Stazione Zoologica Anton Dohrn

Marine Biological Laboratory

marbef

MARBEF Advanced Course on

Chemical ecology and bioassay methods

Tjärnö Marine Biological Laboratory
Sweden, 9-14 September 2006

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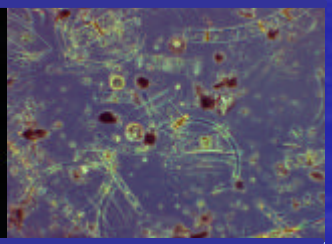
TOPICS: Chemical Ecology, Marine Natural Products, Ecotoxicology, Allelochemicals, Bioassays of natural compounds, Chemical defense, Deterrency, Trophic Interactions, Methods





More info and application forms: **www.marbef.org**
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Deadline for application: June30th, 2006

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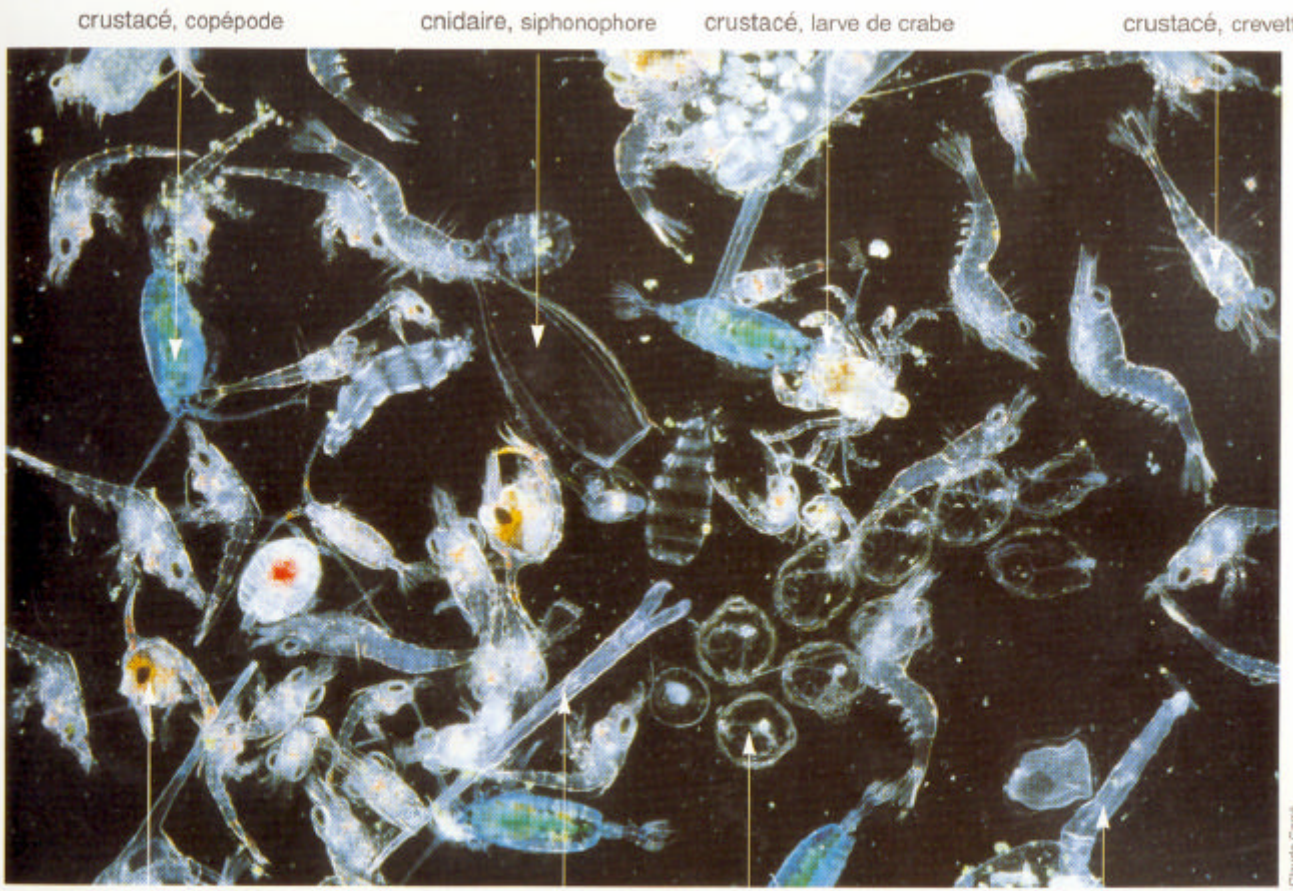
Bioassay Methods to Screen for Toxic Dinoflagellate Metabolites and the Effects of These Toxins on the Feeding and Reproduction of Copepods



Au menu : du plancton (x 8)

Les crustacés - larves et adultes - ainsi que les œufs et larves de poissons dominent dans le repas d'une méduse.

THE MARINE FOOD CHAIN

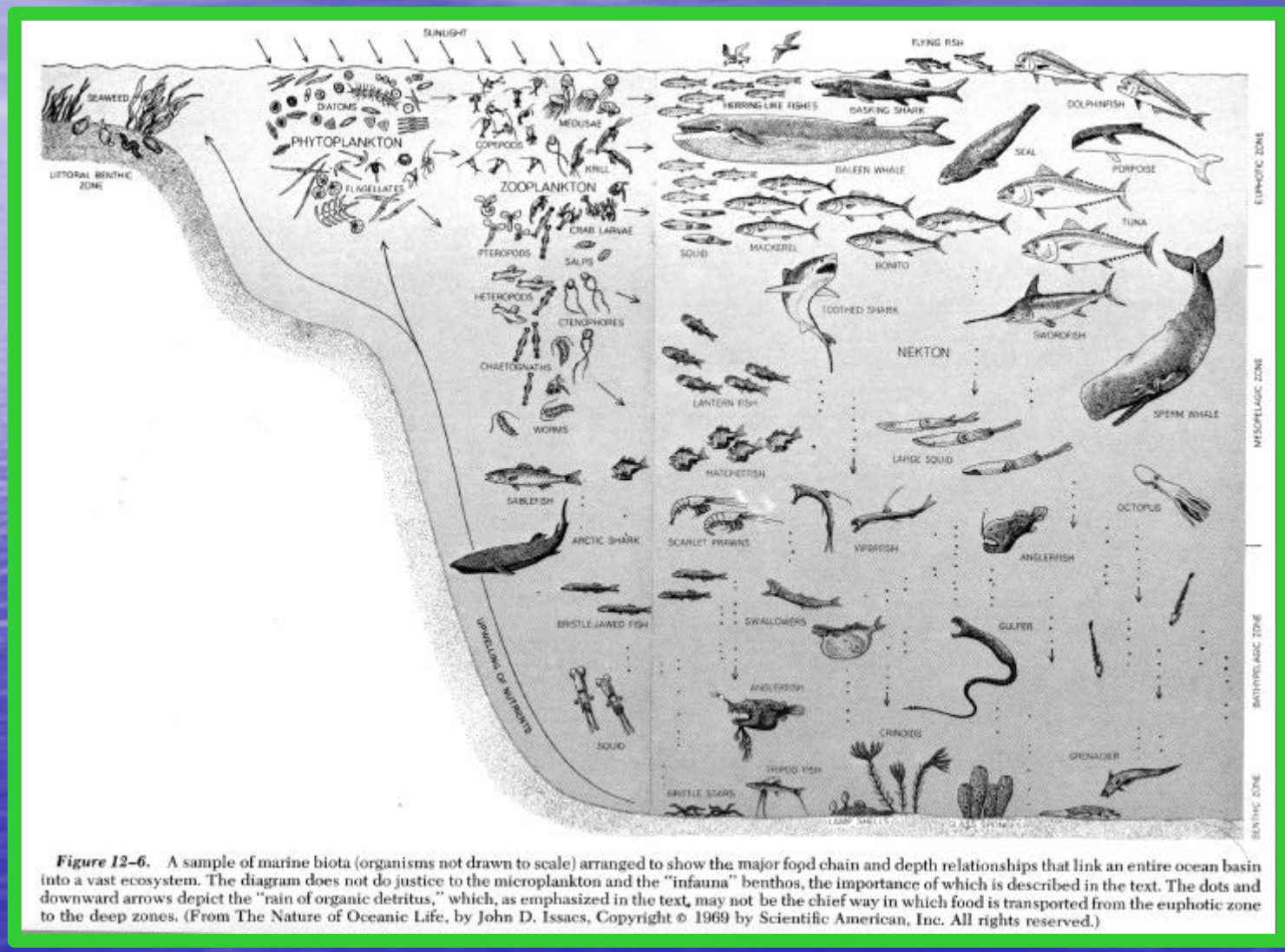
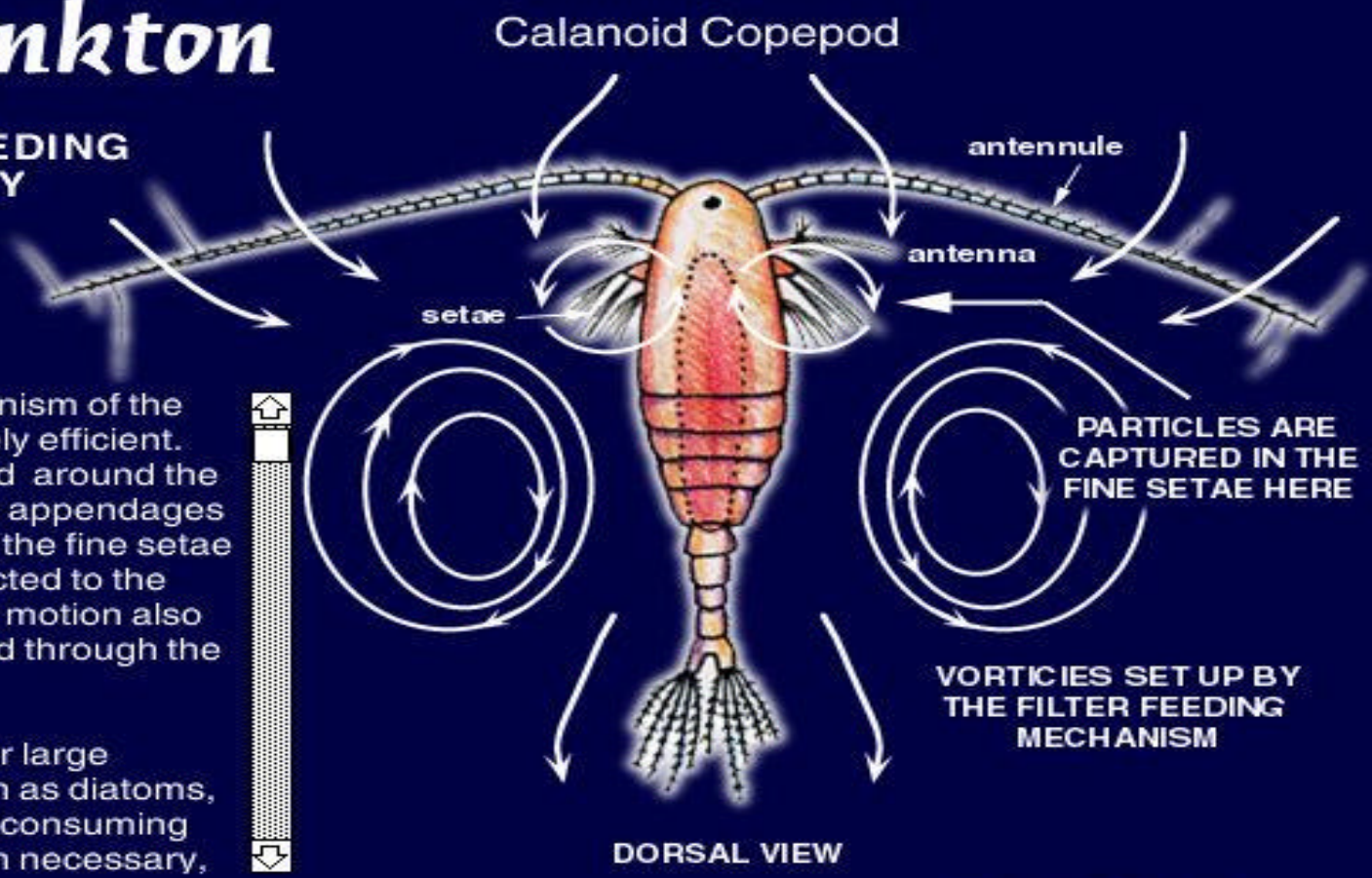


