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Double-labelling methods used to diagnose apoptotic and necrotic cell degradations in copepod nauplii

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Abstract Two double-labelling methods, Tunel + propidium iodide and Annexin V-FITC + propidium iodide, have been tested to diagnose cell degradation processes in N1–N2 nauplius stages produced by spawning females of the copepod *Calanus helgolandicus* fed either non-toxic (PRO: control) or toxic (TR1) diets under laboratory conditions. Observation of labelled samples with a confocal laser-scanning microscope revealed that the maternal-food effect, following absorption of the toxic diet, induced cell apoptosis and necrosis in 80–100% of the offspring, which sooner or later died. Similar cell damages were also observed in 18–50% of the nauplii produced by females incubated in filtered seawater. Such cell degradation processes and high mortality were not observed in nauplii produced by females fed the non-toxic diet. Our protocols allowed detection of cell degradations before death of unhealthy larvae, preferentially with a confocal microscope or a conventional visible-fluorescent-light microscope.

Introduction

The labelling techniques Tunel, initially designed for labelling single cells in vertebrates (Kerr et al. 1972), and Annexin V-FITC have already been applied successfully to thin layer preparations of invertebrate tissues (Harzsch et al. 1999), as well as to the whole body of insects (Nagy and Grabic 1999) and marine invertebrates, such as Cnidaria (Seipp et al. 2001) and copepods (Ianora et al., in preparation). The Tunel-labelling approach has been the most frequently used so far. Recent results obtained with copepods have shown that mor-

tility rates in hatched abnormal nauplii were higher, in relation to the decrease of hatching success and to the maternal diatom–food effect (Carotenuto et al. 2002; Ianora et al., in preparation). Laboratory investigations have further revealed that ingestion of toxic diatoms by spawning copepod females was followed by a series of anomalies in their offspring. Depending on the diatom species, algal cell concentrations and duration of feeding, results have shown that diatom diets could affect cell division in embryos, hatching success of eggs and development and survival of larvae (Chaudron et al. 1996; Ban et al. 1997; Carotenuto et al. 2002; Pohnert et al. 2002; Ianora et al., in preparation). Each of these experimentally induced physiological deficiencies could be easily detected using the standard incubation methods necessary to monitor egg production and hatching success, in addition to microscopic observation. Detection, in turn, allowed evaluation of maternal food effects on the morphological development and survival of embryos and larvae (Poulet et al. 1995; Lacoste et al. 2001). This deleterious effect is related to a chemical defence mechanism induced in diatoms by the production of toxic α -, β -, γ - and δ -unsaturated aldehydes, which inhibit cell division in both embryos and larvae and, thus, further affect their survival rates (Miralto et al. 1999; Pohnert 2000; Carotenuto et al. 2002; Pohnert et al. 2002; Ianora et al., in preparation). Severe symptoms are observed at high food concentrations ($\geq 10^4$ cells ml⁻¹: above naturally occurring diatom blooms). They are characterised by partial or complete hatching failure of eggs and by frequent morphological anomalies in nauplii, resulting from their intoxication by aldehydes ingested by *Calanus helgolandicus* females in feeding periods ≥ 2 –5 days. At lower concentrations ($\leq 10^2$ – 10^3 cells ml⁻¹: below or close to naturally occurring diatom blooms), the maternal diatom–food effect is milder and not easy to detect, because hatching failure and proportions of morphological anomalies in nauplii are not significantly different from controls (a non-diatom diet: *Prorocentrum minimum* at 10^4 cells ml⁻¹; Kang and Poulet 2000). Very often, the morphological aspect

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of hatched nauplii appears normal, although their survival rate is significantly lower than that of controls, suggesting that the maternal diatom–food effect is still influencing the health of the survivors. However, suspected cell degradation processes due to toxic aldehydes, coinciding with premature high mortality of the offspring, cannot be identified with standard detection methods, which become useless at low toxic diatom–food concentrations. The aim of this work was to assay in copepod nauplii two different double-labelling methods, using a confocal laser-scanning microscope. Our main objective was to test if cell degradation processes could be detected in nauplii produced by females fed a toxic diet, in comparison to a non-toxic diet, under laboratory conditions. This approach can be extrapolated to diagnose lethal and sub-lethal intoxication of the offspring by maternal food, or by any other deleterious stimulus, before death of the offspring.

