulate N ingested, 8 and 10%, respectively. This suggests that diatoms contained intracellular pools of NH_4^+ and urea that comprised 8 and 10% of their cellular N. Diatoms contain storage vacuoles (Lomas & Glibert 2000) and possess a complete urea cycle which may cause accumulation of urea in the cytosol (Armbrust et al. 2004), however, *Thalassiosira* spp. accumulate low to undetectable internal pools of NH_4^+ (Conover 1975; Lomas & Glibert 2000) and urea (Conover 1975; Price & Harrison 1988). Thus, there were likely other causes of high release of NH_4^+ and urea during the first 20 minutes of the incubation. Bacterial activity, upon breakage of the algal cells, could have caused rapid

transformation of N, yielding NH_4^+ and urea as byproducts. Bacteria have a strong growth response in the presence of *Acartia tonsa* copepod feeding byproducts (Vargas et al. 2007), and exhibit increases in enzymatic hydrolysis in the presence of feeding zooplankton (Wambeke 1994; Richardot et al. 2001). In addition, although we assumed that sloppy feeding was the only mechanism of release in the first 20 minutes of the incubation, basal excretion of NH_4^+ and urea could have occurred and accounted for a portion of the released N.

Nutrient release by feeding and non-feeding copepods — Release via excretion and fecal pellet leaching was consistently lower in non-feeding copepods compared to feeding copepods. This was also demonstrated by significantly higher NH_4^+ excretion in feeding Antarctic krill compared to those in FSW (Ikeda and Dixon 1984; Atkinson and Whitehouse 2000). Thus, zooplankton excretion rates measured in the absence of food will likely underestimate excretion.

Implications for copepod C and N budgets — While molar DOC:urea-N release ratios were near or above Redfield C:N, DOC:TMN release ratios were below Redfield C:N. This is because unlike N release, where we included organic and inorganic forms, the C term only contains organic dissolved C released (DOC) and excludes inorganic C respired (CO_2). Nonetheless, more N was released as NH_4^+ and urea (via sloppy feeding, excretion, and fecal pellet leaching) or egested as PON than accumulated into growth/egg production (PON). Similarly more N was released than accumulated in a variety of copepod species feeding mainly on diatoms, suggesting reduction in transfer of N to higher trophic levels (Hasegawa et al. 2001). Consequently, the variable partitioning of C and N by copepods results in different fluxes of DOC, NH_4^+ , and urea, and increases

the amount of dissolved forms of N in the water column, which may be readily available to bacteria to fuel the microbial loop (Daly et al. 1999).

Summary and Conclusion

The relative importance of different mechanisms of zooplankton-mediated release (sloppy feeding, excretion, fecal pellet leaching) varied for DOC, NH_4^+ , and urea. Excretion and sloppy feeding were the dominant modes of DOC and NH_4^+ release, while sloppy feeding and fecal pellet leaching were dominant modes of urea release. Urea release via sloppy feeding and fecal pellet leaching has not been reported previously in the literature. The transformation of high proportions of ingested PON to dissolved NH_4^+ and urea, may shunt more of the available N to the microbial food web and less to higher trophic levels. Products of sloppy feeding and excretion (DOC, NH_4^+ , urea) will be rapidly released during feeding activity in the euphotic zone or actively transported via diel vertically migrating zooplankton, and sinking fecal pellets can potentially transfer POC, PON, and urea below the euphotic zone. Released products will support bacterial growth and fuel the microbial loop. Additional studies of the relative magnitude and timing of the various release mechanisms, including how these are affected by diet and how C, N, and P are coupled within the individual mechanisms of release, will be key to our understanding of nutrient dynamics throughout the water column.

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Table 1. Individual carbon (C) and nitrogen (N) content and molar C:N of *Thalassiosira weissflogii* diatoms, *Acartia tonsa* copepods, and *A. tonsa* fecal pellets in this study. C:N ratios converted from weight (g: g⁻¹) to atomic molar (mol: mol⁻¹) according to formulas: C:N (mol: mol⁻¹) = (C:N in g: g⁻¹) x (14/12). bd = below detection. All values are averages (n=2), standard deviation in parentheses.