Figure 12–6. A sample of marine biota (organisms not drawn to scale) arranged to show the major food chain and depth relationships that link an entire ocean basin into a vast ecosystem. The diagram does not do justice to the microplankton and the "infauna" benthos, the importance of which is described in the text. The dots and downward arrows depict the "rain of organic detritus," which, as emphasized in the text, may not be the chief way in which food is transported from the euphotic zone to the deep zones. (From The Nature of Oceanic Life, by John D. Issacs, Copyright © 1969 by Scientific American, Inc. All rights reserved.)

From Giesbrecht's monograph on copepods, 1888

Zooplankton

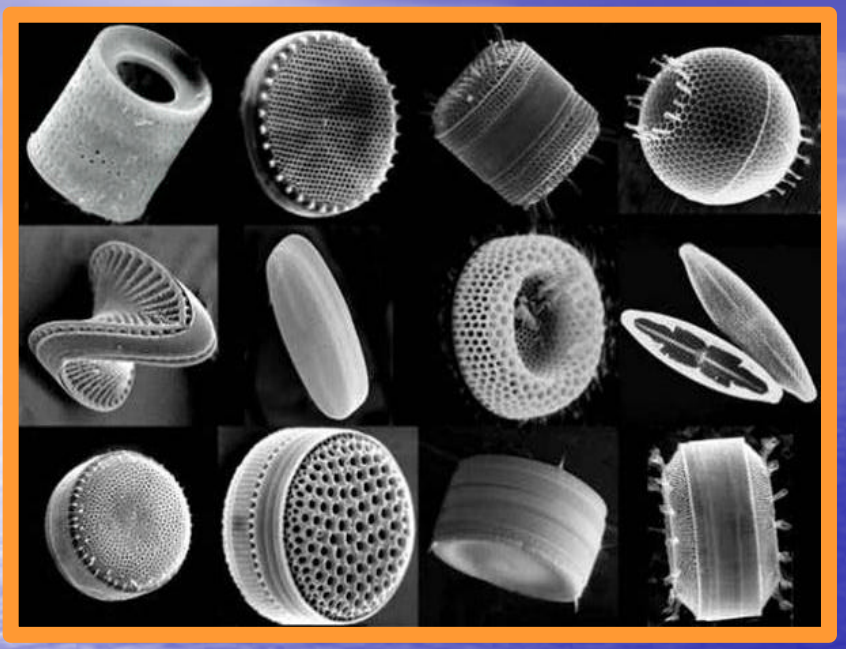
COPEPOD FEEDING STRATEGY

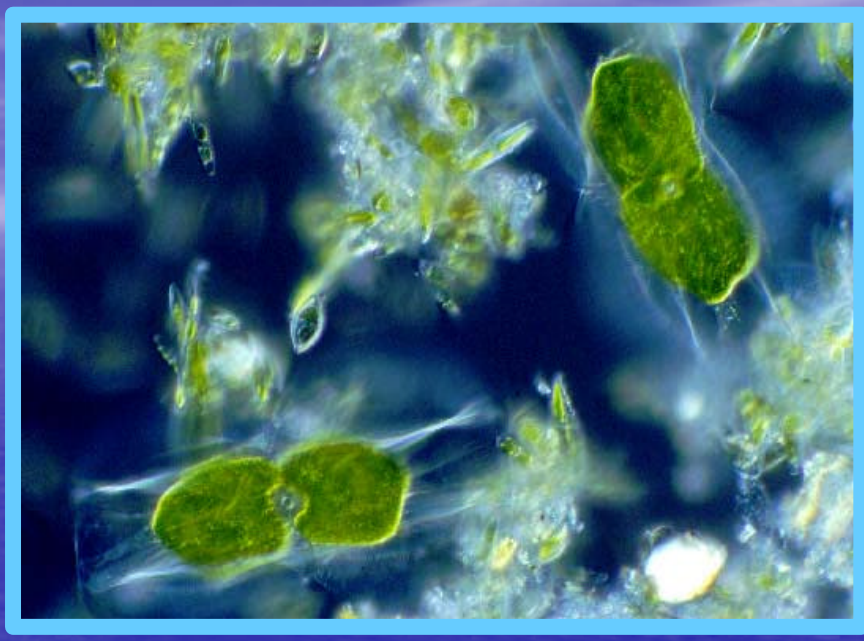


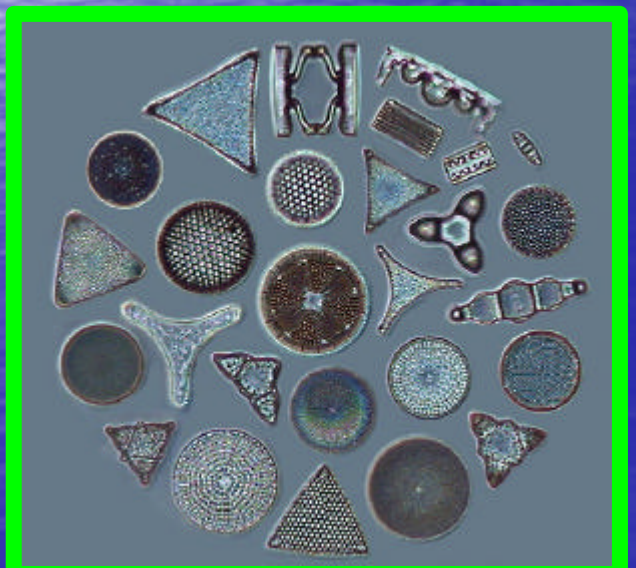
The feeding mechanism of the copepod is extremely efficient. The vorticies formed around the body by the feeding appendages direct particles into the fine setae which are then directed to the mouth. The feeding motion also propels the copepod through the water.

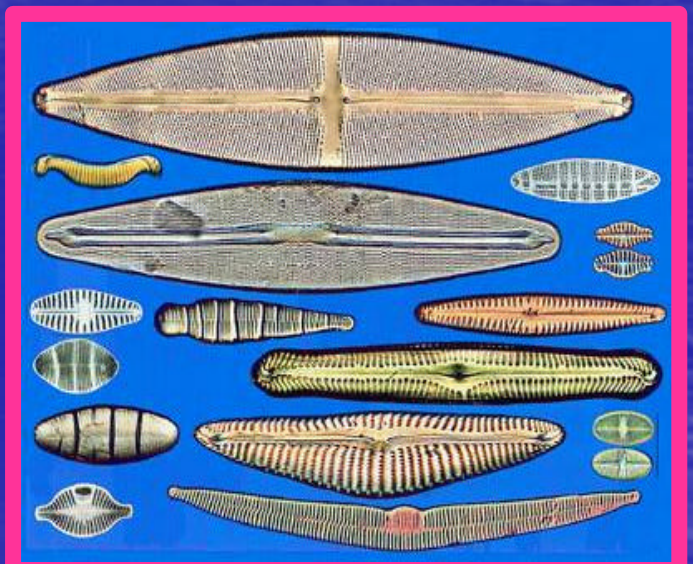
Although they prefer large phytoplankton, such as diatoms, they are capable of consuming small particles when necessary,

