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Simple staining method for differentiating live and dead marine zooplankton in field samples

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Abstract

We describe and evaluate a method for the use of neutral red staining to differentiate live and dead zooplankton in marine field samples. The protocol can be easily incorporated into shipboard zooplankton sampling. The use of neutral red in laboratory studies is common, but its application for quantifying natural live/dead zooplankton composition under field conditions has not been evaluated in detail. We tested the accuracy and precision of the method for a range of salinities and temperatures, and for common estuarine zooplankton groups. Detailed descriptions of staining intensities and patterns are provided. In addition, we evaluated potential artifact mortality due to collection and sample handling. The method produced accurate results under conditions tested, and artifact mortality was negligible using the recommended protocol. Neutral red staining is ideal for quantification of zooplankton carcasses in field samples, which will allow for more systematic study of in situ zooplankton mortality and related processes.

Introduction

A fundamental need in marine zooplankton ecological research is accurate assessment of population abundances. Traditionally researchers collect, preserve, and enumerate zooplankton field samples without evaluating the vital state of the animals. Estimates of animal abundance from field samples are then used to extrapolate per capita ecological rates to population rates (e.g., grazing, metabolism, growth, and reproduction). The underlying assumption in this common practice is that all animals in field samples are live and active; any deviation from such an assumption could result in erroneous understanding of many fundamental processes in the pelagic ecosystem. A number of studies have shown that zooplankton carcasses are prevalent at times in the marine environment (Table 1). Carcasses with visible wounds could be results of partial predation (Genin et al. 1995, Haury et al. 1995), whereas carcasses showing signs of internal decomposition but otherwise intact may represent mortality from different causes, such as parasitism, harmful algal blooms, starvation, and environmental stress (Byron et al. 1984, Hall et al. 1995, Gomez-Gutierrez et al. 2003, Sopanen et al. 2007). Without carefully identifying zooplankton carcasses in samples, researchers may grossly overestimate the abundances of live individuals, especially in cases when a large percentage of the animals are dead in situ.

Inspection of preserved animals for visible signs of damage or decomposition can help to identify carcasses (e.g., Wheeler 1967, Weikert 1977). This method is time consuming and subjective, however, and does not easily distinguish recently dead animals from live animals. A simple method to quickly and reliably differentiate live and dead zooplankton in preserved field samples is therefore needed. Here we describe such a method using the vital stain neutral red. Dressel et al. (1972) first described the use of neutral red staining to differentiate live and dead marine copepods. The method is promising for determining live/dead status of zooplankton in field samples for a number of reasons. It provides a clear color distinction between live and dead animals, making it less subjective and less time consuming than inspecting for signs of injury or decomposition. It also allows for identification of recently dead individuals that may have no visible signs of decomposition. The method is inexpensive, the stain is nontoxic, and the protocol for staining is simple, making it easy to incorporate into routine field sampling.

As with any method, there are likely inherent limitations with the application of neutral red staining to zooplankton in field samples. Dressel et al. (1972) described neutral red staining of Acartia tonsa, Eurytemora affinis, and several other crustacean zooplankton species, but provided no information on...
Elliott and Tang Live/dead determination of zooplankton

duration of staining period, killing method (exposure to vari-
precision of staining results, testing for effects of carcass age,
variables on staining results. They measured the accuracy and
the first to examine the effects of different environmental
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none of these presented any information regarding the

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mortality associated with power plant runoff (Carpenter et al.
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samples, based on a thorough review of the literature. Of
untested effects on the staining results. This includes varia-
tions in temperature, salinity, zooplankton species composi-
tions and abundances, and interference from other particles
within samples (detritus and phytoplankton). There have
been only five studies using neutral red on zooplankton field
samples, based on a thorough review of the literature. Of
the accuracy of the method. Crippen and Perrier (1974)
described a modified protocol for staining additional zoo-
plankton groups, but also did not report a thorough evalua-
tion of the accuracy of the staining. Further, the protocol that
they proposed requires long staining time (1–6 h), making it
impractical for routine field application. Fleming and Cough-
lan (1978) further modified the method to increase sample
storage times, but they also reported no data on the assess-
ment of staining accuracy. Hence, there is still uncertainty
concerning the accuracy and precision of neutral red staining
for differentiating live and dead zooplankton. In addition,
the published protocols require either long staining times or
excessive manipulation of zooplankton and hazardous chem-
icals during staining and preservation, or both, making them
less than ideal for routine shipboard application.

Although neutral red staining has commonly been used to
determine the vital state of zooplankters in laboratory studies,
use of the method for determining live/dead composition of
zooplankton in field samples introduces many variables with
untested effects on the staining results. This includes varia-
tions in temperature, salinity, zooplankton species composi-
tions and abundances, and interference from other particles
within samples (detritus and phytoplankton). There have
been only five studies using neutral red on zooplankton field
samples, based on a thorough review of the literature. Of
these, two were for the specific purpose of assessing copepod
mortality associated with power plant runoff (Carpenter et al.
1974, Hoffmeyer et al. 2005), another two did not describe or
cite the staining protocol used (Vinogradov et al. 1997, 1998),
and none of these presented any information regarding the
accuracy or precision of their protocols. Tang et al. (2006) were
the first to examine the effects of different environmental
variables on staining results. They measured the accuracy and
precision of staining results, testing for effects of carcass age,
duration of staining period, killing method (exposure to vari-
os chemicals), and the possibility of artifact mortality due to
sample handling. They found no significant effects of any of
these factors on the accuracy of live/dead determinations for
copepodes of Acartia tonsa.