Species	C	N	Unit	C:N (molar)
<i>T. weissflogii</i> (diatom)	68.6 (7.0)	11.2 (1.0)	pg	7.1 (0.2)
<i>A. tonsa</i> (copepod)	5.9 (0.2)	1.2 (0.1)	µg	5.7 (0.1)
<i>A. tonsa</i> (fecal pellet)	17.7 (8.2)	bd	ng	-

Table 2. *Acartia tonsa* release rates of dissolved organic carbon (DOC), ammonium (NH₄⁺), and urea from sloppy feeding (SF_{0-3h}), excretion (EXC_{0-3h}), and fecal pellet leaching (FP_{0-3h}) during the 3-hour sloppy feeding/total release experiment (SF/TR). Release rates are averages (n=3) ± 1 SD. (%) = release rate as the proportion of C or N ingestion rate. bd = below detection.

Source of release	DOC	NH ₄ ⁺	Urea
	(ng C ind ⁻¹ h ⁻¹)	(ng N ind ⁻¹ h ⁻¹)	(ng N ind ⁻¹ h ⁻¹)
Sloppy feeding	13.8 ± 13.3 (5%)	3.4 ± 2.5 (8%)	4.5 ± 4.8 (10%)
Excretion	29.2 ± 15.2 (11%)	15.4 ± 7.3 (34%)	0 (0%)
	(ng C pellet ⁻¹ h ⁻¹)	(ng N pellet ⁻¹ h ⁻¹)	(ng N pellet ⁻¹ h ⁻¹)
Fecal pellet leaching	bd (0%)	bd (0%)	1.0 ± 0.1 (6%)

Figure 1. Ingestion rates (I) of *Acartia tonsa* copepods fed *Thalassiosira weissflogii* diatoms in the sloppy feeding/total release experiment (SF/TR) between time points 0 and 20 minutes ($T_{0-20\text{min}}$), 20 minutes and 3 hours ($T_{20\text{min}-3\text{h}}$), and 0 and 3 hours ($T_{0-3\text{h}}$). Ingestion rates were converted from cells individual⁻¹ day⁻¹ (cells ind⁻¹ d⁻¹) using average C and N contents of *T. weissflogii* shown in Table 1. x, y, and z denote significant differences between rates (x>y>z; one-way ANOVA, p < 0.05). Mean of n=3, error bars = 1 standard deviation.