Diatoms

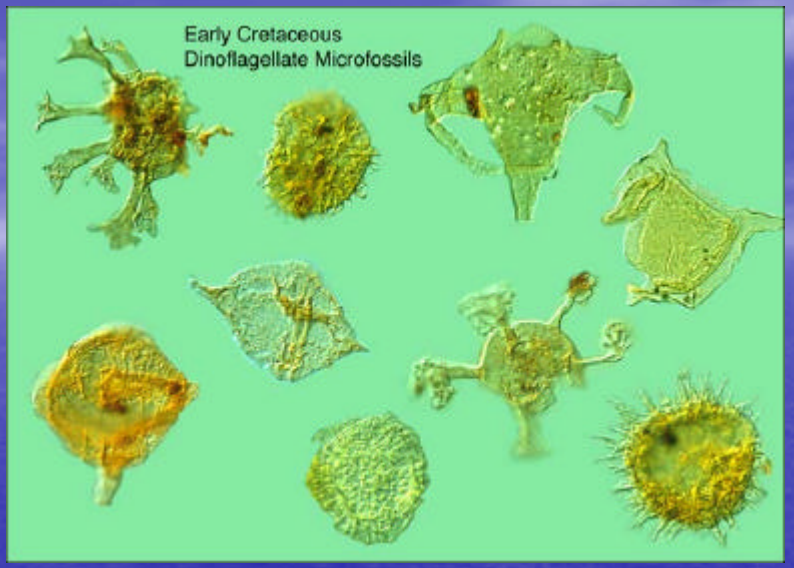


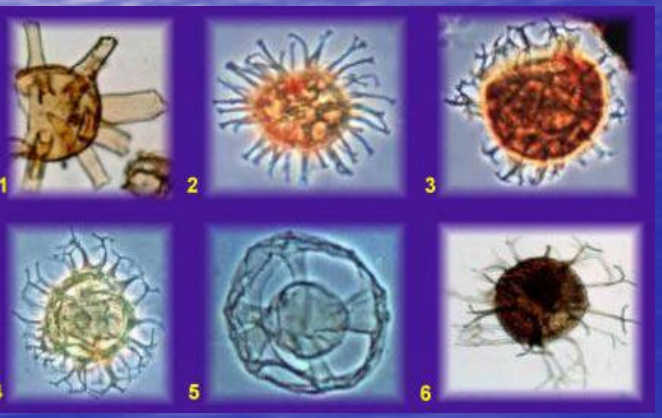


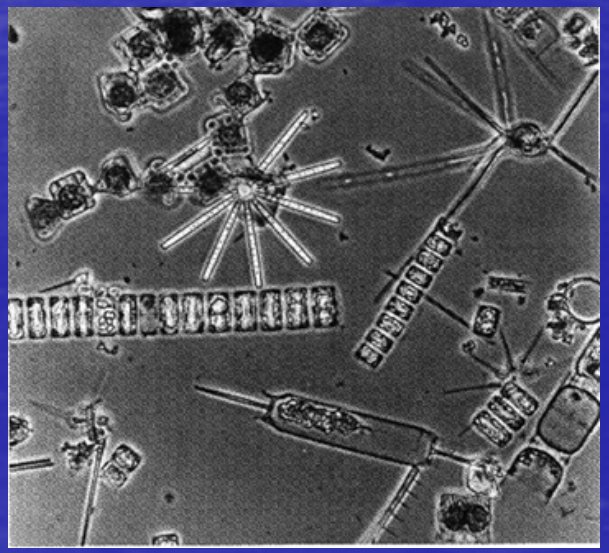




DINOFLAGELLATES

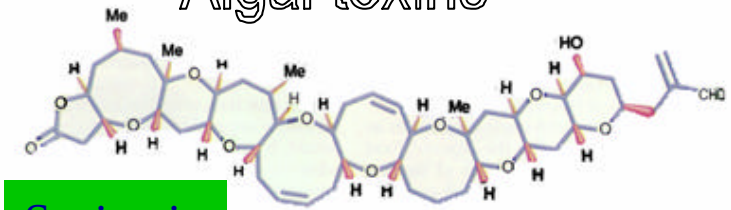






Harmful Algal Blooms

Algal toxins



Saxitoxin

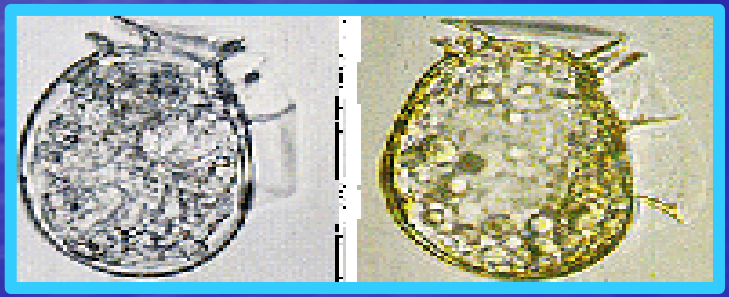
Okadaic acid

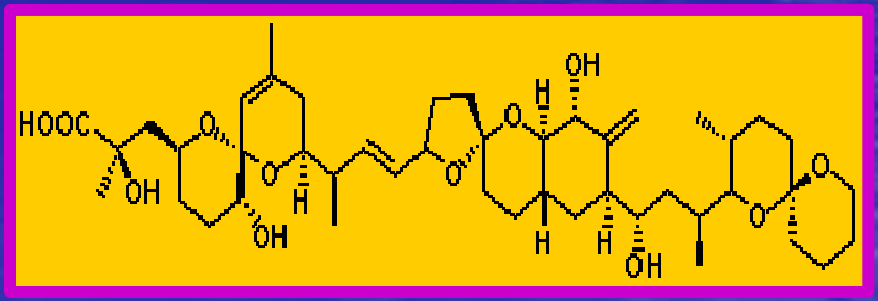
Okadaic acid is produced by several species of dinoflagellates, including species of the genus *Alexandrium*

Diarrhetic shellfish poisoning (DSP) has been reported from every continent except Africa and Australia. DSP has never resulted in a human fatality. As well as diarrhoea, other gastrointestinal symptoms include vomiting, nausea and abdominal cramps, possibly becoming so severe that the patient is incapacitated.

Apart from this acute effect, chronic exposure may promote cancer since it potently inhibits serine/threonine phosphatase enzymes.

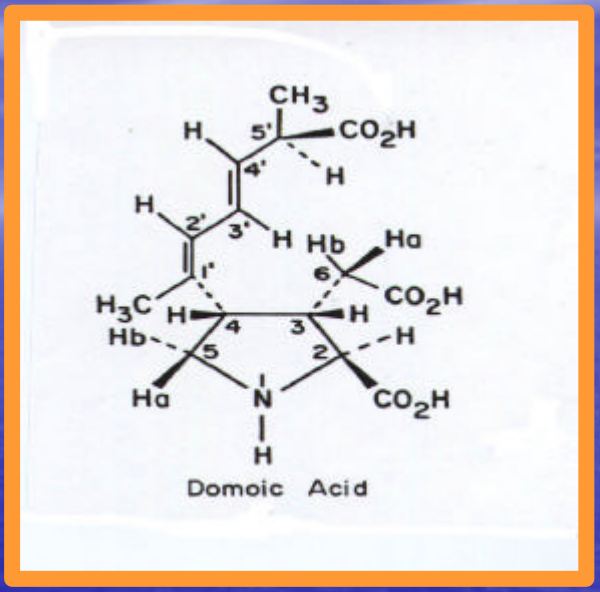
Recent research points towards effects upon testosterone levels in mammals.





DOMOIC ACID

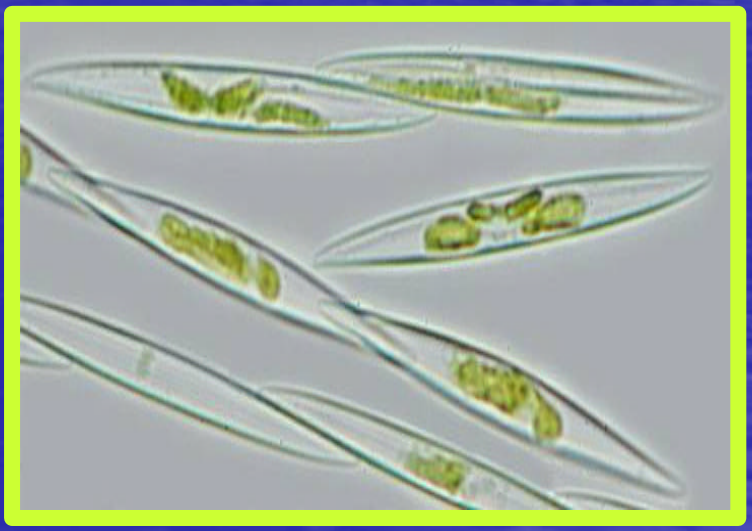
•Domoic acid is a naturally occurring toxin produced by microscopic algae, specifically the diatom species *Pseudo-nitzschia*. Significant amounts of domoic acid can cause Amnesic Shellfish Poisoning in humans



Domoic Acid

•In more severe cases, neurological symptoms develop within 48 hours and include headache, dizziness, confusion, disorientation, loss of short-term memory, motor weakness, seizures, profuse respiratory secretions, cardiac arrhythmias, coma and possibly death.

•Domoic acid has also been responsible for the deaths of hundreds of sea lions in southern California during the last few years. When sea lions (and to a lesser extent, dolphins) eat anchovies and other affected sea life that have fed on toxic diatoms, they become sick.



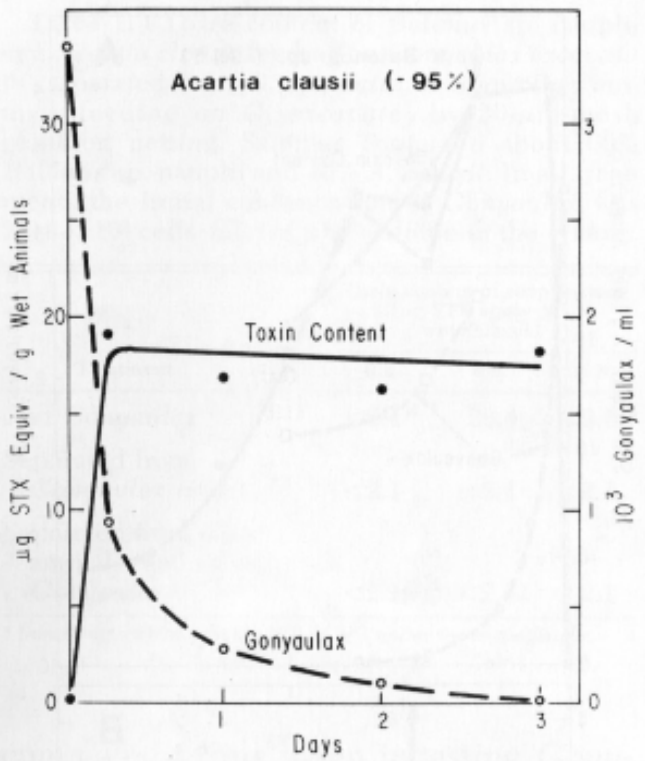


Fig. 1. Toxin content of *Acartia clausii* when feeding on *Gonyaulax excavata*, and removal of dinoflagellate cells. Zooplankton sample used for experiment contained about 95% *A. clausii*.

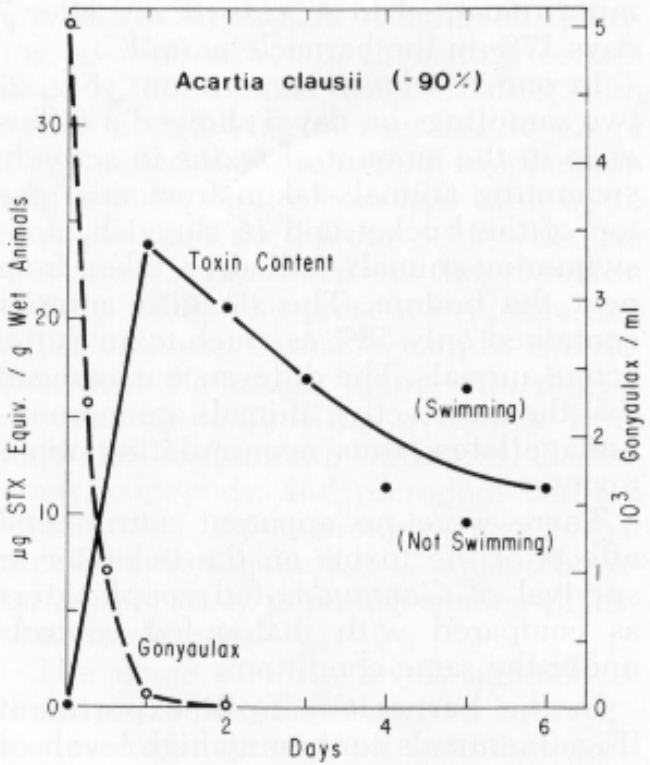


Fig. 2. As Fig. 1, but sample had about 90% *A. clausii*.

