

Use of aniline blue to distinguish live and dead crustacean zooplankton composition in freshwaters

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SUMMARY

1. Traditional zooplankton sampling methods do not account for the presence of zooplankton carcasses, which could lead to a biased understanding of the ecological role of zooplankton.
2. The aniline blue staining method for the differentiation between live and dead freshwater crustacean zooplankton was adapted for field application and the accuracy of the new protocol was tested in the laboratory. Detailed descriptions of the characteristic staining patterns of individuals are provided. The adapted staining method was subsequently applied in a field study to assess the live–dead zooplankton composition in the oligotrophic Lake Stechlin and the eutrophic Lake Dagow in Germany.
3. The modified aniline blue staining method accurately identified percentages of mixed live and dead crustacean zooplankton, making it suitable for quantifying live–dead compositions of copepods and cladocerans in freshwater samples.
4. On average 6% and 8% of the zooplankton were identified as dead in Lake Stechlin and Lake Dagow, respectively. Within each lake there were no significant spatial variations in the horizontal or vertical distribution of zooplankton carcasses. Nor did the percentage of dead zooplankton differ between day and night.
5. The aniline blue staining method provides an effective, simple and economical means for more systematic study of the occurrence and fate of crustacean zooplankton carcasses in freshwaters.

Keywords: aniline blue, cladoceran, copepod, staining method, zooplankton carcasses

Introduction

While predation is considered a major cause of zooplankton mortality, its importance in freshwater systems can fluctuate widely over a short period of time and it often cannot account for all zooplankton mortality (Boersma, van Tongeren & Mooij, 1996; Mehner *et al.*, 1998; Wagner *et al.*, 2004). A number of studies have indicated that non-consumptive mortality of zooplankton due to diseases, pollution, environmental stresses, parasites or harmful algal blooms

can be significant at times (Kimmerer & McKinnon, 1990; Hall *et al.*, 1995; Delgado & Alcaraz, 1999; Duffy *et al.*, 2005), and zooplankton carcasses can maintain a considerable presence within the water column in both marine and freshwater systems (Wheeler, 1967; Weikert, 1977; Terazaki & Wada, 1988; Gries & Güde, 1999; Dubovskaya *et al.*, 2003). Traditionally researchers collect, preserve and quantify zooplankton without distinguishing between live and dead individuals in the samples. Ignoring the presence of carcasses may lead on the one hand to an overestimation of the amount of zooplankton biomass available for grazing and predation, but on the other hand to an underestimation of the amount and quality of organic matter available for flux to the

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benthos or bacterial decomposition within the water column.

Some previous studies of zooplankton carcasses relied on visual examination of the individual animals for signs of internal tissue loss (e.g. Weikert, 1977; Terazaki & Wada, 1988). However, this method is time and labour intensive, and is not practical for handling a large number of samples. In addition, using tissue loss for the identification of carcasses may underestimate the presence of carcasses because tissue decomposition may not be obvious in recently deceased zooplankton (Tang, Freund & Schweitzer, 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 (S.L. Bickel, K.W. Tang and H.-P. Grossart, unpubl. data), using sediment traps may also underestimate carcass abundance if the carcasses are lost (e.g. ingested by fish) before they reach the traps.

An alternative method for assessing the live–dead composition of zooplankton populations is the use of a biological stain such as aniline blue. Seepersad & Crippen (1978) reported that immersion of freshwater zooplankton in a concentrated aniline blue solution (5 g 100 mL⁻¹; 0.068 M) resulted in dead individuals appearing blue, while live individuals remained unstained. This method has been used in toxicology studies (Seepersad, Ramnath & Dyal, 2004) and *in situ* assessment of freshwater zooplankton carcasses (Dubovskaya *et al.*, 2003; Gladyshev *et al.*, 2003). Unfortunately, these authors did not provide a detailed description of staining patterns of the carcasses of different zooplankton species. More importantly, they did not evaluate the accuracy of the method in differentiating between live and dead zooplankton in natural samples. Because no methods are perfect, it is important to evaluate the methods, report the inherent errors and discuss the potential limitations. Without this information, it is not possible to apply the staining method in field studies with sufficient confidence. There are also some basic logistical problems that make the original method of Seepersad & Crippen (1978) impractical for application in the field. Lake researchers often have to operate on small boats, yet the original protocol would require suspension of multiple zooplankton samples in a large staining bath, which

could be difficult to manage on a small vessel or with a large number of samples. While the aniline blue staining method is a promising way to identify live and dead zooplankton in freshwater environments, modifications are needed to make the method more suitable for field applications.

In this study, we describe a modified aniline blue staining protocol that is more applicable for field studies. We evaluate the accuracy of the new protocol in the laboratory for differentiating between live and dead zooplankton, provide detailed descriptions of the staining patterns of the specimens and discuss the potential limitations of the method. After validating the protocol, we applied it in a field study to assess the live–dead crustacean zooplankton composition in two German lakes of contrasting trophic conditions.