Despite their limitations, these published studies provide a
solid foundation for a broadly applicable neutral red staining
method for field use. However, more tests are required to
resolve the accuracy and precision of the method. It is neces-
sary to develop standardized guidelines for interpreting stain-
ing patterns and color intensities, as stain uptake can vary
among individuals (Fleming and Coughlan 1978), species, and
developmental stages (Omori and Ikeda 1984). Furthermore,
the effects on staining results of the variable conditions that
occur in the field are unknown. Finally, the question of arti-
fact collection and handling mortality in field samples
requires more study. The first published study to address the
possibility of artifact mortality was Tang et al. (2006). How-
ever, their conclusions were based on a small sample size
(eight replicate field zooplankton tows), and they did not con-
sider artifact mortality in the more fragile naupliar stages of
copepods. Limitations associated with neutral red staining of
zooplankton field samples can be resolved by testing the accu-
ricy and precision of the results of a single standardized pro-
tocol, and with regard to the factors mentioned above (vari-
able staining pattern/intensity, variable environmental
conditions, and artifact mortality). This will ensure the accu-
racy of in situ live and dead composition data obtained by
neutral red staining. Here we describe a standardized protocol
for collecting, staining, and analyzing zooplankton field sam-
ple s for in situ live and dead determinations of animals. The
protocol was tested across a broad range of environmental
variability, its applicability to a range of common estuarine
zooplankton groups was determined, and the issue of artifact
mortality in naupliar and advanced stages of copepods was
addressed in detail. The result is a simple and reliable protocol

Table 1. Literature reports of the percentage of marine zooplankton identified as dead in field samples.

<table>
<thead>
<tr>
<th>Source</th>
<th>Location</th>
<th>Carcass identification method</th>
<th>% dead</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wheeler 1967</td>
<td>Atlantic off American Coast (20°–38° N)</td>
<td>Visual discrimination</td>
<td>50%–70%</td>
</tr>
<tr>
<td>Weikert 1977</td>
<td>Atlantic off African Coast (10°–20° N)</td>
<td>Visual discrimination</td>
<td>16%–28%</td>
</tr>
<tr>
<td>Roe 1988</td>
<td>N. Atlantic (31.3° N; 25.4° W)</td>
<td>Visual discrimination</td>
<td>25%–50%</td>
</tr>
<tr>
<td>Terazaki and Wada 1988</td>
<td>Sea of Japan (38°–42° N; 132°–140° E)</td>
<td>Visual discrimination</td>
<td>16%–28%</td>
</tr>
<tr>
<td>Böttger-Schnack 1990</td>
<td>Red Sea (21.4° N; 38° E)</td>
<td>Visual discrimination</td>
<td>1%–50%</td>
</tr>
<tr>
<td>Geptner et al. 1990</td>
<td>S.W. Indian Ocean off African Coast</td>
<td>Visual discrimination</td>
<td>10%–90%</td>
</tr>
<tr>
<td>Böttger-Schnack 1995</td>
<td>Throughout Red Sea</td>
<td>Visual discrimination</td>
<td>&lt;10%–29%</td>
</tr>
<tr>
<td>Genin et al. 1995</td>
<td>Gulf of Elat (ca. 29.5° N; 35° E)</td>
<td>Visual discrimination</td>
<td>10%–60%</td>
</tr>
<tr>
<td>Haury et al. 1995</td>
<td>Pacific near California Bight (30°–33° N)</td>
<td>Visual discrimination</td>
<td>10%–60%</td>
</tr>
<tr>
<td>Böttger-Schnack 1996</td>
<td>Arabian Sea</td>
<td>Visual discrimination</td>
<td>5%–70%</td>
</tr>
<tr>
<td>Yamaguchi and Ikeda 2001</td>
<td>North Pacific (42° N; 145.5° E)</td>
<td>Visual discrimination</td>
<td>0%–75%</td>
</tr>
<tr>
<td>Yamaguchi et al. 2002</td>
<td>Across North Pacific (40°–50° N)</td>
<td>Visual discrimination</td>
<td>10%–90%</td>
</tr>
<tr>
<td>Yahel et al. 2005</td>
<td>Gulf of Aqaba (29° N; 34.5°-35° E)</td>
<td>Visual discrimination</td>
<td>10% to 20%</td>
</tr>
<tr>
<td>Tang et al. 2006</td>
<td>Lower Chesapeake Bay and tributaries</td>
<td>Neutral red staining</td>
<td>13%–37%</td>
</tr>
</tbody>
</table>

Visual discrimination was based on microscopic inspection of individual animals for signs of tissue decomposition or injuries.
for determining the vital status of common zooplankton groups in estuarine field samples.

