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Anthropogenic Causes of Copepod Mortality and Bacterial Decomposition of Copepod Carcasses

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ANTHROPOGENIC CAUSES OF COPEPOD MORTALITY AND BACTERIAL DECOMPOSITION OF COPEPOD CARCASSES

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APPROVAL SHEET

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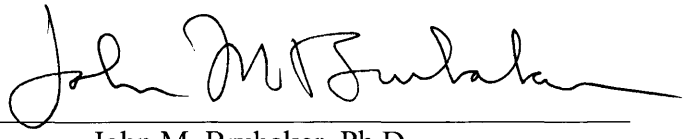

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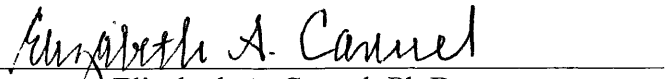

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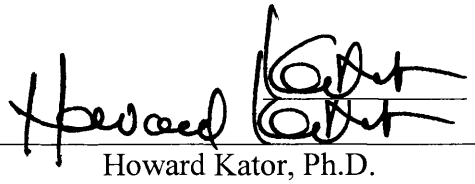

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And most importantly, to my family: I could not have made it this far without your unconditional love and support. Thank you for accepting me and my strange fascination with school, and not asking me (too often) when I was going to get a job.

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ABSTRACT

Although zooplankton carcasses can be quite prevalent within aquatic systems, they have largely been overlooked in most zooplankton population studies. Anthropogenic stressors can potentially increase the overall abundance of carcasses on a local scale. Once a carcass is present within a system, the fate of its biomass is of considerable interest as it may be remineralized within the water column or transported to depth. Through the collection of field samples I assessed the possibility of an anthropogenic stressor (boat-generated turbulence) as a potential source of non-consumptive mortality. I also conducted a series of laboratory experiments to monitor the decomposition of representative crustacean and non-crustacean zooplankton carcasses and determined the fate of carcass-derived organic matter. Higher carcass abundances were found within boat wakes than outside boat wakes, indicating that boat-generated turbulence could have induced non-consumptive mortality of zooplankton. Copepod carcasses decomposed at a much faster rate than rotifer carcasses, suggesting that crustacean zooplankton carcasses would likely be decomposed within the water column and support bacterial production while non-crustacean zooplankton could serve as a transport mechanism of high quality POM to depth.

ANTHROPOGENIC CAUSES OF COPEPOD MORTALITY AND BACTERIAL DECOMPOSITION OF COPEPOD CARCASSES

CHAPTER 1

Introduction to the thesis

Prevalence of zooplankton carcasses

Zooplankton play a critical role in aquatic systems. They can have a significant impact on biogeochemical cycling through diel vertical migration (Longhurst & Harrison 1988, Al-Mutairi & Landry 2001) and fecal pellet production (Bruland & Silver 1981, Urrère & Knauer 1981). Additionally, they provide an integral trophic link between primary production and higher trophic levels. Mortality reduces the overall biomass and number of active individuals thereby influencing the overall impact of zooplankton within the system. In many zooplankton population dynamic models, biomass loss is typically only attributed to predation (Steele & Henderson 1992), which often cannot account for all zooplankton mortality (Boersma et al. 1996, Hirst & Kiørboe 2002, Wagner et al. 2004). A number of studies have indicated non-consumptive mortality due to diseases, pollution, environmental stresses, parasites or harmful algal blooms can be significant at times (e.g. Kimmerer & McKinnon 1990, Hall et al. 1995, Duffy et al. 2005). Consequently carcasses may maintain a considerable presence within the water column (Figure 1.1). In traditional zooplankton research, investigators collect, preserve and quantify zooplankton without distinguishing between live and dead individuals within the samples. This practice can lead to an erroneous understanding of pelagic processes, and under-appreciation of non-consumptive causes of zooplankton mortality.

Methods of carcass identification

Earlier reports of the occurrence of zooplankton carcasses relied on visual examination of individual animals for signs of internal tissue loss (e.g. Weikert 1977, Terazaki & Wada 1988). This method is time and labor intensive, and is not practical for

handling a large number of samples. Additionally, this method may underestimate carcass abundance as tissue decomposition may not be obvious in recently deceased zooplankton (Tang et al. 2006a). Other researchers have quantified zooplankton carcasses by collecting them in sediment traps (e.g., Gries & Güde 1999, Dubovskaya et al. 2003). Because the sinking rate of zooplankton carcasses can vary substantially by species, stage of decomposition and water column condition (Tang et al. unpubl. data); sediment traps may also underestimate carcass abundance if the carcasses are lost (e.g. ingested by fish or transported horizontally) before they reach the traps.

An alternative method for in situ assessment of zooplankton carcasses in marine or estuarine systems is the use of the biological stain Neutral Red (NR). NR is a lipophilic vital stain that has been used for almost 100 years in a variety of plant and animal systems. Within animal tissues, the stain passes through cellular membranes and is retained within lysosomes. When the integrity of the lysosomal membrane is compromised, it is no longer able to retain the NR stain and all color is lost (Svendsen & Weeks 1995). This property has been exploited in a number of terrestrial and aquatic invertebrate ecotoxicology studies (e.g. Svendsen & Weeks 1995, Scott-Fordsmand et al. 1998). The NR staining technique was initially adapted for the identification of live and dead copepods by Dressel et al. (1972), and applied in a limited number of laboratory experiments (Crippen & Perrier 1974, Simon 1974, Fleming & Coughlan 1978), but was not practical for use in the field until an adaptation by Tang et al. (2006a). The efficiency of the field-adapted staining method has since been rigorously evaluated for different copepod life stages and other estuarine zooplankton taxa (Elliott & Tang in press). When applied at the time of sample collection, the adapted NR method effectively stains live

copepods bright red or pink while dead copepods remain unstained. After staining, samples can still be preserved for identification and enumeration at a later date. This fast, simple and cost effective method can easily be integrated into any routine zooplankton sampling protocol to account for the occurrence of carcasses.

The amount of organic material contained within zooplankton carcasses can be substantial. If we assume there is approximately 10^{14} g carbon in zooplankton biomass within the world's ocean (Libes 1992) and carcasses comprise between 15 and 69% of the marine zooplankton population (Haury et al. 1995, Wheeler 1967), anywhere between $1.5x10^{13}$ and $6.9x10^{13}$ g C would be available in the form of zooplankton carcasses for microbial decomposition and/or export to depth. While not examined extensively in earlier studies, anthropogenic stressors have the potential to increase zooplankton mortality (Carpenter et al. 1974, Hall et al. 1995), which may lead to locally elevated amounts of carcass-derived particulate organic matter (POM).

Effects of turbulence on zooplankton

On a daily basis estuarine zooplankton are subject to tide-generated turbulence created by bottom stress. While the intensity of tide-generated turbulence is not constant, it is consistent and predictable. On a more sporadic time scale, zooplankton are also exposed to wind-generated turbulence. The negative effects of turbulence on copepod feeding, behavior, and metabolic rates have been well studied (see Alcaraz 1997). In addition to tide- and wind-generated turbulence, localized areas of increased turbulence can be created through human use of motorized water craft. Although more confined in space and time, turbulence generated by boats can be substantially more intense than tide-

and wind-generated turbulence. If a zooplankter is accustomed to life at a given turbulence level, the sudden and large increase in turbulence caused by boat wakes may be fatal. With the adaptation of the NR method for the identification of live vs. dead zooplankton in field samples, it is now possible to quantify zooplankton carcasses caused by boat-generated turbulence. Once carcasses have been produced, the fate of their biomass, impact on ambient microbial communities and contribution to organic matter fluxes is of considerable interest.

Decomposition and flux of particulate organic matter

The flux of POM to depth plays an integral role in supplying organic constituents to mesopelagic and benthic populations. POM can come from a variety of sources such as phytoplankton detritus (Grossart & Simon 1993, Smith et al. 1992), zooplankton fecal pellets (Bianchi et al. 1992, Hansen et al. 1996), zooplankton molts (Grossart & Simon 1993), discarded larvacean houses (Smith et al. 1992) and zooplankton carcasses (Grossart & Simon 1993). While the production, biotic interactions and contributions to POM fluxes by some of these sources have been well documented (Simon et al. 2002 and references therein), there is a dearth of information regarding these processes in relation to zooplankton carcasses.

Bacterial decomposition is one process working against the transport of POM to depth. As particles sink through the water column, they are quickly colonized by free-living bacteria (Simon et al. 2002) and subjected to dissolution by bacterial hydrolytic enzymes (Azam & Long 2001). Due to molecular size limitations on cellular membrane passage, bacteria are unable to directly incorporate large particulate organic matter into

their biomass. To overcome this limitation bacteria produce exoenzymes which break down POM into smaller, more usable forms that can easily be assimilated across the cellular membrane (Hoppe 1983). This exoenzymatic activity can be estimated through the use of the fluorogenic substrate technique. A saturating concentration of a non-fluorescent fluorogenic substrate analog is added to and incubated within a controlled system. Bacterial-produced exoenzymes cleave an integral bond producing a fluorescent compound (fluorochrome). Fluorochrome production is a proxy for potential enzymatic activity and can easily be measured as fluorescence intensity with a fluorometer. Fluorescence intensity readings are then converted into enzymatic rates based on a calibration curve of known concentrations of the appropriate fluorochrome. The fluorogenic substrate technique has been used in a variety of applications, the most relevant of which deal with the effect of particulate organic matter on bacterial exoenzymatic rates, and subsequent bacterial mediated turnover of POM (Hoppe et al. 1988).

The majority of studies conducted thus far have dealt primarily with exoenzymatic rates accompanying the decomposition of marine or freshwater organic aggregates (i.e. marine or lake snow) or zooplankton fecal pellets. In those studies elevated cell-specific bacterial exoenzymatic activities were observed among aggregate-associated bacteria as compared to free-living bacteria (Karner & Herndl 1992, Smith et al. 1992, Grossart & Simon 1998). Additionally, elevated cell-specific protease activities were observed when bacteria became attached to model organic particles, lending credence to the hypothesis that water column bacteria are able to adjust their enzyme production when attached or detached from particulate substrate (Grossart et al. 2007).

Although aggregate-associated bacteria exhibit higher cell-specific enzymatic rates than their free-living counterparts, hydrolysis and production rates are only loosely coupled. The loose coupling results in the release of a large amount of dissolved organic matter into the surrounding water column, which can contribute to free-living bacterial production (Smith et al. 1992, Thor et al. 2003, Grossart et al. 2007).

Preferential decomposition of particulate organic matter

Preferential decomposition of particulate organic matter constituents occurs through space (water column depth) and time. Labile biomolecules such as proteins are degraded quickly, released into the dissolved organic matter pool or incorporated into bacterial biomass, effectively retaining their constituents in surface waters. Lipids are typically degraded at a slower pace than proteins, increasing their potential to be preserved within sediments (e.g. Canuel & Martens 1996). An increased C: N ratio accompanying decomposition of POM, consistent with the preferential removal of nitrogen rich compounds such as proteins, has been observed in some studies (Müller-Niklas et al. 1994, Fabiano & Pusceddu 1998). Others have found no significant change in the C:N ratio over similar time scales (Fukami et al. 1985, Roy & Poulet 1990). Fabiano & Pusceddu (1998) observed that the labile fraction of proteins and carbohydrates of phytodetritus in Antarctic shelf-waters declined over time while the labile fraction of lipids remained relatively constant, illustrating the rapid use of nitrogen-rich compounds. Similarly, a laboratory decomposition study of artificially created diatom aggregates showed a preferential degradation of organic nitrogen over POC (Grossart & Ploug 2001).

The hydrolysis of a specific substrate often requires a particular enzyme which may only be produced by relatively few heterotrophic organisms; hence, a diverse microbial community is needed to fully exploit the organic constituents of POM (Arnosti 2004). This cooperative decomposition effort is exemplified by the shifting microbial community associated with aggregates as decomposition progresses (e.g. Simon et al. 1999, Bidle & Azam 2001). The preferential decomposition of POM may be a consequence of the presence or absence of key microbial organisms within the system, which can allow specific types of organic material (i.e. proteins) to be rapidly remineralized in surface waters while other organic compounds (i.e. lipids) are transported to depth.

Zooplankton carcasses as "microbial hotspots"

While fecal pellets and particle aggregates have been extensively studied in both freshwater and marine systems (Simon et al. 2002 and references therein), zooplankton carcasses have only recently been considered potential microbial hotspots within the water column. In a study of freshwater copepod carcasses Tang et al. (2006b) documented a shift in the carcass-associated microbial community composition during the decomposition process and an estimated 4 to 6 - fold increase in protease activity associated with the carcasses. Follow-up experiments confirmed the enhancement of carcass-associated bacterial production and enzyme activity over bacteria in the ambient water (Tang et al. in press). It is unknown, however, how this elevated microbial activity alters the biochemical composition of the carcasses thus influencing their effectiveness as a transport mechanism for different organic compounds to depth.

The source of bacteria responsible for the decomposition of POM has been a topic of interest in the past. Some studies have suggested that zooplankton fecal pellets are seeded and decomposed by gut bacteria (Gowing & Silver 1983, Bianchi et al. 1992, Dellile & Razouls 1994, Fabiano et al. 1994), while other studies postulated that free-living bacteria quickly colonize and decompose the sinking fecal pellets (Honjo & Roman 1978, Jacobsen & Azam 1984, Hansen & Bech 1996). If it is possible that fecal pellet decomposition is initiated by gut bacteria, it is also possible that enteric or other zooplankton associated bacteria are responsible for carcass decomposition.

Zooplankton carcasses can potentially act as both a transport mechanism and concentrated substrate to support locally enhanced bacterial abundance, cell-specific enzyme activities and production. If zooplankton carcasses are prevalent within a system, for example due to human activities, aquatic food web models need to be modified to include direct input of zooplankton biomass into the microbial loop. Additionally, this previously unaccounted for bacterial activity can have effects on organic matter transport and elemental cycling within aquatic systems (Cho & Azam 1988, Smith et al. 1992, Azam 1998).

STRUCTURE AND OBJECTIVES OF THESIS

This thesis is comprised of a two-part study, each part written in manuscript format addressing specific research questions:

Chapter 2 assesses the role of boat-generated turbulence as a potential anthropogenic source of non-consumptive mortality among estuarine crustacean zooplankton. The specific question to be addressed is:

> **Q1:** Is the prevalence of zooplankton carcasses higher within boat wakes than outside boat wakes?

Chapter 3 provides a detailed description of microbial responses to crustacean and non-crustacean zooplankton carcasses and assesses how these responses influence the protein and lipid content of the carcasses. The specific questions to be addressed include:

> **Q1:** Will microbial populations respond to crustacean and non-crustacean zooplankton carcasses in a similar manner?
>
> **Q2:** Are zooplankton-associated bacteria (enteric bacteria or attached to body surfaces) or colonizing free-living bacteria responsible for carcass decomposition?
>
> **Q3:** How do bacterial exoenzymatic activity and cell abundance of both carcass-associated and free-living bacteria change during the decomposition process?
>
> **Q4:** How does microbial activity influence the protein and lipid content of crustacean and non-crustacean carcasses?

Chapter 4 summarizes the findings of the thesis and provides some suggestions for avenues of future research.

