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Occurrence of copepod carcasses in the lower Chesapeake Bay and their decomposition by ambient microbes

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Abstract

We tested and refined the Neutral Red staining method for separating live and dead copepods in natural samples. Live copepods were stained red whereas dead copepods remained unstained. The staining results were not affected by method of killing, time of death or staining time. Tow duration had no significant effect on the percent dead copepods collected. The Neutral Red staining method was applied to study the occurrence of dead copepods along the York River and the Hampton River in the lower Chesapeake Bay during June−July, 2005. The zooplankton community was dominated by copepods; on average 29% of the copepod population appeared dead. Recovery of percent dead copepods did not differ between horizontal tows and vertical tows, suggesting that dead copepods were homogenously distributed in the water column. No significant relationship was found between the percent dead copepods and surface water temperature, salinity, Secchi depth or chlorophyll concentration. In laboratory experiments, dead copepods were decomposed by ambient bacteria and the rate of decomposition was temperature-dependent. Combining field and laboratory results we estimated that the non-consumptive mortality (mortality not due to predation) of copepods in the lower Chesapeake Bay was 0.12 d^{-1} under steady-state condition, which is within the global average of copepod mortality rate.
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1. Introduction

Accurate assessment of population abundance is critical to zooplankton ecological research. Traditionally researchers collect, preserve and enumerate zooplankton samples without knowledge of the health status of the zooplankton, and studies on zooplankton mortality are largely focused on predation loss. However, zooplankton could suffer from non-consumptive mortality due to starvation, environmental stresses, diseases, pollution, injuries, parasites, and harmful algal blooms (Carpenter et al., 1974; Murtaugh, 1981; Byron et al., 1984; Burns, 1985; Ianora et al., 1987; Kimmerer and McKinnon, 1990; Hall et al., 1995; Delgado and Alcaraz, 1999; Gomez-Gutierrez et al., 2003). Without differentiating live and dead zooplankton researchers may overestimate zooplankton population recruitment and secondary production, and the available food for the higher trophic levels.

Weikert (1977) observed that in the upwelling region south of Cap Blanc, N.W. Africa, copepod carcasses (*Temora* spp.) amounted to as much as 28% of the copepod population. In Lake Constance, Gries and Güde (1999) estimated, based on sediment trap measurements, that non-consumptive mortality of *Daphnia galeata* accounted for an average of 2.3% of the standing stock per day, or 23% of the estimated production. Similarly, Dubovskaya et al. (2003) observed that in Bugach reservoir, Siberia, high abundance of trap-collected dead *Daphnia* sp. coincided with periods of low pelagic population abundance. These authors further estimated that non-consumptive mortality contributed on average 30%, and at times 100%, to the total mortality of the *Daphnia* population (Dubovskaya

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et al., 2003). Other researchers also reported high abundance of dead copepods in the deep water that appeared to correlate with the abundance of live copepods in the upper water column (Wheeler, 1967; Terazaki and Wada, 1988).

Zooplankton carcasses may become a significant part of the organic sinking fluxes and contribute to the dietary intake by deep-sea fauna (Genin et al., 1995; Haury et al., 1995). High abundances of copepod carcasses have been observed to accumulate near bottom topographic structures and deep water (Terazaki and Wada, 1988; Genin et al., 1995; Haury et al., 1995). A transect study in the N. Atlantic showed that at a depth of 2200–4100 m, the in situ abundance of copepod carcasses exceeded that of live copepods (Wheeler, 1967).

Upon death, the carcass will begin to degrade. Disintegration and decomposition of zooplankton carcasses provides an alternative pathway for nutrient regeneration, elemental recycling and microbial production (Harding, 1973; Lee and Fisher, 1992; Reinfelder et al., 1993). Harding (1973) suggested that decomposition of copepod carcasses is mainly due to the action of external, not internal, bacteria, and the decomposition rate is temperature-dependent. At 4 °C, the decomposition of the carcass of *Calanus finmarchicus* took 11 d to complete, whereas at 20–22 °C, the carcasses were reduced to chitin fragments after only 3 d (Harding, 1973). Terazaki and Wada (1988) estimated that in the deep water of the Japan Sea, where temperature is less than 2 °C, carcasses of the copepod *Calanus cristatus* could drift for over a year and remain largely intact.

Previous observations of dead zooplankton relied on visual signs of body injuries or internal decomposition of the zooplankton bodies, which could be time-consuming and inaccurate, and is often not practical in field settings. Dressel et al. (1972), Crippen and Perrier (1974) and Fleming and Coughlan (1978) applied a method to separate dead and live marine zooplankton based on their differential uptake of the vital stain Neutral Red: Live zooplankton will stain red, whereas dead zooplankton will not stain. The staining method requires minimal handling of the animals on board, and the stained samples can be preserved for later analysis. This staining method, when used in concert with other microscopic observations, allows researchers to record the abundance of dead zooplankton and diagnose their cause of death.

Chesapeake Bay is the largest and economically most important estuary on the east coast of the United States. Calanoid copepods account for the majority of the zooplankton biomass, and the major food source for many planktivorous species in the bay (Kimmel and Roman, 2004). The environmental quality of the bay in recent years has suffered from eutrophication, hypoxia, pollution and nuisance algal blooms (Kiddon et al., 2003). Programs have been established to monitor the status and to restore the health of the bay (e.g. the Chesapeake Bay Program). However, census of the zooplankton community is thus far limited to species composition and abundance, and information on the vital state of the zooplankton is missing.

The objective of the present study is three-fold: (1) to evaluate and improve upon the Neutral Red staining method for application in the field; (2) to assess the abundance of live vs. dead zooplankton in the lower Chesapeake Bay using the Neutral Red staining method; and (3) to investigate the decomposition of zooplankton carcasses by ambient microbes.

