Applicability of the vital dyes neutral red and fluorescein diacetate to differentiate between alive and dead non-copepod zooplankton

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Abstract
It is a common practice for planktonologists to use neutral red (NR) staining to identify viable copepods in marine zooplankton. A new fluorescent dye, fluorescein diacetate (FDA), has also been successfully applied to Copepoda. Meanwhile, almost nothing is known about if NR- and FDA-based viability assays are applicable to many other zooplankton taxa. In this study, efficiencies of NR and FDA staining were evaluated and compared for different taxa and developmental stages of the Black Sea non-copepod zooplankton. Both the dyes were shown to stain well larvae of polychaetes, gastropods and bivalves, rotifers, fish eggs, barnacle nauplii, decapod zoeae, and the heterotrophic dinoflagellate Noctiluca scintillans. Dominant species of Cladocera (Pleopsis polyphemoides, Evadne spinifera, Pseudoevadne tergestina) were stained efficiently with FDA only. Some taxa had sufficient statistics of positive staining only with one of the dyes, NR (e.g. appendicularians and chaetognaths) or FDA (e.g. barnacle cyprids). Correspondingly, more data are demanded to fill the gaps in the target taxa. The dyes proved to be taxa-specific and hence, their reliable application should be based on their target groups.

Key words: viability assay, neutral red, fluorescein diacetate, vital dye, zooplankton.

Introduction
Understanding of structure and functions of aquatic ecosystems, assessing their health status are impossible without exhaustive information about dynamics and physiological state of metazoan zooplankton, including the ratio between alive and dead components of the community, predatory and non-predatory mortality rates of plankton (Tang, Elliott, 2013). The latter can be potentially used to reveal a deterioration of aquatic environments (as a result of pollution, toxic blooms, other kinds of environmental stress), as well as for making predictions of the community (and the whole ecosystem) dynamics under anthropogenic impacts (Di Capua, Mazzocchi, 2017). Methodology to identify and differentiate between alive and dead organisms in marine and freshwater zooplankton is still poorly developed and based mainly on: (i) visual discrimination of carcasses in native (live) and preserved samples (Daase et al., 2014, table 1); (ii) vital or mortal staining protocols. For many decades, the vital dye neutral red (NR) has been successfully used to study live/dead zooplankton, mostly copepods, in marine samples (Tang et al., 2006; Elliott, Tang, 2009, 2011; Ivory et al., 2014; Giesecke et al., 2017). Use of fluorescein diacetate (FDA), a substrate for active enzymes inside viable
cells (Butino et al., 2004), has been recently proposed as an alternative, fluorescent technique for viability assay of marine copepods (Litvinyuk et al., 2009; Litvinyuk et al., 2011; Litvinyuk, Mukhanov, 2012). Earlier, FDA was widely applied in phytoplankton studies (Onji et al., 2000) and, once, for visualization of viable copepod embryos (Butino et al., 2004). The first experience of its application in a field study of copepod zooplankton (Litvinyuk et al., 2011) demonstrated high efficiency of the fluorescent technique and has opened up prospects for its widespread use in marine plankton studies.

Unfortunately, a bulk of marine viability/mortality studies of zooplankton focus mainly on Copepoda, the most dominant and important component of the World Ocean plankton, while very few is known about viability and mortality of other taxa, golo- and meroplankton, features of staining of these groups by the vital dyes. In this study, we have just made an attempt to fill this knowledge gap through collecting and generalizing disparate observations of copepod nauplii and non-copepod zooplankton viability. For a decade, we have been staining the Black Sea zooplankton samples with NR and FDA for identifying alive/dead copepods. The protocol involved taking images of stained/unstained individuals before their analysis so micrographs of other taxa presented in the samples were also obtained and archived. Here, we use this material for: (i) revealing features and efficiency of staining non-copepod zooplankton (and copepod nauplii) with the vital dyes NR and FDA; (ii) compiling lists of applicability of each of the dyes in studies of different taxa.

Material and methods

Mesozooplankton viability assays were performed over the last decade (2009 to 2019) in Sevastopol Bay (the Black Sea) and adjacent coastal waters (Kryglaya Bay, Streleckaya Bay, mouth of Belbek River, etc.), and offshore areas of the Black and Azov seas (four cruises of RV “Prof.Vodyanitsky” in 2016-2017). The samples were collected by vertical net tow with a standard ring net (0.37 m mouth diameter, 150-µm mesh). In the laboratory, the samples were divided into 2 equal subsamples one of which was stained with fluorescein diacetate (FDA, Sigma-Aldrich) according to (Onji et al., 2000): 5 mg/ml stock solution in DMSO (stored at 4⁰C before use), 5 µg/ml final concentration.