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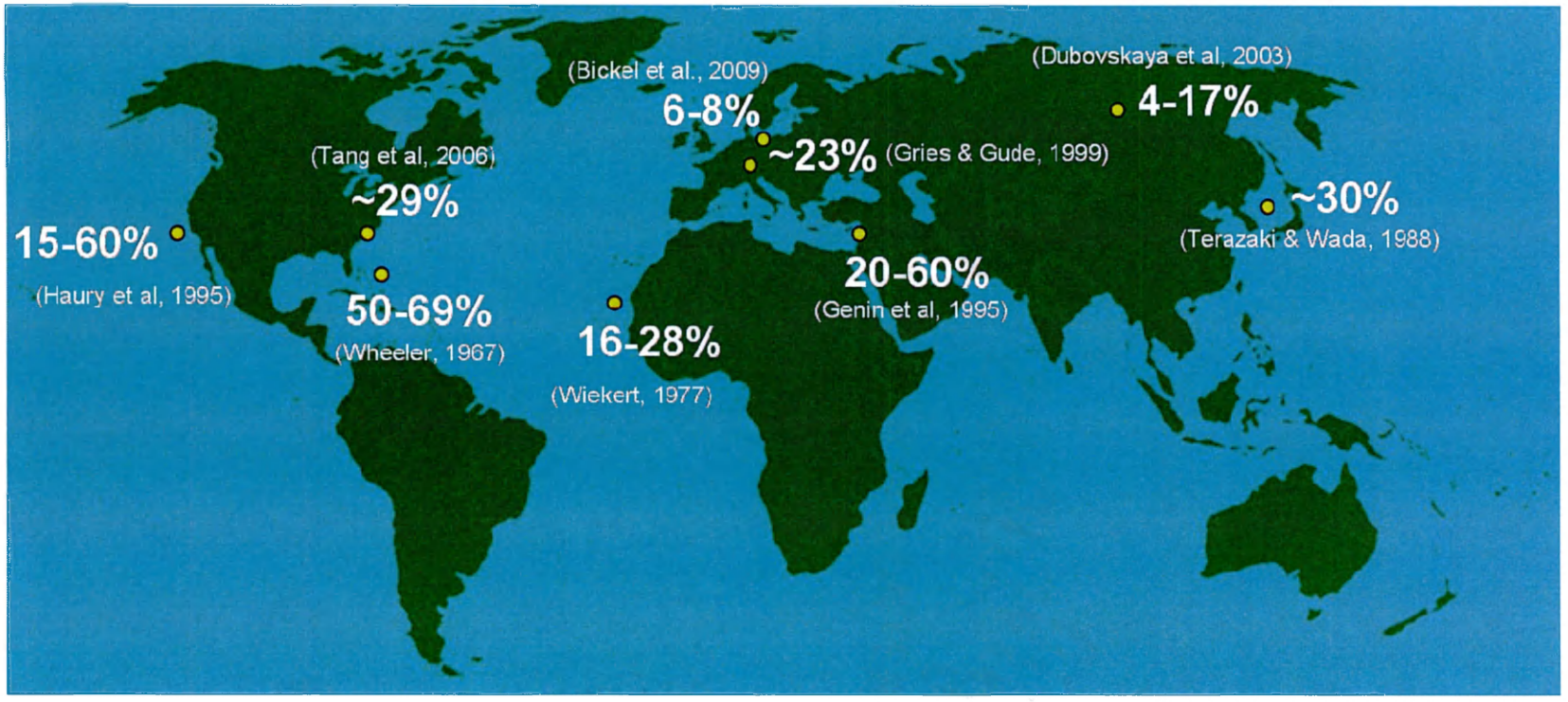
Figure 1.1. Global occurrence of zooplankton carcasses. A summary of previous studies detailing the occurrence of zooplankton carcasses within freshwater and marine systems. Numbers shown are percentages of the sampled populations determined to be dead at the time of sample collection.


(Bickel et al., 2009)
6-8%
(Dubovskaya et al, 2003)
4-17%
~23%
(Gries & Gude, 1999)
(Tang et al, 2006)
~29%
~30%
(Terazaki & Wada, 1988)
15-60%
(Haury et al, 1995)
20-60%
(Genin et al, 1995)
50-69%
(Wheeler, 1967)
16-28%
(Wiekert, 1977)

CHAPTER 2

Increased copepod carcass abundances within boat wakes

ABSTRACT

Zooplankton carcasses can be prevalent within aquatic systems. Prior studies have documented adverse impacts of turbulence on zooplankton; therefore, turbulence-induced mortality may serve as a source of zooplankton carcasses. In this study I documented changes in copepod live/dead composition inside and outside boat wakes to assess the likelihood of boat-generated turbulence as a potential cause of mortality. A higher percentage of copepod carcasses were found within boat wakes than in the ambient water. Possible explanations for the increased carcass prevalence are explored.

INTRODUCTION

Human actions can influence the health and well being of a diverse suite of aquatic organisms, zooplankton included. Among the various anthropogenic stresses, one that is present in practically all marine systems is turbulence. Turbulence is characterized by stochastic movement of water parcels through space and time (Peters and Redondo 1997). Turbulence dissipates through time as energy is transferred from larger to smaller eddies. This process continues until molecular forces become more important, allowing water viscosity to dampen any remaining turbulent energy (Peters & Redondo 1997). Due to the size range of zooplankton, they are subject to both viscous and turbulent (inertial) forces in the environment. While tide-generated and wind-generated turbulence is prevalent naturally, humans may create localized areas of increased turbulence with large motorized water crafts which are commonly used for commercial and recreational purposes.

As a motorized craft is propelled through the water, a large amount of turbulence is generated in its wake. This episodic turbulence suddenly places zooplankton in a potentially stressful environment which could elicit a myriad of responses. In addition to influencing planktonic trophic interactions (e.g. Marrasé et al. 1990, MacKenzie & Legett 1991) and behaviors (e.g. Costello et al. 1990, Waggett & Buskey 2007), strong turbulence can negatively impact zooplankton physiology such as excretion rates (Saiz & Alcaraz 1992a), heart rates (Alcaraz & Saiz 1991, Alcaraz et al. 1994), developmental rates (Saiz & Alcaraz 1991) and gross growth efficiency (Saiz et al. 1992). Prior

experiments applied a relatively stable, non-lethal level of turbulence (turbulent energy dissipation rates ~ 5.0 $mm^2 s^{-3}$). However, turbulence as encountered in boat wakes may be strong enough to cause more significant damage to the zooplankton or even death.

Study of zooplankton mortality due to boat-generated turbulence has been hampered by the lack of suitable methods for identifying zooplankton carcasses in field samples. In studies documenting the occurrence of carcasses in natural systems, some investigators identified zooplankton carcasses through visual examination of wounds and internal tissue loss of individual animals (Wheeler 1967, Weikert 1977, Terazaki & Wada 1988, Genin et al. 1995, Haury et al. 1995), but this method is tedious and time consuming. Other investigators estimated the presence of carcasses from sedimentation losses as determined by sediment traps (Gries & Güde 1999, Dubovskaya et al. 2003), but this approach is not effective in shallow systems with strong advection or high rates of resuspension. An alternative for carcass identification is the Neutral Red (NR) staining method. Initially developed by Dressel et al. (1972), this method was used in a few laboratory experiments (Crippen & Perrier 1974, Simon 1974, Fleming & Coughlan 1978) but was not practical for use in the field until an adaptation by Tang et al. (2006). The NR method was further improved and rigorously evaluated by Elliott & Tang (in press). When applied at the time of sample collection, the NR method stains live copepods bright red or pink while dead copepods remain unstained. After staining, samples can be preserved for identification and enumeration at a later date.

With the development of the NR staining method it is now possible to quantify zooplankton carcasses resulting from environmental and anthropogenic stresses in situ. Within this study we utilized the NR staining method to investigate the potential for boat-

generated turbulence as a cause of non-consumptive mortality in natural systems, consequently increasing the prevalence of zooplankton carcasses.

MATERIALS AND METHODS

In-situ sampling

To determine if higher abundances of copepod carcasses were present within boat wakes, corresponding pairs of 'no wake' and 'wake' samples were collected during multiple sampling trips, with a maximum of 3 corresponding pairs collected behind each boat. All zooplankton samples were taken from the York River estuary, VA, USA. To assess 'no wake' conditions, which represent normal, background in-situ live/dead zooplankton compositions, slow speed (<1 m s^{-1}), one minute horizontal net tows were collected just beneath the surface prior to wake generation. All samples were collected with a 0.5 m mouth diameter, 200 µm mesh zooplankton net with a 200 µm filtering cod end. A flow meter was attached to the mouth of the net to determine the volume of water filtered. The cod end content was concentrated down to 100-200 ml final volume and gently poured into a staining jar through a 2500 µm mesh sieve to remove any jellyfish (which interfere with staining). A 150 µl aliquot of NR stock solution (0.01 g NR powder per ml DI water) per 100 ml of sample was added to the staining jar, which was then capped tightly, gently inverted twice to evenly distribute the stain and placed in an ambient water bath for 15 minutes. Afterwards the sample was filtered onto a 200 µm mesh disk and rinsed copiously with artificial seawater to remove any excess stain. The mesh disks containing the zooplankton were then transferred to a small petri dish and placed on ice. Upon return to the lab the samples were stored at -4° C until analysis.

Two types of 'wake' samples were taken to assess turbulence effects: self-generated and opportunistic wake samples. For self-generated wakes, a large privateer was sped up to about 5500 rpm along a straight line to produce a wake approximately 100 yards long. The boat was then immediately turned around and a one-minute horizontal net tow was taken back through the wake, using the same net and method described previously. The samples were concentrated and stained as detailed above. Opportunistic net tows were taken in wakes generated by larger vessels that happened to pass by during routine sampling trips. The type of vessel was noted in each case. All net tows and staining procedures were the same as described for no wake samples. The sample net and cod end were rinsed well between tows to avoid any carry over of carcasses.

Identification of live and dead zooplankton

In the lab, frozen samples were thawed, back-rinsed into artificial seawater (20 psu), split with a Folsom plankton splitter if necessary, and transferred to a plankton counting wheel. The split sample was acidified (approximately 12 drops of 10% HCl per 5 ml sample) to intensify the color of the stain. Samples were counted on a dissecting microscope under dark field illumination. Members of individual taxa were separated into four groups based on their staining patterns: 1= red or bright pink total staining, 2= patchy red/bright pink staining, 3= light pink staining, 4= unstained. All individuals exhibiting any type of color (categories 1, 2 and 3) were designated as live, while all unstained individuals (category 4) were designated as dead (Elliott & Tang in press). From these observations the live and dead percentages of each zooplankton taxa were determined. The percent dead found in wake and the corresponding no wake samples

were compared to elucidate whether or not boat-generated turbulence could potentially be a source of zooplankton mortality.

Statistical analyses

All percentages were arcsine square root transformed to normalize the data before statistical analyses. A one-tailed paired t-test was used to test the hypothesis that wake samples contained a higher percent dead zooplankton than the corresponding no wake samples. Pearson's correlation coefficients were calculated to assess relationships between observed percent dead, zooplankton abundance, and prevalence of *Acartia tonsa* within the zooplankton population. All statistical tests were performed with Minitab Statistical Software.

RESULTS

A total of 13 pairs of wake and no wake samples were collected from the York River estuary between July 2007 and March 2009. Five of the wakes sampled were self generated with a large privateer while the remaining 8 were sampled opportunistically. The opportunistic wakes were generated by boats that varied in size, shape and speed: One wake was generated by a yacht, one by a commercial fishing vessel, one by a tugboat pushing a barge, two by tugboats travelling at different speeds, and three by the R/V Pelican, which is a military landing craft that has been converted for use as scientific research vessel by the Virginia Institute of Marine Science.

The calanoid copepod *Acartia tonsa* was typically the most common species within the zooplankton samples occurring at abundances between 8.64 and 2514.97 individuals m^{-3} (Table 2.1); it comprised 9-83% of the total zooplankton abundance in wake samples (average of 36%) and 16-65% in no wake samples (average 35%) (Table 2.2). The total abundance of *A. tonsa* was not different between wake and no wake samples ($p=0.197$), and the species accounted for similar percentages of the total zooplankton in both types of samples ($p=0.205$). The efficiency of the NR staining method for the identification of *Acartia tonsa* carcasses has been evaluated exhaustively elsewhere (Elliott & Tang in press); hence, *A. tonsa* was used as the representative species to assess the live/dead zooplankton composition in this study.

The analysis of all net tow samples revealed that there was a significantly higher percentage of *A. tonsa* carcasses present within wakes than what was present outside the

wakes ($p = 0.049$, Table 2.3). Within these data, however, it was noted that half of the tows taken in self-generated wakes actually exhibited a lower fraction of carcasses than were present naturally (no wake). Due to methodological difficulties associated with sampling self-generated wakes (see discussion), only opportunistic samples were used in subsequent statistical analyses. When only opportunistic wake samples were considered, the trend of higher carcass prevalence within wakes was much more noticeable. With the exception of one sample, opportunistic boat wakes consistently had a higher proportion of carcasses than what was found under normal ambient conditions ($p = 0.018$) (Figure 2.1). The fraction of the sample identified as dead within both wake and no wake samples was not correlated to the percentage of the sample population comprised by *A. tonsa* (Figures 2.2 and 2.3) or *A. tonsa* abundance (Figures 2.4 and 2.5).

To better illustrate the impact of wakes, the value of the no wake sample was subtracted from the wake sample for each corresponding pair (Figure 2.6). There was no consistent pattern for either the percentage of the total zooplankton population accounted for by *A. tonsa* (Figure 2.6A) or *A. tonsa* abundances (Figure 2.6B). With one exception, the difference in the percent dead between wake and no wake samples was consistently positive (Figure 2.6C).

DISCUSSION

Sampling limitations

During the sampling process, it was discovered that sampling self-generated wakes posed many problems. While the large privateer used during all sampling events was capable of producing a sizeable wake, difficulties arose when attempting to maneuver the boat to turn around quickly. Often by the time we were able to safely turn the boat around, the self-generated wake had dissipated and was no longer visible. On occasion the foam lines which formed at the edges of the wake were still visible and an attempt was made to tow directly between the two lines. Other times the wake was undistinguishable and tows had to be made in the area where the wake was approximated to have occurred. Sampling within the wake of other boats provided a much better option, allowing us to consistently visualize the wake and sample at a constant distance from the source.

Fields and Yen (1997) estimated that *A. tonsa* typically reside in estuarine conditions with turbulence levels that produce a Kolmogoroff scale of 6.5 mm, which is well above the typical size of the copepod. This suggests that *A. tonsa* actually resides in a relatively calm environment. The intermittent turbulence generated by a boat wake could place the copepod in a suddenly stressful environment to which it is not accustomed. The variable degree of increase in *A. tonsa* carcass prevalence among our opportunistic wakes samples could be attributed to the different intensities of turbulence generated by the different boats. By sampling opportunistically we had no control over

the type or speed of boats producing the wakes. Unfortunately it was not possible to obtain any quantitative measurement of the intensity or scale of the turbulence produced within each of the wakes. Consequently no correlations between absolute turbulence intensity and occurrence of carcasses can be drawn.

Prevalence of copepod carcasses within boat wakes

A higher proportion of copepod carcasses was found within boat wakes than outside the wakes. Two possible mechanisms that could account for this observation include 1) concentration of copepod carcasses within the boat wakes coupled with active avoidance of wakes by live copepods, and 2) mortality induced by a suddenly turbulent and stressful environment.

A number of studies have shown that various copepod species and developmental stages can actively avoid turbulent surface waters through vertical migration (Mackas et al. 1993, Lagaduec et al. 1997, Visser et al. 2001, Maar et al. 2006), and rapidly returned to shallower depths once the turbulent event passes (Incze et al. 2001). Additionally, wind-generated turbulent events have been shown to effectively concentrate weak-swimming zooplankton on a vertical scale (Haury et al. 1990, Haury et al. 1992). It is therefore possible that in our study carcasses were concentrated by boat-generated turbulence, while live copepods were able to avoid the turbulent layer. It should be noted that all previous reports of active turbulence avoidance were observed on a vertical scale with prolonged periods of turbulent activity. To our knowledge no cases of turbulence avoidance on a horizontal scale or during short-term events have been documented.

While copepods may increase their jumping frequency in response to elevated turbulence (Saiz & Alcaraz 1992b), it is uncertain whether they are able to escape sporadic, localized areas of increased turbulence generated by water crafts. *Acartia* reaction times to hydrodynamic stimuli are on the order of milliseconds and the copepod can move approximately 4.5 mm with one escape jump (Buskey et al. 2002). Considering a copepod located along the mid-line of a wake generated by a 2 m–wide motor craft, 222 consecutive escape jumps perpendicular to the direction of wake formation would be necessary to remove the copepod from turbulent conditions. This rough calculation likely underestimates the required escape time and distance, as the copepod would also have to overcome random physical transport due to the turbulence (Yen et al. 2008). Considering these factors, concentration of copepod carcasses by boat wakes is feasible, but avoidance of boat wakes by live copepods is unlikely.