2. Materials and methods

2.1. Neutral Red staining method

We applied the Neutral Red staining method according to Dressel et al. (1972) with some modifications based on Fleming and Coughlan (1978) to make the method better suited for field work. Chemicals of reagent grade or better were purchased from FisherScientific. Neutral Red stock solution (0.01 g ml^{-1}) was prepared by dissolving the chemical in deionized water. An alkaline solution was prepared by dissolving sodium hydroxide in 0.2 μm-filtered seawater and adjusting to pH 9. The basic staining procedure was to add 1.5 ml Neutral Red stock solution to every 1000 ml zooplankton sample volume (final conc. approx. 1:67,000). The sample was kept in a water bath on board for about 15 min, then gently concentrated onto a 200-μm nylon mesh and rinsed repeatedly with filtered seawater to remove excess stain. The rinsed sample was then transferred to a glass sample jar with alkaline filtered seawater (pH 9) and preserved with formaldehyde (final conc. ~4%). An alkaline medium helped stabilize the stain inside the zooplankton tissues for a longer time (Fleming and Coughlan, 1978). Preserved samples were kept cold and dark (cooler with ice on board; refrigerator in the laboratory) for further processing within 4 d. In the laboratory, the preserved samples were transferred to a gridded counting plate, and acidified with dilute hydrochloric acid to a pH <7. Acidification helped develop the color of the stain such that zooplankton that were alive at the time of sampling would appear red, whereas dead ones would appear unstained (Dressel et al., 1972; Fleming and Coughlan, 1978). The color of the stained zooplankton was best observed under a dissecting microscope with dark field. To evaluate the staining method for field applications we conducted a series of tests as described below.

2.2. Test of staining in the laboratory

Field-collected copepods (*Acartia tonsa*) were divided into four subsamples. Three subsamples were killed by exposure to ethanol, formaldehyde or carbon dioxide. The remaining subsample was used as control. The copepods were stained with Neutral Red for 5, 15 and 20 min. The results were tested with 2-way ANOVA after arc-sine transformation with a critical *P*-value of 0.05 for significant differences in methods of killing and duration of staining.

In a separate test, field-collected copepods were killed with 4% formaldehyde and treated with Neutral Red after they had been dead for 1, 5 and 15 min. The dead copepods were stained respectively for 5, 15 and 20 min. The results were tested with 2-way ANOVA after arc-sine transformation with

a critical P-value of 0.05 for significant differences in duration of death and duration of staining.

2.3. Test of duration of tows

Zooplankton are typically collected using plankton nets. Zooplankton trapped within the net may suffer from stress and mortality. We tested how the duration of horizontal net tows may affect the percent dead copepods collected. We did horizontal net tows (standard ring net, 0.5 m dia., 200 μm mesh, filtering cod end) just below the surface at a speed of ~1 knot for 1, 2, 3 and 4 min at a fixed station in the lower Chesapeake Bay (station H-3; see below). Samples were treated with Neutral Red on board and returned to laboratory for species identification and enumeration.

2.4. Field study in the lower Chesapeake Bay

Field sampling was conducted between June and July, 2005 to assess the live vs. dead zooplankton composition along the York River and the Hampton River, a tributary within the James River, in the lower Chesapeake Bay (Fig. 1). We visited 4 stations in the York River three times and 3 stations in the Hampton River two times. In the shallow Hampton River stations (H-1 and H-2; <3 m deep) zooplankton were collected by horizontal net tows as described above. A propeller-type flow meter was attached to the net and the net was hauled for approx. 2 min; filtered volume was calculated based on the flow meter readings. In the other stations (>7 m deep) zooplankton were collected by vertical tows: The net was deployed to near the bottom and retrieved by hand at ~1 m s^{-1}; the filtered volume was calculated as the length of rope deployed multiplied by the net mouth area. To minimize carryover of dead zooplankton, the net was flushed by hauling open-ended through the water column multiple times between samples. In the York River one zooplankton sample was collected at each station; at the Hampton River stations duplicate samples were collected and compared to assess the problem of carryover. In the Hampton River deep station (H-3) we did additional horizontal tows near the surface to compare the percent dead zooplankton collected by vertical tows vs. horizontal tows. Upon retrieval of the net, the cod end content was passed through a 3000-μm mesh to exclude gelatinous zooplankton, then transferred into a glass jar and treated with Neutral Red as described above. In the laboratory the preserved samples were counted for major zooplankton groups and abundances. For the first set of samples (15-June) 25% of each sample was counted; for the other sets each of the entire sample was counted. Copepods were further counted as live (stained red) vs. dead (not stained). Because Neutral Red did not stain the other zooplankton groups well in earlier laboratory tests, the live/dead status of the other zooplankton groups was not evaluated. After counting the total abundance, 1/8 of each sample was removed with a plankton splitter (Wildco) for more detailed examination of copepod species composition.

At each sampling station we measured the surface water salinity with a hand refractometer, and the surface water temperature with a submersible digital thermometer. Water clarity was measured with a Secchi disk. Fifty ml aliquots of the surface water were filtered onto GF/F filters for chlorophyll measurements. The filters were kept in centrifuge tubes and transported back to the laboratory on ice. In the laboratory chlorophyll was extracted with 90% acetone in the dark overnight, and measured with a TurnerDesign TD-700 fluorometer (Parsons et al., 1984).