The second subsample was stained with neutral red (NR) according to [Tang, 2006; Elliott, 2009]: 0.01 g/ml stock solution in distilled water, 15 µg/ml final concentration (1:67000), staining for 15 min to 2-3 h.

After the staining, both the subsamples were gently concentrated onto 100-µm nylon mesh and rinsed twice with 3-µm-filtered seawater to remove excess of stain. Most of the samples were analyzed immediately after the staining. Occasionally, the NR-stained subsamples were concentrated onto nylon mesh, placed in Petri dish, frozen and stored in the dark at −20°C for a few weeks before the analysis.

As NR gives deep red color at pH < 6.8 (Dressel et al., 1972), the stained subsamples were acidified (1 mL of 1 M HCl per 10 mL of subsample, Elliott, Tang, 2009) to develop the color inside the animal. Acidification provided high-quality differentiation between alive and dead animals (Tang et al., 2006).

The stained samples were poured into a modified Bogorov’s chamber, observed and photographed under an inverted microscope Nikon Eclipse TS100-F equipped with a camera (Ikegami ICD-848P) in light (NR) and fluorescent (FDA, blue excitation, green emission) modes. Alive FDA-stained organisms fluoresced bright green under blue light excitation while the dead ones did not produce fluorescence. NR stained alive organisms in deep red.

In total, more than 120 samples were analyzed, and >6000 images of non-copepod zooplankton were taken, with the size of the organisms varying between 60 µm (Bivalvia larvae) and 2.2 mm (Parasagitta setosa).

Results

In total, 17 taxa were identified among non-copepod zooplankton specimens for which viability assay was performed and its results were saved, including Cladocera, nauplii of Cirripedia (barnacle nauplii), larvae of Polychaeta, Bivalvia, Decapoda and Gastropoda, Rotifera, Hydromedusae, embryos and larvae of Pisces. Additionally, Harpacticoida and neretic nauplii of Copepoda were involved in this analysis.
DIFFERENTIATION BETWEEN ALIVE AND DEAD NON-COPEPOD ZOOPLANKTON

For some of the rare taxa, not enough data were available for making reliable conclusions about their staining properties (e.g. for fish larvae, barnacle cyprids, some cladocerans and chaetognaths). However, according to our approach, even few well-stained individuals could indicate the promising use of the dye in studies of their taxonomic group. The results obtained are summarized in Table 1.

The studied plankton representatives differed significantly in their morphology, nutrition and reproduction. As a result, the intensity and localization of staining were even more variable and depended on the taxonomic affiliation of the animals. A tendency of some taxonomic groups to be stained with only one of the dyes confirmed the advisability of using both the markers in the analysis. Only few of the considered taxa have been shown to be suitable for positive staining with both the dyes, NR and FDA (Table 1).

Table 1. Numbers of well-stained (S), unstained (NS) and total (T) organisms of different zooplankton taxa after their treatment with neutral red (NR) and fluorescein diacetate (FDA), and the dye applicability evaluations in the following categories: 1 – reliable application, 2 – potential application, 3 – dye does not work, 4 – insufficient data. Neutral red (NR) applicability evaluations based on the earlier published results: [1] – Dressel et al., 1972; [2] – Crippen & Perrier, 1974; [3] – Tang et al., 2006; [4] – Elliott, Tang, 2009; [5] – Ivory et al., 2014; [6] – Giesecke et al., 2017.