**Materials and procedures**

**Preparation of neutral red stock solution**—Stock solution is prepared by adding 0.1 g neutral red powder (Neutral Red high purity biological stain; Acros Organics) to every 10 mL deionized water and slowly stirring the solution under dim light overnight to completely dissolve the powder. After preparation, the stock solution can be stored in the dark at room temperature in a sealed amber borosilicate glass vial. The exact shelf life of the stock solution was not tested, but we obtained good staining performance using a single stock for a month. It is therefore recommended that the stock solution be replaced monthly or after less than ideal storage conditions (e.g., excessive heat or light exposure).

**Collecting zooplankton**—The protocol for collection and staining of field zooplankton samples, and subsequent live/dead sorting, is outlined in Fig. 1. Sampling is done using a plankton net, and samples are stained before preservation. We used standard conical plankton nets in this study. The net should be towed at a slow speed (≤1 m s⁻¹) to avoid damaging the animals. Before every tow, the net should be rinsed out thoroughly to minimize accidental carryover of dead animals from earlier tows. Tow duration should be kept as short as possible while still collecting an adequate sample size. The concentration of animals can affect the staining process, and samples containing too many animals will result in live individuals being only weakly stained. We obtained good staining results when zooplankton concentration was <75,000 individuals L⁻¹ in the cod-end sample. Assuming a net with 0.5-m mouth diameter and 100-mL final cod-end volume, the recommended maximum tow distance can be calculated as follows:

\[ \text{Tow distance (m)} = \frac{38,200}{\text{in situ zooplankton concentration (individuals m}^{-3})} \]  

This is also equivalent to the tow duration (in seconds) at the recommended maximum tow speed (1 m s⁻¹). Upon net retrieval after each tow, the cod end contents should be carefully transferred into a staining jar (e.g., polyethylene or glass screwcap bottle) and neutral red stain added as described below. The net should not be hosed down before this transfer, as this may kill the animals and inflate the numbers of dead zooplankton.

**Staining and sample storage**—Once a sample has been transferred to the staining jar, neutral red stock solution is added at a volume of 1.5 mL per 1000 mL sample. For samples with an exceptionally high number of animals (or in samples with high concentrations of phytoplankton or detritus), additional neutral red stock may be added to increase stain uptake without causing harm to the animals. As a rough guideline, the water should appear bright red and not pink (too little stain) or brown (too much stain). After stain addition, samples are

**Collection:**

- Thoroughly rinse collection gear to reduce carryover of dead animals
- Collect plankton sample (<1 m s⁻¹ tow speed & minimal tow duration)
- Carefully concentrate tow contents and transfer to staining jar

**Staining and preservation:**

- Add 1.5 mL neutral red stock (10 g L⁻¹ conc.) per 1 L concentrated sample (final concentration approximately 1:67,000)
- Stain for 15 min at in situ temperature (water bath/dark)
- Concentrate stained sample onto nylon mesh
- Rinse briefly with filtered seawater (in situ T & Š) to remove excess stain
- Preserve and store sample immediately (-20°C, <2 months)

**Analysis:**

- Thaw frozen mesh in cold filtered seawater (ca. in situ salinity)
- Acidify sample to pH<7 (1:10 final concentration 1M HCL works well)
- Count sample under dark field microscopy (patchy or uniform red stained tissue = live; pink/unstained=dead; red overhead light facilitates contrast in copepod nauplii and other small individuals)

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**Fig. 1.** Flow diagram of the recommended protocol of the neutral red method for collection, staining, and live/dead sorting of zooplankton samples.
incubated for 15 min at in situ temperature (a dark ambient water bath works well). Afterward, samples are concentrated onto fine nylon mesh disks and rinsed briefly with filtered seawater (near in situ temperature and salinity) to remove excess stain. The mesh disks are then placed flat and sample side up in Petri dishes and stored on ice in the dark. Upon return to the laboratory, samples should be stored at –20°C in the dark until use.

In an earlier test of the effect of preservation and storage on staining results, we took subsamples from a single copepod population using a Folsom Plankton Splitter. Triplicate subsamples were stained and counted immediately; additional triplicate subsamples were preserved by freezing at –20°C or using 3.7% unbuffered formaldehyde followed by refrigeration at 4°C. Preserved samples were counted after 36 days of storage. There was no significant effect of preservation method on the staining results (Table 2). However, preservation with formaldehyde resulted in lower and more variable percentages of stained copepods compared with samples that were counted immediately or preserved by freezing. We also observed that frozen samples retained the stain for more than 2 months when stored properly; after 3 months, samples began to degrade, making counting difficult. We therefore recommend that stained samples be stored at –20°C in the dark and be processed within 2 months of collection.