2.5. Laboratory study of decomposition of zooplankton carcasses

In the laboratory we studied the decomposition of zooplankton carcasses by means of dark-field microscopy. To produce fresh carcasses, live zooplankton collected from the Hampton River were sorted into a glass bowl placed inside an air-tight container with an AnaeroPack (Mitsubishi), which generated an anaerobic environment inside the container to kill the zooplankton. After 4 h batches of dead but intact zooplankton were sorted by groups and transferred to 5 μm-filtered natural seawater in petri dishes. We tested three groups of zooplankton: rotifers (*Synchaeta* sp.), barnacle nauplii, and copepods (*Acartia tonsa*). Dead rotifers and barnacle nauplii were incubated at 20 °C in the dark. Dead copepods were further divided into three batches and incubated at 10, 20 or 30 °C in the dark. At times 5 individuals were randomly transferred from each petri dish onto a glass slide and observed using dark-field microscopy (100–200×, Nikon Eclipse-80i). Images of the carcasses were recorded by a digital camera (Coolsnap ES with ImagePro software). To better illustrate the stage of decomposition we assigned a scale of 0- to 3 based on the coverage of the body cavity by bacteria: 0 indicates no visible bacteria; 1 = up to 30%; 2 = 30–70% and 3 = > 70%.

An additional experiment was conducted to investigate the role of different microbes in the decomposition process. Freshly killed copepods (*Acartia tonsa*) were incubated in 5 μm-filtered seawater, with or without added antibiotics (10 ml l^{-1} penicillin-streptomycin mixture; Sigma Chemicals). The carcasses were incubated at 20 °C in the dark. At times individuals were randomly selected for observation using dark-field microscopy as described before.

3. Results

3.1. Test of the Neutral Red staining method

Dead copepods recovered from natural samples were not stained whereas live copepods were stained red (Fig. 2). When kept refrigerated, preserved samples retained the stain for about 1 week, after which the color began to fade. Other live zooplankton groups, such as barnacle nauplii and polychaetes, were not stained at all (see Section 4). In the test of killing methods, a significantly higher percentage of the copepods were stained in the control group than in the treatment groups, but there was no significant difference among the three

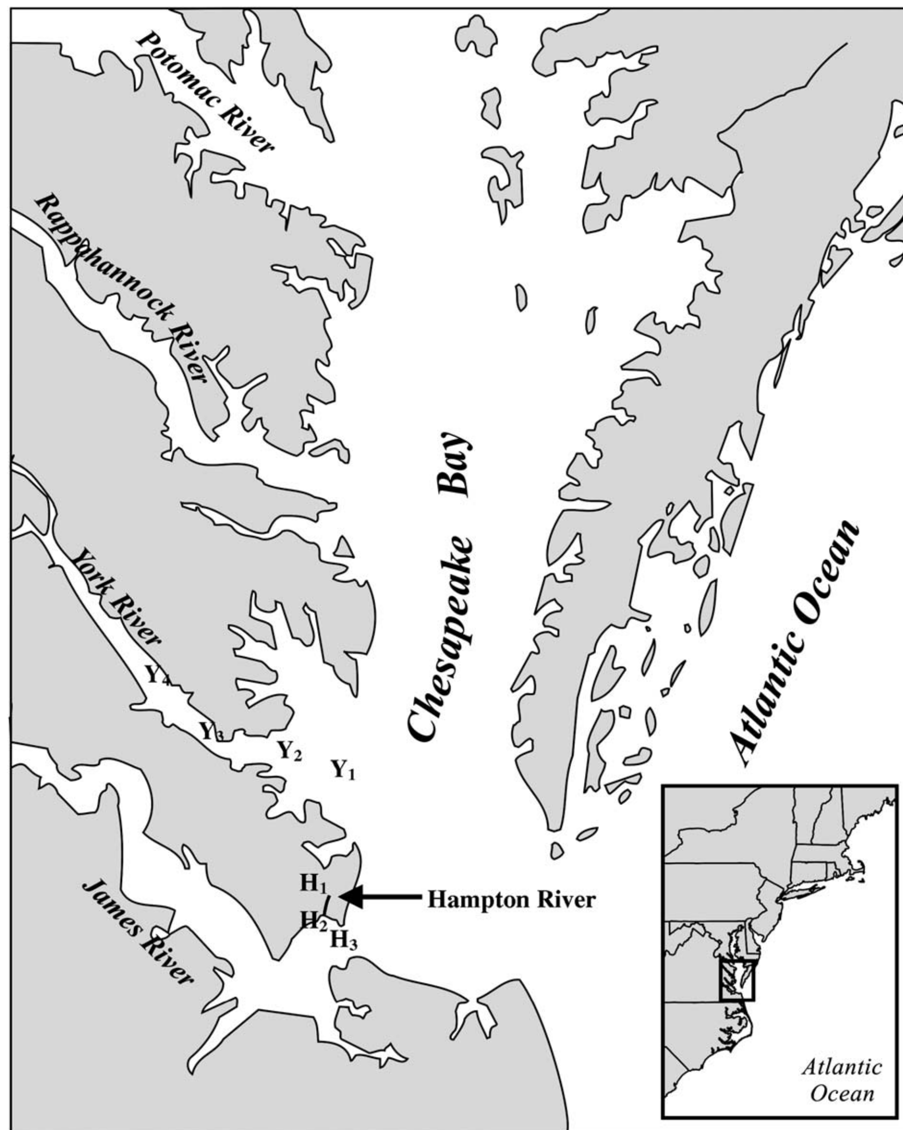

Fig. 1. Sampling stations in the lower Chesapeake Bay for this study.

treatments (Table 1). Duration of death and staining also did not result in significant differences in the percent copepod stained (Table 2). Overall an average of 4% of the killed copepods were stained red. Thus, the formula $0.96U + 0.04$ can be used to correct for the uptake of stain by dead copepods where U is the fraction of unstained copepods in the samples. In the test of plankton net tow duration, the percent copepods identified as dead (not stained) did not increase significantly with tow duration ($r = 0.45$, $P = 0.26$) (Fig. 3).