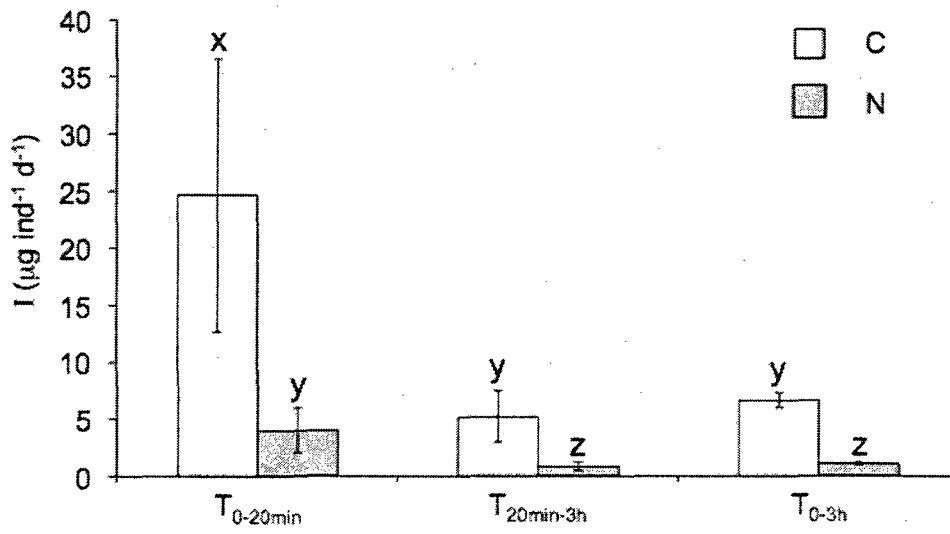


Figure 2. Release rates of a) dissolved organic carbon (DOC), b) ammonium (NH_4^+), and c) urea by the copepod *Acartia tonsa* between time points 0 and 20 minutes ($T_{0-20\text{min}}$), 20 minutes and 3 hours ($T_{20\text{min}-3\text{h}}$), 0 and 3 hours ($T_{0-3\text{h}}$) while feeding on *Thalassiosira weissflogii* in the sloppy feeding/total release experiment (SF/TR), and by non-feeding *A. tonsa* during the post-incubation in filtered seawater (FSW) (Post-Inc). x and y denote significant differences between rates ($x > y$; one-way ANOVA, $p < 0.05$). Mean of $n=3$, error bars = 1 standard deviation.

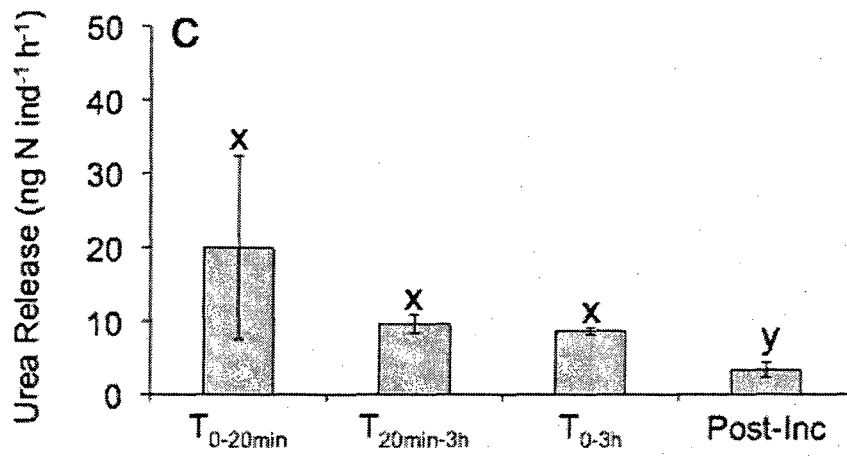
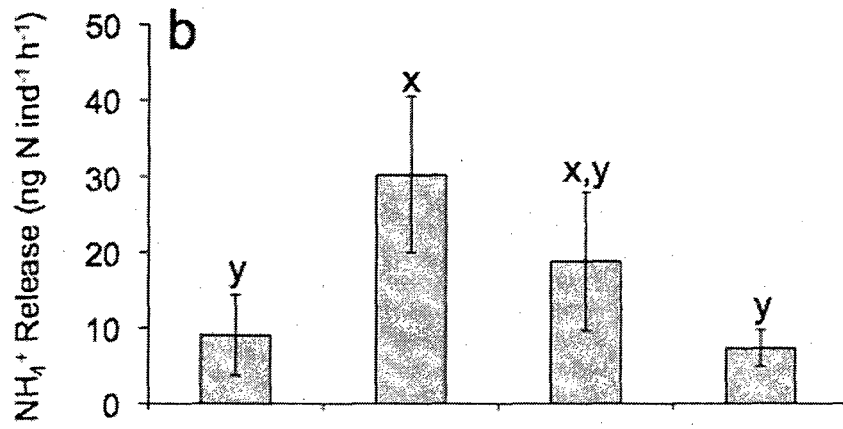
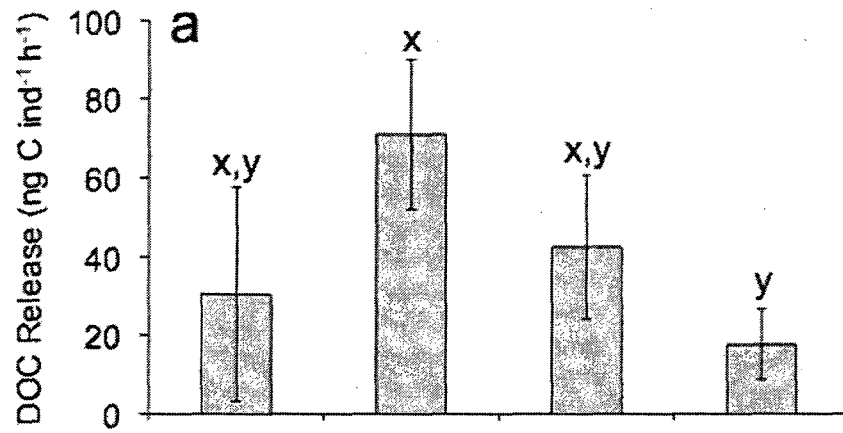