White A.W. – Limnol. Oceanogr. (1981) *Marine zooplankton can accumulate and retain toxins and cause fish kills*

Huntley et al. JEMBE (1982)
Yellow Water in La Jolla Bay, California, July 1980. II. Suppression of zooplankton grazing

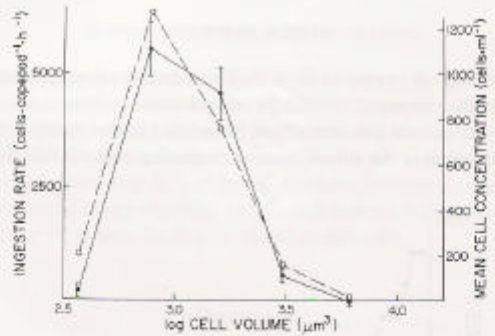


Fig. 3. Ingestion rates of *Calanus pacificus* females in the suspension of *Thalassiosira weissflogii* as a function of particle volume: the ingestion rates (cells · copepod^{-1} · h^{-1}, means (± 1 SD) of nine replicates) are shown as solid lines; dashed lines represent particle concentration (cells · ml^{-1}).

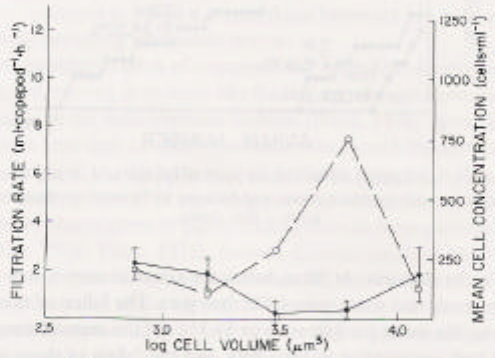


Fig. 4. Filtration rates of *Calanus pacificus* females in the *Gymnodinium flavum* bloom as a function of particle volume: symbols are the same as for Fig. 2.

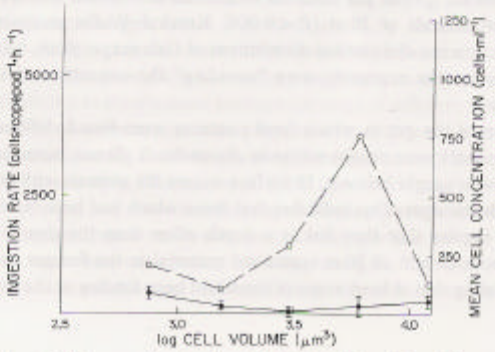


Fig. 5. Ingestion rates of *Calanus pacificus* females in the *Gymnodinium flavum* bloom as a function of particle volume: symbols are the same as for Fig. 3.

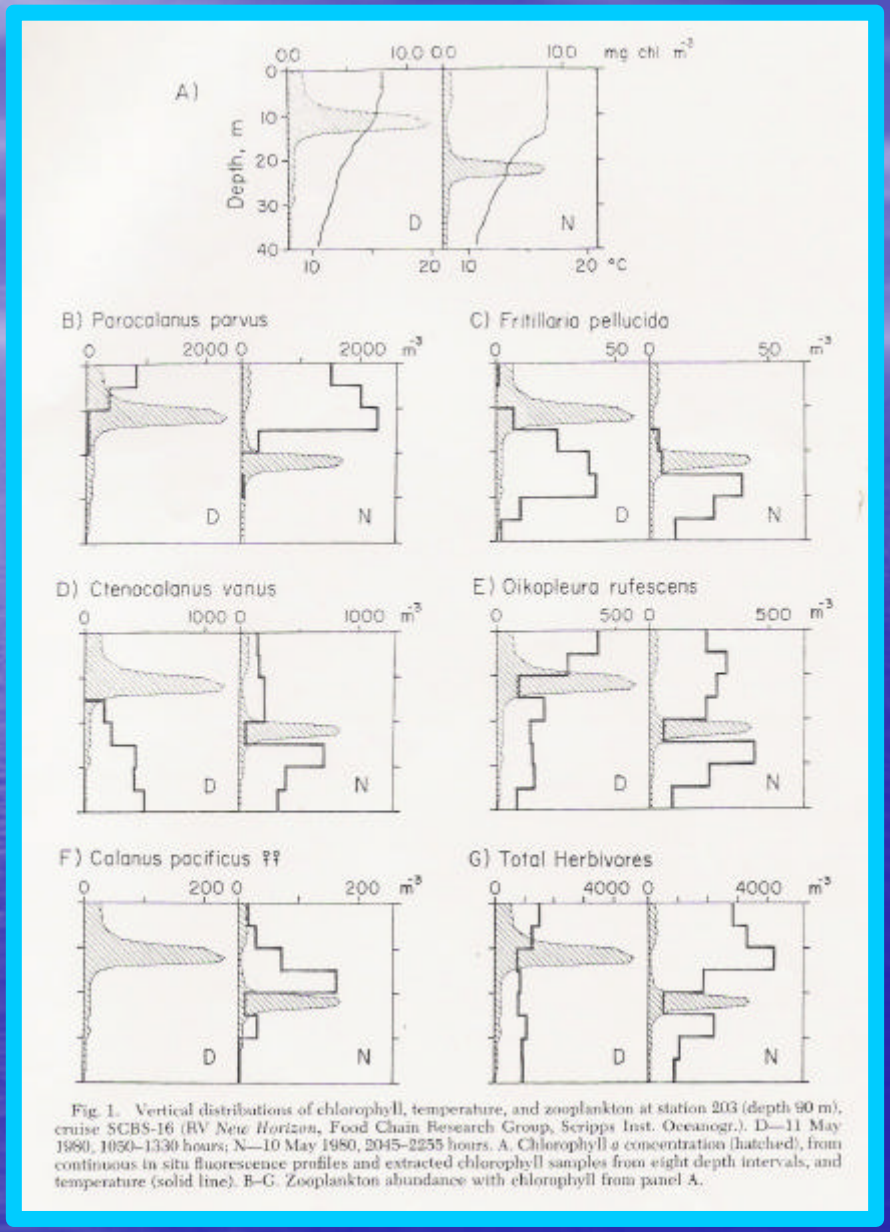


Fig. 1. Vertical distributions of chlorophyll, temperature, and zooplankton at station 203 (depth 90 m), cruise SCBS-16 (RV *New Horizon*, Food Chain Research Group, Scripps Inst. Oceanogr.). D—11 May 1980, 1050–1330 hours; N—10 May 1980, 2045–2255 hours. A. Chlorophyll *a* concentration (hatched), from continuous in situ fluorescence profiles and extracted chlorophyll samples from eight depth intervals, and temperature (solid line). B–G. Zooplankton abundance with chlorophyll from panel A.

Paul Fiedler Limnol. Oceanogr. (1982) *Zooplankton avoidance and reduced grazing responses to Gymnodinium splendens*

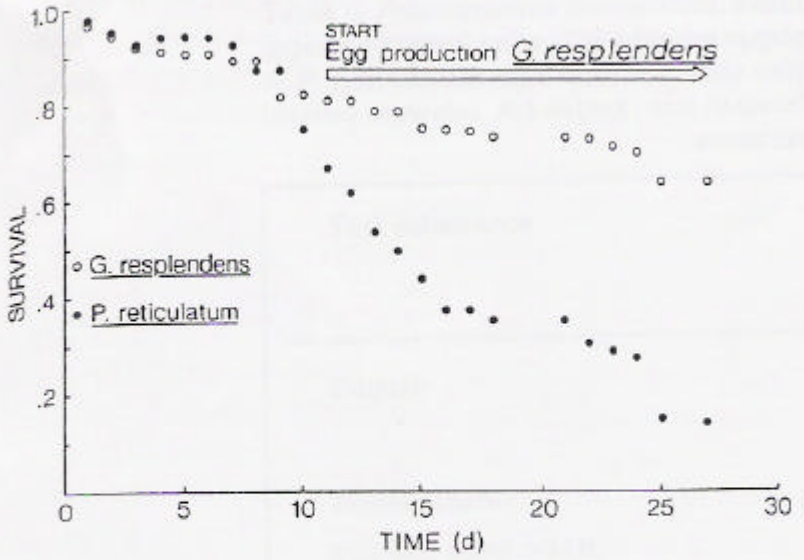


Fig. 4. *Calanus pacificus*. Comparative effects of bloom concentrations (500 µg C l^{-1}) of *Protoceratium reticulatum* and *Gyrodinium resplendens* on survival and egg production rate of adult females. Over a 30 d period, copepods exposed to *P. reticulatum* produced no eggs and experienced significantly greater mortality

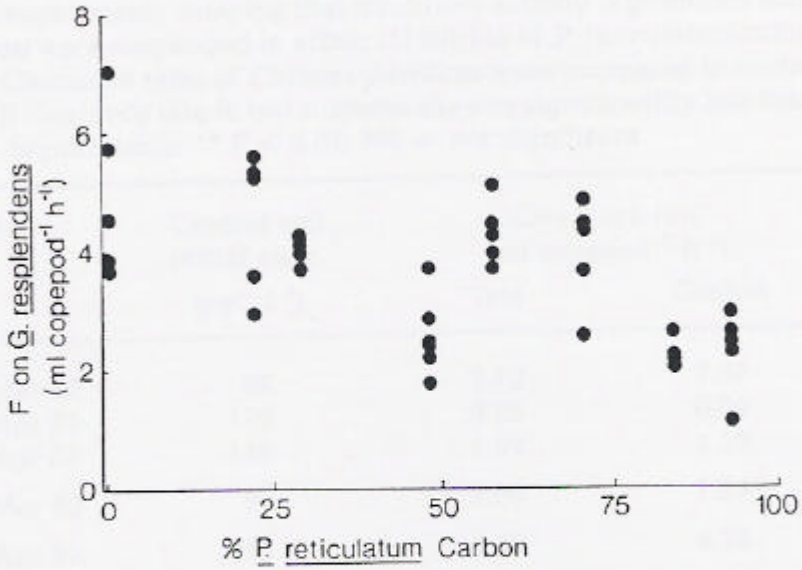


Fig. 5. *Calanus pacificus*. The presence of *Protoceratium reticulatum* suppresses feeding (F) on a normally edible dinoflagellate *(Gyrodinium resplendens)*. The total cell concentration was kept constant at approximately 200 µg C l^{-1} while varying the proportion of both cells in the suspension. As the proportion of *P. reticulatum* was increased the copepods removed edible cells at progressively lower rates