3.2. Live/dead copepods in the lower Chesapeake Bay

The environmental conditions of the sampling stations are summarized in Table 3. We excluded gelatinous zooplankton from the zooplankton samples to avoid interference of their mucous with the staining and counting. In both tributaries copepods accounted for over 90% of the zooplankton in most stations (Tables 4 and 5), of which 70–100% (average 93%) were *Acartia tonsa*. The other common copepods were *Eurytemora affinis*, *Paracalanus* sp., *Centropages* sp., cyclopoids and harpacticoids. Other numerically important zooplankton included barnacle nauplii, polychaetes and decapods.

On one occasion our boat drifted across a front in the Hampton River mouth during a horizontal net tow; an unusually high abundance of copepods were caught (>10,000) and as much as 78% of them appeared unstained. This sample taken in the front was excluded in subsequent data analysis but will be addressed in the discussion section. In the York River, 21–33% of the copepods were dead based on Neutral Red staining (Table 6). In the Hampton River, 13–37% of the copepods were dead (Table 6). Neither space nor time had a significant influence on the percentage of dead copepods (2-way ANOVA after arc-sine transformation, $P > 0.05$). Duplicate tows in the Hampton River were compared using pair *t*-test after arc-sine transformation: there was no significant difference between the first and the second tows

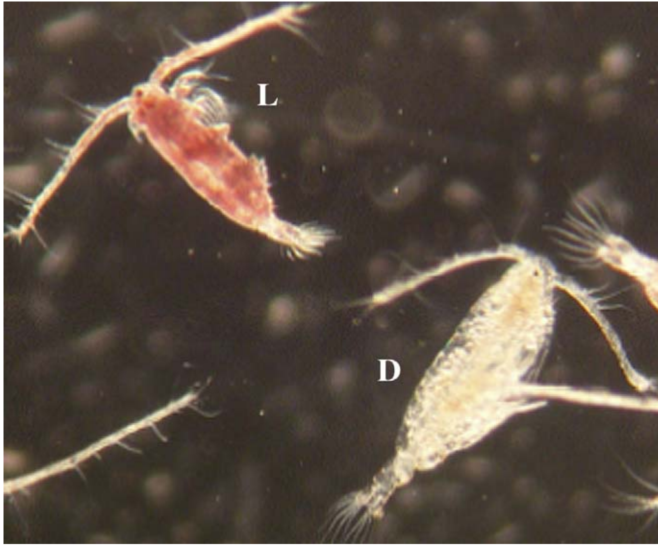

Fig. 2. Appearance of Neutral Red-treated copepods (*Acartia tonsa*) under a stereomicroscope with dark-field (Nikon SMZ1000). Copepods that were alive at the time of staining appeared red (L); copepods that were dead at the time of staining appeared unstained (D). Picture was taken with a Nikon Coolpix 4300 digital camera.

($P = 0.434$), indicating that carryover of dead copepods between tows was not significant. At the deep station in the Hampton River (H-3) we compared the percent dead copepods collected by horizontal tows and vertical tows, and no significant difference was found (Table 7). Disregarding the sample taken in the front, on average 29% of the copepods were dead in this study, and to correct for the uptake of Neutral Red by dead copepods, the adjusted average would be 32%. The percent dead copepods did not correlate with any of the individual environmental parameters (linear regressions, $P > 0.05$).

3.3. Decomposition of zooplankton carcasses

For each zooplankton group tested, the decomposition pattern was similar among the replicate individuals observed at each time point. Freshly killed rotifers and barnacle nauplii appeared mostly transparent with clear and intact internal tissues under dark-field. Bacteria became visible after 5 h inside the dead rotifers, and in some individuals the internal tissues appeared to have lost integrity. By 18 h, dead rotifer bodies began to collapse and dense internal bacterial mass appeared. At the end of 2 d, the bodies of dead rotifers were hardly recognizable. Dead barnacle nauplii were sampled after 15 h, and bacteria were visible inside the bodies. By 39 h, the dead barnacle nauplii were infested by dense swarms of bacteria and appeared opaque, yet the carapace remained largely intact.

Freshly killed copepods had transparent bodies and clearly visible internal tissues. As decomposition progressed at 30 °C the carcasses turned opaque due to dense bacterial mass after 6 h (Fig. 4). Bacterial mass was first concentrated in and around the gut, then spread to the entire body cavity. In the mid-stage of decomposition (12–30 h) the internal tissues and the carapace began to lose integrity, and dense bacterial mass could be seen spouting from fractures of the carcass (Fig. 4). In the late stage of decomposition (>42 h) the carcass was reduced to a largely empty carapace or carapace fragments with little internal tissues and bacteria (Fig. 4). The decomposition process was similar but at a lower pace at 20 °C, and the carcasses did not reach the late stage of decomposition until 78 h (Fig. 5). At 10 °C the decomposition

Table 1
Percent copepods (*Acartia tonsa*) stained by Neutral Red under different killing methods and staining duration. Field-collected copepods were killed by ethanol, formaldehyde or CO_2; untreated samples were used as control. Samples were stained with Neutral Red for 5, 15 and 20 min. Numbers of copepods counted are given in parentheses. The percent copepods stained was significantly higher in the control, but not different among the other treatments or with staining duration (2-way ANOVA on arc-sine transformed data, $P > 0.05$)

	Staining duration		
	5 min	15 min	20 min
Treatment			
Control	74 (2379)	77 (2922)	71 (1260)
Ethanol	2 (458)	2 (489)	2 (531)
Formaldehyde	1 (529)	2 (453)	15 (597)
CO_2	2 (507)	3 (426)	4 (450)