Figure 3. Rates of various modes of a) dissolved organic carbon (DOC), b) ammonium (NH_4^+), and c) urea release by the copepod *Acartia tonsa*, including sloppy feeding between time points 0 and 20 minutes ($\text{SF}_{0-20\text{min}}$), 20 minutes and 3 hours ($\text{SF}_{20\text{min}-3\text{h}}$), and 0 and 3 hours ($\text{SF}_{0-3\text{h}}$); fecal pellet leaching ($\text{FP}_{0-3\text{h}}$); excretion ($\text{EXC}_{0-3\text{h}}$); total release in the sloppy feeding/total release experiment (SF/TR) ($\text{Total}_{0-3\text{h}}$), and during the post-incubation in FSW (Post-Inc). $\text{SF}_{20\text{min}-3\text{h}}$ rates were normalized according to ingestion rates (Eq. 4), and sloppy feeding rates over the 3 hour SF/TR experiment, $\text{SF}_{0-3\text{h}}$, were calculated according to Eq. 5. $\text{FP}_{0-3\text{h}}$ was estimated using release rates calculated from the FP experiment according to equation 3, and converted from $\text{nmol pellet}^{-1} \text{h}^{-1}$ to nmol h^{-1} by multiplying by 101, the number of pellets 36 copepods will produce in 1 hour at our measured fecal pellet production rate of 2.8 pellets copepod $^{-1} \text{h}^{-1}$. $\text{EXC}_{0-3\text{h}}$ is calculated by subtracting the sum of $\text{SF}_{0-3\text{h}}$ and $\text{FP}_{0-3\text{h}}$ from $\text{Total}_{0-3\text{h}}$. Values in parentheses are molar ratios of DOC:total measured nitrogen (TMN; NH_4^+ + urea). bd = below detection.