**Microscopic analysis of stained samples**—Frozen samples can be thawed by resuspension in filtered seawater. Samples are then acidified to pH <7 to develop the stain’s color inside the animals. Acidification can be done using any acidic solution, and the addition of 1 mL of 1 M HCl per 10 mL sample works well in our experience. Samples are then viewed with a dissecting microscope. We used a Nikon SMZ1000 stereomicroscope with C_DSD diascopic stand. Microscopy lighting is an important factor, and excessive lighting may cause stained animals to appear pale and unstained animals to appear pink. For adult copepods, dark field lighting should be used. This same lighting in combination with a red overhead light aids stain visibility for copepod nauplii and small copepodites. Animals alive at the time of staining are stained bright red in part or all of their tissues (mainly prosome tissue for copepods); animals dead before the staining will appear unstained, cloudy white, or light pink. Color will begin to fade in 1 h after resuspension and acidification; this is particularly problematic for smaller animals such as copepod nauplii. The use of cold seawater for thawing and resuspension reduces this problem compared to using water at room temperature.

**Assessment**

Although a protocol for neutral red staining of zooplankton was described as early as Dressel et al. (1972), questions still remain as to the potential limitations of the method. Our goal was to describe and test a standardized protocol for staining and live/dead sorting that can be applied easily to field samples. For the method to be incorporated as a regular part of zooplankton field sampling, it is important to assess the accuracy and precision of staining results when applied to different zooplankton groups and across a range of environmental conditions. In coastal and estuarine environments, both salinity and temperature can vary greatly over space and time, and may influence the staining process. Temperature directly influences cellular activity, and could therefore influence stain uptake. We also observed that neutral red did not work well for freshwater zooplankton samples (see also Bickel et al. 2009). One possible explanation for this is the effect of pH. Neutral red forms a hydrophilic cation in acidic solution, but a lipophilic anion under alkaline conditions (Horobin and Kiernan 2002). Cells might take up more readily the lipophilic form that is present in slightly alkaline solutions such as seawater. Finally, for application in field studies, it is important that the staining results are representative of the natural live/dead compositions of the zooplankton. If artifact mortality occurs due to collection or handling of the samples, it must be quantified and live/dead composition data corrected accordingly.

**General staining patterns**—To test if the method would present false results (false-positive or false-negative staining), a large number (>600) of live and active *Acartia tonsa* copepodites were collected. Of these, some were killed by immersion in 0.2 µm filtered seawater at 50°C for 5 min. Several other killing methods were also used, including the use of dilute HCl, NaN₃, and freezing. The heat method was verified on multiple occasions to quickly and effectively kill the zooplankton, whereas survival of some individuals was commonplace after freezing and sodium azide and acid exposure. One problem does occur when using heat to kill the zooplankton: Animals killed by heat will initially stain quite brightly, as described by Crippen and Perrier (1974). This is perhaps due to residual cellular and enzymatic activities. For this reason, heat-killed animals were held in water at 20°C for 5 min before staining, after which no visible stain uptake was observed. Assemblages of live animals and heat-killed animals were treated with neutral red separately. The results were unequivocal: 100% of the live and active animals were stained bright red, and 100% of heat-killed individuals appeared unstained (Fig. 2A). Live individuals were stained throughout part or all of their prosome, and often in the antennules and urosome as

<table>
<thead>
<tr>
<th>Treatment</th>
<th>n</th>
<th>Mean % stained (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Immediate count</td>
<td>3</td>
<td>74.0 (7.0)</td>
</tr>
<tr>
<td>Freezing (–20°C)</td>
<td>3</td>
<td>72.4 (11.9)</td>
</tr>
<tr>
<td>3.7% formaldehyde</td>
<td>3</td>
<td>61.9 (20.6)</td>
</tr>
</tbody>
</table>

Copepodites were counted immediately after staining or preserved by freezing or with formaldehyde for 36 days before counting. There was no significant difference between treatments (ANOVA of arcsine-transformed data; F_{2,6} = 3.92, P = 0.08).
Fig. 2. Appearance of neutral red–treated zooplankton under a stereomicroscope (Nikon SMZ1000) and recommended lighting. Shown are 100% dead and 100% live *Acartia tonsa* (A), patchily stained and pink individuals (B), and the live and dead individuals of various developmental stages and groups tested (C). Pictures were taken with a Nikon Coolpix 4300 digital camera.
well. Overall, a bright red concentration of stain in protosome tissues indicated a live individual, regardless of whether the entire prosome was stained or staining was patchy.

**General methodology**—To assess the performance of the neutral red method, laboratory tests were done with mixtures of known numbers of live and dead zooplankton. For each test, a large number (approximately 300 or more) of live and active animals were collected from a single station in the York River estuary, USA (37.24°N, 76.45°W). Of these, approximately half were killed by heat as described earlier. Following the preparation of dead zooplankton, known numbers of live and dead animals were mixed and treated with the neutral red method. For all the tests described in this study, samples were stained, preserved, and analyzed according to the protocol described in “Materials and procedures,” with storage time ranging from several hours to less than 2 days. Staining efficiency is defined as 100 – % E, where % E is the difference between the known percent live animals (expected) and the counted percent live animals (observed). A staining efficiency of 100% represents perfect staining. A value >100% indicates that a lower percentage of dead individuals were observed relative to the expected value (dead animals incorrectly stained or were preferentially lost). A value <100% indicates a higher percentage of dead individuals observed relative to the expected value (live animals failed to stain or were preferentially lost).