Methods

Laboratory testing of aniline blue staining method

Laboratory tests were conducted in June and July 2007 with zooplankton collected from Lake Matoaka, VA, U.S.A. (37°15'55'N, 76°43'22'W), and in August 2007 with zooplankton collected from Lake Dagow (53°09'01'N, 13°03'60'E) and Lake Stechlin (53°09'03'N, 13°01'40'E) in northeastern Germany. Crustacean zooplankton were collected via slow speed, short duration net tows, and were transported back to the laboratory in ambient water. In the laboratory, fresh carcasses of copepods and cladocerans were produced by briefly exposing live zooplankton to weak (<3%) hydrochloric acid. The carcasses were rinsed with 0.2- μ m filtered lake water to remove excess acid, and were then transferred to beakers filled with 0.2- μ m filtered lake water for testing. A 0.45 M stock solution of aniline blue was created by adding 10 g of aniline blue powder (Sigma-Aldrich, St Louis, MO, U.S.A.) to 30 mL deionized water; the mixture was stirred under dim light at low speed overnight until all the powder was dissolved. Attempts to create a more concentrated stock solution were unsuccessful, as some aniline blue powder remained undissolved. The stock solution was kept at room temperature in a borosilicate glass bottle, sealed with a polypropylene cap and was used within 3 days. We did not test the shelf-life of the stock solution; it is therefore recommended that fresh stock solution is always prepared before use. Instead of

suspending zooplankton samples in a concentrated aniline blue solution as described by Seepersad & Crippen (1978), aliquots of the stock solution were added to glass beakers containing the carcasses in known volumes of filtered lake water. To determine if staining results vary with stain concentration, we tested three final stain concentrations: 0.0214, 0.0131 and 0.0045 M, which were created by adding 5, 3, and 1 mL of the aniline blue stock solution, respectively, to every 100 mL of lake water. The highest concentration tested (0.0214 M) was lower than that used by Seepersad & Crippen (1978), but similar to that used by Seepersad *et al.* (2004). The carcasses were stained for 15 min at room temperature under ambient laboratory light conditions. The percentages of copepod and cladoceran carcasses stained at each concentration were assessed by dark field visual examination with a Nikon SMZ1000 dissecting microscope (Nikon, Tokyo, Japan) with illumination from below. All microscopic counts were performed with overhead laboratory lighting turned off. A carcass displaying bright blue colouration on any part of the body was considered stained. A one-way ANOVA was performed on arcsine transformed percentage data to assess differences in staining efficiencies among the different concentrations of aniline blue.

To evaluate the staining accuracy, experiments were conducted in the laboratory with only live or only dead zooplankton to test if the method accurately stains the specimens. To assess the suitability of the method for field applications where a mixture of live and dead zooplankton would be expected, additional laboratory experiments were conducted where known numbers of live and dead zooplankton were mixed and subjected to the staining. For these tests the zooplankton were divided into copepods and cladocerans, which are the two dominant broad zooplankton groups in most lakes and reservoirs. The copepod category included members of the orders Calanoida and Cyclopoida; the cladoceran category included members of the families Daphniidae, Bosminiidae and Sididae. In each test a known number of live individuals, freshly killed individuals, or a mixture of the two were added to a beaker containing 100 mL of 0.2 μ m-filtered lake water. A 5 mL aliquot of 0.45 M aniline blue stock solution was added to the beaker (final conc. 0.0214 M) and gently swirled to mix the stain evenly. This stain concentration was chosen based on the results of earlier tests (see Results). After staining

for 15 min at room temperature under ambient laboratory light conditions, each sample was gently collected on a 47 mm diameter, 200 μ m nylon mesh disc and rinsed well with deionized water to remove any excess stain. The disc was then transferred to a petri dish and kept frozen at -15°C until the sample was counted, typically within 24 h. Before counting, samples were thawed at room temperature and back rinsed into a plankton counting wheel with 0.2- μ m filtered lake water, then acidified with approximately 10 drops of 10% hydrochloric acid. Acidification helped develop and intensify the stain, facilitating the differentiation between stained and unstained individuals. During the counting process, each individual was classified as live or dead based on its characteristic staining pattern. A paired *t*-test was performed on the arcsine transformed percentage data to determine if the amount of dead zooplankton added was significantly different from the amount identified as dead by the staining method.

In situ application of aniline blue staining

Field sampling was conducted during August 2007 to determine the prevalence of zooplankton carcasses in Lake Dagow and Lake Stechlin, located in northeastern Germany. While the two lakes are very close to each other, and are connected via a small outflow, they exhibit very different nutrient statuses and bathymetries. Lake Stechlin is a deep (max. 69 m), oligotrophic lake with no reported hypoxia even in the hypolimnion. In contrast, Lake Dagow is a shallow (max. 12 m), highly eutrophic lake that experiences seasonal hypoxia (Gonsiorczyk, Casper & Koschel, 1998).

Day and night duplicate vertical net tows through the epilimnion and the whole water column were taken at five stations in Lake Stechlin and three stations in Lake Dagow. Station S2 in Lake Stechlin was very shallow and not stratified; consequently only 'epilimnion' samples were collected at this station. The two lakes were sampled three days apart; however, day and night samples from each lake were taken on the same day. A 58 cm mouth diameter, 90 μ m mesh net was deployed and retrieved by hand. The net and cod end were rinsed well between each net tow to minimize carryover of zooplankton between tows. Each tow was concentrated down to 100–200 mL volume and gently transferred to a glass

jar for immediate staining. Aliquots of 0.45 M aniline blue stock solution were added to the jars to attain a final stain concentration of 0.0214 M. Afterward the sample jars were capped and gently swirled to evenly mix the stain, then placed in an ambient water bath to prevent any post-collection death due to temperature fluctuations. After staining for 15 min, samples were collected onto 47 mm diameter 200 μm mesh discs and rinsed with 0.2- μm filtered lake water to remove any excess stain. The mesh discs were then transferred to petri dishes, and placed on ice until returned to the laboratory, at which point they were stored at $-20\text{ }^{\circ}\text{C}$ until further processing. In the laboratory each sample was thawed, back rinsed and acidified as described previously. If necessary, the sample was split with a Folsom plankton splitter (Wildco, Buffalo, NY, U.S.A.) before identification and enumeration. The crustacean zooplankton community was identified to the genus level whenever possible, and categorized as live or dead based on characteristic staining patterns. Estimates of hypolimnion species abundance and live–dead percentages were derived by a subtraction of epilimnion raw counts from whole water raw counts taken at the same station.