Table 2
Percent copepods (*Acartia tonsa*) stained by Neutral Red after different death duration and staining duration. Field samples of copepods were killed by formaldehyde and stained with Neutral Red for 5, 15 and 20 min after they had been dead for 1, 5 and 15 min. Numbers of copepods counted are given in parentheses. The percent copepods stained was not different among treatments (2-way ANOVA on arc-sine transformed data, $P > 0.05$)

	Staining duration		
	5 min	15 min	20 min
Death duration			
1 min	7 (127)	6 (137)	7 (241)
5 min	5 (326)	4 (364)	4 (297)
15 min	1 (529)	2 (453)	15 (597)

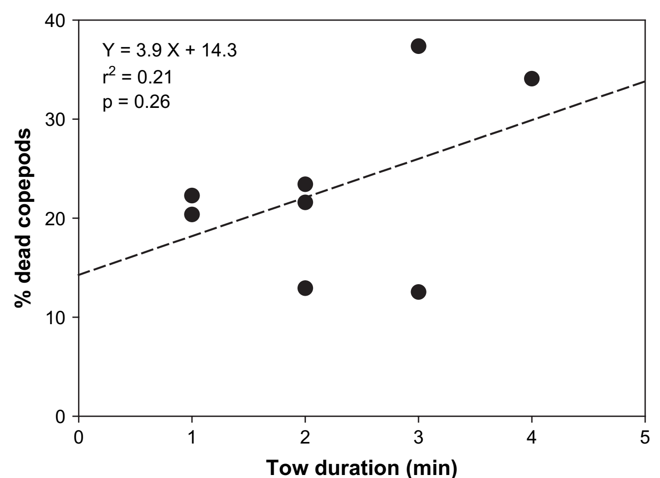

Fig. 3. Test of tow duration. Plankton net was towed just under the surface for 1, 2, 3 and 4 min, and the percent dead copepods was determined by the Neutral Red staining method. A linear regression function fitted to the data showed a positive, but not significant, trend.

Table 3
Water depths and environmental conditions during the study period

Date in 2005	Station	Water depth (m)	Water temp. (°C)	Salinity (psu)	Secchi depth (m)	Chl*a* (μg l^{-1})
York River						
15-June	Y-1	13.3	24.3	14	1.7	0.36
	Y-2	11.7	27.1	14	2.0	0.48
	Y-3	27.3	27.0	13	1.1	0.27
	Y-4	7.3	28.4	11	0.6	0.14
24-June	Y-1	13.3	20.6	17.5	0.8	no data
	Y-2	11.7	24.7	17	0.8	2.64
	Y-3	27.3	23.4	19	1.1	13.98
	Y-4	7.3	24.7	16	0.5	no data
12-July	Y-1	12.7	27.2	18	1.1	6.88
	Y-2	11.3	28.2	18	0.9	8.62
	Y-3	25.3	29.6	17	0.8	9.40
	Y-4	11.3	29.5	15	1.0	13.42
Average for York River			26.2	15.8	1.0	5.62
Hampton River						
5-July	H-1	2.2	30.1	15	0.2	19.65
	H-2	2.2	29.8	19	0.3	5.05
	H-3	10.3	27.2	18	1.3	10.25
25-July	H-1	2.2	33.4	8	0.3	57.66
	H-2	2.2	33.0	16.5	0.6	32.59
	H-3	10.3	27.2	20	1.4	20.04
Average for Hampton River			30.1	16.1	0.7	24.2

was even slower, and epiphytes became visible on the external body surface after 54 h and continued to increase (Fig. 5). By comparison epiphytes were not prominent at 20 and 30 °C.

In an experiment to test the effects of antibiotics, copepod bodies in the control (no antibiotics) decomposed in a similar fashion as described previously and the decomposition was almost complete after 91 h. In contrast, the decomposition process was different and much slower in the antibiotic treatment: The carcasses showed no sign of decomposition after 67 h. By 114 h, the carcasses were colonized by protozoans and internal tissues began to disappear (Fig. 6). By the end of the experiment (163 h) most of the internal tissues had disappeared and a few protozoans remained; some carcasses also had epiphytes on the external surfaces.

Table 4
Zooplankton abundance (ind m^{-3}) and composition in the York River

Station		15-June	24-June	12-July
Y-1	Copepods	86.92	1411.07	428.03
	Decapods	0	0	0
	Polychaetes	24.47	21.95	5.10
	Others	0	6.27	0
Y-2	Copepod	33.95	0	222.35
	Decapods	0	0	3.71
	Polychaetes	19.52	31.06	7.41
	Others	2.97	0	3.71
Y-3	Copepods	173.16	645.96	623.11
	Decapods	0	0	8.74
	Polychaetes	2.12	32.93	2.91
	Others	0	0	0
Y-4	Copepods	75.38	310.83	1471.59
	Decapods	0	10.19	12.23
	Polychaetes	4.84	7.64	36.69
	Others	0	0	0

Table 5
Zooplankton abundance (ind m^{-3}) and composition in the Hampton River

Station		5-July	25-July
H-1	Copepods	51.78	127.15
	Decapods	2.55	5.59
	Polychaetes	1.70	1.40
	Barnacle nauplii	215.60	92.92
	Others	2.55	2.10
H-2	Copepod	21.89	323.97
	Decapods	2.00	4.24
	Polychaetes	0	2.83
	Barnacle nauplii	98.36	74.98
	Others	0	0
H-3	Copepods	642.84	1622.96
	Decapods	24.90	40.74
	Polychaetes	6.79	13.58
	Barnacle nauplii	43.01	0
	Others	4.53	0