As copepods were essentially trapped within the boat-generated wakes, the primary concern shifts to whether or not turbulence levels were strong enough to cause mortality. In addition to altering copepod behaviors, increased turbulence levels adversely impact copepod physiological processes (Saiz & Alcaraz 1992a, Alcaraz & Saiz 1991, Alcaraz et al. 1994). It is possible that boat-generated turbulence created a suddenly stressful environment to which the copepods were unable to adapt, resulting in death. Laboratory experiments which expose copepods to increasing levels of turbulence could be conducted to quantify the intensity at which turbulence becomes lethal to copepods.

Boat-generated turbulence may also resuspend carcasses from sediments, similar to how elevated levels of suspended sediments have been observed after episodic wind

events (Bloesch 1995). The amount of resuspension would depend on water column depth, wake intensity and stability of the sediments (Leuttich et al. 1990). If carcass resuspension from sediments occurred, an increase in the total *A. tonsa* abundance would be expected in the wake samples, but was not observed in our data.

While the exact cause for the elevated prevalence of copepod carcasses within boat wakes is unknown, the presence of a larger number of carcasses within a localized area has a number of ecological implications and raises multiple corollary questions. If boat-generated turbulence causes mortality, increased carcass abundances would be expected during summer months when recreational boat traffic increases. Carcass abundances should also be higher in ports and other areas with heavy boat traffic, thereby reducing zooplankton biomass and altering trophic interactions within these systems. Conversely, if boat-generated turbulence simply serves as a concentrating mechanism, wakes could represent a localized, concentrated food source for planktivorous organisms similar to plankton aggregations in an estuarine turbidity maximum (Roman et al. 2005). The fate of carcass biomass is also of particular interest. If not consumed, carcasses may transport organic material to depth or be subject to microbial decomposition, shunting zooplankton biomass into the microbial loop; either way influencing the biogeochemical cycling of the system.

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Table 2.1. Total abundances of *Acartia tonsa* (live and dead) inside and outside of boat wakes. O = opportunistic, SG = self-generated; the R/V Pelican is a military landing craft which has been converted into a research vessel.

Date	Tidal Condition	Station Depth (m)	Boat Type	Wake Type	Abundance (individuals m^{-3}) No Wake	Wake
16 July 2007	Ebb	7.6	Yacht	O	46.01	27.24
16 July 2007	Slack	13.7	Barge/tugboat	O	140.11	592.38
16 July 2007	Flood	21.3	Fishing boat	O	153.29	109.43
10 Oct 2007	Flood	22.9	R/V Pelican	O	285.15	182.76
10 Oct 2007	Flood	22.9	R/V Pelican	O	373.07	645.53
10 Oct 2007	Flood	22.9	R/V Pelican	O	508.51	1030.87
17 Feb 2009	Flood	21.3	Tugboat	O	2514.97	2192.07
31 Mar 2009	Flood	21.3	Tugboat	O	304.35	238.21
16 July 2007	Flood	12.2	Large privateer	SG	536.93	86.96
9 July 2007	Flood	7.6	Large privateer	SG	38.47	16.88
9 July 2007	Slack	13.7	Large privateer	SG	78.30	9.76
9 July 2007	Ebb	21.3	Large privateer	SG	32.81	17.40
9 July 2007	Ebb	12.2	Large Privateer	SG	8.64	37.92
Mean ± SD					386.20 ± 664.59	399.03 ± 624.37

Table 2.2. Percentage of the total sampled zooplankton population comprised of *Acartia tonsa* (live and dead) inside and outside of boat wakes. O = opportunistic, SG = self-generated; the R/V Pelican is a military landing craft which has been converted into a research vessel.

Date	Tidal Condition	Station Depth (m)	Boat Type	Wake Type	Percent of total population	
					No Wake	Wake
16 July 2007	Ebb	7.6	Yacht	O	47.60	53.20
16 July 2007	Slack	13.7	Barge/tugboat	O	41.66	83.17
16 July 2007	Flood	21.3	Fishing boat	O	49.67	29.86
10 Oct 2007	Flood	22.9	R/V Pelican	O	22.60	18.73
10 Oct 2007	Flood	22.9	R/V Pelican	O	26.91	27.55
10 Oct 2007	Flood	22.9	R/V Pelican	O	24.85	39.54
17 Feb 2009	Flood	21.3	Tugboat	O	64.97	70.10
31 Mar 2009	Flood	21.3	Tugboat	O	No Data	No Data
16 July 2007	Flood	12.2	Large privateer	SG	62.33	21.85
9 July 2007	Flood	7.6	Large privateer	SG	17.95	9.34
9 July 2007	Slack	13.7	Large privateer	SG	23.25	12.07
9 July 2007	Ebb	21.3	Large privateer	SG	21.26	23.66
9 July 2007	Ebb	12.2	Large Privateer	SG	16.47	42.60
Mean ± SD					34.96 ± 17.44	35.97 ±22.93

Table 2.3. Percentage of dead *Acartia tonsa* inside and outside of boat wakes. O = opportunistic, SG = self-generated; the R/V Pelican is a military landing craft which has been converted into a research vessel.

Date	Tidal Condition	Station Depth (m)	Boat Type	Wake Type	Percent Dead	
					No Wake	Wake
16 July 2007	Ebb	7.6	Yacht	O	1.58	6.33
16 July 2007	Slack	13.7	Barge/tugboat	O	7.54	4.58
16 July 2007	Flood	21.3	Fishing boat	O	5.72	11.17
10 Oct 2007	Flood	22.9	R/V Pelican	O	12.9	22.37
10 Oct 2007	Flood	22.9	R/V Pelican	O	7.01	10.94
10 Oct 2007	Flood	22.9	R/V Pelican	O	2.33	25
17 Feb 2009	Flood	21.3	Tugboat	O	15.81	27.33
31 Mar 2009	Flood	21.3	Tugboat	O	12.86	14.69
16 July 2007	Flood	12.2	Large privateer	SG	7.81	38.19
9 July 2007	Flood	7.6	Large privateer	SG	23.42	13.69
9 July 2007	Slack	13.7	Large privateer	SG	8.97	1.49
9 July 2007	Ebb	21.3	Large Privateer	SG	5.23	8.39
9 July 2007	Ebb	12.2	Large privateer	SG	8.45	5.49
Mean ± SD					9.20 ± 5.89	14.59 ± 10.71

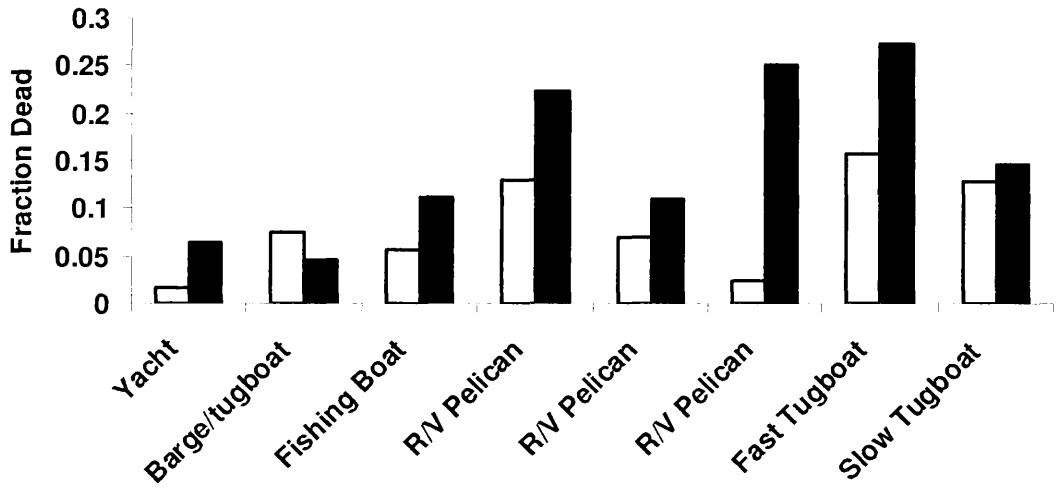


Figure 2.1. Fraction of the sampled *Acartia tonsa* population identified as dead under natural turbulence conditions (white bars) and within the wakes generated by various motor crafts (black bars).

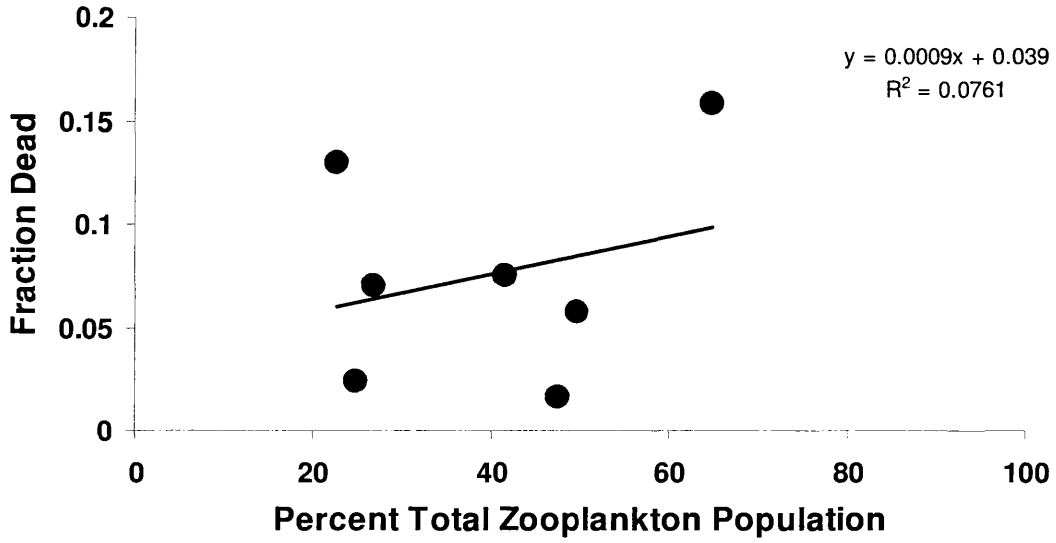


Figure 2.2. Fraction of *Acartia tonsa* identified as dead as a function of percent total zooplankton population composed of *A. tonsa* under natural turbulence conditions (no wake). No correlation between the two parameters was found (Pearson's correlation coefficient = 0.216, p = 0.642).

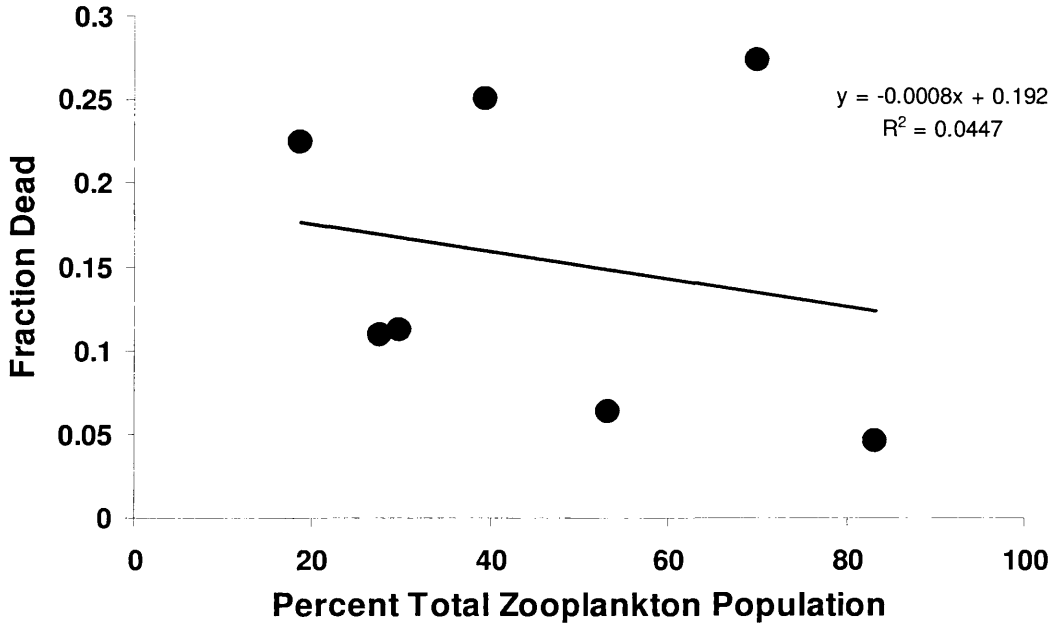


Figure 2.3. Fraction of *Acartia tonsa* identified as dead as a function of percent total zooplankton population composed of *A. tonsa* within boat wakes. No correlation between the two parameters was found (Pearson's correlation coefficient = -0.3, p = 0.513).

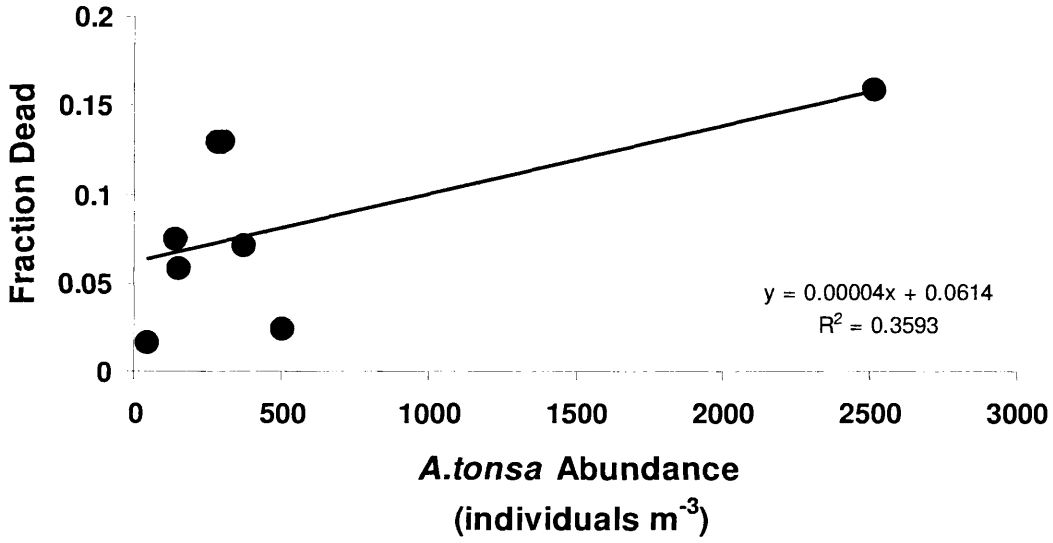


Figure 2.4. Fraction of *Acartia tonsa* identified as dead as a function of *A. tonsa* abundance under natural turbulence conditions (no wake). No correlation between the two parameters was found (Pearson's correlation coefficient = 0.535, p = 0.172).