Immediately before the daytime zooplankton tows at each station, vertical profiles of pH were measured with a WTW pH197 probe, and dissolved oxygen and temperature were measured with WTW Oxi 197-S probe (BO & GA Medikal Kimya Elektronik Gida, Istanbul, Turkey).

Results

Characteristic staining patterns

A variety of staining patterns was observed among dead zooplankton in the initial trials (Fig. 1a–d). Dead calanoid and cyclopoid copepods showed two common staining patterns: (i) bright blue staining of the entire body, including antennae but not caudal setae and (ii) patchy, bright blue staining confined predominantly to the lateral portions of the cephalosome and urosome. There were a few instances when dead copepods did not take up the stain at all. Dead cladocerans also exhibited two predominant staining patterns: (i) all internal tissues and the second antennae were stained bright blue and (ii) patchy, incomplete stain, occurring when only portions of internal tissues took up the stain. For example, legs and the

surrounding tissues were typically stained bright blue while tissues in the head region did not take up the stain. There were also some instances when the freshly killed cladocerans did not take up the stain at all, or only the anal tip of the gut showed a bright blue colour.

Live copepods exhibited very different staining patterns (Fig. 1e,f). The majority of live copepods were unstained. However, some individuals of both calanoid and cyclopoid copepods showed bright blue staining of the entire gut or anterior portions of the gut, which would suggest ingestion of the stain. Also, a small patch of bright blue was noted near the spermatophore region of some live female copepods. Similar to copepods, live cladocerans were either unstained, or had their entire gut tract appear bright blue (Fig. 1g,h). We also observed that brood carrying females occasionally appeared unstained, while the embryos inside the brood chamber took up the stain and appeared either bright or pale blue. Seepersad & Crippen (1978) observed similar gut staining among live copepods and cladocerans as well as the staining of spermatophores in live female copepods, and consequently cautioned about misinterpreting these stained individuals as dead. In our initial tests, a visual examination of live copepods and cladocerans after the staining process but before freezing verified that the stained individuals were still alive and actively swimming. Although some live zooplankton were stained, their staining patterns were so different from that of dead zooplankton that they could be easily identified.

Laboratory staining tests

In the tests of the staining efficiency, there was a slight, but not significant, increase in staining efficiency for copepods with increasing concentration (one-way ANOVA of arcsine transformed percentage data, $F = 3.71$, $P = 0.089$) (Table 1). Likewise, increasing stain concentration did not significantly affect the staining efficiency for cladocerans (one-way ANOVA of arcsine transformed percentage data, $F = 0.61$, $P = 0.576$). Based on these results, a final concentration of 0.0214 M was used in all subsequent tests and field samplings. This concentration is similar to what has been used for toxicology applications (Seepersad *et al.*, 2004).

To assess the efficiency of the aniline blue staining method further, laboratory tests were conducted in