| Phyllum Annelida | Class Polychaeta | polychaete larvae | FDA S | FDA NS | FDA T | NR S | NR NS | NR T | NR (earlier published) |
|-----------------|-----------------|------------------|-------|--------|-------|------|--------|------|-----------------------|
| Phylum Cnidaria | Class Hydrozoa | medusae | 0 | 0 | 23 | 14 | 1 | 15 | - |
| Phylum Chordata | Superclass Pisces | fish larvae | 0 | 0 | 0 | 2 | 0 | 2 | - |
| Fish eggs | 15 | 7 | 22 | 10 | 2 | 12 | - | - |
| Phylum Arthropoda | Infraclass Cirripedia | barnacle nauplii | 64 | 59 | 123 | 450 | 209 | 689 | - |
| Barnacle cyprids | 5 | 4 | 9 | 0 | 0 | 0 | - | - |
| Class Branchiopoda | Superorder Cladocera | Eudanhe spinifera | 15 | 2 | 17 | 0 | 25 | 25 | - |
| Penilia avirostris | 15 | 25 | 40 | 0 | 102 | 102 | - | - |
| Pseudovadne tergestina | 2 | 3 | 5 | 0 | 30 | 30 | - | - |
| Pleopis polyphemoides | 18 | 41 | 59 | 85 | 180 | 265 | - | - |
| Podon spp., Beosmina sp. | 0 | 0 | 0 | 0 | 0 | 0 | - | - |
| Subclass Copepoda | copepod nauplii | 314 | 37 | 351 | 1023 | 97 | 1120 | - |
| subclass Copepoda | Order Harpacticoidea | harpacticoiids | 58 | 13 | 71 | 7 | 1 | 8 | - |
| Phylum Mysxozoa | Class Dinophyceae | Noculica scintillans | 80 | 57 | 137 | 189 | 73 | 262 | - |
| Phylum Chordata | Class Appendicularia | Oikopleura dioica | 9 | 0 | 9 | 66 | 3 | 69 | - |
| Phylum Chaetognatha | Class Sagittoida | chaetognaths | 1 | 2 | 3 | 97 | 10 | 107 | - |

Polychaeta larvae demonstrated perfect, bright staining of alive individuals with both the dyes (Fig. 1, 18, 20) while their dead organisms stayed well-unstained (Fig. 1, 19, 21) thus providing high-quality differentiation between alive and dead individuals. The intensity of the staining was comparable with one observed in our earlier experiments with a culture of the copepod Calanipeda aquaedulcis (Litvinyk et al., 2009).

Rotifera produced positive results for a wide range of species including Synchaeta spp. and Brachionus sp. Active and motile specimens exhibited the most bright FDA fluorescence of the entire body (Fig. 1, 17). The dead individuals were poorly distinguishable from the dark background. NR stained well stomach, intestine and reproductive tissue (Fig. 1, 16) but not the entire rotifer body (like in copepods). Intensity, localization and size of the NR-stained area varied sufficiently in different specimens so the viability assay of rotifers was not easy.

Gastropoda larvae showed satisfactory staining with both the dyes (Fig.1, 38-41) which seemed to penetrate the shell well. FDA gave a bright staining (Fig. 1, 40) with a perfect differentiation of alive individuals from dim and colorless dead ones (Fig. 1, 39) that is a reason to apply this dye in gastropod viability assay.

Decapoda larvae were rare in the plankton samples that explains so small sample size – 19 specimens (FDA). However, bright green fluorescence of 13 of them after FDA staining (Fig. 1, 22) gives enough reason to argue that FDA is satisfactory for this group. NR statistics was also insufficient (13 specimens) but positive in terms of the dye efficiency (Tabl. 1).
Fish eggs and larvae. Despite insufficient statistics (22, 12 specimens for FDA, NR, respectively), fish eggs seemed to be a good target for both the dyes (Fig. 1, 25, 26). We have got no data on FDA staining of fish larvae and only two specimens were stained with NR. The latters had deep red color of the entire body (Fig. 1, 27).

Cirripedia larvae. Both the dyes intensively stained larvae of barnacles (Fig. 1, 12, 14) but the color of NR staining varied from ‘classical’ deep magenta (Fig. 1, 14) to light pink that made it difficult to differentiate between alive and dead specimens. FDA staining was more reliable as it allowed one to avoid the controversial situations (Fig. 1, 12, 13).

Bivalvia larvae were stained with both the dyes but the quality of the results was too low for a reliable classification of a specimen to alive or dead (Fig. 1, 36, 37). Weak intensity of the staining made the major problem, probably due to calcareous shell being a barrier for the dye. This group should be avoided if NR or FDA are used for viability assay.

Oikopleura dioica is a small pelagic holoplankton chordate whose body was well stained with both the dyes, FDA and NR (Fig. 1, 30, 31) whereas the tail often remained colorless. We defined an individual alive if its body was well stained irrespective of the tail color (Fig. 1, 30). Despite a good statistics of NR application (69 ind.), only few (3) organisms were classified as unstained/dead (Tabl.1) that may indicate some nonspecific (false-positive) staining of organisms irrespective of their physiological status. The same problem was detected with FDA (0 unstained versus 9 stained individuals) but the sample size was too small to make any conclusions about applicability of FDA in O. dioica viability assay.