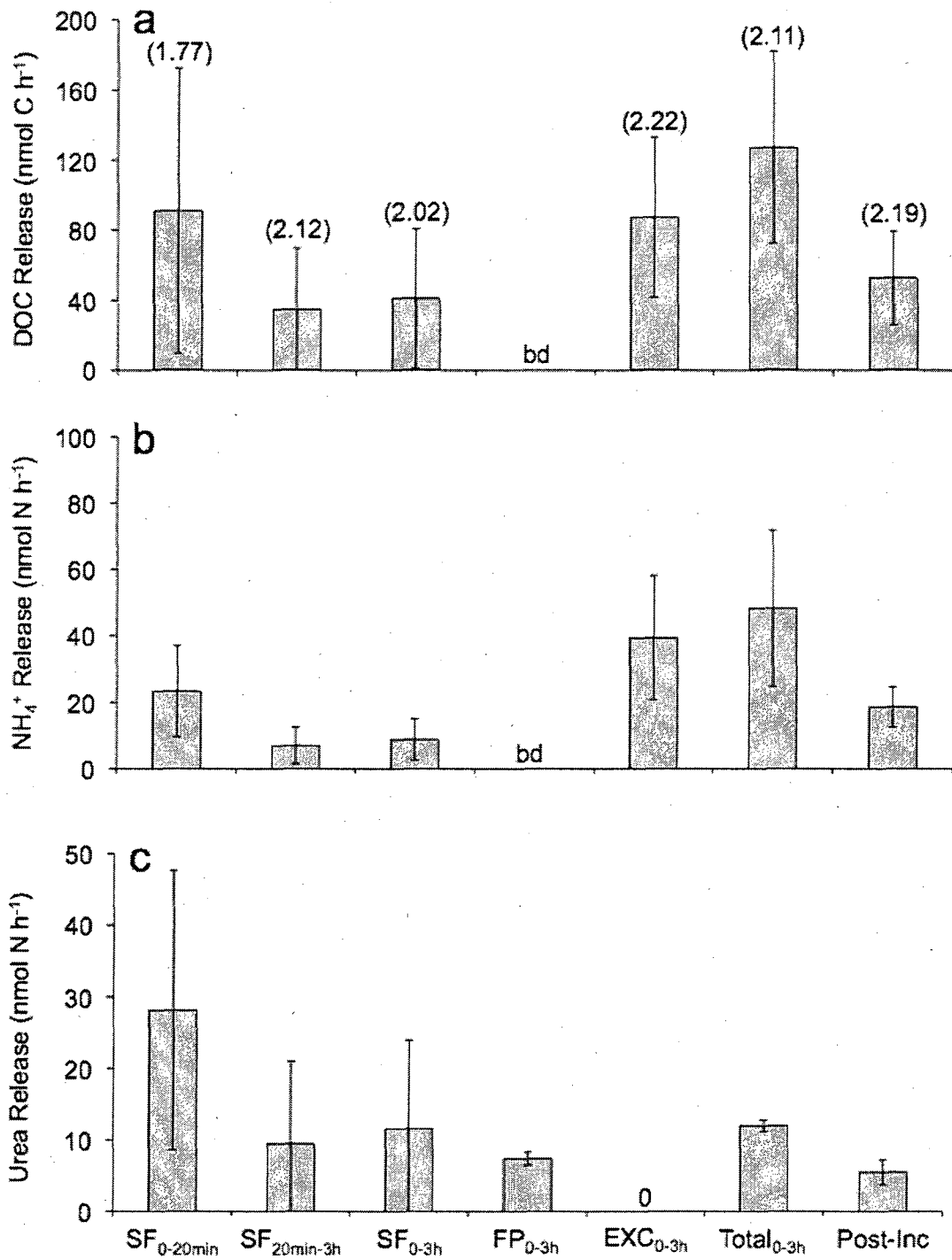


Figure 4. *Acartia tonsa* carbon (C) flow during feeding on *Thalassiosira weissflogii* in the sloppy feeding/total release experiment (SF/TR). The sloppy feeding (SF) and excretion (EXC) estimates were based on normalized release rates during the 3-hour incubation, or SF_{0-3h} and EXC_{0-3h}, respectively. Egestion estimates were calculated from measured fecal pellet carbon content and production rates. Respiration and growth/egg production was calculated by subtracting excretion and egestion from particulate organic C (POC) ingested. The first value shown is the calculated average rate of release or assimilation (ng C individual⁻¹ h⁻¹) from which the percentage of C removed from suspension was calculated (second value). Modified from Møller et al. (2003) and Steinberg & Saba (2008).