Materials and methods

Copepod samples

Sexually mature females of the copepod *Calanus helgolandicus* were sampled offshore Roscoff, North Brittany, France, from February to June 2002. For each test, 50–100 single females were placed in filtered seawater for 24 h and then incubated individually in beakers filled with filtered seawater, enriched with one algal diet [a toxic diet: the diatom *Thalassiosira rotula* strain CCMP 1647 (TR1) or a non-toxic diet: the dinoflagellate *Prorocentrum minimum* (PRO: control), Pohnert et al. 2002], at low concentrations comprised of between 10^2 and 10^3 cells ml^{-1} . We know from previous results that these algae are ingested well by *C. helgolandicus* females (Kang and Poulet 2000). From day 1 to 7, eggs spawned by females fed the two single diets were collected daily, three to five batches of 20–30 eggs each were transferred to vials with 3 ml of filtered seawater and incubated at 17°C for 24 h until hatching. Three categories of hatched nauplii, belonging to naupliar stage N1 or N2, were sampled. Samples in controls consisted of nauplii with both normal morphological appearance, normal swimming and low mortality (<5%). Sub-normal samples in tests corresponded to nauplii with apparently normal body morphology, but with higher mortality (50–80%) during the second day following hatching. Strong abnormal samples corresponded to nauplii showing mild or heavy external, morphological anomalies on their body and/or appendages, with both defective swimming behaviour and high mortality rates (80–100%). The majority of normal and abnormal nauplii were produced by females fed PRO and TR1, respectively. Experiments with each food category were performed three times. Another category of nauplius samples, reflecting the in situ food conditions, was produced on two occasions during the same period by batches of unfed females, incubated for 24 h in filtered seawater (Millipore filter, 0.22 μm). These females produced eggs with high hatching rates ($\geq 80\%$), producing nauplii without apparent external anomalies and with lower mortality rates (30–50%).

Labelling-method characteristics

The two methods (Tunel+propidium iodide and Annexin V-FITC+propidium iodide) used here with copepods have been widely utilized (Kerr et al. 1972; Abrams et al. 1993; Yasuda et al. 1995; Schrevels et al. 1998). Three categories of cells can be monitored in the body of the fixed nauplii when using two double-labelling methods: intact (Tunel⁻/pi⁺; A-fitc⁻/pi⁻), apoptotic (Tunel⁺/pi⁺; A-fitc⁺/pi⁻) and necrotic (A-fitc⁺/pi⁺) cells

(Abrams et al. 1993; Vermes et al. 1995). The novelty lies in the fact that the methods were adapted and applied in order to distinguish cell degradation processes in the whole body of copepod nauplii.

The principle of the double-labelling method Tunel+propidium iodide has been described earlier by Kaufman et al. (2000). Briefly, when cells enter apoptosis, endonuclear lysis triggers the cleavage of DNA into oligonucleosomic fragments. Such fragments can be labelled on the free radical 3'-OH at its extremity by a fluorescent marker, dUTP. Fixation of dUTP to the 3'-OH radicals is achieved with a desoxynucleotidyle transferase (TdT) (Kaufman et al. 2000). In the 488–563 nm excitation wavelength range, dUTP emits a green fluorescent light, which allows detection of apoptotic cells by their green nuclei. DNA in damaged cells (necrosed), or in cells with permeable plasmatic membranes (chemically fixed), can be specifically labelled with propidium iodide, which emits red fluorescent light at the same excitation wavelength. DNA in normal, living cells is not labelled by propidium iodide, and, thus, nuclei are not visible with the confocal microscope (control, see Fig. 1C).