**Application to other zooplankton groups**—To date, neutral red has been applied mainly to calanoid copepods (Dressel et al. 1972, Tang et al. 2006), whereas other zooplankton groups reportedly require staining time longer than what is convenient for field applications (Crippen and Perrier 1974). As part of the method development, we assessed the applicability of our protocol to several common estuarine zooplankton groups: copepod nauplii, copepodites of *Acartia tonsa* (Calanoida) and *Oithonasp.* (Cyclopoida), barnacle nauplii, and planktonic polychaete larvae. For each zooplankton group, replicates of known numbers of live and dead individuals (approximately 100 or more individuals per replicate) were mixed, stained, and counted. The appearances and staining patterns of the groups tested are shown in Fig. 2B and C. The results show that the neutral red method worked for all tested groups (Table 3), with a mean staining efficiency of 99.1% (SD 1.5%). However, the usefulness of the method depends not only on the initial staining efficiency, but also on the ease of visibility of absorbed stain and retention of stain after uptake. Live polychaete larvae initially stained bright red, but the stain faded to barely visible levels in 10 min after thawing and acidification. Live barnacle nauplii also took up the stain, but the staining was confined to weak pink coloration at joints. Copepod nauplii and small copepodites both stained efficiently. Due to the small size of these individuals, the staining result was more difficult to see than for larger copepodites. Also, the stain faded noticeably as soon as 0.5 h after thawing and acidification. Large calanoid copepods stained the most clearly and retained the stain for a long time after thawing. In conclusion, the use of neutral red staining for some zooplankton groups requires special attention. Samples of polychaete larvae need to be analyzed quickly after thawing, and barnacle nauplii need to be inspected closely for stain uptake. Other groups, such as copepod nauplii and copepodites, can be confidently determined as live or dead with relative ease. For copepod nauplii and small copepodites, samples can be carefully counted on high magnification promptly after acidification, and with the aid of a red overhead light.

**Effects of environmental conditions**—To evaluate the effects of environmental conditions on staining efficiency, tests were conducted on *Acartia tonsa* (copepodite stage IV through adult) across a range of salinities and temperatures analogous to field conditions. *A. tonsa* is commonly found in meso- and polyhaline environments (salinity 5–30) and can tolerate salinities between 0 and 52 (Cervetto et al. 1999). The geographic range of this species is mainly restricted to the temperate zone (approximately 5–25°C), although it can tolerate temperatures between −1 and 32°C (Gonzalez 1974). Field-collected *A. tonsa* were acclimated to laboratory conditions similar to the in situ conditions when they were caught (10°C; salinity 20). Groups of animals were then transferred to water of the desired conditions, adjusting by 5°C or 5 salinity increments, and with 24-h acclimation periods between adjustments. Temperature treatments were set up by adjusting the incubation temperature and allowing the water to equilibrate naturally. Salinity was adjusted by adding deionized water or brine (made with Instant Ocean) to the containers until the desired salinity was achieved. DI water or brine solution was added very slowly and carefully with constant mixing to achieve uniform salinity within the containers. After 24 h of acclimation, live and active copepods were selected from each treatment (salinity 10–30 maintained at 10°C; temperature 5–30°C maintained at 20 salinity). Approximately half were killed by heat exposure, and known numbers of live and dead individuals were mixed, stained with neutral red, and counted in triplicate (approximately 100 or more individuals per replicate). There was no significant effect of salinity on staining efficiency (Table 4), with a mean staining efficiency of 100.2% (SD 1.5%). The effect of temperature on staining efficiency was significant (Table 4). Post hoc pairwise comparisons showed that staining efficiency was significantly lower for the lowest temperature (mean 98.5%) than for higher temperatures (99.6–100.8%). A temperature of 5°C is at the lower end of the temperature range that *A. tonsa* experiences in the Chesapeake Bay mainstem, and it is likely that cellular and enzymatic activities, hence stain uptake, are reduced at this low temperature. However, even at 5°C, the error of the staining method was less than 2%.

Additional tests were done to determine if salinity fluctuations might affect neutral red staining results. A “salinity shock” was administered to simulate the effect of passing the animals through a strong halocline, or rinsing them with water of salinity very different from the in situ salinity. For
this test, field-collected *A. tonsa* copepodites were acclimated to laboratory conditions as described above. Duplicate groups of live animals acclimated to a salinity of 20 were quickly transferred to 25 and stained immediately. Additionally, duplicate groups acclimated to a salinity of 15 were transferred to 10 and stained immediately. These treatments would not cause immediate death of the copepods, as *A. tonsa* has been observed to survive salinity shocks of similar or greater magnitudes, at least over short time periods (hours) (Cervetto et al. 1999). Neither an upward nor downward salinity shock resulted in strong bias in staining efficiency. We obtained good staining efficiency with the upward-shock treatment (mean 100.0%, SD 1.3%), which was not significantly different from 100% according to one-sample t-test (*t* = –0.03, d.f. = 1, *P* = 0.98). The downward-shock treatment yielded a somewhat lower staining efficiency (mean 97.5%, SD 2.5%), but this was not significantly different from 100% (*t* = 1.42, d.f. = 1, *P* = 0.39). A slightly lower staining efficiency in downward-salinity shock treatment is expected, as animals could be removing solutes from their cells to maintain osmotic equilibrium, which could work against the uptake of charged neutral red molecules.