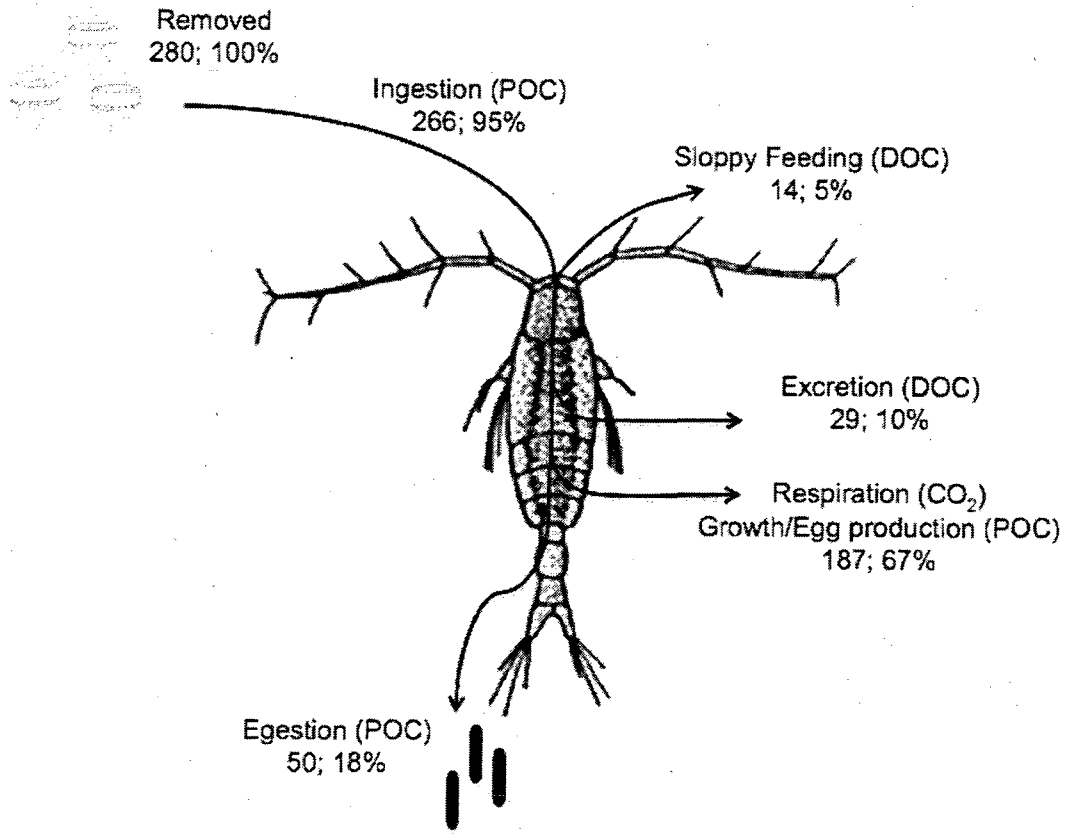
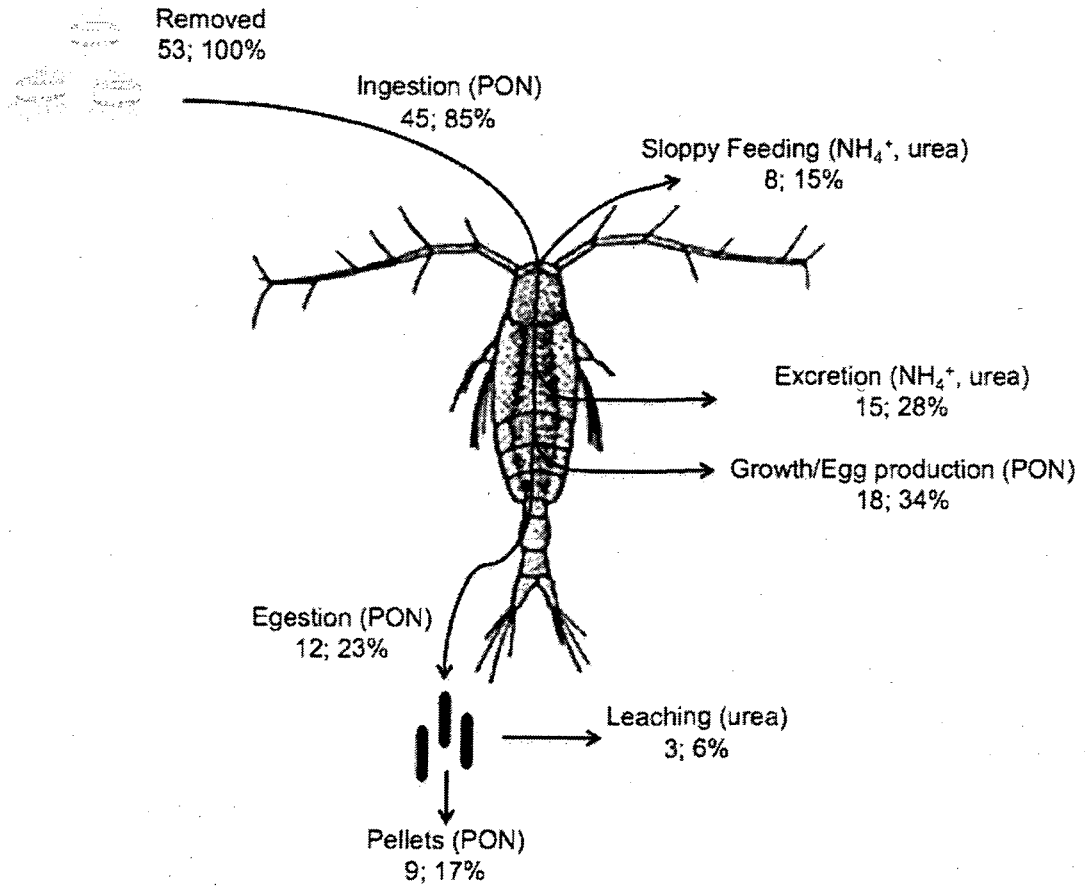