According to Walker and Quirke (2001), the Tunel (TdT-mediated dUTP nick end labelling) method is not entirely specific to apoptosis, but can also label necrotic cells, where partial DNA fragmentation occurs (Kaufman et al. 2000). Thus, it seems that the Tunel+propidium iodide double-labelling method is most appropriate to labelling the end phase of the apoptotic process



Fig. 1A–C *Calanus helgolandicus*. Three normal, anomaly-free, nauplii (stage N1) produced by copepod females fed the control diet, a non-toxic dinoflagellate, *Prorocentrum minimum* (PRO). **A** Black and white picture of a nauplius observed with a conventional microscope under visible light. Colour pictures of the same type of nauplius labelled with Tunel+propidium iodide (**B**) and with Annexin V-FITC+propidium iodide (**C**), both observed with the confocal microscope at 488 nm excitation wavelength. Size of nauplii: $220 \pm 10 \mu\text{m}$

(Darzynkiewicz et al. 1995); this is one reason why we also used the Annexin V-FITC method, specific to cell apoptosis entering its early phase (Bossy-Wetzel and Green 2000). The second reason is that chemically preserved cells in copepods are all permeable to propidium iodide. In fixed samples, non-fragmented DNA is thus labelled with propidium iodide in normal, apoptotic and necrosed cells, which therefore cannot be safely differentiated with the double staining of the TUNEL method (Fig. 1B). Thus, our protocol with TUNEL + propidium iodide differentiates only between apoptotic and normal cells.

The Annexin V-FITC method relies on the principle that a certain group of phospholipids, called phosphatidylserine (PS), located inside the plasmatic cell membrane in normal, living cells, are translocated to the external surface of the membrane during the early apoptotic phase (Bossy-Wetzel and Green 2000). Presence of PS outside the membrane is a specific criteria of cells entering the apoptotic phase. A specific protein, Annexin V, able to specifically fix to phospholipids in the presence of Ca^{2+} , is labelled with fluorescein isothiocyanate (FITC). Translocation of PS outside the cell membrane occurs before DNA condensation, or fragmentation, and permeabilisation of the membrane, related to cell necrosis. The double labelling with propidium iodide allows us to specifically label DNA in necrosed, permeable cells. Consequently, this method allows the simultaneous labelling of apoptotic (with Annexin V-FITC: green light emission) and necrotic (with propidium iodide: red light emission) cells in non-preserved samples (Vermees et al. 1995). Moreover, nuclei and cell membranes in control samples (apoptosis, necrosis free) are not visible, simply because these markers do not label intact DNA or PS in healthy cells (Fig. 1C).

In summary, a series of preliminary tests on samples of copepod nauplii, designed to assay these methods, have revealed that the TUNEL approach must be used with chemically pre-fixed samples, which makes the double labelling with propidium iodide useless; whereas the Annexin V-FITC + propidium iodide method is strictly applicable to live samples, prior to their chemical fixation (Fig. 1A, B, C). Therefore, each method offers its own specific advantages and constraints, as reported below.

Preparation of samples

Pretreatment of samples and preparation of buffers and reagents

Samples of whole nauplii, 12–30 specimens each, were fixed for a minimum of 24 h in 4% paraformaldehyde buffered with 0.2 M NaCl in phosphate-buffered saline (PBS) at pH 7.4. In order to render the carapace permeable to TUNEL reagents, fixed samples were further rinsed for a few seconds, three times, in vials, into which liquid nitrogen was gently poured. Samples were incubated for another 24 h at 25°C in Eppendorf tubes containing 50 µl of a chitinase solution (Sigma, concentration 1 U ml⁻¹ in phosphate buffer, stored at -30°C before assays). These preparations followed a procedure used earlier by Ianora et al. (in preparation). The problem is that small fragile nauplii can be lost or damaged in liquid nitrogen, but once fixed they can be stored in the refrigerator (5°C) for days or weeks prior to labelling. With the Annexin method, preliminary assays designed to compare nitrogen/chitinase-treated and non-treated samples revealed that such pretreatments are useless with copepod nauplii, which have a thin carapace permeable to this marker. With the Annexin method, samples were never treated with liquid nitrogen or chitinase solution, which induced damage in the live, non-fixed specimens beyond 30 min and led to complete decomposition in 1 h.