**Artifact mortality in the field**—Another potential problem with neutral red staining of field zooplankton samples is artifact mortality associated with animal collection and handling. Zooplankton net tows likely impose high mechanical stresses on the captured animals. The turbulent and barrier-ridden environment inside the net, and the subsequent confinement to the cod end, may cause mortality to the animals. Handling of the samples prior to staining may also cause stress and mortality to the animals. Although steps should be taken to minimize these stresses, it is still possible that artifact mortality of zooplankton could occur during capture (cod-end mortality) and handling (handling mortality).

To quantify cod-end mortality, we conducted field tests that consisted of a series of successive net tows of different durations at a single location in the field. If cod-end mortality occurred, longer tow duration would result in an increase in the percentage of dead zooplankton. Two of these field experiments were done at a single location in the York River estuary, one for copepodites (with 200-µm mesh net) and one for copepod nauplii (with 63-µm mesh net). Linear least-squares regression was used to test for a relationship between tow duration and % dead copepods. In neither of these experiments was the slope of the regression line significantly different from 0 (Fig. 3). In addition, we compared tows with filtering cod-end and tows with nonfiltering cod-end. Nonfiltering cod-end is designed to reduce the stress experienced by the trapped animals. If cod-end mortality occurred, for two analogous tows, the one using a nonfiltering cod-end would have lower % dead copepods compared to the one using a filtering cod-end. The resulting means were 8.3% dead for filtering cod-end and 12.9% dead for nonfiltering cod-end. There was no significant difference between cod-end types in % dead copepods according to two-sample t-test (arcsine transformed data, *t* = –1.92, d.f. = 22, *P* = 0.07).

Additional laboratory experiments were conducted to test whether artifact mortality could occur during collection and

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**Table 3. Results of staining efficiency tests for the various zooplankton groups.**

<table>
<thead>
<tr>
<th>Group</th>
<th>n (added, recovered)</th>
<th>% dead expected</th>
<th>% dead observed</th>
<th>Staining efficiency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Copepod nauplii</td>
<td>(136,121)</td>
<td>61.8</td>
<td>62.8</td>
<td>99.0</td>
</tr>
<tr>
<td></td>
<td>(100,89)</td>
<td>81.0</td>
<td>82.0</td>
<td>99.0</td>
</tr>
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<td>(94,87)</td>
<td>43.6</td>
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<td>99.9</td>
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<tr>
<td><em>Acartia tonsa</em></td>
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<td>100.3</td>
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<td>46.0</td>
<td>97.2</td>
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<td>(173,173)</td>
<td>49.7</td>
<td>51.4</td>
<td>98.3</td>
</tr>
<tr>
<td></td>
<td>(159,159)</td>
<td>57.2</td>
<td>57.1</td>
<td>100.2</td>
</tr>
<tr>
<td></td>
<td>(173,173)</td>
<td>61.3</td>
<td>63.6</td>
<td>97.7</td>
</tr>
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<td></td>
<td>(213,213)</td>
<td>39.9</td>
<td>40.2</td>
<td>99.7</td>
</tr>
<tr>
<td><em>Oithona</em> sp.</td>
<td>(98,91)</td>
<td>76.5</td>
<td>78.0</td>
<td>98.5</td>
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<tr>
<td></td>
<td>(92,92)</td>
<td>46.7</td>
<td>44.1</td>
<td>102.7</td>
</tr>
<tr>
<td></td>
<td>(96,96)</td>
<td>65.6</td>
<td>64.6</td>
<td>101.0</td>
</tr>
<tr>
<td>Barnacle nauplii</td>
<td>(90,89)</td>
<td>50.0</td>
<td>49.4</td>
<td>100.6</td>
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<td></td>
<td>(96,96)</td>
<td>49.0</td>
<td>50.0</td>
<td>99.0</td>
</tr>
<tr>
<td></td>
<td>(96,95)</td>
<td>52.1</td>
<td>53.7</td>
<td>98.4</td>
</tr>
<tr>
<td></td>
<td>(262,255)</td>
<td>64.9</td>
<td>66.3</td>
<td>98.6</td>
</tr>
<tr>
<td></td>
<td>(362,358)</td>
<td>50.0</td>
<td>50.6</td>
<td>99.4</td>
</tr>
<tr>
<td></td>
<td>(173,171)</td>
<td>58.4</td>
<td>60.2</td>
<td>98.1</td>
</tr>
<tr>
<td>Polychaete larvae</td>
<td>(40,38)</td>
<td>22.5</td>
<td>26.3</td>
<td>96.2</td>
</tr>
</tbody>
</table>