Figure 5. *Acartia tonsa* nitrogen (N) flow during feeding on *Thalassiosira weissflogii* in the sloppy feeding/total release experiment (SF/TR). The sloppy feeding (SF), excretion (EXC), and fecal pellet leaching (FP) estimates were based on normalized release rates during the 3-hour incubation, or SF_{0-3h}, EXC_{0-3h}, and FP_{0-3h}, respectively. Egestion of particulate organic N (PON) was calculated using carbon (C) egestion rates and applying a fecal pellet C:N ratio of 4.3 (g g⁻¹) measured by Butler & Dam (1994) for *A. tonsa* feeding on exponential phase *T. weissflogii* at 2405 cells ml⁻¹. Growth/egg production was calculated by subtracting excretion and egestion from PON ingested. The first value shown is the calculated average rate of release or assimilation (ng N individual⁻¹ h⁻¹) from which the percentage of N removed from suspension was calculated (second value). Modified from Møller et al. (2003) and Steinberg and Saba (2008).



CHAPTER 5
Summary and Future Direction

Zooplankton play a central role in marine food webs as grazers of primary production, as prey for higher trophic levels, and in the cycling of organic and inorganic material (Miller & Landry 1984, Steinberg et al. 2000, 2002, Carlson 2002, Schnetzer & Steinberg 2002, Steinberg & Saba 2008). Via feeding on phytoplankton and other zooplankton, zooplankton recycle DOM and inorganic nutrients that become available for uptake by bacteria and phytoplankton. The effects of different food sources on copepod feeding rate and reproduction has been the focus of a number of studies (Stoecker & Egloff 1987, Stoecker & Capuzzo 1990, Kleppel & Burkart 1995, Bonnet & Carlotti 2001, Broglio et al. 2003). My research demonstrated that copepod nutrient release rates, composition, and stoichiometry are significantly affected by diet (Ch. 2, Ch. 3). Despite similar ingestion rates while feeding on an exclusively carnivorous diet, an exclusively herbivorous diet, and an omnivorous mixed diet, all with similar C:N ratios, DOC, urea, DOP, NH_4^+ , and PO_4^{3-} release rates of *Acartia tonsa* copepods were extremely variable (Ch. 2). The highest DOC, NH_4^+ , and TDN release rates occurred while copepods were feeding carnivorously, while the lowest release rates occurred while feeding on a mixed omnivorous diet, likely due to higher copepod C and N gross growth efficiencies (GGE) in the more 'well-balanced' diet (Ch. 2). Additionally, dissolved organic phosphorus (DOP) release was only detectable when copepods were feeding carnivorously on heterotrophic dinoflagellates. Because microzooplankton are an important component of the diet of many mesozooplankton, future studies examining copepod metabolism, growth efficiency, and inorganic and organic nutrient release should include mixed diets consisting of phytoplankton and microzooplankton in order to better model the role of zooplankton in nutrient and C budgets.