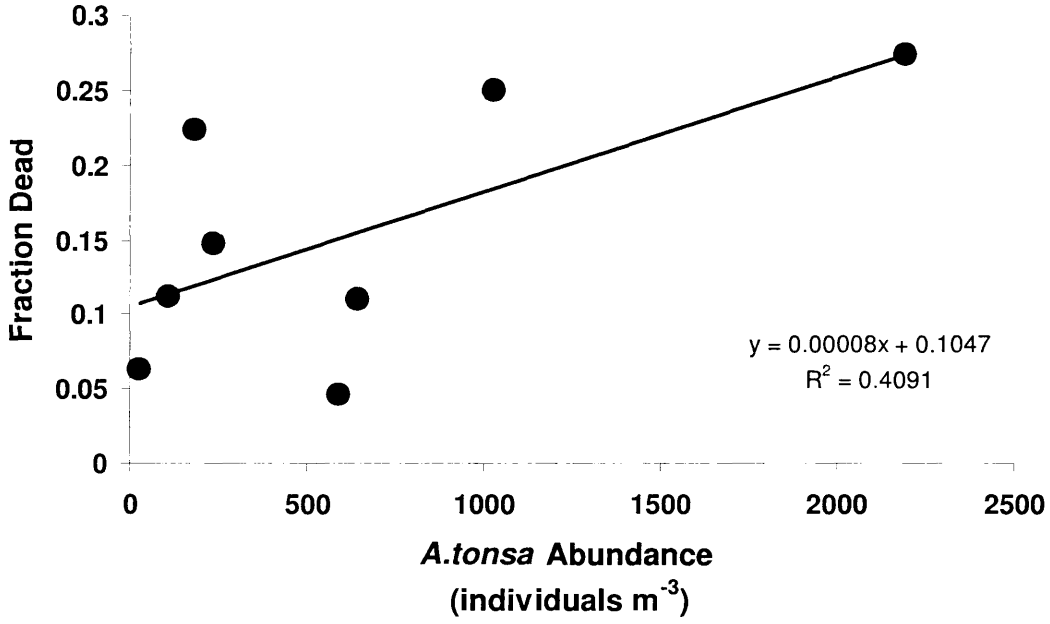
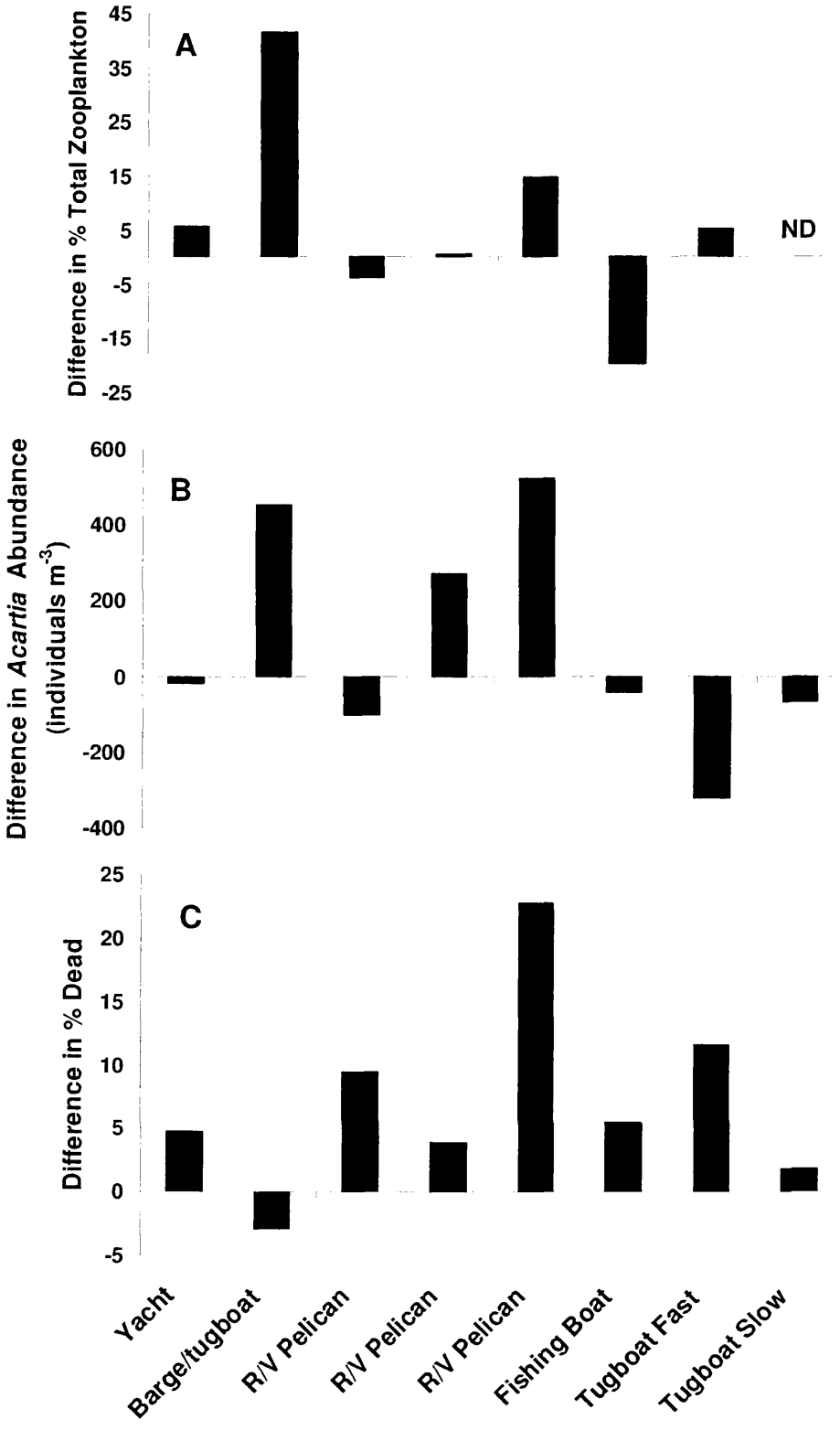


Figure 2.5. Fraction of *Acartia tonsa* identified as dead as a function of *A. tonsa* abundance within boat wakes. No correlation between the two parameters was found (Pearson's correlation coefficient = 0.593, p = 0.121).

Figure 2.6. Differences between wake and no wake samples in (A) the percent total zooplankton population comprised of *Acartia tonsa*, (B) abundance of *A. tonsa* and (C) the percentage of *A. tonsa* identified as dead. Positive values indicate that the parameter was higher within wake samples than in no-wake samples. ND indicates no data.


A
Difference in % Total Zooplankton
45
35
25
15
5
-5
-15
-25
ND
B
Difference in Acartia Abundance (individuals m-3)
600
400
200
0
-200
-400
C
Difference in % Dead
25
20
15
10
5
0
-5
Yacht
Barge/tugboat
R/V Pelican
R/V Pelican
R/V Pelican
Fishing Boat
Tugboat Fast
Tugboat Slow

CHAPTER 3

Microbial decomposition of proteins and lipids in crustacean vs. non-crustacean zooplankton carcasses

ABSTRACT

Zooplankton carcasses can be common within aquatic systems and potentially serve as organic-rich substrates for water column bacteria and transport mechanisms of particulate organic matter to depth. Previous studies of zooplankton carcass decomposition have focused solely on crustacean zooplankton, and changes in the biochemical composition of carcasses have not been investigated. In this study I compared the decomposition of carcasses of representative crustacean and non-crustacean zooplankton. Changes in carcass protein and lipid content were also monitored during the decomposition process. Bacteria associated with both crustacean (the calanoid copepod *Acartia tonsa*) and non-crustacean (the rotifer *Brachionus plicatilis*) carcasses displayed higher cell-specific protease and lipase activities than bacteria in the ambient water. When incubated with ambient microbial communities *A. tonsa* carcasses lost approximately 70% of their initial protein within 8 hours, while protein lost from *B. plicatilis* carcasses over 24 hours was insignificant. The lipid content of both copepods and rotifers did not change over the span of 24 hours. Our results suggest that crustacean zooplankton carcasses decompose quickly and may support microbial loop processes within the water column, while non-crustacean zooplankton carcasses decompose at a lower rate and could play an important role in the transport of high quality organic matter to depth.

INTRODUCTION

Organic aggregates play an important role in shaping microbial food web dynamics and transporting surface derived particulate organic matter (POM) to the mesopelagic and benthos. The formation, prevalence, and decomposition of organic aggregates, as well as the associated microbial processes, have been studied extensively in freshwater and marine systems (reviewed in Simon et al. 2002). One source of POM that has been largely overlooked is zooplankton carcasses. A number of studies in both freshwater and marine systems have found that zooplankton carcasses can comprise anywhere between 4 and 69% of the sampled population (e.g., Wheeler 1967, Weikert 1977, Terazaki & Wada 1988, Dubovskaya et al. 2003). If we assume there is approximately 10^{14} g of zooplankton carbon biomass in the world's ocean (Libes 1992), anywhere between $4x10^{12}$ and $6.9x10^{13}$ g C would be available in the form of zooplankton carcasses for microbial decomposition and/or export to depth. In comparison, approximately $3x10^{16}$ g C is currently present in the world's oceans as detritus (Libes 1992). While zooplankton carcasses contribute less than 1% to the global detrital carbon pool, processes associated with zooplankton carcasses could be significant on a local scale. Organic aggregate carbon: nitrogen ratio ranges from 5 to 20, and particulate combined amino acids account for 8-51% of aggregate bound POM (Simon et al. 2002). By comparison, calanoid copepods, the most abundant marine mesozooplankton taxa, have an average C: N ratio close to 3 and proteins commonly account for 30-70% of their dry weight (Båmstedt 1986). This suggests that zooplankton

carcasses provide higher quality POM than detrital aggregates. Also, unlike phytoaggregates which rarely become anoxic (Ploug 2001), decomposing zooplankton carcasses can quickly become suboxic or anoxic, and support anaerobic microbial processes that otherwise could not occur in the water column (Glud et al. unpubl. data).

During the decomposition of estuarine zooplankton carcasses, there was visible internal tissue loss and accumulation of bacteria within the carapace (Tang et al. 2006a). A shift in the carcass-associated microbial community composition and a 4 to 6-fold increase in protease activity associated with the carcasses was also observed during the decomposition of freshwater copepods and cladocerans (Tang et al. 2006b). Follow-up experiments confirmed a significant shift in the microbial community and an enhancement of carcass-associated bacterial production and enzyme activity relative to free-living bacteria (Tang et al. in press). While the most recent study by Tang et al. (in press) observed a change in carcass C and N contents, no measurements of biomolecules (i.e. proteins or lipids) were made. Therefore it is unknown whether elevated microbial activity or autolysis of zooplankton tissues alters the biochemical composition of carcasses, thus influencing the effectiveness of carcasses as transport mechanisms for high quality organic materials to depth.

All previous studies of carcass decomposition have focused solely on crustacean zooplankton. Non-crustacean zooplankton are common in freshwater and marine systems and at times can dominate the zooplankton community (Loeb et al. 1997, Holst et al. 1998, Chiba et al. 1998), but decomposition of their carcasses has rarely been studied. The exoskeleton of crustacean zooplankton is composed primarily of chitin, which is relatively resistant to bacterial degradation (Kirchner 1995) and may hinder

bacterial colonization and decomposition of the carcasses. In comparison, non-crustacean zooplankton lack a chitinous exoskeleton, and earlier qualitative observations suggested that their carcasses may decompose faster, although quantitative measurements were not available (Tang et al. 2006a).

In the present study I hypothesize that the absence of a chitinous exoskeleton allows non-crustacean zooplankton carcasses to be decomposed more rapidly than crustacean zooplankton carcasses. Incubations were conducted in the presence and absence of natural bacterial communities to determine the relative importance of free-living bacteria, attached bacteria and zooplankton tissue autolysis in the decomposition process. Additionally, we monitored microbial activity and changes in carcass protein and lipid contents to determine the change in quantity and quality of carcass-derived POM during the decomposition process.

MATERIALS AND METHODS

Zooplankton collection and carcass production

Individual incubation experiments were conducted with one representative species of estuarine crustacean and non-crustacean estuarine zooplankton, respectively. The calanoid copepod *Acartia tonsa* was used as the model crustacean zooplankton while the rotifer *Brachionus plicatilis* served as the non-crustacean zooplankton. *A. tonsa* is dominant among the mesozooplankton within the Chesapeake Bay, can often be found year-round and reaches peak abundances during the summer months (Kimmel & Roman 2004). Likewise, *B. plicatilis* is common in the Chesapeake Bay during the spring and summer and plays an integral trophic role in the system (Dolan & Gallegos 1992). *A. tonsa* were collected from the mesohaline portion of the York River estuary, Virginia, USA. Upon return to the lab, live zooplankton were gently concentrated onto a 200 μm mesh sieve and then killed by brief exposure to weak acid (approximately 10 seconds in <3% HCl). The carcasses were rinsed extensively with 0.2 μm filtered artificial seawater (FASW, 20 psu) to remove the acid and then back rinsed into a petri dish with FASW. Adult *A. tonsa* were identified under a dissecting microscope and transferred into a second petri dish containing 0.2 μm FASW. No differentiation between male and female copepods was made. Laboratory cultures of *B. plicatilis* were hatched from resting eggs, maintained in 0.2 μm filtered York River water (FYRW) at 25°C and fed a diet of the cryptophyte *Rhodomonas salina*. For carcass production and collection, rotifer cultures were concentrated onto an acid-washed 35 μm mesh sieve, rinsed with 0.2 μm FASW,

back-rinsed into an acid-washed beaker and brought to a known volume with 0.2 µm FASW. The beaker was gently swirled to evenly distribute the rotifers and three aliquots of 5 ml were removed and preserved with Lugol's stain to determine rotifer abundance via direct counts. To produce carcasses for the experiments, aliquots of the rotifer culture were transferred to sterile, polystyrene centrifuge tubes and placed in a 55°C water bath for 10 minutes. Heat was used instead of acid because the latter was found to be ineffective at killing rotifers. The same procedures were followed to produce copepod and rotifer carcasses for lipid decomposition experiments with one exception: pre-combusted borosilicate glass culture tubes were used in place of plastic during heat-killing of rotifers.

Laboratory incubation experiments

To assess microbial response to zooplankton carcasses and subsequent changes in carcass biochemical composition, a series of laboratory incubation experiments was conducted. Incubation water was collected from the York River estuary one to two days prior to each experiment, gravity filtered through a series of decreasing pore size sieves and stored at 15°C. All incubations, except those for lipid decomposition, were performed with two different water filtrates. A 5 µm filtration was used to retain the natural bacterial assemblages while excluding a majority of larger metazoans and protozoans which might feed on bacteria. A 0.2 µm filtration was used to remove most free-living bacteria.

To monitor bacterial abundance and exoenzyme activity 25 similarly sized *A. tonsa* or 250 *B. plicatilis* carcasses were gently pipetted into a sterile polystyrene petri

dish filled with 25 ml of 5 μm or 0.2 μm FYRW. For carcass protein measurements 200 freshly prepared copepod carcasses or approx. 10,000 rotifer carcasses were incubated in acid washed, sterilized, borosilicate glass bottles with 200 or 1,000 ml of 5 μm or 0.2 μm FYRW respectively. To determine the change in lipid content of carcasses during the decomposition process 700 freshly prepared copepod carcasses or approx. 20,000 rotifer carcasses were incubated in acid washed, pre-combusted, borosilicate glass bottles with GF/D (nominal pore size 2.7 μm) filtered YRW at a final concentration of 1 copepod carcass or 10 rotifer carcasses per ml. Within each experiment, triplicate incubations containing carcasses and corresponding controls of FYRW (no carcasses added) were established for each time point. All incubation bottles were placed in a 15°C environmental chamber on orbital shaker tables at low speeds to provide mixing. Exoenzyme activities and bacterial abundances were measured for up to four days. Protein content of copepod and rotifer carcasses was measured at 0, 8, 16 and 24 hours while lipid content was measured at 0 and 24 hours.

Exoenzymatic activity

To assess the enzyme activity attributed to free-living bacteria, 3 ml aliquots of incubation water were removed from each replicate treatment and control and transferred to separate cuvettes. Two aliquots were taken from each replicate: one for protease measurements, the other for lipase. Two additional 3 ml aliquots were taken from any one of the replicates and 300 μl of 35% formaldehyde was added to create killed-control samples for protease and lipase measurements respectively. The killed-control samples,

where all exoenzymatic activities had been stopped, were used to correct for non-enzyme related fluorescence.

To determine enzyme activity of copepod carcass-associated bacteria, five carcasses were removed from the incubation vessel and transferred to a 1 ml glass tissue grinder. It was unavoidable that a small amount of incubation water was also transferred with the carcasses; however, our calculations indicate that the bacterial abundance within this water would be negligible compared to the number of bacteria associated with the carcass itself. The carcasses were ground vigorously to break up zooplankton tissues and release bacteria from within the carcasses. Carcass homogenate was then transferred to a cuvette; the tissue grinder was rinsed three times with 0.2 μm FASW into the same cuvette and 0.2 μm FASW was added to bring the final volume to 3 ml. Two additional samples of 5 copepod carcasses were taken from any of the replicates, ground as described previously and processed as killed-control samples. Due to the smaller size of rotifers, 50 carcasses were used for each carcass-associated measurement to ensure detectable signals. Rotifer carcasses were processed in the same manner as copepods.