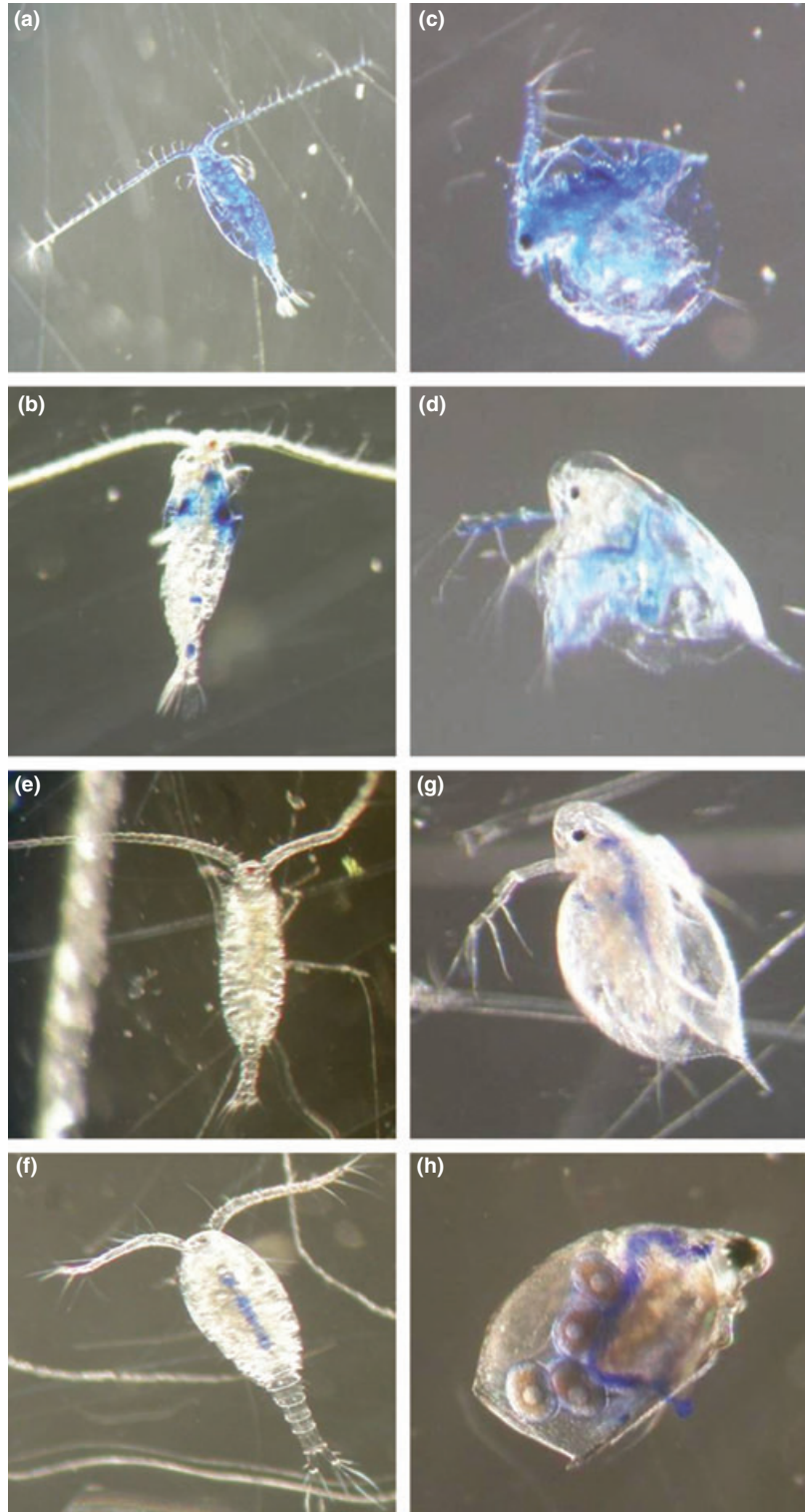


Fig. 1 Characteristic aniline blue staining patterns of dead (a–d) and live (e–h) freshwater zooplankton. Dead copepods (a and b) stained entirely bright blue or exhibited bright patchy staining; the majority of dead cladocerans (c and d) exhibited whole or patchy bright blue staining. Live copepods (e and f) appeared unstained or had portions of the gut appearing bright blue; live cladocerans (g and h) appeared unstained or showed a blue gut.

which only live, only dead, or a known mixture of live and dead zooplankton were subjected to the staining procedure. Among all laboratory trials, the number of

animals recovered after the staining was equal to or better than 75% of the original sample size. In the trials with only live individuals, $17.1 \pm 15.9\%$

Table 1 Percentage of dead zooplankton stained after a 15 min immersion in different concentrations of aniline blue

| Final stain concentration (mol L ⁻¹) | Copepod | Cladoceran |
|--|------------|------------|
| 0.0045 | 82.7 (133) | 68.6 (188) |
| 0.0045 | 89.8 (128) | 48.2 (112) |
| 0.0045 | 85.4 (164) | 39.9 (178) |
| 0.0131 | 94.2 (139) | 72.7 (165) |
| 0.0131 | 95.7 (93) | 53.8 (171) |
| 0.0131 | 84.6 (39) | 56.2 (169) |
| 0.0214 | 96.9 (96) | 67.1 (225) |
| 0.0214 | 100 (17) | 68.1 (248) |
| 0.0214 | 92.7 (151) | 50.9 (163) |

Numbers of individuals counted are given in parentheses. There is no significant difference in the percentage stained among the different stain concentrations for either copepods or cladocerans (one-way ANOVA of arcsine transformed percentage data, $P > 0.05$).

(mean \pm SD) of cladocerans ($n =$ number of trials = 3) and $0.9 \pm 0.7\%$ of copepods ($n = 6$) were incorrectly identified as dead based on the characteristic staining patterns described previously. Paired t -tests of the arcsine transformed percentage data indicated that the percentage of the population misidentified as dead by the staining method was not significant for cladocerans ($t = -3.08$, $P = 0.091$), but was significant for copepods ($t = -4.17$, $P = 0.009$).

The staining trials consisting of only dead individuals showed that $70.6 \pm 30.6\%$ (mean \pm SD) of cladocerans ($n =$ number of trials = 7) and $62.2 \pm 19.9\%$ of copepods ($n = 13$) showed characteristic dead staining patterns. A comparison of observations before and after staining indicated that aniline blue did not stain all carcasses of cladoceran or copepod (paired t -test of arcsine transformed per cent; cladoceran: $t = 3.46$, $P = 0.013$; copepod: $t = 10.18$, $P < 0.001$).

In the live–dead mixture tests, live and dead individuals of cladocerans or copepods were added in known proportions. After staining, the sample population was examined for the characteristic staining patterns to identify carcasses. The expected and observed percentages of dead individuals are presented in Table 2. For both cladocerans ($n = 3$) and copepods ($n = 14$), paired t -test of the arcsine transformed percentage data indicated that the percentage of individuals identified as dead by the staining was not significantly different from the actual percentage of dead individuals (cladoceran: $t = -3.48$, $P = 0.74$; copepod: $t = 1.38$, $P = 0.191$).