Cladocera. Four species of cladocerans were found in the samples, which are dominant in the Black Sea zooplankton: Penilia avirostris, Evadne spinifera, Pseudevadne tergestina and Pleopis polyphemoides. Three of them (P. avirostris, E. spinifera and P. tergestina) were either not stained with NR or stained faded.
All of 157 specimens of these species found in the samples were classified as unstained, i.e. dead (Fig. 1, 2, 9, 11), although some of them were motile, i.e. exactly alive. The fourth species, *Pleopsis polyphemoides*, had weak color of the body but quite bright and deep staining of eggs in brood chambers (Fig. 1, 5). Unlike other species, the percentage of unstained specimens of *P. polyphemoides* was relatively high (about 30%) so taking into account sufficient statistics (285 specimens in total), this species can be reliably analyzed with NR.

After FDA staining, *P. polyphemoides* exhibited bright green fluorescence (Fig. 1, 3) while unstained specimens were poorly distinguished from the background (Fig. 1, 4) that was perfect for viability assay. Alive individuals of *E. spinifera* had not so bright fluorescence, and only 2 (of 17) specimens were classified as unstained, that may cause some concern and requires additional data for more reliable analysis. Even worse situation was with *P. tergestina* (5 specimens) however 2 alive animals had perfect, bright fluorescence (Fig. 1, 10). Staining of alive *P. avirostris* was not bright, and in some individuals, the intestine was the only source of FDA fluorescence (Fig. 1, 1).

*Parasagitta setosa*, holoplanktonic chaetognaths, were well stained with NR. In most cases, the entire body was red (Fig. 1, 29) but sometimes, only head and dorsal ganglion were well stained. Weak FDA fluorescence was revealed only in one specimen while the other 2 animals had no staining (Fig. 1, 28). So, at least NR can be efficiently used in chaetognaths viability assay while representative sample is required for FDA applicability evaluation.

*Noctiluca scintillans*, a heterotrophic dinoflagellate, were analyzed in native, unsupervised samples. NR stained well the whole cell of *N. scintillans* whereas FDA fluorescence was localized in the region of nucleus and central cytoplasm (Fig. 1, 32). Good contrast between stained and unstained (Fig. 1, 33) cells guaranteed a reliable analysis however, its duration had to be as short as possible because of a leakage of the dye from the cell and a subsequent fading of the fluorescence.

*Hydromedusae* were never stained with FDA while NR was localized only in tentacles and appendages (Fig. 1, 35). Only 1 of 15 specimens was not stained with NR, that may evidence either high percentage of alive animals or false-positive staining. If FDA cannot be exactly used in this taxon studies, then more data are demanded to evaluate the applicability of NR for hydromedusae viability assay.

*Copepoda nauplii* (mainly *Acartia spp.*, *Calanus sp.*) were perfectly stained with both the dyes. Color saturation and sufficient statistics guaranteed high-quality differentiation between live and dead animals (Fig. 2), like it was achieved for copepods and adults (Litvinyuk et al., 2009; Litvinyuk et al., 2011; Litvinyuk, Mukhanov, 2012).

**Discussion**

Despite most of the taxa involved in the analysis (excepting copepod nauplii) were relatively rare in the Black Sea zooplankton samples, sufficient statistics of stained/unstained specimens have been accumulated for some of them (Table 1), in particular, polychaete and gastropod larvae, rotifers, barnacle nauplii, some of cladocerans (for NR only), the dinoflagellate *Noctiluca scintillans*, appendicularians (for NR only) and chaetognaths (for NR only). Realizing the subjectivity of our analysis, we had to define some limiting, minimal sample size (about 40-50 specimens) for which the applicability of the dye would be considered reliable. However, even if this upper limit of statistics was achieved, the number of positively-stained specimens had to be nonzero. Otherwise, the dye was concluded to be unable to stain the taxon. Extremely low statistics (single specimens) prevented making any conclusions about the dye efficiency while the other cases implied good prospects for the dye to be applied in the viability assay but demanded more data to confirm the reliable application.

As we suggested earlier, even few well-stained individuals could indicate potential applicability of the dye to a particular taxon. At the same time, absence of unstained specimens (like in NR-treated fish larvae and FDA-treated *O. dioica*, Table 1) was a problem as it may be a result of non-specific (false-positive) staining (all organisms are stained, regardless of whether they are alive or dead). So, it is important to build-up statistics of unstained specimens to avoid false-positive staining.