Preparation of the PBS buffer solution has been reported before (Ausubel 1998). This buffer can be stored at 5°C in the dark for weeks. Before usage, aliquots of the buffer solution were diluted 1/10 with MilliQ distilled water, pH 7.3–7.4.

The phosphate buffer solution 0.1 M, pH 6 was prepared as reported by Ausubel (1998). This buffer can be stored at 5°C in the dark for weeks.

Propidium iodide solution (Sigma) was prepared at a concentration of 100 µg ml⁻¹ of HEPES buffer. HEPES buffer (Sigma; pH 7.4, 1100 mOsmol l⁻¹, similar to seawater osmolarity) was prepared according to Ausubel (1998). This solution can be stored at 5°C in the dark for weeks.

Double labelling with TUNEL + propidium iodide

For each sample, 100 µl of the mixed TUNEL (Roche) solutions 1 and 2 was placed in an Eppendorf tube, where fixed nauplii samples following chitinase treatment and first pre-rinsed three times with PBS, were further bathed for 90 min at 37°C. Samples were rinsed again three times with PBS and bathed in a new Eppendorf tube containing 100 µl of propidium iodide solution for 5 min at room temperature. Samples were rinsed once again with PBS and then mounted on slides, each with a small cavity filled with PBS, and covered with a cover slip before observation under the confocal microscope. Microscope observation was carried out within few minutes or could be delayed a few days after double labelling. The samples remained in good optical condition and could be observed over 3 days if kept at 5°C in the dark.

Double labelling with Annexin V-FITC + propidium iodide

Samples of live nauplii were pipetted into an Eppendorf tube with a minimum of seawater (<100 µl). Excess water was eliminated. Each sample (20–30 nauplii per sample) was promptly added to a solution with 100 µl of Annexin V-FITC (Sigma) and 10 µl of propidium iodide and incubated for 10 min at 5°C in the dark. Samples were then promptly rinsed three times with the HEPES buffer, in order to remove excess of reagents, and were fixed further with the 4% paraformaldehyde solution (60 min at 5°C in the dark). Samples were rinsed once again with HEPES buffer and then mounted on slides as described above. We noticed that non-preserved samples were quickly damaged by the laser beam, 10–25 min after setting the slide on the microscope. The fixed samples could be stored at 5°C in the dark for 3–4 weeks without optical modification.

For each sample, adding or removing reagents was promptly achieved under the binocular microscope with a Gilson pipette. Pipetting nauplii from one Eppendorf tube to another, or to the slide, was carefully done using cones pre-cut at the tip, to avoid damage and lost of specimens.

Observations with the confocal microscope

We used an inverted confocal laser-scanning microscope, model Olympus Fluoview, equipped with a laser set at 488–563 nm excitation wavelengths, which induces a green fluorescence emission (channel 1) with both TUNEL and Annexin, and a red fluorescence emission (channel 2) with propidium iodide. The two fluorescent signals were digitised and visualised with the Fluoview 2.1 program. The settings of each channel (photomultiplier voltage, laser intensity) were adjusted in such a way as to provide a good signal without too much photobleaching on the labelled samples. In these conditions, the autofluorescence of the carapace was almost undetectable (see Figs. 1C, 4B, 5). Under such settings, bright green (Annexin) or yellow-green (TUNEL, superposition of red and green lights) spots, observed above the channel 1–2 thresholds, were assumed to correspond to apoptotic cell degradations. With Annexin, yellow-green spots (superposition of red and green lights) corresponded to necrotic processes. Nauplii were observed under $\times 10$ or $\times 20$ objective magnification. Higher magnifications of cells were not achieved with these samples. Genetically induced “natural” apoptosis has been reported during embryogenesis in the nematode *Caenorhabditis elegans* (131 “natural” apoptotic cells were detected among a total of 1090 cells; Yuan et al. 1993). Judging from the results in controls (Fig. 1) and tests (Figs. 2, 3, 4, 5), it was assumed that the proportion of cell degradations,