4. Discussion

4.1. Evaluation of the Neutral Red staining method

The Neutral Red staining method developed by Dressel et al. (1972) allows researchers to separate live and dead copepods, making it possible to study naturally occurring dead copepods in the marine environment. In previous studies different researchers used different staining time (from 5 min to 6 h) in the laboratory, but there was no mention whether the duration of staining affected the results (Crippen and Perrier, 1974). We tested the method under different working conditions to verify its applicability for field study. Major findings are: (1) Duration of staining (5–20 min) did not affect the results (Table 2); (2) Non-copepod zooplankton, such as barnacle nauplii and polychaetes, did not take up the stain in our tests. Crippen and Perrier (1974) used a lower stain concentration and reported a required staining time of 6 h for barnacle nauplii. Thus, the negative results for non-copepod zooplankton could have been due to the short staining time, and their live/dead status in our field samples could not be

Table 6
Percent dead copepods in the York River and the Hampton River as determined by the Neutral Red staining method

York River	15-June	24-June	12-July
Y-1	30.77	31.17	28.45
Y-2	38.89	27.01	21.24
Y-3	42.66	20.88	33.94
Y-4	36.24	41.30	53.57
Average for York River = 33.84			
Hampton River	**5-July**	**25-July**	
H-1	28.70	25.07	
H-2	31.07	30.52	
H-3	19.09	36.88	
Average for Hampton River = 28.56			

Table 7
Comparison of percent dead copepods collected by vertical tows vs. horizontal tows. No significant difference was detected (Mann–Whitney test; $P = 0.28$)

	Vertical	Horizontal
Mean	27.60	23.06
S.D.	4.67	8.86
N	4	8

evaluated; (3) For net tows of short duration at low speed, cod-end mortality was negligible (Fig. 3); and (4) Carryover of carcasses between tows could be minimized by flushing the net repeatedly between tows.

The Neutral Red method is based on the uptake of the vital stain by live copepods, and has an advantage over mortal stain methods that are based on the uptake of stains by dead zooplankton (e.g. Aniline Blue; Seepersad and Crippen, 1978). Upon death a zooplankton body begins to decompose and lose its internal tissues, which may interfere with its ability to take up stains. Thus, depending on the stage of decomposition a dead zooplankter may not be successfully stained.

4.2. Environmental conditions in the lower Chesapeake Bay

Southern Virginia had an unusually warm and dry summer in 2005. Air temperature averaged 27.3 °C and reached over 40 °C on certain days, whereas precipitation was only about 100 mm for the month of July. This weather condition led to relatively high surface water temperature and salinity. The average and maximum surface water temperatures were respectively 27.5 °C and 33.4 °C in our study area. The high water temperature may induce physiological stresses to the zooplankton community. On the other hand, the salinity gradient along the two rivers was not very strong due to limited freshwater input. Secchi depth and chlorophyll measurements indicated the presence of a very high phytoplankton biomass in the Hampton upriver stations, and the water appeared brownish. Eutrophication of the Chesapeake Bay has been well documented, and the unusually warm temperature may have further promoted rapid phytoplankton growth in the bay.

4.3. Zooplankton composition in the lower Chesapeake Bay

The zooplankton community in the study area was dominated by copepods in the more saline regions, whereas barnacle nauplii were more restricted to the less saline portions of the Hampton River. Among the copepods, the calanoid species *Acartia tonsa* was the most abundant, which is typical for the Chesapeake Bay during spring and summer. Not all the copepods caught were alive. There were no spatial or temporal