According to the final evaluations of the dyes’ applicability, which are summarized in Table 1, NR has the highest number of taxa with sufficient statistics and unambiguous diagnosis (categories 1 + 3), including three cladoceran species for which it cannot be used exactly. More data are required for another four taxa, fish larvae, barnacle cyprids, hydromedusae and harpacticoids. The latter two have insufficient
numbers of unstained animals. The others (fish eggs and decapod zoeae) can be potentially involved in the NR-based viability assay.

Some of our conclusions about the applicability of NR proved to be in a good agreement with earlier published data (Table 1). In particular, positive staining was registered in such taxa as polychaete larvae (Crippen, Perrier, 1974; Elliott, Tang, 2009), rotifers, gastropod larvae, chaetognaths (Crippen, Perrier, 1974), barnacle nauplii (Crippen, Perrier, 1974; Elliott, Tang, 2009; Giesecke et al., 2017) and copepod nauplii (Crippen, Perrier, 1974; Ivory et al., 2014; Giesecke et al., 2017), Oikopleura spp. (Giesecke et al., 2017) (Table 1). For some of these taxa, negative results were occasionally obtained, like for polychaete larvae and barnacle nauplii (Tang et al., 2006) and harpacticoids (Ivory et al., 2014). The same problem was with fish eggs (Crippen, Perrier, 1974) for which we have got positive result, as well as with Podon polyphemoides (Leuckart), a cladoceran taxonomically close to well-stained Pleopsis polyphemoides (Dressel et al., 1972). All these faults were likely due to methodical problems. In earlier studies, too low concentrations of the dye or too short staining duration were used (Dressel et al., 1972; Crippen, Perrier, 1974). Improvements in the NR staining protocol and its complementation with the techniques of sample preservation (e.g. freezing, Elliott, Tang, 2009) and developing the color under acidic conditions (Crippen, Perrier, 1974; Tang et al., 2006) had changed the situation for the better. However, no special methodical research have been conducted to evaluate applicability of NR to the non-copepod zooplankton although the idea to expand the list of NR-stained species was proposed in the earliest publications. Unfortunately, none of the publications cited above provided any statistics of the analyzed organisms that is why all the positive observations in Table 1 have been defined as ‘potential application’. To be exact, the negative observations could not be also classified in Table 2 as the category 3 (dye does not work) as the data were fragmentary and insufficient.

FDA has been reliably confirmed to be efficient (categories 1 + 3) in staining of 8 taxa (Table 1), including polychaete larvae, rotifers, barnacle nauplii, P. avirostris, P. polyphemoides, copepod nauplii, harpacticoids and N. scintillans. 6 taxa were categorized as ‘potential application’: gastropod and bivalve larvae, fish eggs, barnacle cyprids, decapod zoeae and a cladoceran (E. spinifera). Hydromedusae were never stained with FDA.

Thus, FDA covered the same groups as NR but gave positive results for the cladocerans which were not stained with NR (Table 1). This fluorescent dye looks highly promising for zooplankton viability assay not only because its application covers most of the community but also due to much better brightness and color contrast between well-stained and unstained specimens. Very modest experience of applying FDA in marine zooplankton studies (Litvinyuk et al., 2009; Litvinyuk et al., 2011; Litvinyuk, Mukhanov, 2012) looks surprising against the background of long history of its intensive application in phytoplankton viability assay. Advantages of the new fluorescent marker will hopefully help to fill the methodical gap.

Conclusions

1. Neutral red (NR) can be efficiently applied in viability assay of the following Black Sea meroplankton: polychaete, gastropod and bivalve larvae, rotifers, barnacle nauplii; and the holoplankton: chaetognaths, the appendicularian Oikopleura dioica and the heterotrophic dinoflagellate Noctiluca scintillans. 2. More data are required for fish eggs and decapod zoeae, for which NR can be potentially used. 3. NR should be applied to cladocerans with care as their species staining gives inconsistent results. FDA is more preferable and reliable dye for this taxon. 4. Fluorescein diacetate (FDA) can be reliably applied in viability assay of the following Black Sea meroplankton: polychaete larvae, rotifers and barnacle nauplii; and the holoplankton: harpacticoids, the heterotrophic dinoflagellate Noctiluca scintillans and cladocerans. 5. More statistics are required for gastropod and bivalve larvae, fish eggs, barnacle cyprids, decapods zoeae and some of cladocerans, for which FDA can be potentially used. 6. FDA never stained hydromedusae and should not be applied to this group. 7. Both the dyes, NR and FDA, can be efficiently used in viability assay of copepod nauplii. 8. The dyes proved to be taxa-specific and hence, their reliable application should be based on their target groups.

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