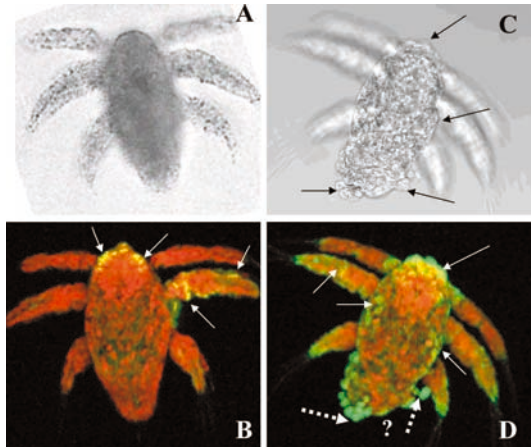


Fig. 2A–D *Calanus helgolandicus*. Nauplii (stage N1), labelled with Tunel+propidium iodide, produced by females fed the toxic diatom *Thalassiosira rotula* (TR1). **A, B** Pictures illustrating a case in which external, morphological malformations of the larva are not distinguished, or do not exist at all, while anomalies, corresponding to apoptotic cell degradations, are visible inside the body or in several appendages (yellow-green, white arrows). **C, D** Pictures showing both external, morphological malformations on the distal part of the body (black arrows) and anomalies scattered inside the whole body, corresponding to apoptotic cell degradations (yellow-green, white arrows). The great majority of normal cells are stained red by propidium iodide, while the presence of necrotic cells cannot be safely discriminated with this method. Green light spots in many similar damaged larvae (panel D, dashed white arrows) were not identified. Size of nauplii: $220 \pm 10 \mu\text{m}$

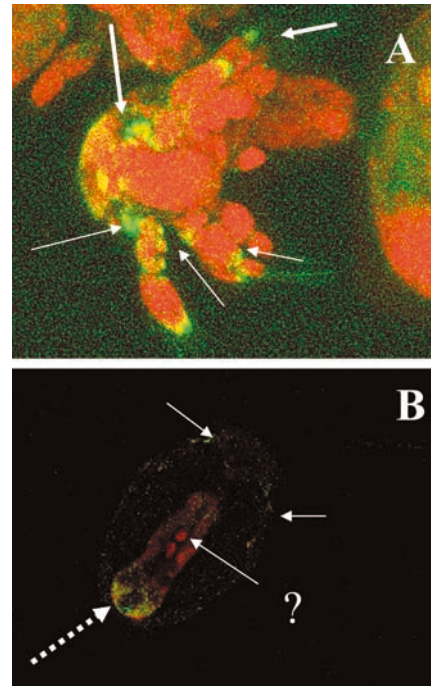


Fig. 4A, B *Calanus helgolandicus*. Abnormal nauplii produced by wild females incubated 24 h in filtered seawater in the laboratory. **A** Nauplius (stage N4, $280 \pm 10 \mu\text{m}$), labelled with Tunel+propidium iodide; **B** nauplius (stage N2, $220 \pm 10 \mu\text{m}$), labelled with Annexin V-FITC+propidium iodide. Anomalies detected inside the whole body and appendages correspond to apoptotic (green+yellow-green, in panel A; green, in panel B; white arrows) and to necrotic (yellow-green, dashed white arrows, in panel B only). Red light spots along the gut track were not identified