The fluorogenic substrate analogs 4-methylumbelliferyl heptanoate (MUH) (Izquierdo & Henderson 1998) and L-leucine 7-amido-4-methylcoumarin (Leu-MCA) (Hoppe 1983) were used to assess lipase and protease activities, respectively (Figure 3.1). Concentrated stocks (5 mM) of the lipid and protein analogs were prepared by dissolving MUH or Leu-MCA powder into 2-methoxyethanol or Milli-Q water respectively. A 60 μl aliquot of 5 mM MUH or Leu-MCA stock was added to each cuvette to obtain a final concentration of 0.1 mM. All cuvettes were incubated in the dark for one hour at 15 °C. Fluorescence intensity was measured immediately afterward on a Shimadzu RF-1501

spectrofluorophotometer (Shimadzu Corp., Japan) at λ_{ex}= 380 nm and λ_{em}= 440 nm for protease, λ_{ex}= 365 nm and λ_{em}= 445 nm for lipase. Fluorescence intensities were converted into substrate cleavage rates based on calibration curves prepared with 7-amido-4-methylcoumarin (AMC) for protease and 4-methylumbelliferone (MUF) for lipase. Fluorescence measurements were normalized to DAPI or SYBR-Gold total direct counts (see *Bacterial enumeration*) from the same replicate to determine potential cell-specific enzyme activities.

Bacterial enumeration

From each replicate treatment and control, a 1ml aliquot of water was stained with 4',6-diamidino-2-phenylindole (DAPI) and filtered onto a 0.2 µm black polycarbonate filter to enumerate free-living bacteria (Porter & Feig 1980). Carcass-associated bacterial abundances were determined by grinding 5 copepod or 50 rotifer carcasses as described previously. The carcass homogenates were filtered onto 0.2 µm black polycarbonate filters and stained with SYBR-Gold using a method based on Chen et al. (2001). Staining with SYBR-Gold provided greater contrast between bacteria and carcass detritus than DAPI, and was therefore preferable for counting carcass-associated bacteria.

Carcass protein content and molecular weight distribution

The contents of each incubation bottle for protein decomposition were gravity filtered onto a separate 25 mm diameter (for copepods) or 47 mm diameter (for rotifers) 5 µm pore size polycarbonate filter. Afterward the actual number of copepod carcasses on the filters was examined under a dissecting microscope. The small size and large number

of rotifer carcasses used prevented visual verification of the exact number of carcasses. The total number of rotifer carcasses used for protein measurement was therefore estimated from the concentration of carcass stock and aliquot volume. All filters were cut into small pieces with a surgical scalpel, placed into individual microcentrifuge tubes and stored at -40°C until processing.

To prepare the samples for total protein measurement, 600 µl of 0.2 µm FASW was added to each microcentrifuge tube, which was then sonicated on ice continuously for 60 seconds with a Microson Ultrasonic cell disruptor XL2000 (Misonix Inc, Farmingdale, NY) microprobe and centrifuged for 15 minutes at 14,000g in an accuSpin micro 17R (Fisher Scientific Inc.) refrigerating microcentrifuge at -9°C to pellet out the filter. Total protein of the supernatant was determined colorimetrically with the Bradford method (Bradford 1976) and quantified against a standard curve of bovine serum albumen. Changes in the size composition of carcass proteins were monitored by sodium dodecylsulfate – polyacrylamide gel electrophoresis (SDS-PAGE). Protein samples from different stages of decomposition were run on a 12% acrylamide resolving gel with a 4% acrylamide stack for 45 minutes at 200 volts. Within each gel one lane was devoted to a protein standard (EZ-Run ^{TM} *Rec* Protein Ladder, Fisher BioReagents), which provided a visual estimate of the sizes of proteins present in each sample. Each gel was dual stained with coomassie blue and silver stain to visualize the protein bands. Gels were photographed using Alpha Innotech software.

Carcass lipid content

The contents of each incubation bottle for lipid decomposition were gravity filtered onto pre-combusted 47 mm diameter, GF/D filters. Filters were stored in foil packets at -40°C until analysis. All tools were thoroughly rinsed with Milli-Q water and hexane between samples. Frozen filters were thawed, cut into small pieces and mixed with hydromatrix (Varian Inc., Palo Alto, CA) to remove excess water. Neutral and polar lipid fractions were extracted from each filtered sample using the accelerated solvent extraction "silica sandwich" method described by Poerschmann & Carlson (2006). Neutral and polar extracts were concentrated down to 1 ml with a Turbo-Vap (Zymark, Germany), blown dry under nitrogen gas, and brought to 100 µl final volume with hexane (for neutral lipids) or chloroform (for polar lipids). Total extracted neutral and polar lipids were determined gravimetrically.

Statistical analyses

A one-way ANOVA was performed on bacterial abundances, cell-specific enzyme activities and carcass protein content to detect temporal changes for each of the parameters. A post-hoc Tukey test of 95% confidence intervals was performed to compare individual time points. To assess differences in the abundances and enzymatic activities among control, free-living and carcass-associated bacterial populations, two-way nested ANOVAs were performed to help eliminate any bias of time within the measurements. A one-sample t-test was performed to assess any changes in the lipid content over the span of 24 hours, and a 2-sample t-test was performed to detect shifts in

the neutral to polar ratio of the lipid content. All statistical analyses were performed using Minitab Statistical software.

RESULTS

Bacterial abundance

From here on "carcass-associated" refers to bacteria found inside or attached to the external surface of a carcass; the term "free-living" is used in reference to bacteria in ambient water when carcasses were present, and "control" refers to bacteria in ambient water when no carcasses were present.

When copepod carcasses were incubated in 5 μm FYRW, free-living bacterial abundance was highly variable but significantly decreased over the incubation (Figure 3.2A; ANOVA, $p = 0.006$; Tukey test $p = 0.009$). In contrast, free-living bacterial abundance increased over the incubation period with rotifer carcasses under the same conditions (Figure 3.3A; ANOVA, $p = 0.016$; Tukey test, $p = 0.003$). Carcass-associated bacterial abundance increased significantly on copepods incubated in 5 μm FYRW (Figure 3.2C; ANOVA, $p = 0.029$) while it did not increase significantly for incubations with rotifers (Figure 3.3C). When incubated in 0.2 μm FYRW free-living bacterial abundances in both copepod (Figure 3.2B) and rotifer (Figure 3.3B) incubations increased by 24 hours (ANOVA, $p<0.001$; Tukey test, $p<0.001$ for both copepods and rotifers). Carcass-associated bacteria in the 0.2 μm FYRW incubations increased on copepods (Figure 3.2D; ANOVA, $p = 0.035$) but did not change significantly on rotifer carcasses (Figure 3.3D). Bacterial abundances per copepod carcass were on average an order of magnitude higher than abundances per rotifer carcass. When initial carcass-associated bacterial abundances (5 μm FYRW incubation) were normalized to literature C-content values (4.6

μg C copepod $^{-1}$, Tang et al. 1999; 170 ng C rotifer^{-1}, Øie et al. 1997), copepods carcass also contained more bacteria than rotifer carcasses: 8206 ± 5506 cells μg C^{-1} vs. 2914 ± 990 cells μg C^{-1} (mean ± SD).

Exoenzymatic activity

The potential cell-specific protein analog hydrolysis activity and lipid analog hydrolysis activity were calculated for each of the bacterial groups (control, free-living, and carcass-associated) in each incubation experiment. These enzymatic measurements will henceforth be referred to as protease and lipase activities. Among the 5 μm FYRW incubations, free-living bacteria in the copepod treatment increased their protease activity to maximum within 24 hours (Figure 3.4A; ANOVA, $p = 0.009$); no change was detected in the rotifer treatment (Figure 3.5A). Copepod carcass-associated bacteria in 5 μm FYRW incubations showed no change in protease activity through time, whereas their lipase activity rapidly peaked by 8 hours (ANOVA, Tukey test, $p<0.001$; Figure 3.4). Rotifer carcass-associated bacteria demonstrated high initial protease and lipase activities which declined rapidly by 8 hours (ANOVA, Tukey test, $p<0.001$ for both protease and lipase; Figure 3.5).

When copepod carcasses were incubated in 0.2 μm FYRW, the protease and lipase activities of free-living bacteria dropped after the initial measurements and remained low for the duration of the incubation (Figure 3.6). In the similar experiment with rotifer carcasses, free-living bacteria showed no change in protease activity throughout the experiment, while their lipase activity increased to a maximum at 16 hours

before decreasing (Figure 3.7). There was no change in the protease or lipase activity of carcass-associated bacteria in either copepod or rotifer treatments (Figures 3.6 and 3.7).

Enhancement factors (EF) were calculated for protease and lipase activity (EF = activity of carcass-associated bacteria: activity of free-living bacteria). Mean EF for incubations in 5 μm FYRW and 0.2 μm FYRW are presented in Table 3.1 and Table 3.2, respectively. In general, carcass-associated enzyme activities were highly enhanced for both copepod and rotifer carcasses in 5 μm FYRW. Within 0.2 μm FYRW incubations rotifer EF were much lower and copepod EF increased later in the incubations.

Carcass protein content

Approximately 70% of the initial total copepod protein was lost within the first 8 hours of incubation in 5 μm FYRW (Figure 3.8), after which there was no change in the total carcass protein (ANOVA, Tukey test, $p = 0.013$). This overall decrease as well as three main trends were noted in the SDS-PAGE results (Figure 3.9A): 1) higher molecular weight proteins (e.g. 150 and 100 kD) present at the initial time point disappeared as decomposition progressed; 2) intermediate sized proteins (approximately 60 kD) appeared at 16 hours and then disappeared by 24 hours; 3) there were protein bands (e.g., 45 kD) that persisted throughout the decomposition process. Copepod carcasses incubated in 0.2 μm FYRW showed no significant decrease in the amount of particulate protein observed per carcass over the span of 24 hours (Figure 3.8). SDS-PAGE confirmed that proteins of all sizes (in particular the large sizes) persisted throughout the entire experiment (Figure 3.9B). In direct contrast to copepods, there was no significant change in the total protein content of rotifer carcasses incubated in 5 μm

FYRW (Figure 3.10), and the majority of the protein bands present initially remained throughout the 24 hours of incubation (Figure 3.11A). Likewise, rotifer carcasses incubated in 0.2 μm FYRW showed no change in the total particulate protein content (Figure 3.10) or in the protein size distribution (Figure 3.11B).

Carcass lipid content

On average the neutral and polar fractions of lipids within copepod carcasses declined over the span of 24 hours when incubated in GF/D FYRW, although the decrease was not significant (Figure 3.12). No shift in the ratio of neutral to polar lipids per carcass was noticed over the 24 hours of incubation. Similarly, there was no significant decrease in the neutral or polar lipid content of rotifer carcasses over the 24 hour incubation period (Figure 3.13). There was also no change in the ratio of neutral to polar lipids contained within rotifer carcasses.

DISCUSSION

Crustacean zooplankton carcasses within freshwater lakes have been shown to serve as microbial hotspots, supporting elevated bacterial exoenzyme activity, production and abundances (Tang et al. 2006b, in press). The patterns and levels of bacterial abundances and cell-specific protease activity associated with the decomposition of copepod carcasses within this study are consistent with previous studies (Tang et al 2006b, in press). However, bacterial cell-specific lipase activity observed in this study was three orders of magnitude higher than that observed in the freshwater decomposition studies. As the production of specific exoenzymes is highly dependent upon microbial community structure (Arnosti 2004), this difference in activity may be a consequence of differences in the microbial communities between the two systems.

When carcasses were incubated with natural bacteria (5 μm FYRW), carcass-associated bacteria exhibited enhanced protease and lipase activities over their free-living counterparts in both copepod and rotifer treatments (Table 3.1). There were, however, differences in the decomposition process between copepods and rotifers. Bacterial abundance on copepods increased as decomposition progressed, while there was no change in bacterial abundance on rotifer carcasses. The enzyme activity of copepod carcass-associated bacteria either remained constant (protease) or increased rapidly (lipase). Initial protease and lipase activities on rotifer carcasses were 22 and 4 times higher, respectively, than the corresponding enzyme measurements of copepods. By 8 hours lipase activity on rotifer carcasses dropped to levels comparable to that on copepod

carcasses, while protease activities remained approximately three times higher than that on copepods. The high enzyme activity on rotifers immediately after death may be attributed to residual digestive enzymes produced by the rotifers (de Araujo et al. 2000, Štrojsová & Vrba 2005). Enzyme production by rotifers and their epizootic bacteria are highly localized (Štrojsová & Ahlrichs 2009) and the fluorogenic substrate analogs used were not specific to bacteria-produced enzymes. Given such, it was not possible to differentiate between the two enzyme sources. Variations in the starting microbial community may have also contributed to the different microbial responses to copepod and rotifer carcasses. Bacterial communities within the Chesapeake Bay have been shown to exhibit strong seasonal shifts (Kan et al. 2006). While both carcass types were incubated in filtered York River water, copepod and rotifer experiments were conducted at different times of the year.

Although lipase activity observed with copepod carcasses was up to three orders of magnitude higher than that of protease, copepod lipid content remained constant while protein content rapidly declined. Preferential decomposition of proteins and nitrogen-rich substrates has been observed among other organic aggregates (e.g. Grossart & Ploug 2001), but at a much slower rate than measured in this study. I found that approximately 70% of initial protein content was lost from copepod carcasses over the span of 8 hours compared to 50% of initial PON lost from diatom aggregates over 5.4 days (Grossart & Ploug 2001). In contrast, Fukami et al. (1985) found the protein loss to be similar for diatoms and copepods. The enhanced protease activity, disappearance of large protein molecules and ephemeral appearance of intermediate sized protein molecules indicate the rapid hydrolysis of larger proteins into smaller forms through bacterial exoenzymatic

activity. In comparison, rotifer carcasses subject to free-living bacterial enzymes lost insignificant amounts of protein despite elevated exoenzyme activities. It has been considered that the proteins conserved throughout the decomposition process may be attributed to bacterial biomass. Within these experiments it was not possible to separate bacterial and carcass proteins. A back-of-the-envelope calculation using carcass-associated bacterial abundances from the corresponding enzyme experiments and an average value of 34.4 fg protein per bacterial cell (Simon & Azam 1989) suggest that bacterial biomass could account for a maximum of 3% measured copepod or rotifer protein at any given time. From these calculations it is expected that bacterial contribution to total protein measurements would be negligible.

The selective decomposition of some protein sizes and retention of others suggests that there are two separate protein pools contained within copepod carcasses. The first pool is likely highly labile and rapidly consumed by bacteria, while the second pool is more refractory and likely to be transported to depth. Reinfelder et al. (1993) also noted two separate protein fractions released from carcasses of the copepod *Anomalocera patersoni*: approximately 64% of the total protein was comprised of a rapidly exchanging pool with a half-life of 1.1 d, and the remainder of the protein belonged to a slowly exchanging pool with a half-life of 5.5 d. Contrary to our initial expectations, it was found that proteins contained within crustacean zooplankton were decomposed at a much faster rate than those of non-crustacean zooplankton. This indicates that the presence of a chitinous exoskeleton does not impede the decomposition process in any way. Rather, the decomposition rate may be regulated by the overall quality of the protein. Through an examination of the SDS-PAGE results it was noted that copepods

(Figure 3.9) initially contained multiple proteins larger than 70 kD and only three prominent protein bands in the intermediate size range (40-60 kD). In contrast, rotifers (Figure 3.11) had fewer large proteins and 5 prominent bands of proteins in the 40-60 kD range. In both copepods and rotifers larger proteins tended to disappear as decomposition progressed, while the intermediate sized proteins persisted. It is possible that the rapidly consumed protein pool discussed previously is primarily comprised of larger protein sizes and the refractory protein pool is dominated by intermediate sized proteins. The dominance of intermediate sized proteins in rotifers may explain the insignificant loss of carcass protein during the experiment. Rotifer integument contains a layer of tightly-packed keratin which is likely responsible for maintaining rotifer body shape (Kleinow & Wratil 1995). A portion of the protein bands retained throughout the incubations may be keratin proteins which typically range in size from 40-70 kD (e.g. Lee et al. 1979, Kleinow 1993) and are highly resistant to decomposition (Kleinow 1993).