Table 2 Expected and observed percentages of dead zooplankton in laboratory tests where known proportions of live and dead individuals were mixed

| Taxa | Expected | Observed |
|------------------|----------|----------|
| Copepod (553) | 38.8 | 39.1 |
| Copepod (325) | 33.3 | 34.2 |
| Copepod (504) | 56.8 | 55.8 |
| Copepod (466) | 53.2 | 54.5 |
| Copepod (433) | 50.0 | 46.4 |
| Copepod (333) | 50.0 | 52.0 |
| Copepod (382) | 35.4 | 29.6 |
| Copepod (177) | 35.0 | 34.5 |
| Copepod (222) | 47.5 | 40.1 |
| Copepod (228) | 48.7 | 33.3 |
| Copepod (936) | 45.5 | 29.8 |
| Copepod (216) | 47.7 | 54.9 |
| Copepod (194) | 52.1 | 56.2 |
| Copepod (217) | 54.2 | 57.6 |
| Cladoceran (112) | 54.3 | 59.8 |
| Cladoceran (183) | 50.3 | 56.8 |
| Cladoceran (177) | 50.0 | 63.3 |

Dead zooplankton were identified based on characteristic staining patterns after being treated with aniline blue. Numbers of individuals counted are presented in parentheses. There is no significant difference between expected and observed percentages for either copepods or cladocerans (paired t -test, copepods: $t = 1.38$, $P = 0.191$; cladocerans: $t = -3.48$, $P = 0.74$).

In situ application of aniline blue staining

The zooplankton communities in both Lake Stechlin (Table 3) and Lake Dagow (Table 4) were dominated by cladocera (*Daphnia* sp. and *Bosmina* sp.), cyclopoid copepods [*Mesocyclops leuckarti* (Claus), *Thermocyclops oithonoides* (Sars)] and calanoid copepods [*Eudiaptomus gracilis* (Sars), *Eurytemora lacustris* (Poppe)]. The species abundances were quite different between lakes, however. In Lake Stechlin cladocerans comprised 50–86% (average 67%) of the zooplankton in all samples, of which 43–89% (average 72%) were *Daphnia* sp. and 1–30% (average 13%) were *Bosmina* sp. In contrast, in Lake Dagow copepods accounted for 52–96% (average 73%) of all zooplankton, of which 59–99% (average 81%) were cyclopoids and 0.3–41% (average 19%) were calanoids. The cladocerans *Ceriodaphnia* sp. and *Diaphanosoma brachyurum* (Lievin) were also found in both lakes.

The aforementioned aniline blue characteristic staining patterns were used to determine the live–dead composition of the zooplankton populations at the time of collection. Within each sample, only taxa with more than 100 individuals were used in live–

Table 3 Average abundance (individuals m⁻³) of the four most common zooplankton groups in Lake Stechlin

| | Epilimnion | Hypolimnion | Day | Night |
|--------------------|------------|-------------|-----|-------|
| Station S1 | | | | |
| <i>Daphnia</i> sp. | 971 | 92 | 486 | 1036 |
| <i>Bosmina</i> sp. | 49 | 22 | 27 | 119 |
| Cyclopoid copepods | 400 | 140 | 291 | 241 |
| Calanoid copepods | 152 | 69 | 118 | 115 |
| Station S2 | | | | |
| <i>Daphnia</i> sp. | 379 | ND | 379 | 484 |
| <i>Bosmina</i> sp. | 45 | ND | 45 | 79 |
| Cyclopoid copepods | 168 | ND | 168 | 128 |
| Calanoid copepods | 80 | ND | 80 | 58 |
| Station S3 | | | | |
| <i>Daphnia</i> sp. | 261 | 122 | 170 | 229 |
| <i>Bosmina</i> sp. | 114 | 24 | 54 | 44 |
| Cyclopoid copepods | 136 | 219 | 184 | 100 |
| Calanoid copepods | 45 | 28 | 35 | 42 |
| Station S4 | | | | |
| <i>Daphnia</i> sp. | 1000 | 206 | 473 | 479 |
| <i>Bosmina</i> sp. | 264 | 91 | 116 | 116 |
| Cyclopoid copepods | 394 | 188 | 266 | 283 |
| Calanoid copepods | 142 | 46 | 75 | 113 |
| Station S5 | | | | |
| <i>Daphnia</i> sp. | 967 | 33 | 236 | 264 |
| <i>Bosmina</i> sp. | 152 | 34 | 42 | 45 |
| Cyclopoid copepods | 412 | 74 | 202 | 204 |
| Calanoid copepods | 113 | 21 | 57 | 57 |

ND, no data.

Station S2 was a shallow, unstratified water column. There was no differentiation between epilimnion and whole water samples. Consequently hypolimnion abundances could not be calculated.

Table 4 Average abundance (individuals m⁻³) of the four most common zooplankton groups in Lake Dagow

| | Epilimnion | Hypolimnion | Day | Night |
|--------------------|------------|-------------|-----|-------|
| Station D1 | | | | |
| <i>Daphnia</i> sp. | 62 | 66 | 61 | 42 |
| <i>Bosmina</i> sp. | 653 | 986 | 463 | 218 |
| Cyclopoid copepods | 1141 | 1232 | 998 | 796 |
| Calanoid copepods | 199 | 1812 | 660 | 302 |
| Station D2 | | | | |
| <i>Daphnia</i> sp. | 339 | 1.4 | 105 | 14 |
| <i>Bosmina</i> sp. | 811 | 88 | 293 | 48 |
| Cyclopoid copepods | 1205 | 429 | 845 | 795 |
| Calanoid copepods | 591 | 3.6 | 139 | 123 |
| Station D3 | | | | |
| <i>Daphnia</i> sp. | 252 | 7.9 | 32 | 14 |
| <i>Bosmina</i> sp. | 469 | 239 | 312 | 153 |
| Cyclopoid copepods | 1595 | 103 | 888 | 1231 |
| Calanoid copepods | 254 | 119 | 173 | 59 |

dead analyses. All live–dead composition percentages were arcsine transformed before statistical analyses. The live–dead percentage of each major zooplankton