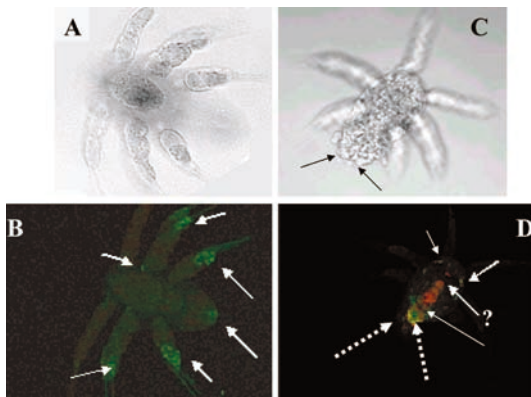


Fig. 3A–D *Calanus helgolandicus*. Nauplii (stage N2), labelled with Annexin V-FITC+propidium iodide, produced by females fed the toxic diatom *Thalassiosira rotula* (TR1). **A, B** Pictures illustrating a case in which external, morphological malformations of the larvae are not distinguished, or do not exist at all, while anomalies, corresponding to apoptotic cell degradations (green, white arrows) are visible inside the body and appendages. **C, D** Pictures showing both external, morphological malformations on the distal part of the body (black arrows) and anomalies scattered inside the whole body and appendages, corresponding to apoptotic (green, white arrows) and to necrotic (yellow-green, dashed white arrows) cell degradations, which are clearly discriminated with this method. Red light spots along the gut track were not identified. Size of nauplii: $220 \pm 10 \mu\text{m}$

mediated by the maternal diatom–food effect, was obviously higher than the “natural” apoptotic cell stock, which is still unknown in copepods. Each sample was scanned into a number of optical sections, varying between 14 and 30 layers per nauplii, 5–10 μm apart, depending on the position of larvae and appendages in the slide chamber and the distribution of abnormal cells inside the whole body. Each layer, corresponding to one image, was stored in 12 bit, tif format, and could be further processed with the Photoshop 5 programme. Complementary black and white pictures of samples were taken, in order to relate the external morphological aspects with the cell patterns inside the body. For preliminary tests, we used about 20 samples, each with 10–12 nauplii produced by females fed TR1 or PRO. For comparative tests, we screened five, six and three replicate samples, with 10–30 nauplii each, produced in three consecutive bioassays, by females fed PRO or TR1 and by females incubated in filtered seawater, respectively. Each bioassay was performed three times with PRO and TR1 and only twice with filtered seawater. One half of the nauplii was labelled with Tunel+propidium iodide, while the second half was labelled with Annexin V-FITC+propidium iodide.

Results and discussion

Figure 1 shows that samples of normal, healthy nauplii are stained red with Tunel+propidium iodide, whereas they are almost transparent with Annexin V-FITC+propidium iodide. These specimens, used as controls, were produced by females fed the non-toxic diet (PRO). In such assays, 80–100% of the new-born

larvae were normal in the three replicate tests. Red spots scattered inside the whole body were due to intact DNA in cell nuclei coloured with propidium iodide in samples preserved with paraformaldehyde (Fig. 1B). In live, normal, impermeable cells, labelled with Annexin V-FITC + propidium iodide prior to fixation of samples with paraformaldehyde, DNA was not labelled by propidium iodide (Fig. 1C). Abnormal nauplii produced by females fed the toxic diatom TR1 are shown in Figs. 2, 3 and 5. Cell degradations detected with TUNEL (apoptosis: yellow-green) or Annexin (apoptosis: green; necrosis: yellow-green) were observed in $\geq 70\%$ of the new-born nauplii. This proportion was similar in all three replicate assays performed with TR1. Similar results with TUNEL + propidium iodide were obtained by Ianora et al. (in preparation).

In mussels, the presence of apoptotic and secondary necrotic cells is known to result from the induction of