There was no observed change in the lipid content of either copepod or rotifer carcasses over the span of 24 hours despite high lipase activities. Bacteria capable of hydrolyzing lipids are quite common within aquatic systems (Mudryk & Skórczewski 2006) but decomposition of lipids tends to occur over a longer time scale than that of proteins. Within Antarctic shelf waters Fabiano and Pusceddu (1998) found little change in the proportion of hydrolysable phytodetritus lipid with depth, and two to three weeks were necessary to observe decomposition of algal fatty acids within sediments (Ding & Sun 2005). While our study did not monitor change in lipid content over longer time periods, the decomposition of copepod and rotifer lipids could proceed in a manner similar to phytodetrital lipids.

When incubated without ambient free-living bacteria (0.2 μm FYRW), both copepod and rotifer carcasses demonstrated insignificant loss of proteins, and a minimal or delayed enhancement of carcass-associated enzyme activity. This suggests that both autolysis of zooplankton tissue and the activity of enteric or previously attached bacteria are minimal. A comparison of the 5 μm and 0.2 μm FYRW experiments indicates that carcass decomposition is largely mediated by the colonization of free-living bacteria, similar to what has been observed in the decomposition of zooplankton fecal pellets (e.g. Harding 1973, Honjo & Roman 1978, Jacobsen & Azam 1984, Hansen & Bech 1996).

Further detailed studies are necessary to determine if the currently observed patterns of protein and lipid decomposition are consistent among other crustacean and non-crustacean zooplankton taxa. If the patterns of decomposition are consistent across taxa, the fate of zooplankton carcass biomass could be largely dependent upon zooplankton community composition. In systems dominated by copepods, the relative contribution of proteins and lipids to total biomass could potentially affect the amount and quality of organic matter transported to depth by carcasses. For example, during a mass mortality event, carcasses of a high latitude copepod species with lipids comprising up to 73% of dry weight (Båmstedt 1986) could transport a much higher amount of carbon to depth than carcasses of lipid deficient species in lower latitudes. In contrast, a mass mortality event among a protein rich copepod population could result in a large fraction of copepod biomass being incorporated into the microbial loop within surface waters.

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Table 3.1. Mean enhancement factors for protease and lipase activities of zooplankton carcasses incubated with a natural ambient microbial community (5 μm FYRW). ND = no data.

	Copepod				Rotifer			
	Protease		Lipase		Protease		Lipase	
Time (h)	Mean	SD	Mean	SD	Mean	SD	Mean	SD
0	85.6	97.3	187.8	176.7	448.3	52.3	121.36	10.28
8	7.5	5.4	127.3	21.9	39.3	15.7	33.4	6.3
16	5.1	0.2	90.5	43.9	26.9	12.0	21.9	12.0
24	1.5	1.7	16.1	13.0	9.4	4.2	15.9	9.2
48	17.0	9.8	79.0	48.3	33.2	19.9	62.3	42.9
72	20.1	9.1	ND	ND	ND	ND	ND	ND
96	11.5	7.4	ND	ND	ND	ND	ND	ND

Table 3.2. Mean enhancement factors for protease and lipase activities of zooplankton carcasses when the natural ambient microbial community is removed (0.2 µm FYRW). ND = no data.

	Copepod				Rotifer			
	Protease		Lipase		Protease		Lipase	
Time (h)	Mean	SD	Mean	SD	Mean	SD	Mean	SD
0	0.4	0.3	0.2	0.08	5.8	2.8	2.6	2.1
8	ND	ND	ND	ND	4.6	2.4	1.9	2.0
12	36.2	27.5	8.5	ND	ND	ND	ND	ND
16	ND	ND	ND	ND	3.8	1.1	0.4	0.09
24	263.1	147.8	66.4	38.7	1.9	0.3	0.6	0.3
36	200.1	155.3	46.0	17.3	ND	ND	ND	ND
48	74.8	28.1	87.7	67.6	6.4	5.9	3.5	2.1
72	172.7	49.0	56.0	20.0	ND	ND	ND	ND

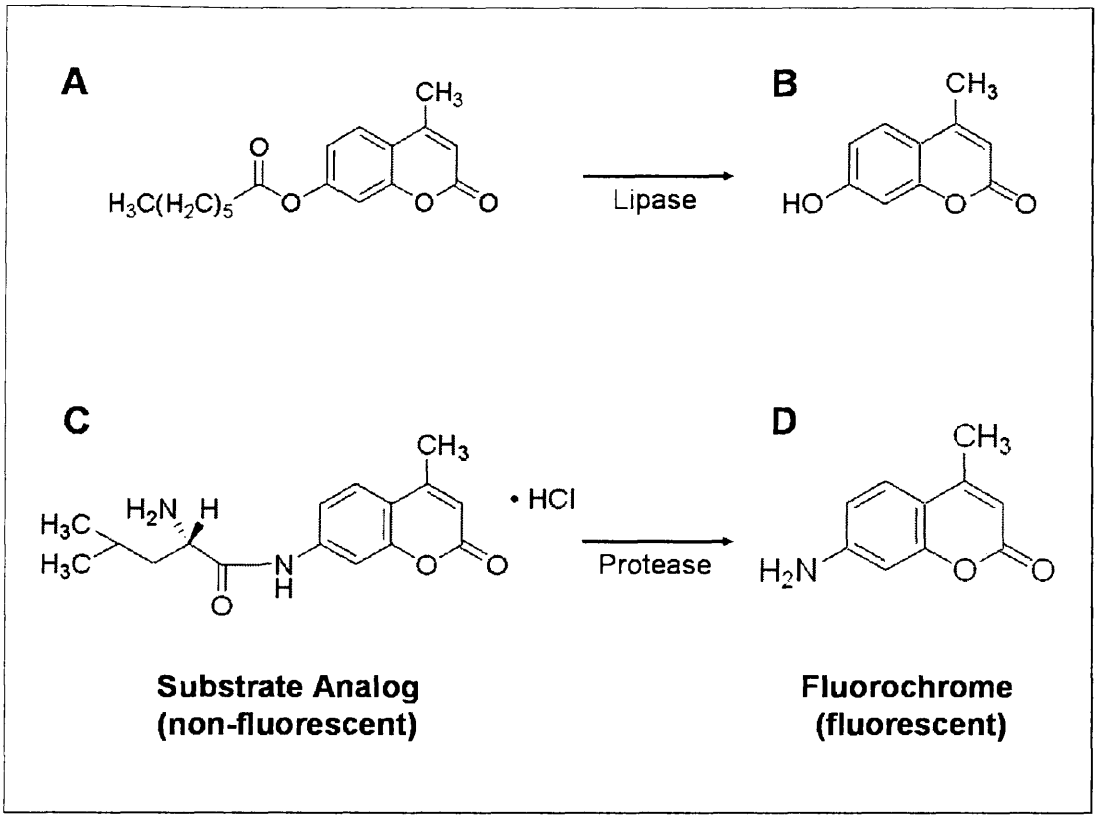

Figure 3.1. Substrate analogs and corresponding fluorochromes used to assess potential bacterial exoenzyme activity. Lipase activity was approximated by measurement of 4-methylumbelliferone (B) produced from the cleavage of 4-methylumbelliferyl heptanoate (A). Protease activity was approximated by measurement of 7-amido-4-methylcoumarin (D) produced from the cleavage of L-leucine 7-amido-4-methylcoumarin (C).

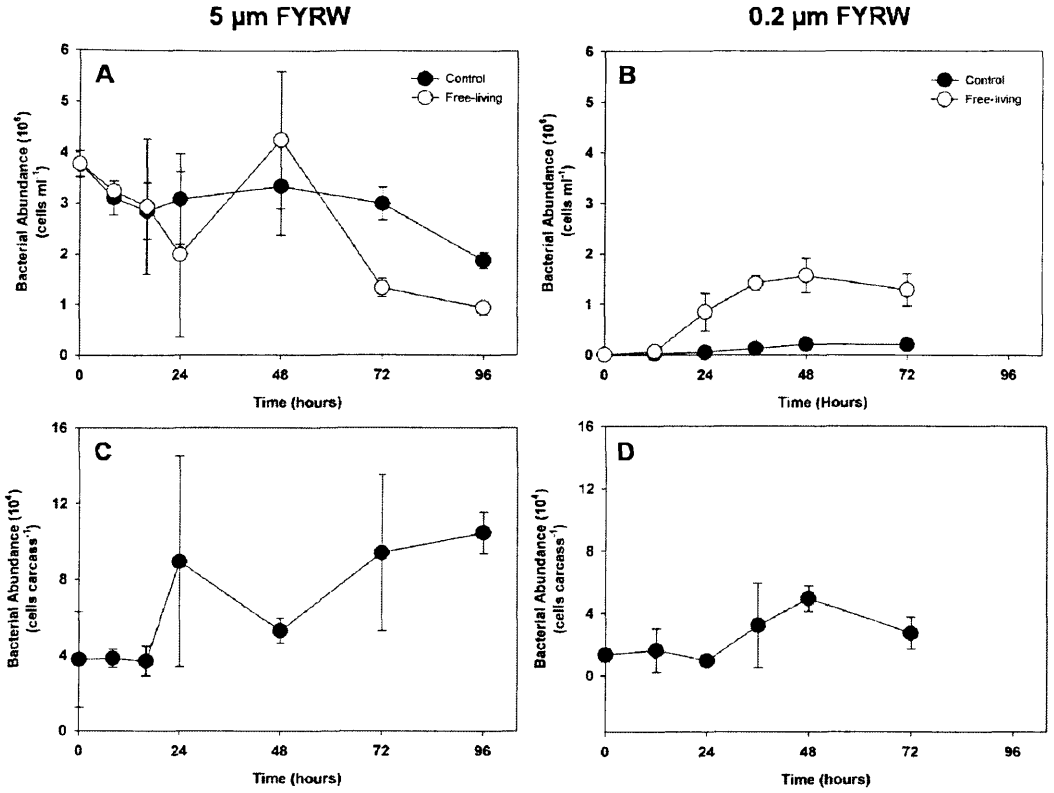

Figure 3.2. Abundances of free-living and control bacterial populations (A, B) and carcass-associated bacterial abundances (C, D) during the decomposition of copepod carcasses in 5 µm FYRW (A, C) and 0.2 µm FYRW (B,D).

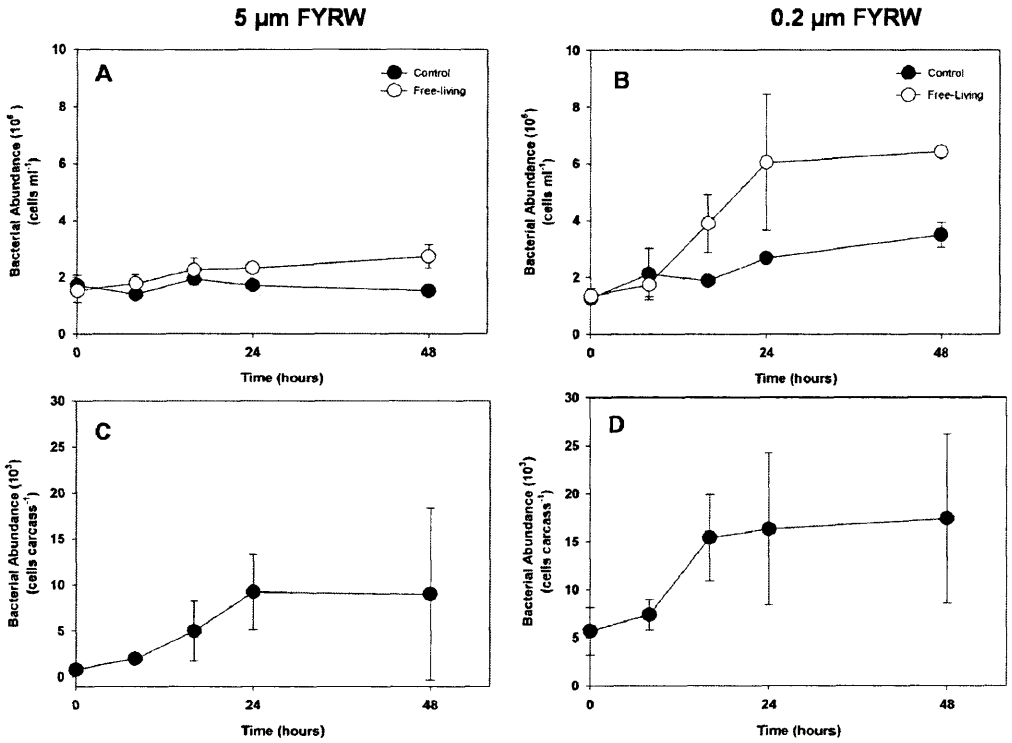

Figure 3.3. Abundances of free-living and control bacterial populations (A, B) and carcass-associated bacterial abundances (C, D) during the decomposition of rotifer carcasses in 5 µm FYRW (A, C) and 0.2 µm FYRW (B,D).

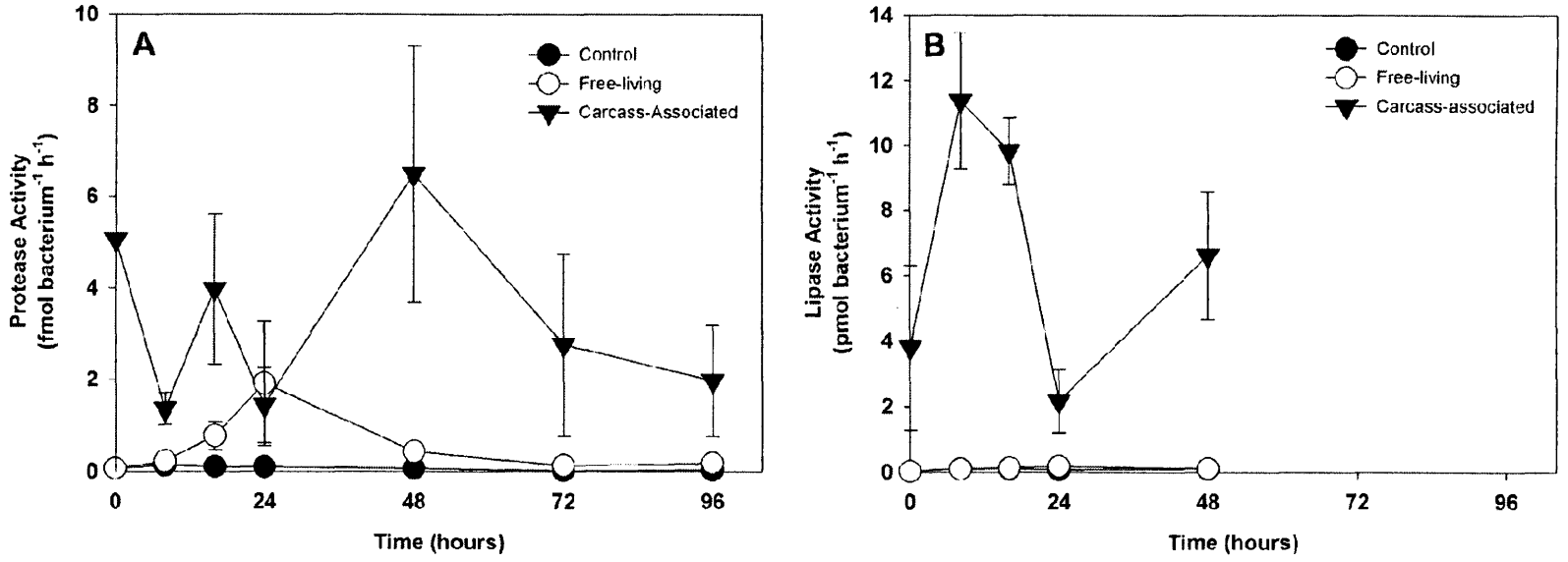

Figure 3.4. Mean ± SD (n = 3) cell-specific protease (A) and lipase (B) activities for control, free-living and carcass-associated bacteria measured during incubations of copepod carcasses in 5 μm FYRW. Scale and units are different for protease and lipase measurements respectively.