Huntley et al. MEPS (1986)
Chemically mediated rejection of dinoflagellate prey by the copepod Calanus pacificus

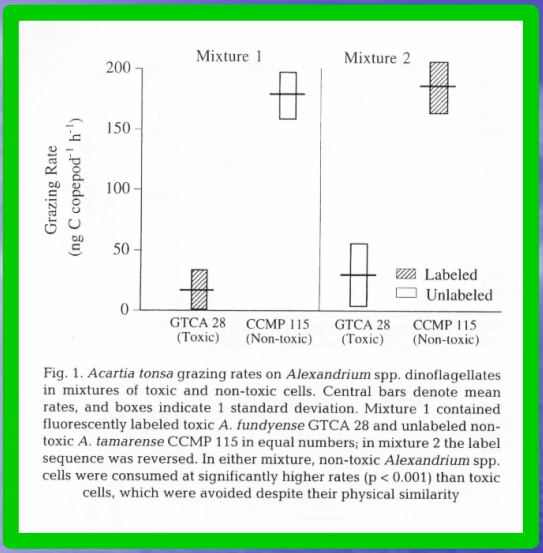


Fig. 1. *Acartia tonsa* grazing rates on *Alexandrium* spp. dinoflagellates in mixtures of toxic and non-toxic cells. Central bars denote mean rates, and boxes indicate 1 standard deviation. Mixture 1 contained fluorescently labeled toxic *A. fundyense* GTCA 28 and unlabeled non-toxic *A. tamarense* CCMP 115 in equal numbers; in mixture 2 the label sequence was reversed. In either mixture, non-toxic *Alexandrium* spp. cells were consumed at significantly higher rates ($p < 0.001$) than toxic cells, which were avoided despite their physical similarity

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Mixed dinoflagellate species grazing experiments

All 3 copepod species exhibited significantly different grazing responses in mixtures of dinoflagellate species depending on the presence or absence of toxin

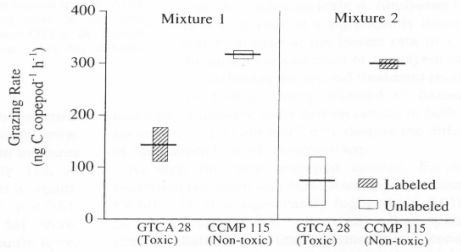


Fig. 2. *Centropages hamatus* grazing rates on *Alexandrium* spp. dinoflagellates in mixtures of toxic and non-toxic cells. Central bars denote mean rates, and boxes indicate 1 standard deviation. Non-toxic *A. tamarense* cells were consumed at significantly higher rates than toxic *A. fundyense* cells ($p < 0.001$). Nevertheless toxic cells constituted up to a third of the diet

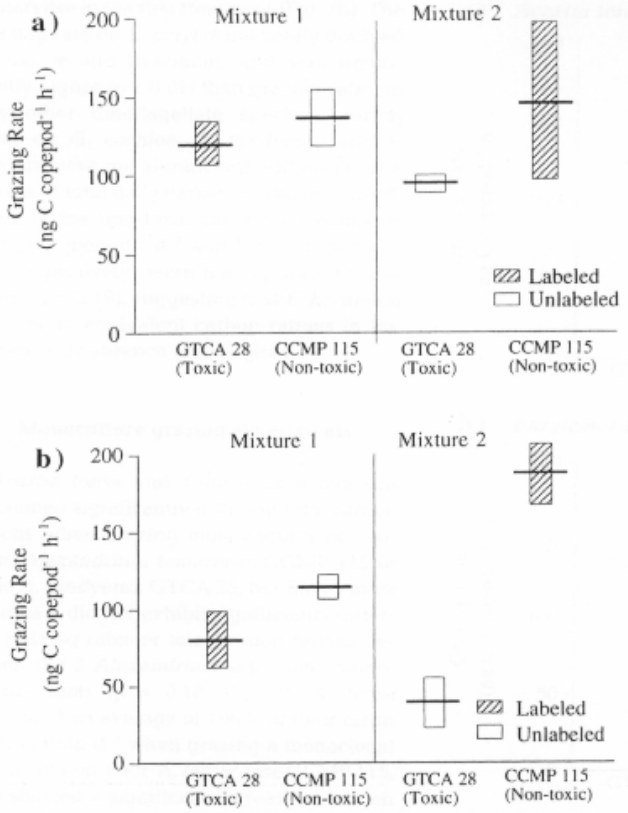


Fig. 3. *Eurytemora herdmani* grazing rates on *Alexandrium* spp. dinoflagellates in mixtures of toxic and non-toxic cells. Central bars denote mean rates, and boxes indicate 1 standard deviation. (a) The first trial resulted in no significant differences between grazing rates on toxic and non-toxic cells in a mixture ($p = 0.073$). (b) In the second trial, *E. herdmani* demonstrated significantly higher grazing rates on non-toxic *A. tamarense* CCMP 115 over toxic *A. fundyense* GTCA 28, though variability was high and the 2 mixture treatments were not identical (see 'Discussion')

Teegarden MEPS (1999) *Grazing of the toxic dinoflagellate Alexandrium sp. By adult copepods*

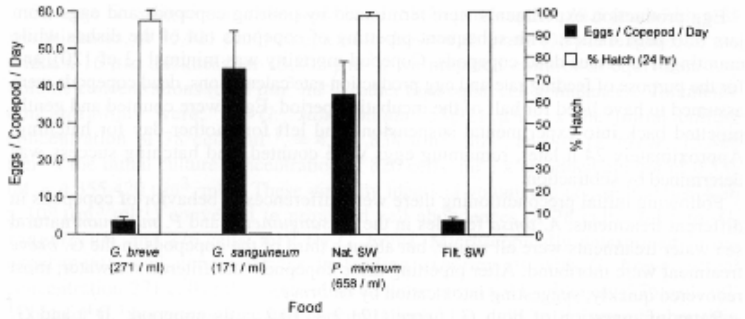


Fig. 1. Egg production and hatching rates for the copepod *Acartia tonsa* feeding upon diets of toxic *Gymnodinium breve*, non-toxic *G. sanguineum*, natural seawater dominated by *Prorocentrum minimum*, and filtered seawater, Beaufort, North Carolina, March, 1995. Microscopically-determined cell concentrations for food suspensions are given in parentheses.

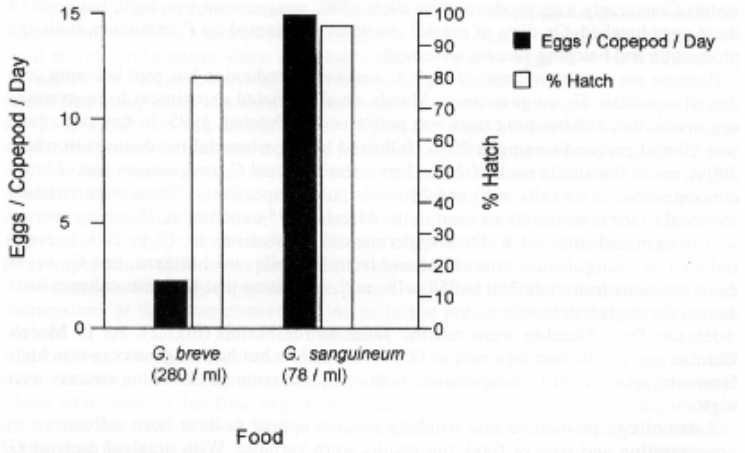


Fig. 2. Egg production and hatching rates for the copepod *Acartia tonsa* feeding upon diets of toxic *Gymnodinium breve*, non-toxic *G. sanguineum*, Beaufort, North Carolina, October, 1995. Microscopically-determined cell concentrations for food suspensions are given in parentheses.

Turner NATO ASI Series (1998) ***Interactions between toxic marine phytoplankton and the marine copepod Acartia tonsa***

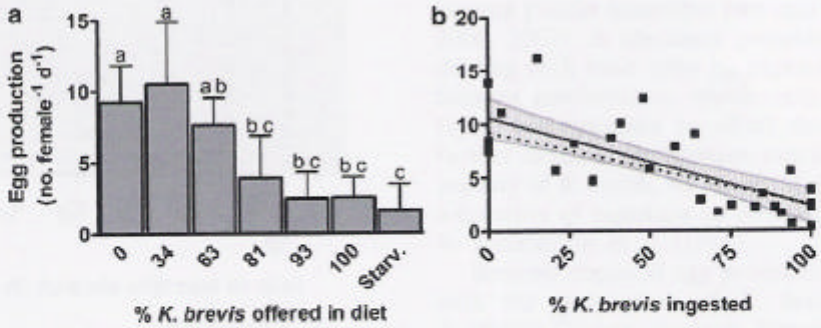


Fig. 4 Egg production rates for the copepod *A. tonsa* **a** fed combinations of *K. brevis* and *R. lens*, measured on Day 5 (mean ± SD, $n = 5$) and **b** as a proportion of *K. brevis* in the diet. *Letters* indicate significant grouping of treatments (ANOVA and Tukey–Kramer test) at $P \leq 0.05$. The *solid line* in **b**. represents linear regression of all data ($r^2 = 0.57$, $P < 0.0001$), with the *shaded area* representing 95% confidence intervals for this regression. The *dashed line* connects mean egg production of copepods fed 0% *K. brevis* to mean egg production of copepods fed 100% *K. brevis*

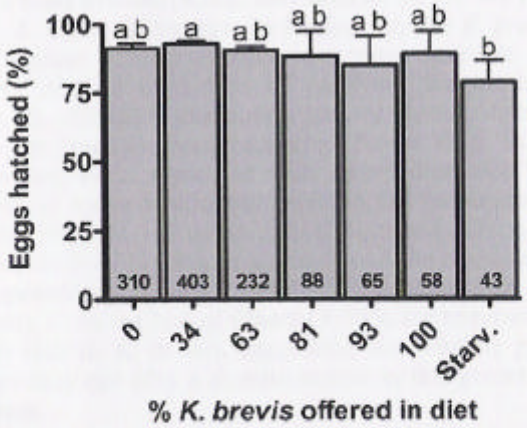


Fig. 6 Egg hatching success for the copepod *A. tonsa* fed combinations of *K. brevis* and *R. lens*, measured from eggs laid on Day 4 (mean ± SD, $n = 5$). *Letters* indicate significant grouping of treatments (ANOVA and Tukey–Kramer test) at $P \leq 0.05$. The total number of eggs used to determine hatching success is shown within the *bars*

Prince et al. Oecologia (2006) *Fitness consequences for copepods feeding on a red tide dinoflagellate Karenia brevis*