cell death by deleterious, toxic chemical compounds, including pollutants (Micic et al. 2001). We know from previous results (Miralto et al. 1999; Pohnert 2000; d'Ippolito et al. 2002; Pohnert et al. 2002) that several types of unsaturated aldehydes, produced by diatoms and ingested by spawning females, are deleterious to both embryogenesis and morphogenesis of the offspring. Cell division anomalies during embryogenesis were previously observed with a fluorescent light microscope, using two DNA markers (Hoesch 33232 or Tripian blue; Poulet et al. 1995), or simply deduced through the incubation method aimed to estimate the rates of egg-hatching success (Laabir et al. 1995). These methods are not suitable for the study of cell degradations in nauplii. Two cases can arise. First, when the intoxication is low, the response is usually not characterised by external, morphological malformations (Figs. 2A, 3A; Kang and Poulet 2000) and, thus, the impact of toxins remains undetected. At the stage of sub-lethal diatom contamination, labelling methods, combined with confocal microscopy, become extremely useful to detect cell degradations inside the larvae (Figs. 2B, 3B). Second, when the intoxication is high, the response is characterised by various morphological anomalies on the body of the larvae, which are easily visible with a normal microscope (Figs. 2C, 3C; Poulet et al. 1995; Ban et al. 2000). However, such malformations were associated with cell degradations observed inside the body of $> 70\%$ of the total hatched nauplii (Figs. 2D, 3D). Several pieces of evidence suggested that the cell degradations shown in Figs. 2, 3, 4 and 5 result from the maternal food effect, in relation to primary or secondary induction by aldehydes from diatoms (Miralto et al. 1999; Pohnert et al. 2002; Carotenuto et al. 2002; Ianora et al., in preparation). However, until very recently (Ianora et al., in preparation; Figs. 2, 3, 4, 5), the presence and nature of such cell anomalies in copepod nauplii had not been determined. Although the mechanism of the cell degradation process observed in copepod nauplii is still unknown, induction of apoptosis by aldehydes is strongly suspected, judging from similar results obtained with bioassays on human and mouse cell lines (Nappey et al. 1996; Miralto et al. 1999).

Figures 3 and 5 illustrate some major advantages of labelling methods used together with the confocal microscope. Furthermore, screening optical layers allows visualisation of the position of cell degradations in the body or appendages of the larvae, which sooner or later induce death in these specimens (Poulet et al. 1995; Carotenuto et al. 2002; Ianora et al., in preparation). In summary, results obtained with these protocols (Figs. 3B, D, 5A, B, C) have clearly identified the nature and scattering layers of cell degradations in the offspring in relation to the maternal diatom–food effect.

In nauplii, produced by females incubated in filtered seawater and with unknown past feeding history, apoptotic and necrotic processes were also detected with these methods (Fig. 4). Only 18%, 20% and 50% of the nauplii screened in three batches of 20–30 specimens

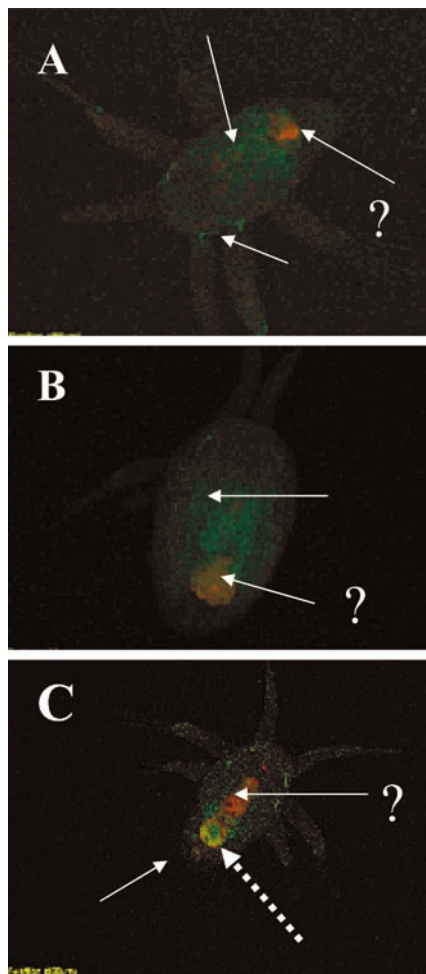


Fig. 5A–C *Calanus helgolandicus*. Abnormal nauplius larvae (stage N2), labelled with Annexin V-FITC + propidium iodide. **A, B, C** These examples correspond to cell degradations observed in nauplii produced by different batches of females fed the toxic diet TR1 (apoptosis: green, white arrows; necrosis: yellow-green, dashed arrow). Red light spots in the gut track were not identified. Size of nauplii: $220 \pm 10 \mu\text{m}$