*n*, number of animals added versus recovered in each replicate; % dead expected, % dead animals initially present in the sample; % dead observed, % dead animals determined by the neutral red method.
handling. Large numbers (100–460) of live and active *A. tonsa* copepodites and copepod nauplii were collected from the York River estuary and placed in ambient water. These animals were then siphoned through tubing (12 mm internal diameter) into submerged miniature reproductions of plankton nets (for copepodites, 200-µm mesh, 2.27-cm² mesh surface area, 0.9-cm mouth diameter, 5.5-mL cod-end volume; for nauplii, 63-µm mesh, 2.54-cm² mesh surface area, 0.5-cm mouth diameter, 3-mL cod-end volume). The siphoning lasted for 1.5–2 min at a flow rate of 1.25–2.5 L min⁻¹. Based on the surface area of the collection nets and the speed of the flow, these laboratory conditions were equivalent to towing a 0.5-m-diameter plankton net for the same duration (1.5–2 min) at a speed of ≥1 m s⁻¹. The final concentrations of animals in the collection containers were 2.3–8.4 × 10⁷ individuals m⁻³, comparable to the typical cod-end concentration of zooplankton in our field tows. After siphoning, the collected animals were diluted to ensure good staining efficiency and stained with neutral red. During the entire process the animals were transferred, sieved, and pipetted multiple times, with the total time and

Table 4. Results of staining efficiency across the range of salinities and temperatures tested for *Acartia tonsa* (copepodite stage IV through adult).

<table>
<thead>
<tr>
<th>Salinity treatments at 10°C</th>
<th>n (added, recovered)</th>
<th>% dead expected</th>
<th>% dead observed</th>
<th>Staining efficiency</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>(110,110)</td>
<td>52.7</td>
<td>53.6</td>
<td>99.1</td>
</tr>
<tr>
<td></td>
<td>(132,128)</td>
<td>55.3</td>
<td>56.3</td>
<td>99.1</td>
</tr>
<tr>
<td></td>
<td>(191,189)</td>
<td>71.2</td>
<td>67.7</td>
<td>103.5</td>
</tr>
<tr>
<td>15</td>
<td>(130,120)</td>
<td>73.8</td>
<td>73.3</td>
<td>100.5</td>
</tr>
<tr>
<td></td>
<td>(96.96)</td>
<td>68.8</td>
<td>68.8</td>
<td>100.0</td>
</tr>
<tr>
<td>20</td>
<td>(101,99)</td>
<td>48.5</td>
<td>50.5</td>
<td>98.0</td>
</tr>
<tr>
<td></td>
<td>(150,149)</td>
<td>66.0</td>
<td>65.8</td>
<td>100.2</td>
</tr>
<tr>
<td></td>
<td>(144,138)</td>
<td>72.2</td>
<td>71.7</td>
<td>100.5</td>
</tr>
<tr>
<td></td>
<td>(416,405)</td>
<td>26.9</td>
<td>25.9</td>
<td>101.0</td>
</tr>
<tr>
<td></td>
<td>(325,325)</td>
<td>32.9</td>
<td>32.6</td>
<td>100.3</td>
</tr>
<tr>
<td></td>
<td>(356,356)</td>
<td>21.6</td>
<td>20.5</td>
<td>101.1</td>
</tr>
<tr>
<td>25</td>
<td>(266,256)</td>
<td>75.2</td>
<td>75.8</td>
<td>99.4</td>
</tr>
<tr>
<td></td>
<td>(169,169)</td>
<td>59.8</td>
<td>62.1</td>
<td>97.6</td>
</tr>
<tr>
<td></td>
<td>(159,154)</td>
<td>49.7</td>
<td>51.3</td>
<td>98.4</td>
</tr>
<tr>
<td>30</td>
<td>(88,86)</td>
<td>47.7</td>
<td>47.7</td>
<td>100.1</td>
</tr>
<tr>
<td></td>
<td>(103,101)</td>
<td>52.4</td>
<td>52.5</td>
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<tr>
<td></td>
<td>(108,106)</td>
<td>70.4</td>
<td>71.7</td>
<td>98.7</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Temperature treatments at 20 salinity</th>
<th>n (added, recovered)</th>
<th>% dead expected</th>
<th>% dead observed</th>
<th>Staining efficiency</th>
</tr>
</thead>
<tbody>
<tr>
<td>5°C</td>
<td>(266,256)</td>
<td>75.2</td>
<td>75.8</td>
<td>99.4</td>
</tr>
<tr>
<td></td>
<td>(169,169)</td>
<td>59.8</td>
<td>62.1</td>
<td>97.6</td>
</tr>
<tr>
<td></td>
<td>(159,154)</td>
<td>49.7</td>
<td>51.3</td>
<td>98.4</td>
</tr>
<tr>
<td>10°C (same as 20 salinity above)</td>
<td>(110,110)</td>
<td>50.0</td>
<td>50.0</td>
<td>100.0</td>
</tr>
<tr>
<td></td>
<td>(110,110)</td>
<td>47.3</td>
<td>47.3</td>
<td>100.0</td>
</tr>
<tr>
<td></td>
<td>(114,114)</td>
<td>60.5</td>
<td>59.6</td>
<td>100.9</td>
</tr>
<tr>
<td>20°C</td>
<td>(94,94)</td>
<td>43.6</td>
<td>43.6</td>
<td>100.0</td>
</tr>
<tr>
<td></td>
<td>(148,148)</td>
<td>58.1</td>
<td>58.1</td>
<td>100.0</td>
</tr>
<tr>
<td></td>
<td>(138,138)</td>
<td>67.4</td>
<td>66.7</td>
<td>100.7</td>
</tr>
<tr>
<td>25°C</td>
<td>(133,133)</td>
<td>60.9</td>
<td>60.9</td>
<td>100.0</td>
</tr>
<tr>
<td></td>
<td>(104,104)</td>
<td>55.8</td>
<td>55.8</td>
<td>100.0</td>
</tr>
<tr>
<td></td>
<td>(154,154)</td>
<td>58.4</td>
<td>59.1</td>
<td>99.4</td>
</tr>
<tr>
<td>30°C</td>
<td>(137,137)</td>
<td>58.4</td>
<td>57.7</td>
<td>100.7</td>
</tr>
<tr>
<td></td>
<td>(134,133)</td>
<td>61.9</td>
<td>63.2</td>
<td>98.8</td>
</tr>
<tr>
<td></td>
<td>(180,180)</td>
<td>57.8</td>
<td>58.3</td>
<td>99.4</td>
</tr>
</tbody>
</table>