Zooplankton also have complex interactions with HABs, which can affect grazing, reproduction, and ultimately, nutrient regeneration (Sunda et al. 2006). Contrary to the previous hypothesis that grazer deterrence caused by HABs will subsequently decrease grazer-mediated nutrient recycling (Sunda et al. 2006), my study showed that grazer deterrence of all cultures of HAB species *Prorocentrum minimum* and *Karlodinium veneficum* by the copepod *Acartia tonsa* and the heterotrophic dinoflagellate *Oxyrrhis marina* led to starvation, which subsequently caused higher DOM and inorganic nutrient release rates via catabolism of body tissues (Ch. 3). Additionally, I suggest the enhanced nutrient release of *A. tonsa* feeding on karlotoxin-producing *K. veneficum* was due to direct disruption of copepod membranes from the toxin. Low ingestion rates coupled with high nutrient release rates by the grazers could intensify HAB bloom proliferation, especially HAB species with high uptake affinities for organic forms of nutrients, or alternatively lead to feedback mechanisms by which non-harmful algae are able to outcompete HABs. These complex feedbacks have the potential to cause substantial changes in nutrient cycling dynamics in estuarine and coastal systems, and thus, warrant further investigation. Additionally, because certain zooplankton can selectively feed on alternate food sources during harmful algal blooms, future studies examining grazer-HAB nutrient dynamics should include mixed diets containing HAB and non-HAB species.

Finally, previous studies have demonstrated that sloppy feeding and fecal pellet leaching are important mechanisms for the release of DOM (Jumars et al. 1989, Roy et al. 1989, Roy & Poulet 1990, Urban-Rich et al. 1998, Urban-Rich 1999, Møller & Nielson 2001, Møller et al. 2003, Thor et al. 2003, Møller 2007; Vincent et al. 2007), yet no

previous studies have attempted to simultaneously isolate all mechanisms of zooplankton-mediated release (sloppy feeding, fecal pellet leaching, and excretion). Thus, little is known of the relative importance of each process to carbon and nitrogen production. Through a series of experiments and calculations, I isolated the individual release mechanisms and found that the relative importance of sloppy feeding, excretion, and fecal pellet leaching varied for DOC, NH_4^+ , and urea (Ch. 4). Excretion and sloppy feeding were the dominant modes of DOC and NH_4^+ release, while sloppy feeding and fecal pellet leaching were dominant modes of urea release. These results have implications for the rapidity and location at which the regenerated nutrients are recycled in the water column. Additionally, copepods transformed a high proportion of PON to dissolved NH_4^+ and urea, yielding low molar ratios of released DOC:TDN, which may ultimately provide more of the regenerated N to the microbial food web and less to higher trophic levels. Additional studies of zooplankton-mediated nutrient release mechanisms, including how these are affected by food quantity and quality and how C, N, and P are coupled within the individual mechanisms, would be beneficial to our understanding of nutrient dynamics throughout the water column.

The results of this dissertation emphasize the importance of diet and release mechanism on the regeneration of nutrients, particularly DOM, by zooplankton. DOM was a significant proportion of total dissolved nutrients released by zooplankton in multiple experiments reported here. Future studies measuring organic release, as well as what factors regulate the composition and bioavailability of the organic material released, would provide valuable additional information on the role of zooplankton in ocean carbon and nutrient budgets.

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