each, produced by unfed females, presented such cell degradations. Previous records have shown that on an annual basis, average hatching failure and abnormal nauplii were about 30%, but could increase at times up to 75% during spring diatom blooms in nature (Laabir et al. 1998). These cell degradations are totally missed when observations, aimed to estimate the mortality of juvenile copepods, are limited to the measurement of hatching success (Irigoién et al. 2002). Even though we ignored the causes of anomalies and mortality observed in samples reflecting natural conditions (Ban et al. 2000), the symptoms shown in Fig. 4 resembled those induced by toxic diatoms (Figs. 2, 3, 5). Even when fed with PRO, these larvae do not survive in the laboratory (Ianora et al., in preparation). In the field, apart from aldehydes produced by diatoms, various toxic chemical and physical stimuli (e.g. tri-*n*-butyltin chloride; uv, see Lacuna and Uye 2001; Micic et al. 2001) are known to induce mortality. Therefore, these labelling methods could be useful to estimate the occurrence of lethal and sub-lethal anomalies in juveniles directly sampled with plankton nets in the sea, in relation to studies on mortality and recruitment processes.

With Tunel + propidium iodide, green fluorescence was detected at the distal end of the body of several severely damaged larvae (Fig. 2D). The reason why these cells did not uptake propidium iodide remains unclear. With Annexin V-FITC + propidium iodide, large, red fluorescent spots were detected in the vicinity of the gut track in abnormal nauplii (Figs. 3D, 4B, 5A, B, C). Again, the presence of these red spots was puzzling. They meant that propidium iodide was taken up, but not Annexin. In the non-feeding larvae (stages N1, N2) shown in Figs. 3D, 4B and 5A, B, red spots were assumed to be non-copepod cells, mainly bacteria. In older naupliar stages (N3–N6), known to feed on phytoplankton, these spots could be confused with natural fluorescence due to chlorophyll *a* in the phytoplankton ingested by the organisms. This aspect should be examined with caution in field samples analysed routinely.

Our first objective in comparing these methods was to apply the best method possible to laboratory and/or field investigations of juvenile copepod mortality. However, the suitability of these methods to zooplankton species is not clear cut and must be determined by the operator, who is familiar with their characteristics. With Tunel (Roche), the cost is high (20 Euros per sample) and the number of samples that can be treated is low (25 samples treated with 5 kits per box). The complete duration of the chemical preparation of samples (fixation + liquid nitrogen + chitinase + double labelling + rinsing) is > 48 h. In total, this method requires six steps with copepods and, probably, with any chitinous zooplankton. Liquid nitrogen and chitinase pretreatments may not be necessary for soft-bodied zooplankton. Once ready and mounted on the slides under a cover slip made air tight with nail varnish, samples can be stored at 5°C in the dark for 3 days and observed with the confocal microscope

several times, without bleaching. If the necrosis process can be ignored, this method is suitable for zooplankton (Appendicularian, Cladoceran, fish larvae, etc.) collected during oceanographic surveys; samples can be fixed on board the ship and stored for weeks in paraformaldehyde 4% prior to pretreatment, double labelling and fluorescent microscope examination in the laboratory. With Annexin V-FITC (Sigma), the cost is lower (3 Euros per sample) and the number of samples that can be treated is higher (100 per dose). In order to optimize the preparation of good-quality samples, the duration of both labelling and rinsing must be short (15–20 min). Samples must be fixed with paraformaldehyde 4% prior to microscope observation. Once preserved, these samples can be stored 3–4 weeks as specified above. We recommend this method for laboratory investigations of copepods and any small-sized zooplankton organisms. Its major advantage lies in the fact that it is cheaper and faster and that apoptotic and necrotic cell degradations can be diagnosed simultaneously.

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