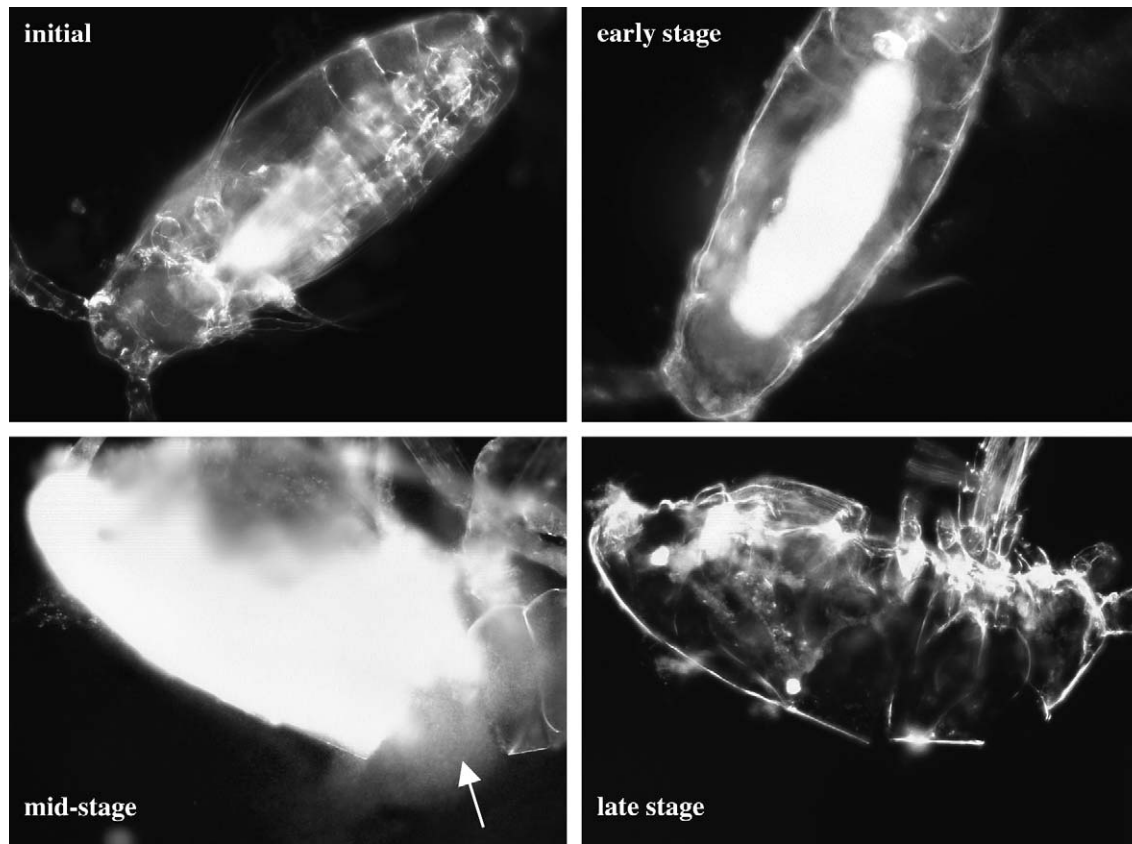

Fig. 4. Decomposition of copepod (*Acartia tonsa*) as observed by dark-field microscopy. The copepod body was transparent with intact internal tissues at the beginning. At the early stage of decomposition, the gut regions turned opaque by dense bacteria. At mid-stage bacteria filled the entire body cavity; carapace began to fracture and release bacteria from the inside (arrow). At late stage most of the internal tissues and bacteria had disappeared and mainly the carapace remained.

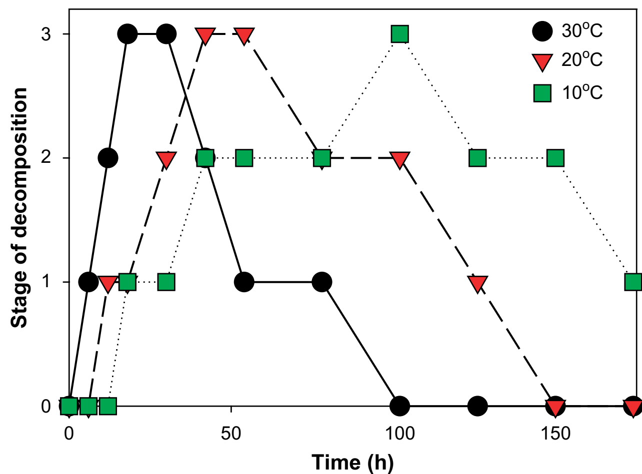

Fig. 5. Course of decomposition of copepod carcasses (*Acartia tonsa*) at different temperatures. The stage of decomposition is described by a scale of 0 to 3 based on the coverage of body cavity by bacteria: 0 = no visible bacteria; 1 = up to 30%; 2 = 30–70%; 3 = > 70%.

trends in percent dead copepods along the two rivers, and the live/dead composition of the copepod populations did not vary within the range of the environmental factors observed. Several researchers have reported that zooplankton carcasses accumulate in the deep water in open oceans (Wheeler, 1967; Terazaki and Wada, 1988), but in estuaries carcasses would be resuspended more readily by turbulence. In station H-3 where we compared horizontal tows and vertical tows, there was no significant difference in percent dead copepods recovered, suggesting that dead copepods were distributed rather homogenously throughout the water column.

A sample collected in a front in the lower James River (near H-3, 23-July) had a very high copepod abundance (∼11,000), of which 78% was unstained. Because the amount of Neutral Red added was based on sample volume and not total number of copepods, it is possible that the amount of stain added was not sufficient to stain all the live copepods in the sample, resulting in overestimation of dead copepods. However, another sample collected outside the front on the same day also had a high number of copepods (∼9000), yet the percent unstained was within the range, at 34%. This comparison leads us to believe that the high percentage of unstained copepods in the sample taken in the front is not an artifact, but reflects a real presence of an unusually high number of dead copepods within the front. Fronts in estuaries are known to retain high plankton abundances, and are believed to be favorable feeding grounds for larval fish (Roman et al., 2005). However, our results suggest that a high percentage of the copepods trapped within a front could be carcasses. Live copepods such as *Acartia tonsa* may be able to avoid a front by swimming downward whereas carcasses would be trapped within a front, leading to elevated proportion of dead copepods in the sample.

4.4. Decomposition of zooplankton carcasses

Zooplankton carcasses represent a rich organic carbon source for bacteria. In our laboratory experiments, bacteria colonized these carcasses and decomposed their internal tissues. Among the zooplankton carcasses tested, rotifers lacked a hard covering and the carcasses were reduced to unrecognizable remains within 2 d. In contrast, crustacean carcasses (barnacle nauplii and copepods) decomposed more slowly under the same environmental condition, and at the end of the decomposition the carapace was still recognizable. Microbes with chitinolytic enzymes are capable of breaking down crustacean carapace, but the process tends to be slow (Kirchner, 1995; Kirchman and White, 1999). Our experiments showed that most of the carcass' internal tissues were prone to microbial degradation, but the chitinous carapace was relatively resistant.