Table 5 Average percentages of dead zooplankton in Lake Stechlin as determined by the aniline blue staining method

| | Epilimnion | Hypolimnion | Day | Night |
|--------------------|------------|-------------|------|-------|
| Station S1 | | | | |
| <i>Daphnia</i> sp. | 3.9 | 36.3 | 4.7 | 8.7 |
| <i>Bosmina</i> sp. | 3.9 | NA | 12.1 | 10.1 |
| Cyclopoid copepods | 1.8 | 0.2 | 0.5 | 1.6 |
| Calanoid copepods | 0.7 | 0.0 | 0.4 | NA |
| Station S2 | | | | |
| <i>Daphnia</i> sp. | 23.2 | ND | 23.2 | 7.7 |
| <i>Bosmina</i> sp. | 3.7 | ND | 3.7 | 27.1 |
| Cyclopoid copepods | 0.5 | ND | 0.5 | 3.2 |
| Calanoid copepods | 0.2 | ND | 0.2 | 0.0 |
| Station S3 | | | | |
| <i>Daphnia</i> sp. | 6.9 | 0.0 | 1.5 | 3.7 |
| <i>Bosmina</i> sp. | 20.9 | 0.0 | 12.1 | 9.2 |
| Cyclopoid copepods | 4.6 | 0.6 | 0.8 | 1.4 |
| Calanoid copepods | 0.4 | 0.0 | 0.0 | 0.0 |
| Station S4 | | | | |
| <i>Daphnia</i> sp. | 5.7 | 35.3 | 9.4 | 0.2 |
| <i>Bosmina</i> sp. | 5.6 | 47.6 | 26.4 | 4.9 |
| Cyclopoid copepods | 2.3 | 0.8 | 1.2 | 0.7 |
| Calanoid copepods | 0.1 | 0.0 | 0.2 | 0.0 |
| Station S5 | | | | |
| <i>Daphnia</i> sp. | 27.4 | 0.0 | 5.5 | 12.9 |
| <i>Bosmina</i> sp. | 4.1 | 8.3 | 10.6 | 3.0 |
| Cyclopoid copepods | 4.0 | 4.3 | 1.9 | 0.6 |
| Calanoid copepods | 0.2 | 0.0 | 0.0 | 0.5 |

NA, not applicable; ND, no data. (See Table 3)

Samples containing fewer than 100 individuals of the specific taxonomic group were not included in data analysis.

taxon was compared between duplicate net tows using paired *t*-test after arcsine transformation. There was no significant difference between duplicate tows for any of the taxa in Lake Stechlin or Lake Dagow ($P > 0.05$), indicating that the aniline blue staining method produced consistent results and there was no carryover of carcasses between net tows. The average percentage of each of the four most common zooplankton groups identified as dead by staining at each station is presented in Tables 5 & 6 for Lakes Stechlin and Dagow, respectively.

In Lake Stechlin no spatial differences in the live–dead composition of the four dominant zooplankton groups (*Daphnia* sp., *Bosmina* sp., Cyclopoid copepods and Calanoid copepods) were observed among stations (nested ANOVA $P = 0.338$). Additionally, within each group at each station there were no differences in live–dead composition between epilimnion and hypolimnion samples (Tukey pair wise comparisons, $P > 0.05$) with the exception of *Daphnia* sp. at S1 ($P = 0.02$). A comparison of daytime and night time

Table 6 Average percentage of dead zooplankton in Lake Dagow as determined by the aniline blue staining method

| | Epilimnion | Hypolimnion | Day | Night |
|--------------------|------------|-------------|------|-------|
| Station D1 | | | | |
| <i>Daphnia</i> sp. | 1.3 | NA | 9.8 | 3.7 |
| <i>Bosmina</i> sp. | 2.9 | 19.0 | 19.3 | 13.7 |
| Cyclopoid copepods | 4.5 | 34.4 | 20.0 | 3.2 |
| Calanoid copepods | 3.5 | 1.2 | 1.8 | 0.8 |
| Station D2 | | | | |
| <i>Daphnia</i> sp. | 2.7 | NA | 3.4 | NA |
| <i>Bosmina</i> sp. | 7.0 | NA | 20.4 | 4.3 |
| Cyclopoid copepods | 5.9 | 18.5 | 9.3 | 3.1 |
| Calanoid copepods | 1.5 | NA | 1.6 | 1.4 |
| Station D3 | | | | |
| <i>Daphnia</i> sp. | 0.4 | NA | NA | NA |
| <i>Bosmina</i> sp. | 1.1 | 60.8 | 18.1 | 16.8 |
| Cyclopoid copepods | 2.5 | NA | 7.1 | 4.8 |
| Calanoid copepods | 1.4 | 0.0 | 2.4 | 2.8 |

NA, not applicable. (See Table 5)

whole water samples indicated that time of day did not influence the live–dead composition of any of the major zooplankton groups at any of the stations (Tukey pair wise comparison, $P > 0.05$). Combining all the data an average of 6% of the zooplankton in Lake Stechlin were estimated to be dead.

In Lake Dagow there were no spatial differences in the live–dead composition of the four major zooplankton groups, either among the stations (nested ANOVA, $P = 0.498$), or between epilimnion and hypolimnion within each station (Tukey pair wise comparisons, $P > 0.05$). Additionally, there were no diurnal differences in the live–dead composition (nested ANOVA, $P = 0.245$). Combining all the data an average of 8% of the zooplankton in Lake Dagow were estimated to be dead.