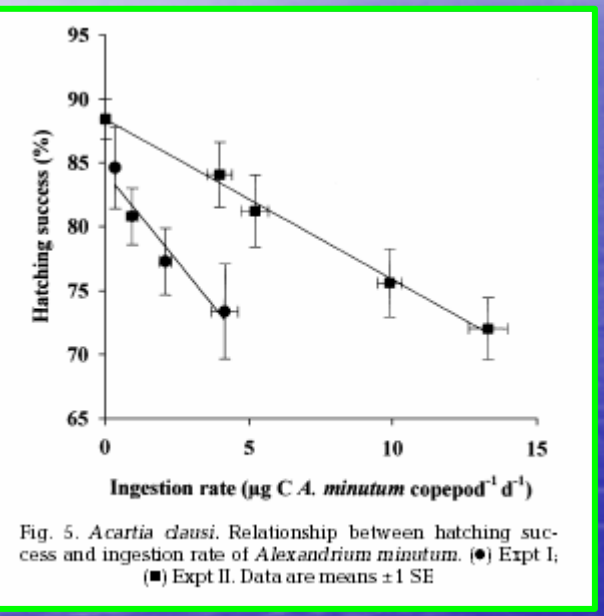


Fig. 5. *Acartia clausi*. Relationship between hatching success and ingestion rate of *Alexandrium minutum*. (●) Expt I; (■) Expt II. Data are means ±1 SE

rangopulos et al. MEPS 2000

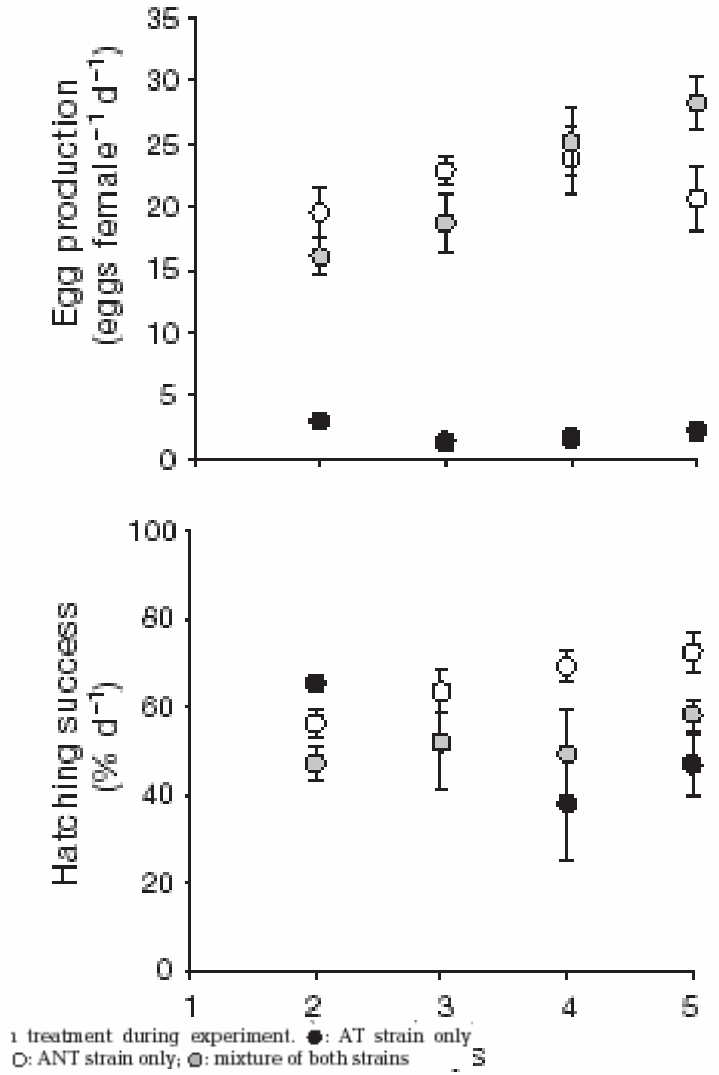


Fig. 6. *Acartia clausi*. Egg production and egg-hatching success duringexperimental period (mean ± SD, n = 3). Symbols as in Fig. 4

Barreiro et al. MEPS 2006

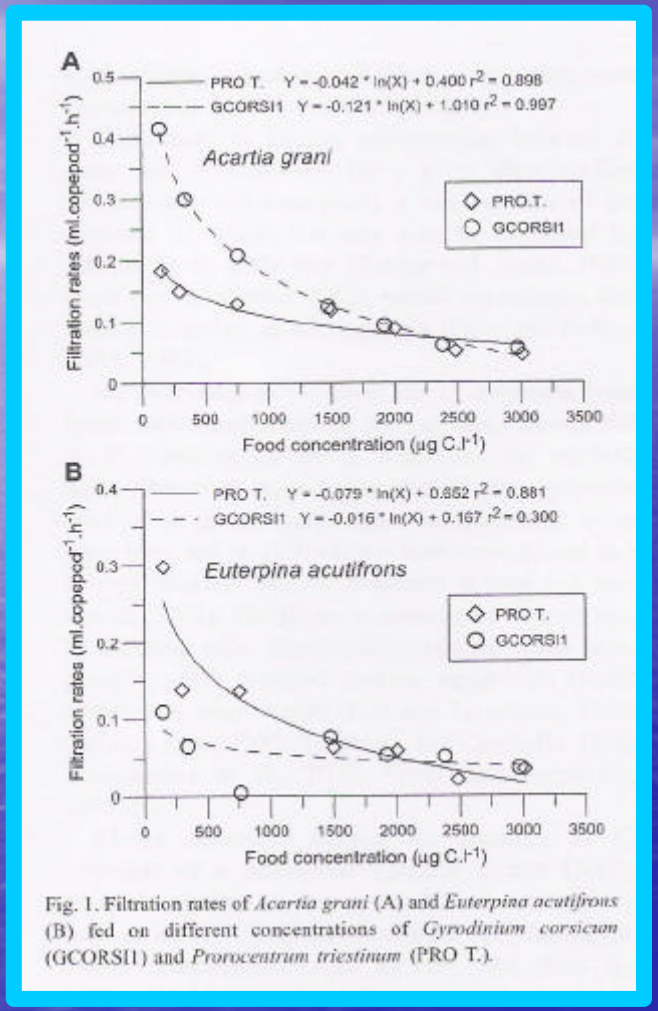


Fig. 1. Filtration rates of *Acartia grani* (A) and *Euterpina acutifrons* (B) fed on different concentrations of *Gyrodinium corsicum* (GCORSI1) and *Prorocentrum triestinum* (PRO T.).

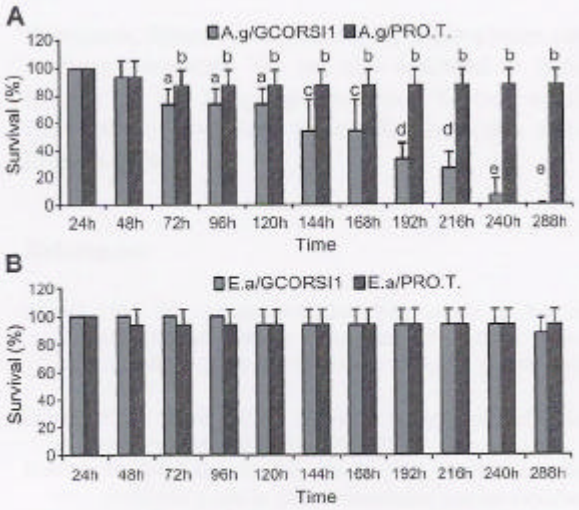


Fig. 2. Survival rates of *Acartia grani* and *Euterpina acutifrons* exposed to *Gyrodinium corsicum* and *Prorocentrum triestinum* for 288 h. Different letters represent statistical difference ($p < 0.05$) between treatments.

Marinho da Costa et al. JEMBE (2005) *Toxin content and toxic effects of the dinoflagellate Gyrodinium corsicum on the ingestion and survival rates of the copepod Acartia grani and Euterpina acutifrons*

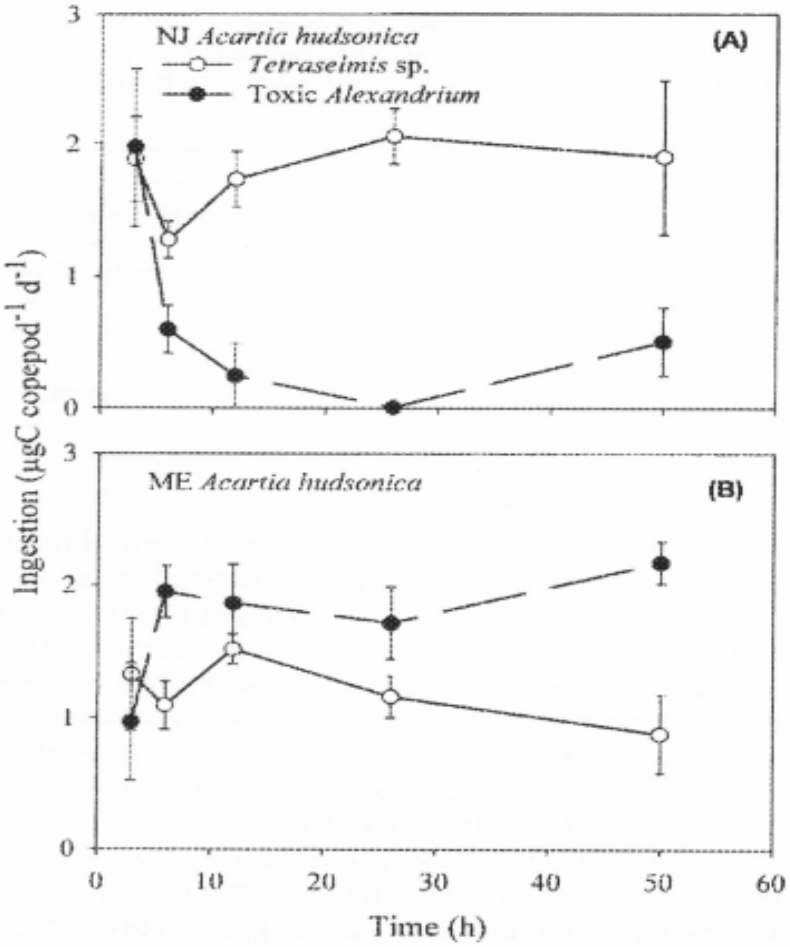


Fig. 1. Sole diet experiments. Mean ingestion rates vs time of (A) New Jersey (NJ), and (B) Maine (ME) female *Acartia hudsonica* offered sole diets of toxin-containing *Alexandrium fundyense* and the control alga *Tetraselmis* sp. measured from 3 h incubations. Points on plots represent the end time of each 3 h incubation. Error bars are SEs (n = 3)

S.P.Colin and H.G.Dam – Mar.Ecol.Prog.Ser. (2003)

Summary of Results

1) There is a high variability in the effects of dinoflagellate toxins on grazing and selectivity with effects ranging from severe physical incapacitation and even death, to more subte effects on swimming and feeding behavior, to no apparent physiological effects

2) Major variations have also been observed in the deleterious long-term effects of grazing upon toxic dinoflagellates in terms of reduced fecundity, hatching success and larval survival

3) Most of the acute effects demonstrated in laboratory experiments (death, incapacitation, altered swimming behavior, reduced fecundity and gamete viability) have not been observed directly in nature

4) Some toxins (domoic acid) have never been shown to induce negative effects on planktonic organisms that consume cells that produce such toxins

5) Some copepods show greater resistance to dinoflagellate toxins than others (i.e. detoxification mechanisms)

So what is the function of these toxins?