Zooplankton naturally carry bacteria, particularly on their body surfaces and inside their guts (Nagasawa and Nemoto, 1988; Carman and Dobbs, 1997; Tang, 2005). Our experiments did not determine whether the decomposition was initiated by internal bacteria or bacteria in the surrounding water. Harding (1973) found that sterilized copepod carcasses decomposed just as readily as untreated carcasses, and concluded that the decomposition was likely due to ambient bacteria instead of internal bacteria. A more valid test would be to

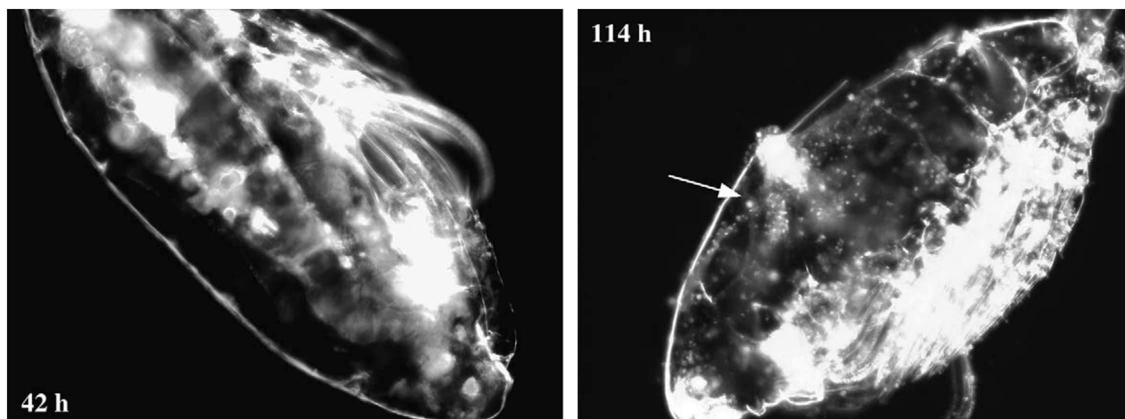

Fig. 6. Decomposition of copepod carcass (*Acartia tonsa*) in the presence of antibiotics. The carcass showed no sign of decomposition after 42 h. By 114 h large protozoans appeared inside the carcass (arrow) and most of the internal tissues had disappeared.

observe the decomposition of untreated carcasses in a sterile environment. It is, however, difficult to completely separate a zooplankton carcass from the ambient bacteria. One may use disinfectant to rid the carcass of ambient bacteria, but the disinfectant may alter the carcass surfaces or diffuse into the body cavity. While the contribution of ambient bacteria to decomposing zooplankton carcasses is certain, the possible role of internal bacteria remains to be studied.

Consistent with the results by Harding (1973), the rate of decomposition in our experiments was positively related to temperature. Besides motile bacteria, other microbes such as epiphytes and protozoans were also able to colonize and decompose the carcasses but at a slower pace.

4.5. Copepod mortality in the Chesapeake Bay and its ecological implications

By combining the field data and the laboratory results we estimated the in situ non-consumptive mortality of copepods in the lower Chesapeake Bay. The majority of the copepod populations were *Acartia tonsa*, a broadcast-spawning calanoid copepod species. Thirty-two percent (adjusted average) of the copepods were dead. In our field study the surface water temperature averaged 27.5 °C. Based on the laboratory experiments, at this temperature, a copepod carcass would be reduced to a nearly empty carapace in about 4 d. Assuming that the copepod population was in steady state, the in situ non-consumptive mortality rate can be estimated as $D/[4(1-D)]$, where D is the fraction of dead copepods in the samples. The so estimated mortality rate is $0.12\ d^{-1}$, within the reported mortality rate for broadcast-spawning copepods (Hirst and Kiørboe, 2002). Previous studies of copepod mortality tend to focus on predation, whereas our results suggest that non-predatory factors could be equally important in causing copepod mortality.

To estimate bacterial production supported by zooplankton carcasses, we use a body carbon content of $4.6\ \mu gC\ ind^{-1}$ for female *Acartia tonsa* (Tang et al., 1999 data for Long Island Sound in the summer). Assuming that 80% of the carcass carbon was lost to bacterial decomposition, the total bacterial carbon consumption during the 4 days' decomposition would be $0.92\ \mu gC\ d^{-1}$. If we further assume a carbon content of $150\ fgC\ cell^{-1}$ (Vrede et al., 2002 for exponentially growing cell) and a growth efficiency of 50% for the bacteria (del Giorgio and Cole, 1998 for eutrophic system), decomposition of an *A. tonsa* carcass would support a production of 3×10^6 cells d^{-1}. In situ bacterial abundance and specific growth rate in the lower Chesapeake Bay are $7 \times 10^9\ l^{-1}$ and $1.75\ d^{-1}$, respectively (Smith and Kemp, 2003). In our field study the zooplankton population abundance averaged $0.5\ ind\ l^{-1}$. If 32% of all the zooplankton were dead, and if all dead zooplankton followed the same course of decomposition as what we observed in the laboratory, the dead zooplankton population would contribute ~0.001% to the ambient bacterial production. Thus, the contribution of zooplankter carcasses to the water column bacterial production is negligible.

4.6. Neutral Red staining method as a monitoring tool

Zooplankton sample collection is a routine practice in many environmental monitoring programs in coastal and open oceans. However, without knowing the live/dead composition of the zooplankton community important information could be lost. In situ mortality is a critical, but often overlooked, parameter in the study of zooplankton population dynamics (Ohman and Wood, 1995). We found that the Neutral Red staining method can be used in field studies to quantify live vs. dead copepods, and with improvement, may apply to other zooplankton groups. The protocol is inexpensive and simple, and the subsequent sorting of live/dead individuals does not add much to the conventional counting process. Although we did not observe a significant relationship between percent dead copepods and the measured environmental factors, it should be noted that the environmental factors varied within a rather narrow range in our field study. We recommend this method as a tool for studying the responses of zooplankton communities to long-term environmental changes as well as episodic stresses such as pollution and storm events. The observation of an exceptionally high percent dead copepods associated with a front deserves further investigation. Other stains, such as Aniline Blue for dead zooplankton, may be combined with Neutral Red to further improve the method. Finally, the ecological importance of zooplankton carcasses, particularly their contribution to microbial processes in the water column, awaits further study.

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