To compare Lake Stechlin and Lake Dagow, taxon-specific data from all stations within each lake were pooled. Within the epilimnion, there was a significantly higher percent dead *Daphnia* sp. in Lake Stechlin than in Lake Dagow (Student's *t*-test, $t = -4.04$, $P = 0.002$). There was no significant difference between the two lakes in the prevalence of carcasses within the hypolimnion for any of the major zooplankton groups (Student's *t*-tests, $P > 0.05$). Among daytime whole water samples, carcasses were more prevalent in Lake Dagow than in Lake Stechlin for both calanoid and cyclopoid copepods (Student's *t*-test, $t = 6.47$, $P < 0.001$; $t = 4.27$, $P = 0.008$ respectively). Among night time whole water samples only

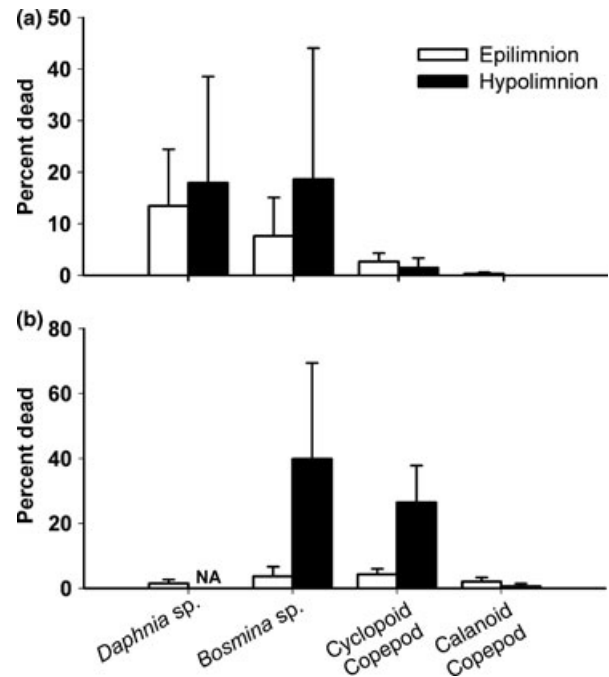


Fig. 2 Percentages of dead zooplankton (mean + SD) in different strata in Lake Stechlin (a) and Lake Dagow (b) as identified by the aniline blue staining method. For each taxon only samples with more than 100 individuals were included in data analysis. NA, not applicable.

cyclopoid copepods showed a significantly higher percentage of carcasses in Lake Dagow (Student's *t*-test, $t = 3.25$, $P = 0.008$). The taxon-specific percentages of dead individuals as determined by the staining method in Lake Stechlin and Lake Dagow are shown in Fig. 2 (epilimnion and hypolimnion) and Fig. 3 (day and night).

In the epilimnion of Lake Stechlin cladoceran carcasses were more abundant than copepod carcasses. This is not surprising, given that Stechlin is a cladoceran dominated lake. However, paired *t*-test of arcsine transformed percentage data showed that cladocerans were over-represented among the carcasses relative to their abundance in the entire zooplankton community ($t = 5.26$, $P = 0.001$). In Lake Dagow both copepods and cladocerans comprised the same proportions of the carcasses as they did within the whole zooplankton community (paired *t*-tests of arcsine transformed data, $P > 0.05$). The relative proportions of copepods and cladocerans among the carcasses were not different between epilimnion and hypolimnion in Lake Dagow.

The relationships between live–dead zooplankton compositions and water column conditions (dissolved

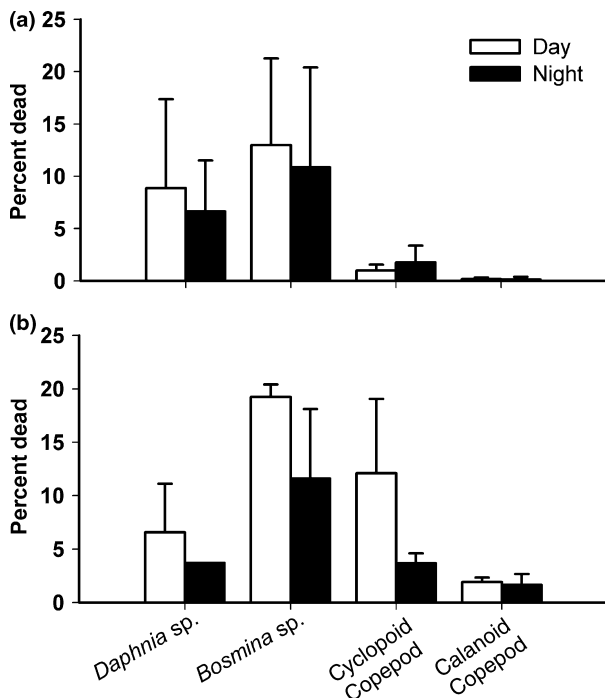


Fig. 3 Percentages of dead zooplankton (mean + SD) at different sampling times in Lake Stechlin (a) and Lake Dagow (b) as identified by the aniline blue staining method. For each taxon only samples with more than 100 individuals were included in data analysis.

oxygen, pH and integrated temperatures) were also examined. In Lake Stechlin the percentage of dead zooplankton was significantly and positively correlated with both temperature (Pearson's correlation $P = 0.036$) and pH (Pearson's correlation, $P = 0.044$). In Lake Dagow, the percentage of dead zooplankton population was negatively correlated with dissolved oxygen levels (Pearson's correlation, $P = 0.049$).