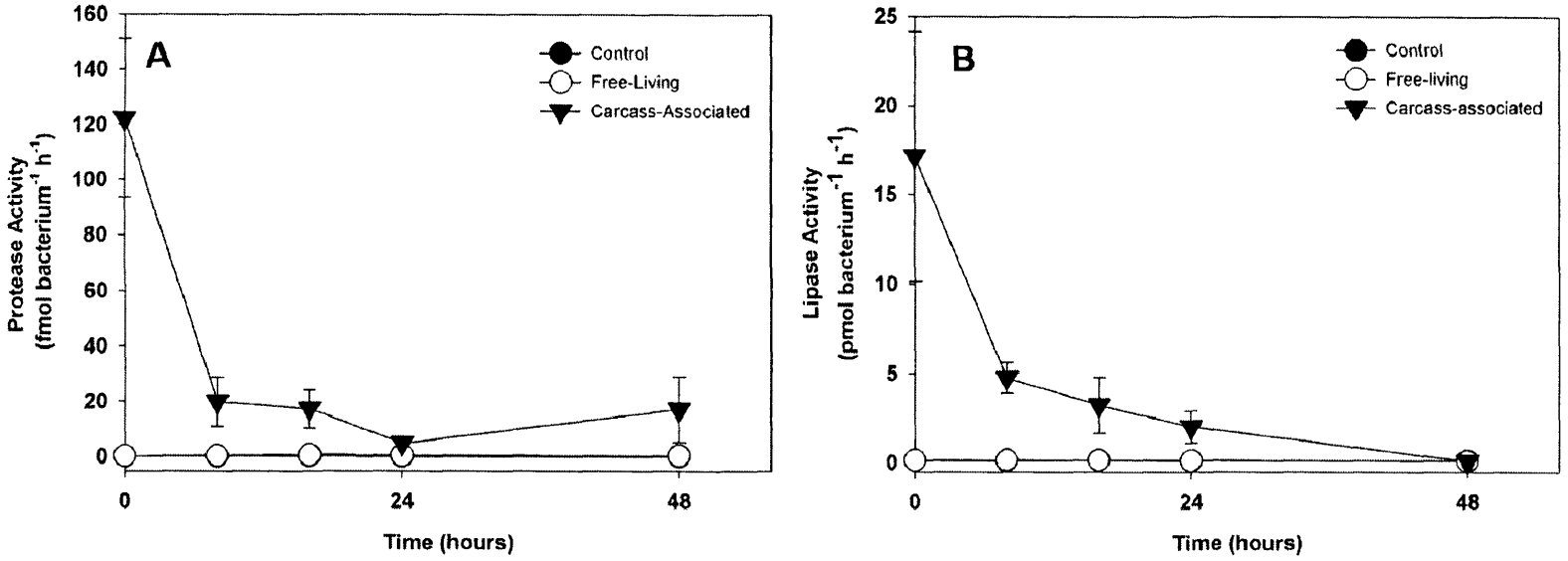

Figure 3.5. Mean ± SD (n = 3) cell-specific protease (A) and lipase (B) activities for control, free-living and carcass-associated bacteria measured during incubations of rotifer carcasses in 5 μm FYRW. Scale and units are different for protease and lipase measurements respectively.

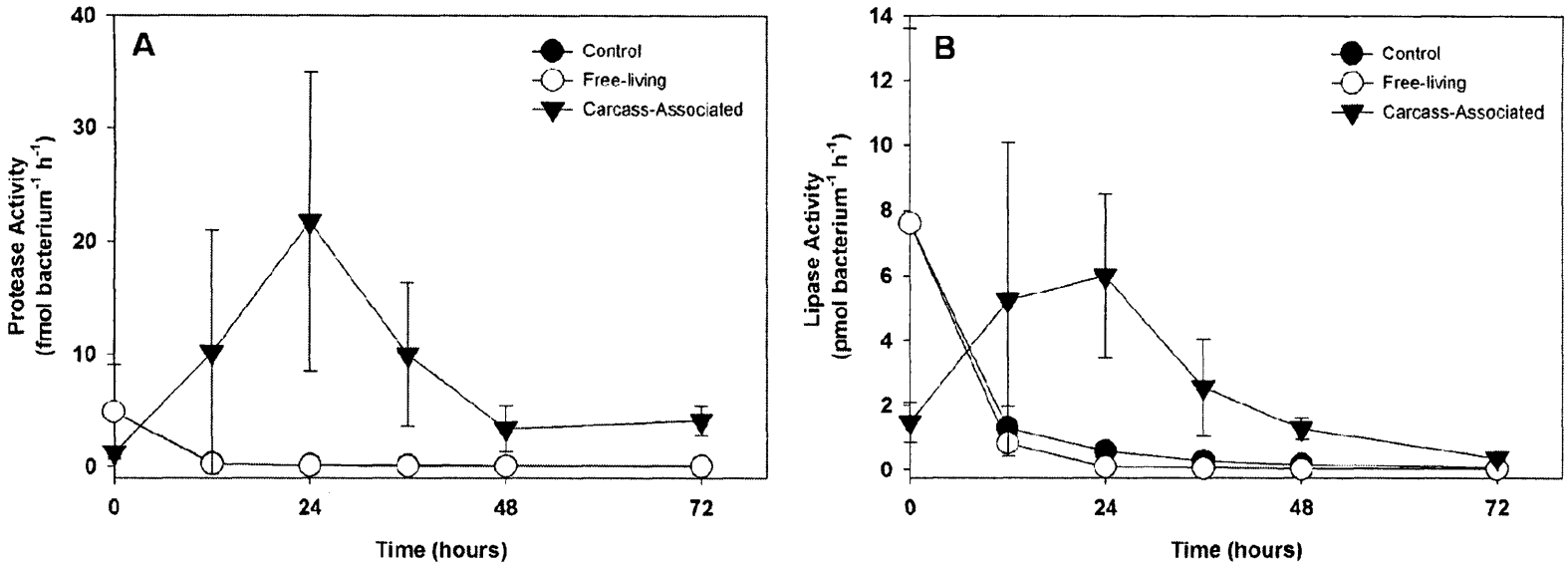

Figure 3.6. Mean ± SD (n = 3)cell-specific protease (A) and lipase (B) activities for control, free-living and carcass-associated bacteria measured during incubations of copepod carcasses in 0.2 μm FYRW. Scale and units are different for protease and lipase measurements respectively.

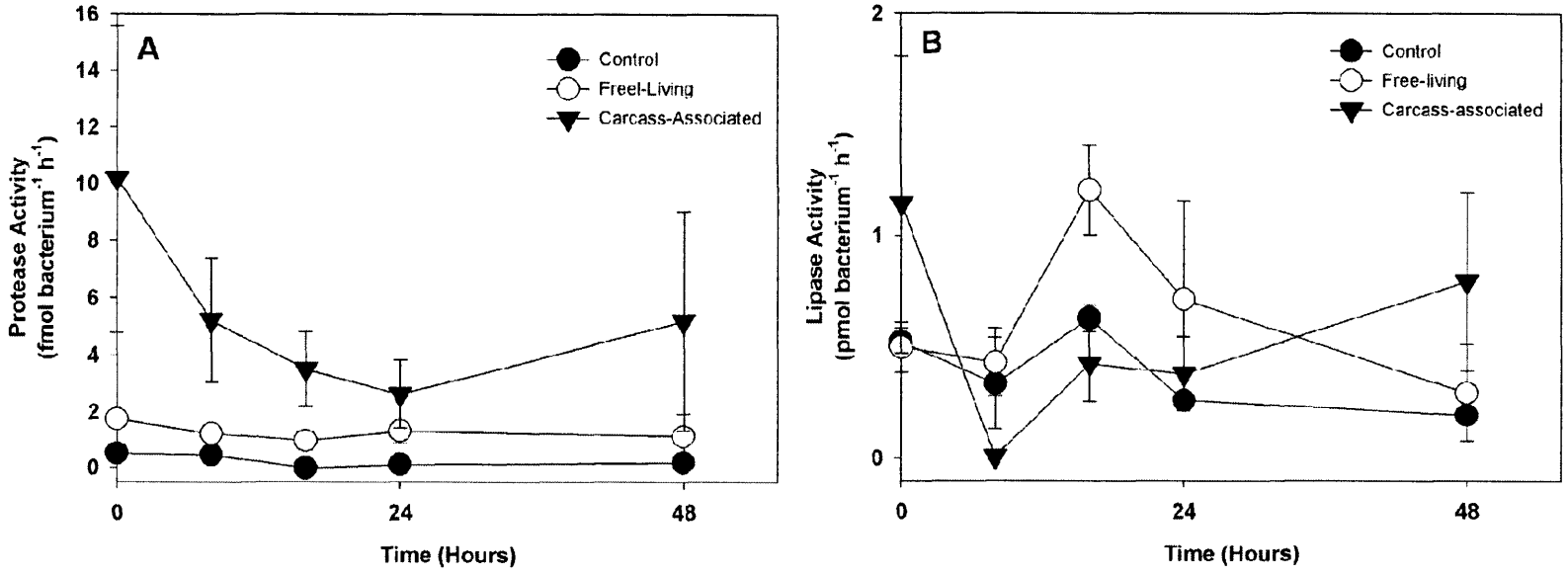

Figure 3.7. Mean ± SD (n = 3) cell-specific protease (A) and lipase (B) activities for control, free-living and carcass-associated bacteria measured during incubations of rotifer carcasses in 0.2 μm FYRW. Scale and units are different for protease and lipase measurements respectively.

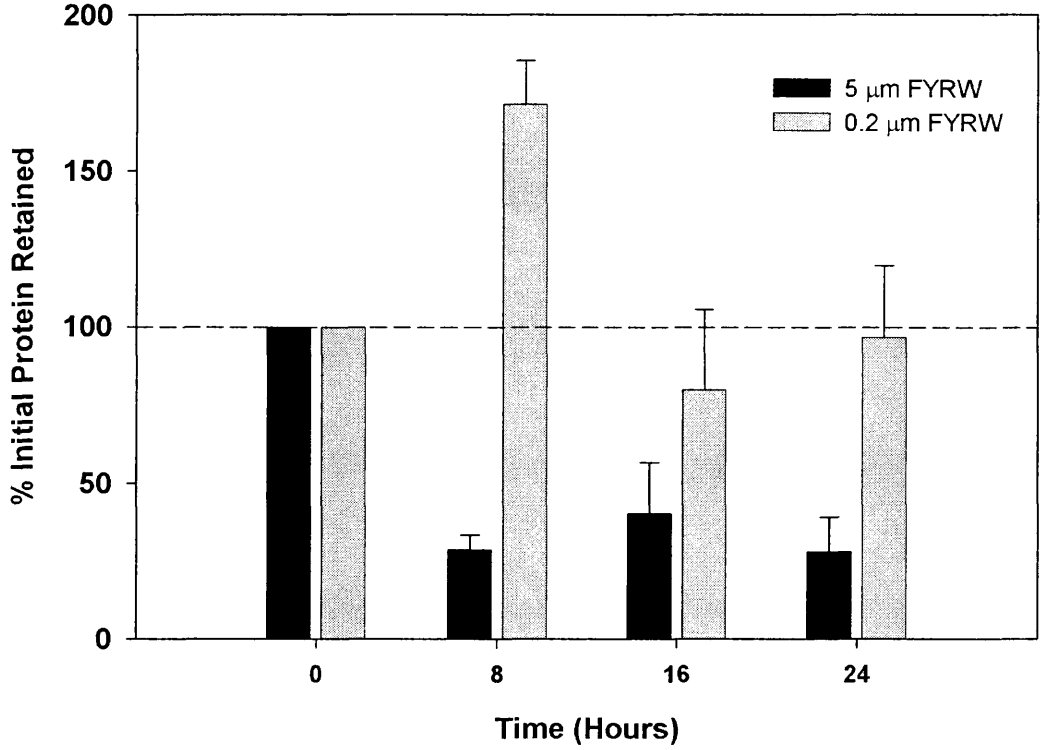

Figure 3.8. Mean ± S.D. percent initial protein retained within copepod carcasses incubated in two different water filtrates. Dashed horizontal line is 100% of initial protein for reference.

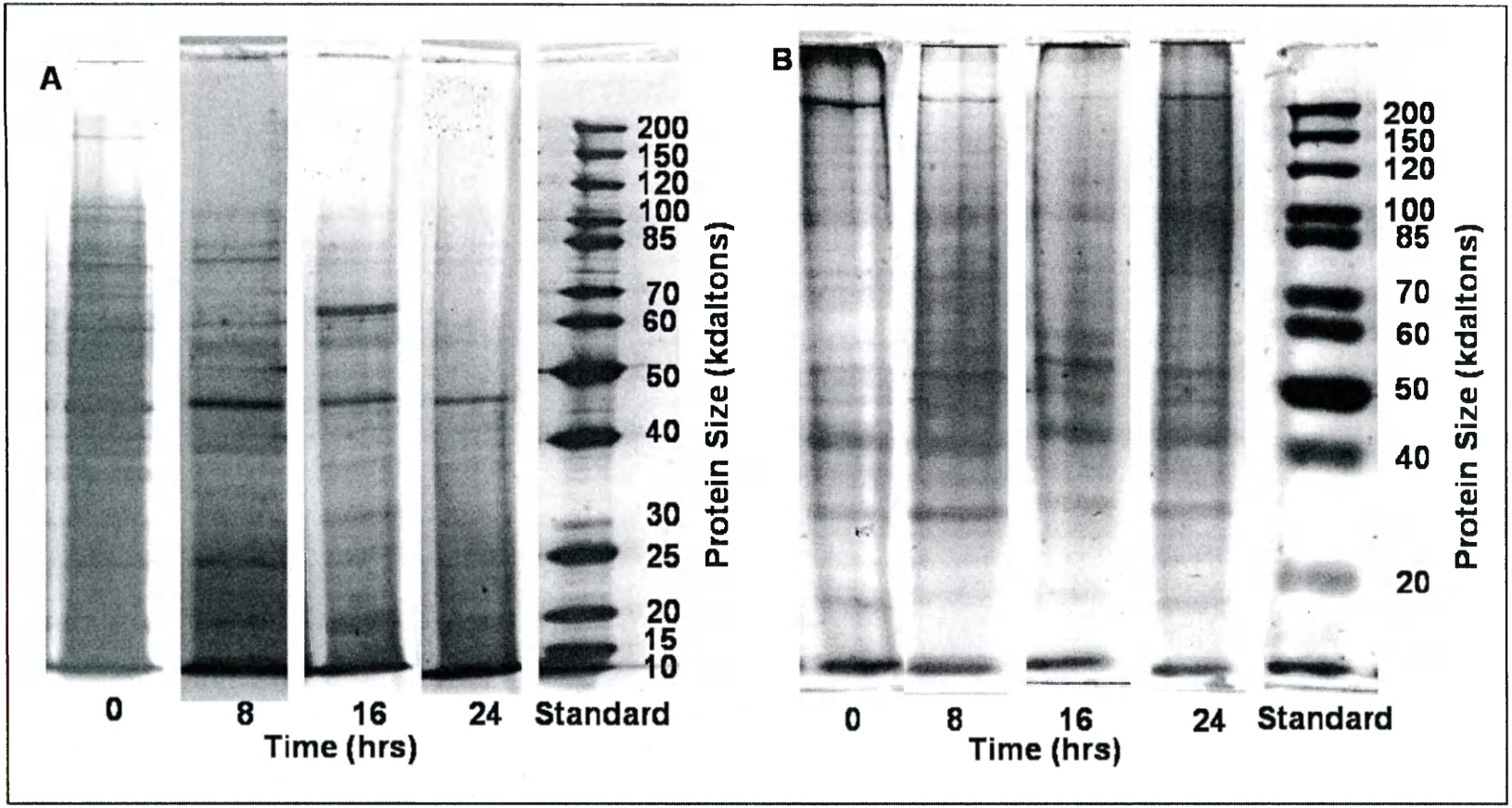

Figure 3.9. Protein size composition of copepod carcasses incubated in (A) 5 µm FYRW or (B) 0.2 µm FYRW.