The putative function of dinoflagellate toxins is often assumed to be chemical defense

- Noxious compounds could be produced to act as feeding deterrents.

- Often their main purpose would not be to intoxicate the predator but to discourage "tasting" or to initiate rapid release of the prey following physical handling and capture.

- Such contact signals could be sequestered on the surface of the cell or released into the medium

- Deterrence could also be expressed at the post-ingestive stage after crushing of prey cells. The predator response could be regurgitation or at least discouragement of further consumption

On the other hand……

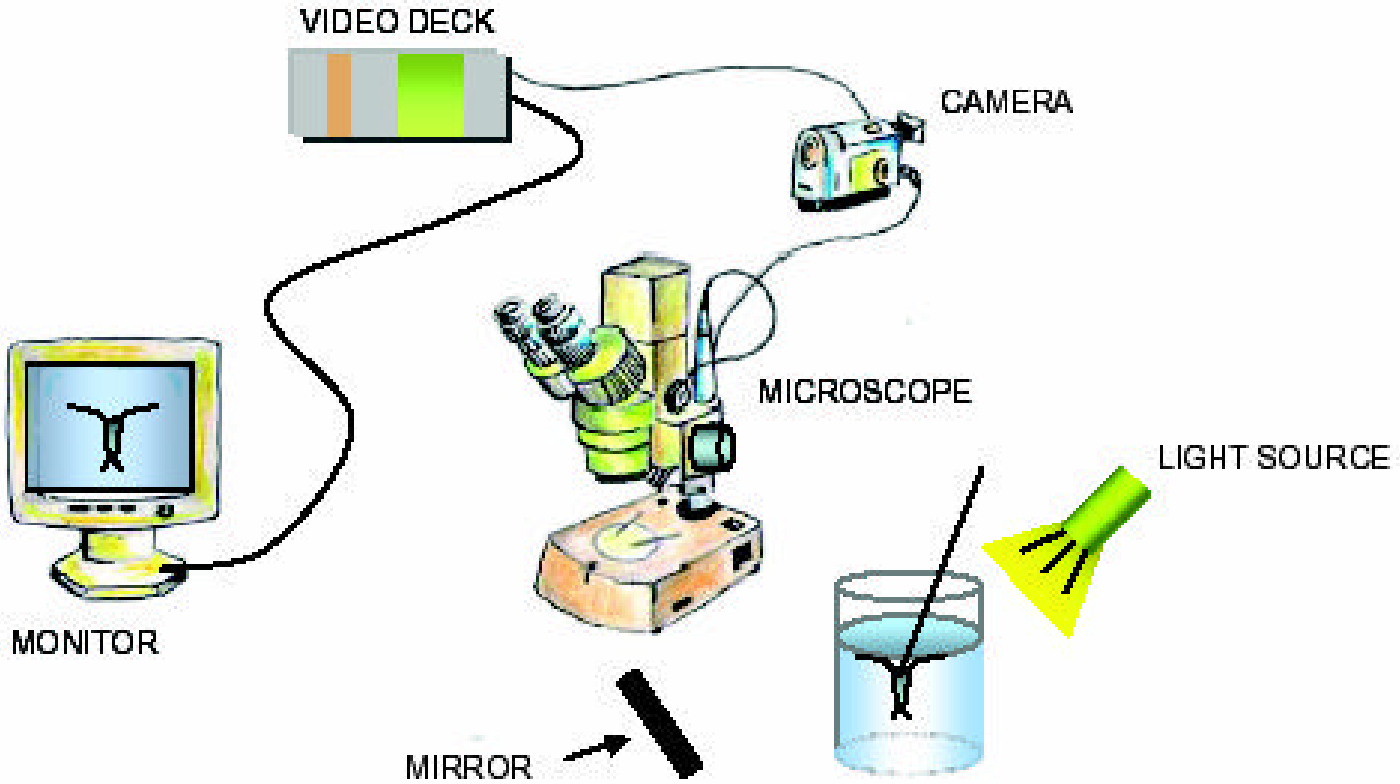
Postingestive release of toxic compounds from the prey cells may also result in physical incapacitation (paralysis, altered swimming behaviour etc..) or mortality of the predator

Finally, "stealth compounds" of low acute toxicity to adult or mature predators could lead to postingestive reduction in fecundity or depressed viability of gametes ("kill the children" selection, according to Cembella 2003)

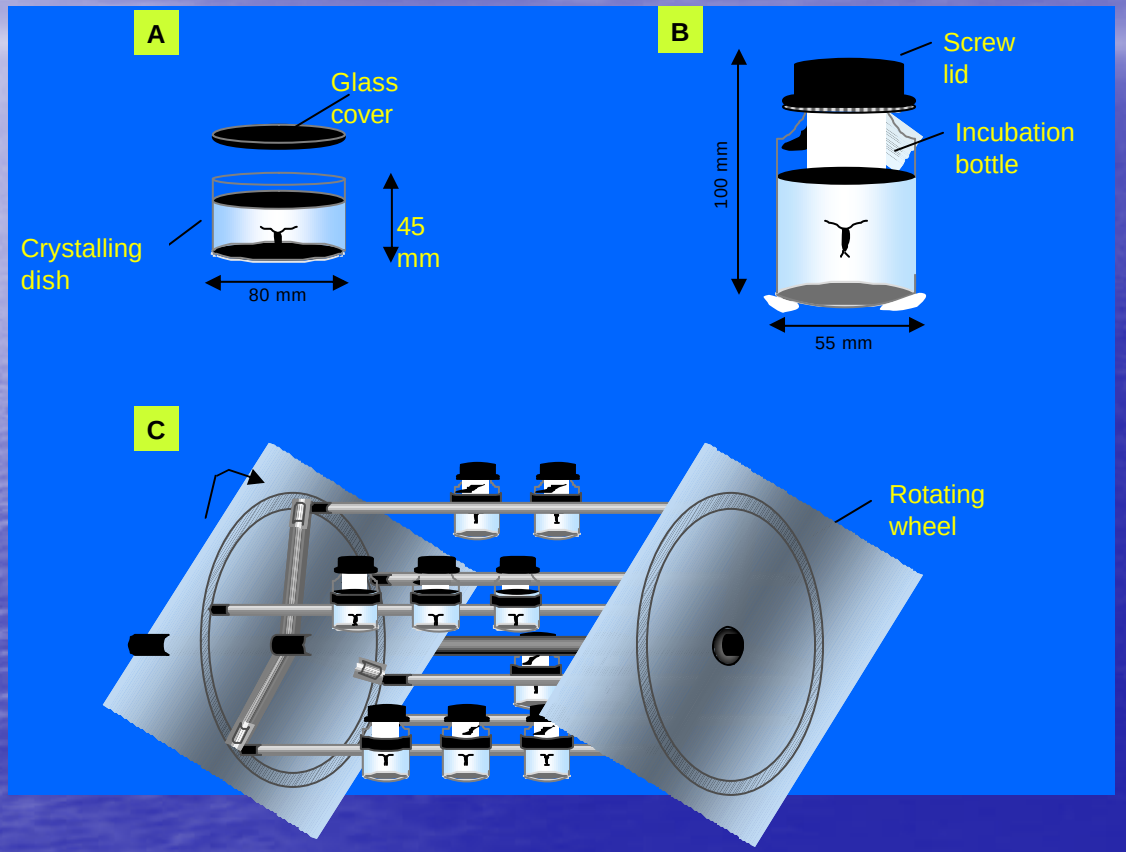
Postingestive deterrence would obviously be ineffective for the protection of the individual cell but group defence would be maintained on a community basis

The end result would be that blooms would grow and persist when grazing pressure would otherwise have caused them to crash

Bioassay method to study copepod feeding behavior



Bioassays to study copepod feeding, egg production, egg hatching success and larval development



In these assays copepods are incubated either in (A) crystallizing dishes or in (B) jars filled with known quantities of food. Experimental jars are mounted on a (C) rotating wheel (0.5 rpm) in a controlled temperature room and on a fixed dark:light cycle.

Common methods used to quantify feeding rates in copepods

1) **Food removal methods**
   main pros: direct method, allows inferences on size and taxon selectivity
   main cons: bottle effects (crowding, sedimentation, food chain effects)

2) **Gut fluorescence methods**
   main pros: not too labor intensive
   main cons: difficulty of determining realistic values of pigment destructio and gut evacuation constants

3) **Radiotracer methods**
   main pros: minimized incubation stress, useful when grazers and food is small
   main cons: variable uptake rates in natural assemblages causing problems in conjunction with selective grazing

For further information consult ICES Zooplankton Methodology

Common methods to determine egg production rates in copepods

1) "Egg production" or "Direct observation" method has its origin in the early work of Marshall and Orr (1950s) who studied egg layin of Calanus sp. by observing females placed singly into small dishe containing seawater
   Underpinning the egg production method is the postulate that the egg laying observed immediately after capture accurately reflects the spawning behaviour of counterparts in the sea during the same period
   Investigators have concluded that egg laying observed in the first 24h is a valid refelction of in situ behaviour

2) Edmondson egg ratio method estimates egg production rates per female from egg and female densities at sea at the time of collection
   This method is generally not adopted because it often underestimates production

Egg viability

1) After the incubation period, the females are removed and a known quantity of eggs are left for a period sufficient to allow hatching - generally 72h or more, depending on the temperatur

2) At the end of this period, nauplii and any remaining eggs can be fixed in alcohol or formaldehyde and counted

3) Because eggs without properly formed membranes may disintegrate quickly, the number of eggs at the beginning of the incubation rather than the sum of eggs and nauplii at the end, more accurately represents the initial number in the calculation of hatching success

4) In order to attribute hatching failure to accumulation of toxins in females, the possibility of unfertilized or resting eggs must first be ruled out

Incubation containers and density of females

1) Cannibalism is potentially a major source of error in the estimation of egg production rates (i.e. can result in an underestimation of egg production rates by a factor of 5-10)

2) Error due to cannibalism can be minimized by use of containers equipped with a partition screen but such containers may damage eggs and are not recommended for studies on egg viability

3) Several studies have reported a "crowding" effect (the higher the female density, the lower the egg production rate) due to several factors (higher rates of predation on eggs, increased physical disturbance, chemical cues)

4) There is no evidence of an effect of incubation volume on egg laying but very small containers are not recommended

Duration of incubation

1) 24h experiments have generally been employed to study the effects of secondary metabolites on feeding behavior and selectivity

2) Longer incubations become increasingly dependent on incubation conditions (food, temperature etc...)