Column labels are the same as for Table 3. For the salinity treatments, there was no significant effect of salinity on staining efficiency according to ANOVA (F₄,₁₂ = 1.12, P = 0.41). For the temperature treatments, there was a significant effect of temperature on staining efficiency according to ANOVA (F₅,₁₅ = 4.51, P = 0.02), with a significantly lower staining efficiency in the 5°C treatment (according to post hoc pairwise Bonferroni comparisons).
amount of handling far exceeding what our protocol recommends. In all cases, less than 2% of the animals appeared unstained following this process (copepodites, \( n = 3 \), mean 1.6% dead, SD 0.5%; nauplii, \( n = 3 \), mean 1.2% dead, SD 0.6%). These results suggest that artifact mortality associated with collection and handling of copepods per our protocol is negligible. This is perhaps not surprising, given that neutral red stain is taken up intracellularly. Even copepods damaged during collection and handling should continue to take up stain until activity at the cellular level has ceased. Indeed, one important observation of the siphoning tests was the occurrence of several stained copepods with severe wounds. The small amount of tissue remaining inside the carapace was stained bright red, indicating that animals torn apart by the rough handling were still active enough at the cellular level to take up the stain. Conversely, the few animals that appeared unstained (dead) during the siphoning tests were completely intact, and may have represented either false-negative staining or an accidental carryover of dead animals between replicates. Regardless, artifact mortality associated with collection and handling of field samples can be avoided if our protocol is followed properly.

**Discussion**

The lack of information on the vital state of the animals represents a major oversight and limitation in traditional zooplankton sampling. Neutral red vital staining is a promising method that provides this missing information. With the neutral red method described in this article, researchers can now easily and reliably quantify live/dead zooplankton compositions in situ as part of routine field sampling. This information will improve estimates of live zooplankton population abundance, as well as estimates of ecological rates at the population level. Ability to quantify carcasses in field samples will also make it more feasible to study natural zooplankton mortality and its causes. In addition to improvement of future studies, insights into recurring patterns of carcass abundance and distribution within a system will allow for re-examination of past studies of the same system where live/dead zooplankton composition was not considered. Such a retrospective effort could change some of our long-held understandings in marine zooplankton ecology.

**Comments and recommendations**

In this study, the neutral red method was evaluated for its applicability under coastal and estuarine conditions and for common estuarine zooplankton groups. It is particularly well suited for copepods, the most abundant zooplankton in many marine environments (Humes 1994). However, application of the method to untested zooplankton taxa (including untested copepod species) should be attempted only after testing to ensure that the targeted species take up the stain and retain it for an adequate period during preservation and microscopic analysis. This is because stain uptake varies with different taxa (Omori and Ikeda 1984). For those taxa that begin to lose stain shortly after acidification (e.g., polychaete larvae), it is suggested that color photographs be taken for later detailed analysis. Use of the method in environments differing greatly from estuarine settings requires additional considerations. For example, long tow duration may be needed to collect sufficient sample in the open ocean, and the effects of long tow duration (>5 min) on artifact mortality need to be evaluated carefully. Also, rigorous washing of the net down into the cod end is a common practice in open ocean net tows, but should be avoided for samples intended for use in live/dead determination. Although this may influence the accuracy of abundance estimates in these samples, duplicate tows can be collected, one for live/dead determinations and another for abundance estimation. Another untested factor is the complications associated with bringing zooplankton up from great depths. Drastic changes in temperature and pressure may be experienced by zooplankton brought up from deep water, and the consequences on artifact mortality and staining efficiency are unknown. Finally, the neutral red method was found to significantly underestimate the number of live *Acartia tonsa* individuals near the lower temperature limit for this species. It is also possible that very high temperatures could inflate the
number of live individuals, as implied by the observed uptake of stain after heat killing of zooplankton. Overall, extreme temperatures appear to affect stain uptake, and this factor should be accounted for if the method is to be used in such environments.

References
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