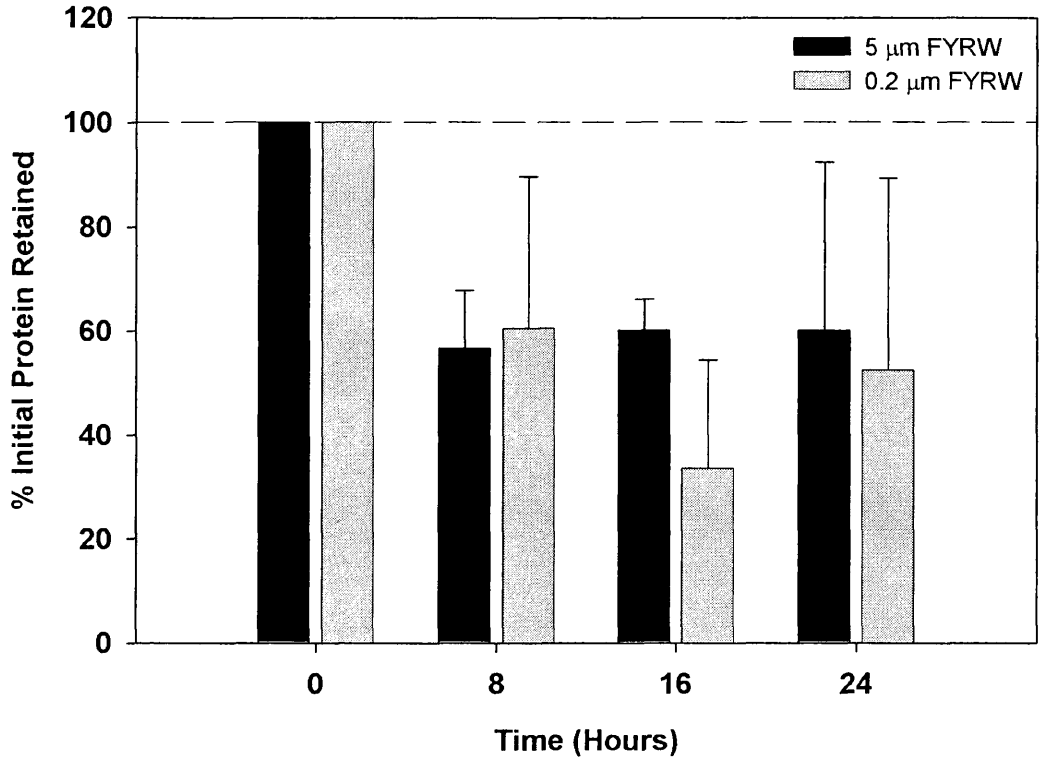

Figure 3.10. Mean ± S.D. percent initial protein retained within rotifer carcasses incubated in two different water filtrates. Dashed horizontal line is 100% of initial protein for reference.

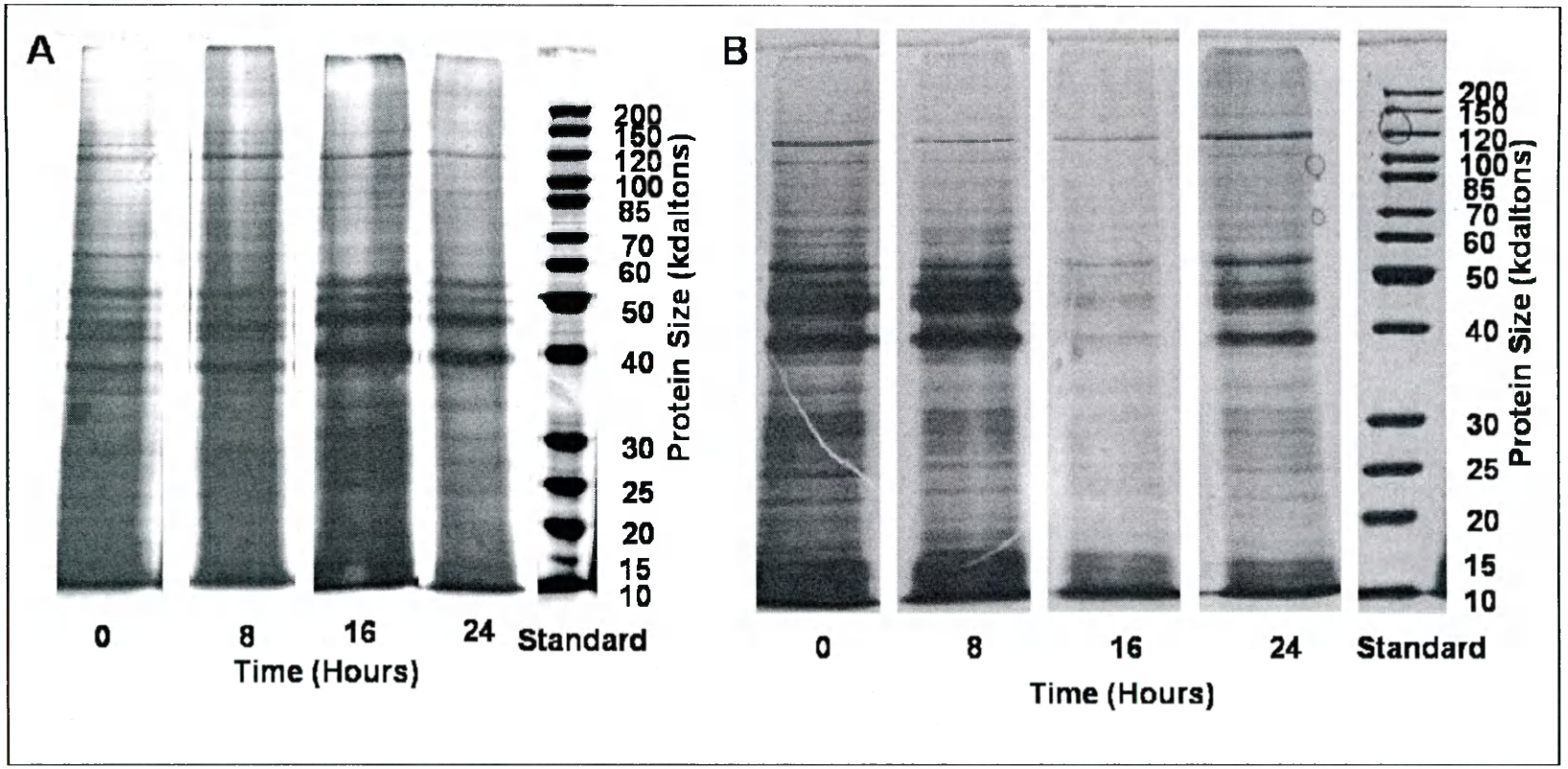

Figure 3.11. Protein size composition of rotifer carcasses incubated in (A) 5 µm FYRW or (B) 0.2 µm FYRW.

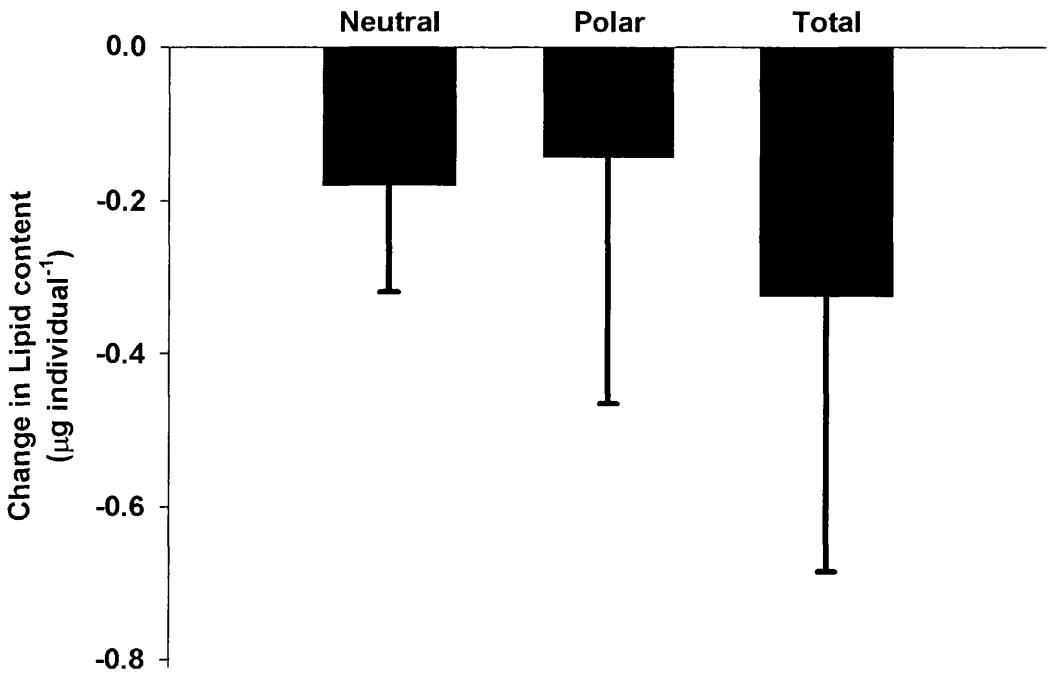

Figure 3.12. Change in lipid content of copepod carcasses. Values represent the mean value of the difference between carcass lipid content at 24 hours and 0 hours. Error bars represent one standard deviation.

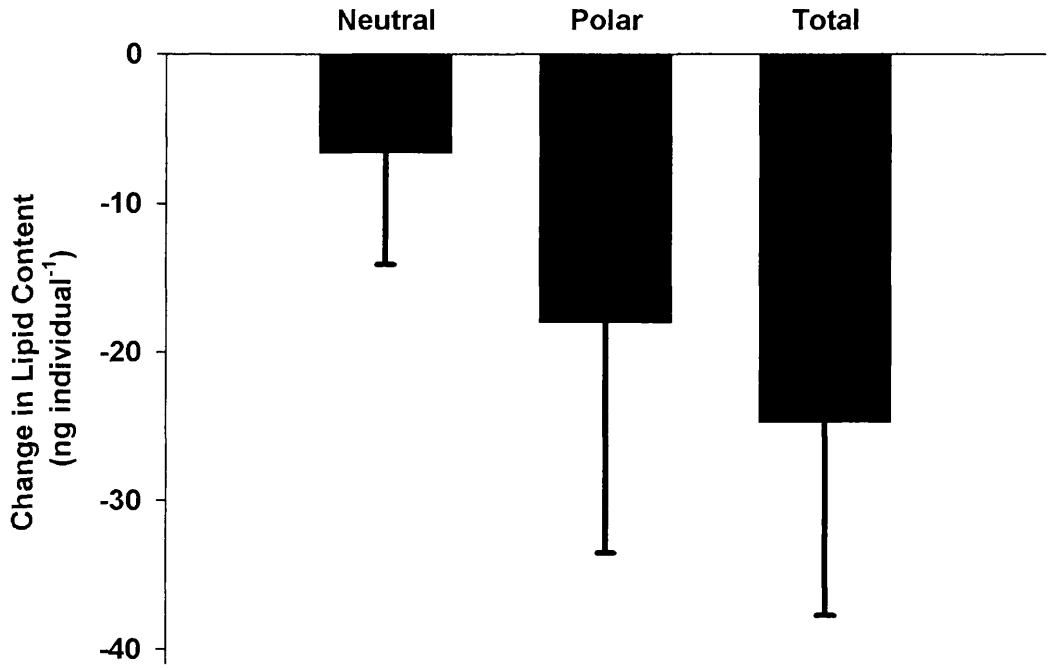

Figure 3.13. Change in lipid content of rotifer carcasses. Values represent the mean values of the difference between carcass lipid content at 24 hours and 0 hours. Error bars represent one standard deviation.

CHAPTER 4

Summary and Concluding Remarks

Zooplankton carcasses can be abundant within aquatic systems (e.g. Wheeler 1967, Weikert 1977, Terazaki & Wada 1988, Genin et al. 1995) but are rarely accounted for during traditional zooplankton sampling. While non-consumptive mortality due to natural factors such as diseases and harmful algal blooms has been explored (Kimmerer & McKinnon 1990, Delgado & Alcaraz 1999, Duffy et al. 2005), few studies have addressed anthropogenic causes of non-consumptive mortality among zooplankton (Hall et al. 1995). Through field sampling and the use of a vital staining technique I was able to demonstrate that some boat wakes contained a higher fraction of copepod carcasses than what was found naturally in the system (Chapter 2). While turbulence within boat wakes is capable of concentrating zooplankton carcasses, active avoidance of turbulence by live copepods is highly improbable. Although it remains to be tested, it is possible that the intensity of turbulence within boat wakes is high enough to cause mortality. As higher carcass abundances were noted within boat wakes, areas which experience heavy boat traffic may produce elevated concentrations of zooplankton carcasses. In addition to the removal of an important trophic link from the traditional food web, increased zooplankton mortality could potentially enhance microbial loop processes and transport of POM to depth on a local scale.

It is well established that organic aggregates function as transport mechanisms for POM to depth and serve as microbial hotspots, supporting elevated bacterial production (reviewed by Simon et al. 2002). Recent studies have indicated that zooplankton carcasses, like organic aggregates, are able to function as microbial hotspots (Tang et al. 2006, in press), but have not addressed the change in biochemical composition within carcasses during decomposition. Results from my laboratory experiments corroborate

previous findings that zooplankton carcasses serve as microbial hotspots. More importantly, my research suggests that biomolecules contained within crustacean and non-crustacean zooplankton carcasses are subject to different fates (Chapter 3). My research indicated that proteins contained within crustacean zooplankton carcasses were more likely to be remineralized and support microbial production within the water column. In contrast, biomolecules within non-crustacean zooplankton carcasses were more resistant to microbial degradation and could therefore contribute to POM flux. Considering these findings, the fate of zooplankton carcasses biomass would largely be dependent upon the zooplankton community composition and their non-consumptive mortality rates.

The results of my thesis bring about a number of corollary questions and present multiple areas for future research. First and foremost, it is integral to evaluate the decomposition of other crustacean and non-crustacean zooplankton to determine if the observed decomposition patterns are consistent across taxa. If these decomposition patterns can be applied to other taxonomic groups, systems which display a spatial separation of crustacean and non-crustacean species (e.g. Chiba et al. 1998) or in which the dominant zooplankton alternates between crustacean and non-crustacean species (e.g. Lampert & Rothhaupt 1991) may experience localized or pulsed intervals of intense surface-water microbial production during times of crustacean dominance or POM transport to depth in times of non-crustacean dominance.

The Southern Ocean is one area where strong interactions between crustacean (krill) and non-crustacean zooplankton (salps) has been documented. Intense feeding competition during the spring bloom coupled with a decrease in winter ice extent can lead

to the dominance of salps within Antarctic systems (Loeb et al. 1997). It is known that the relative abundances of salps and krill already influence carbon flux in the Southern Ocean through fecal pellet production (e.g. Pakhomov et al. 2002). As salps are not a major component of Antarctic predator diets (Foxton 1966), non-consumptive mortality may be important and salp contribution to carbon flux could extend beyond fecal pellet production.

Microbial decomposition of gelatinous zooplankton carcasses is another avenue of future research. Gelatinous zooplankton blooms are a common occurrence globally and are responsible for many negative impacts on ecosystem and socioeconomic levels (Purcell et al. 2007). Considering the sudden establishment and collapse of jellyfish blooms (e.g. Graham et al. 2001), the fate of this biomass is of great interest. Observed accumulations of jellyfish carcasses on the sea floor (Billet et al. 2006, Yamamoto et al. 2008) suggest that microbial decomposition may proceed at a slower rate for gelatinous zooplankton as well. If mass deposition on the benthos is common, jellyfish carcasses may contribute to benthic food webs and support production in higher trophic levels, or be decomposed by sediment bacteria and possibly contribute to bottom water hypoxia.

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