Discussion

Evaluation of aniline blue staining method

In laboratory tests with only live individuals, a variable percentage of cladocerans was misidentified as dead by the staining method, although the result was not statistically significant. Conversely, a very small, albeit significant, percentage of live copepods was misidentified as dead. These observations suggest that aniline blue staining is appropriate for estimating the portion of the zooplankton population that is alive at time of collection. One possible explanation for the false positive (live individuals being stained) is that

mortality occurred due to handling of the animals during the experiments. Earlier tests showed that the stain itself did not cause mortality among the zooplankton.

The trials containing only dead individuals showed that the method did not stain 100% of the dead cladocerans or copepods, which would result in an underestimation of carcasses. During staining trials with heat-killed zooplankton, Seepersad & Crippen (1978) also noted that some dead individuals stained only partially or did not take up the stain at all. They attributed this to the zooplankton entering a moribund state after exposure to a stressor (such as heat) although it was technically still physiologically alive. This moribund state, however, was not assigned any physical qualifications, which makes distinction from physiologically dead zooplankters quite difficult. Our results suggest that even in a highly unlikely situation where all the zooplankton in a sample are dead, the staining method would underestimate the cladoceran and copepod carcasses by at most 29–38%.

The primary goal of the staining trials with only live or only dead zooplankton was to provide a detailed description of live and dead staining patterns. In natural samples there will most likely be a mixture of live and dead zooplankton. As such, the ultimate test of the accuracy of the staining method was the use of known mixtures of live and dead zooplankton. Our results show that the live–dead compositions identified by the staining were not statistically different from the expected values for both cladocerans and copepods. All laboratory staining tests considered together indicate that while the method will stain dead individuals, it produces more consistent results for copepods than for cladocerans. Overall the method is an effective way for estimating live–dead compositions of copepods and cladocerans in natural freshwater samples. Because the staining method is simple and inexpensive, and the stained samples can be frozen until analysis, it is particularly appropriate for field studies where a large number of samples need to be collected.

In situ live–dead zooplankton compositions

Previous studies that included the live–dead composition of zooplankton populations in natural systems have reported a wide range in the prevalence of carcasses. Studies in estuarine and marine systems

have found that carcasses comprised 15–69% of the sampled populations, with the higher abundances of carcasses occurring in areas of hydrographic or hydrodynamic interest such as over seamounts (Haury *et al.*, 1995), over coral reefs (Genin, Gal & Haury, 1995), below the thermocline (Terazaki & Wada, 1988), in upwelling regions (Weikert, 1977) or along tidal fronts (Tang *et al.*, 2006a). The few studies investigating zooplankton carcasses in freshwater systems reported that carcasses comprise a much smaller portion (4–23%) of the total populations (Gries & Güde, 1999; Dubovskaya *et al.*, 2003; Gladyshev *et al.*, 2003). While the abundances of zooplankton carcasses within Lake Stechlin (c. 6%) and Lake Dagow (c. 8%) as determined by the aniline blue staining method are lower than those reported in marine and estuarine studies, they are similar to carcass abundances in other freshwater systems assessed by sediment trap (Gries & Güde, 1999) or a combination of sediment trap and staining (Dubovskaya *et al.*, 2003; Gladyshev *et al.*, 2003).

Between the two lakes, there was a slight difference in the distribution of carcasses within the water column. In Lake Dagow, cladoceran and copepod carcasses tended to be more concentrated in the hypolimnion, where the water was near anoxic. This anoxic condition might cause higher zooplankton mortality while minimizing the removal of carcasses by fish predation and microbial decomposition, leading to accumulation of carcasses at depth. In contrast, in Lake Stechlin cladoceran carcasses appeared to be more concentrated in the epilimnion, which suggests that there may be some mechanisms causing increased mortality among cladocerans in the epilimnion, or removing their carcasses from the hypolimnion. This postulation remains to be confirmed in future study.

Concluding remarks

Freshwater zooplankton play an essential role as consumers of primary production, as a food source for planktivorous fish, and as potential substrates for bacteria. Unfortunately most field studies have ignored the live–dead composition of the zooplankton; as such, our understanding of the ecological functions of zooplankton may have been biased. We have shown that the biological stain aniline blue, previously described by Seepersad & Crippen (1978), can be used for differentiating between live and dead

cladocerans and copepods in freshwater samples. We modified the staining protocol to make the method more convenient for field applications. This simple and inexpensive method is compatible with routine field sampling, and does not add much extra work to traditional sample processing in the laboratory. However, our laboratory tests showed that the method tends to underestimate zooplankton carcasses in extreme cases where only dead zooplankton are present. Despite this limitation, under most natural conditions where a mixture of live and dead individuals is present, the method does reliably quantify the live–dead composition of the zooplankton. To minimize error due to false positive or false negative staining, we recommend that a minimum sample size of 100 individuals should be used when estimating live–dead percentages.

Another similar staining method with neutral red has been successfully used in marine field studies (Tang *et al.*, 2006a). These methods together should allow for more systematic studies of zooplankton carcasses in the natural environment. In many lakes a mid-summer decline of the zooplankton populations is observed (Threlkeld, 1979), of which diseases and starvation are among the possible causes (Hülsmann & Weiler, 2000; Benndorf *et al.*, 2001; Hülsmann & Voigt, 2002). The aniline blue method will allow researchers to better characterize the increase in zooplankton carcasses during the mid-summer decline, and study their contributions to biogeochemical fluxes and microbial productions within the systems (Tang, Hutalle & Grossart, 2006b).

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