3) Longer incubations are generally necessary to study the the long-term effects of secondary metabolites on feeding and egg production rates

Temperature and Light Regime

1) Protocols for egg production measurements usually call for incubations at ambient temperatures

2) There is no evidence that immediate post-capture egg laying is affected over a wide range of incubation temperatures

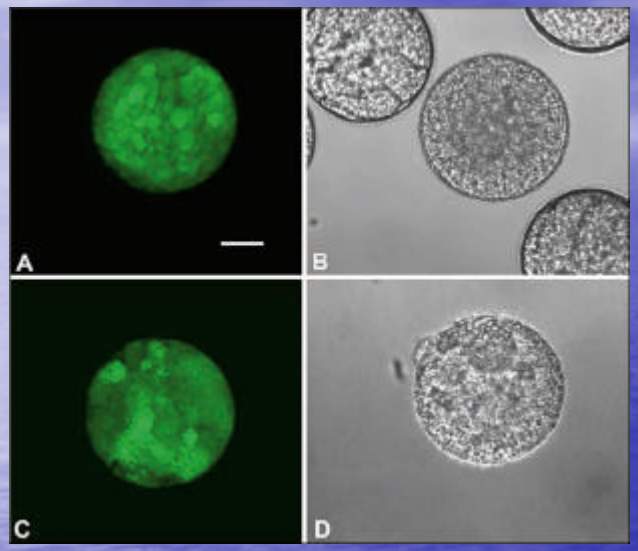
3) Longer incubations require preliminary experiments to determi the temperature range over which egg laying is stable

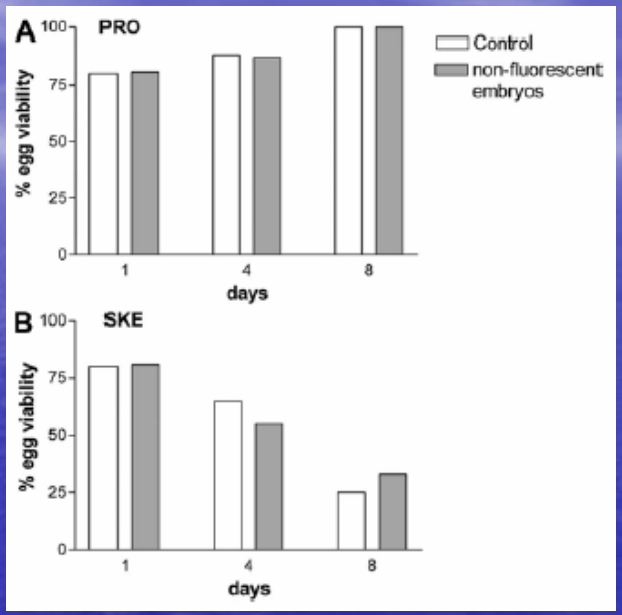
4) There is little indication that the light regime during incubation influences the 24h egg laying

5) Most investigators simulate the ambient light cycle or 12L:12D regime

Molecular probes for the detection of viability and apoptosis

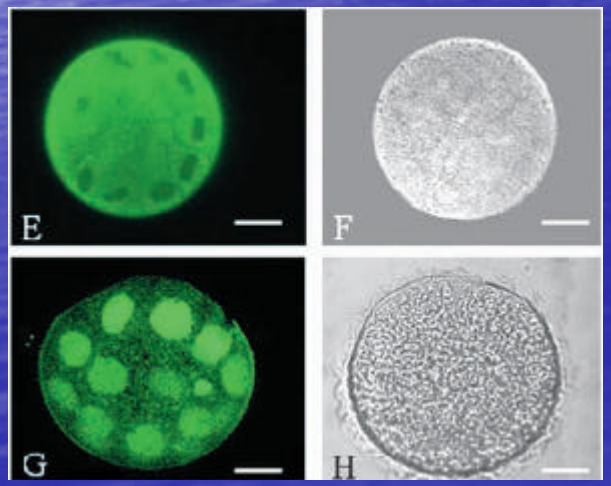
Copepod egg stained with Sytox Greeen



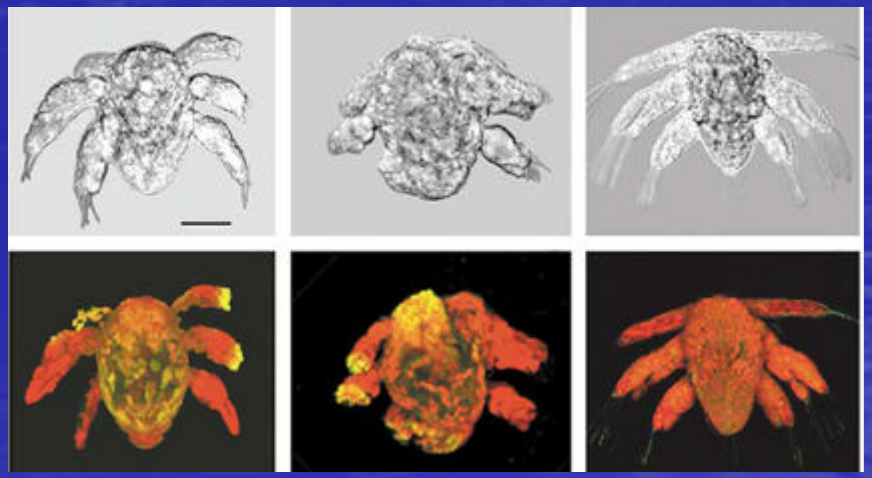


Buttino et al. 200

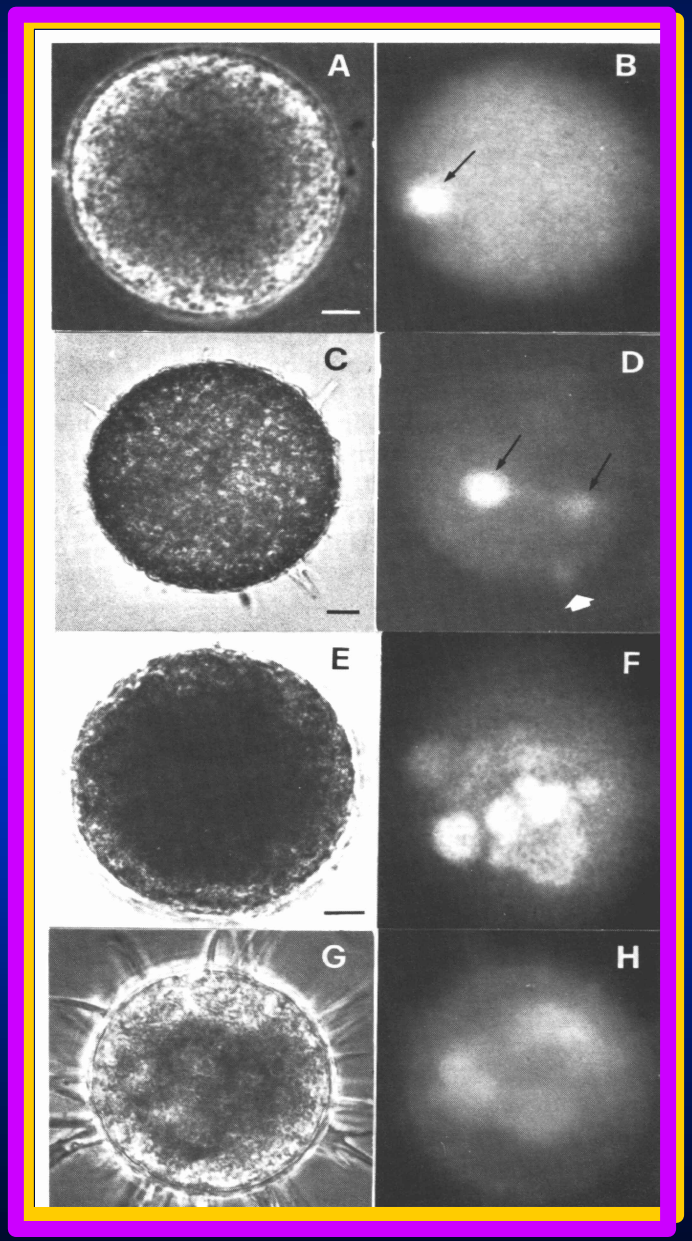
Copepod egg stained with TUNEL



Copepod nauplii stained with TUNEL

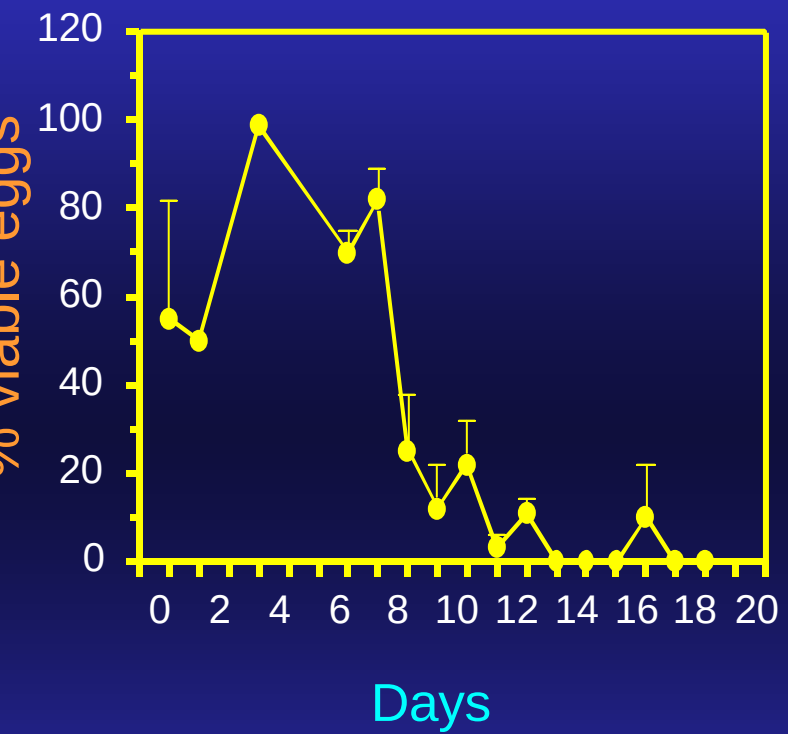


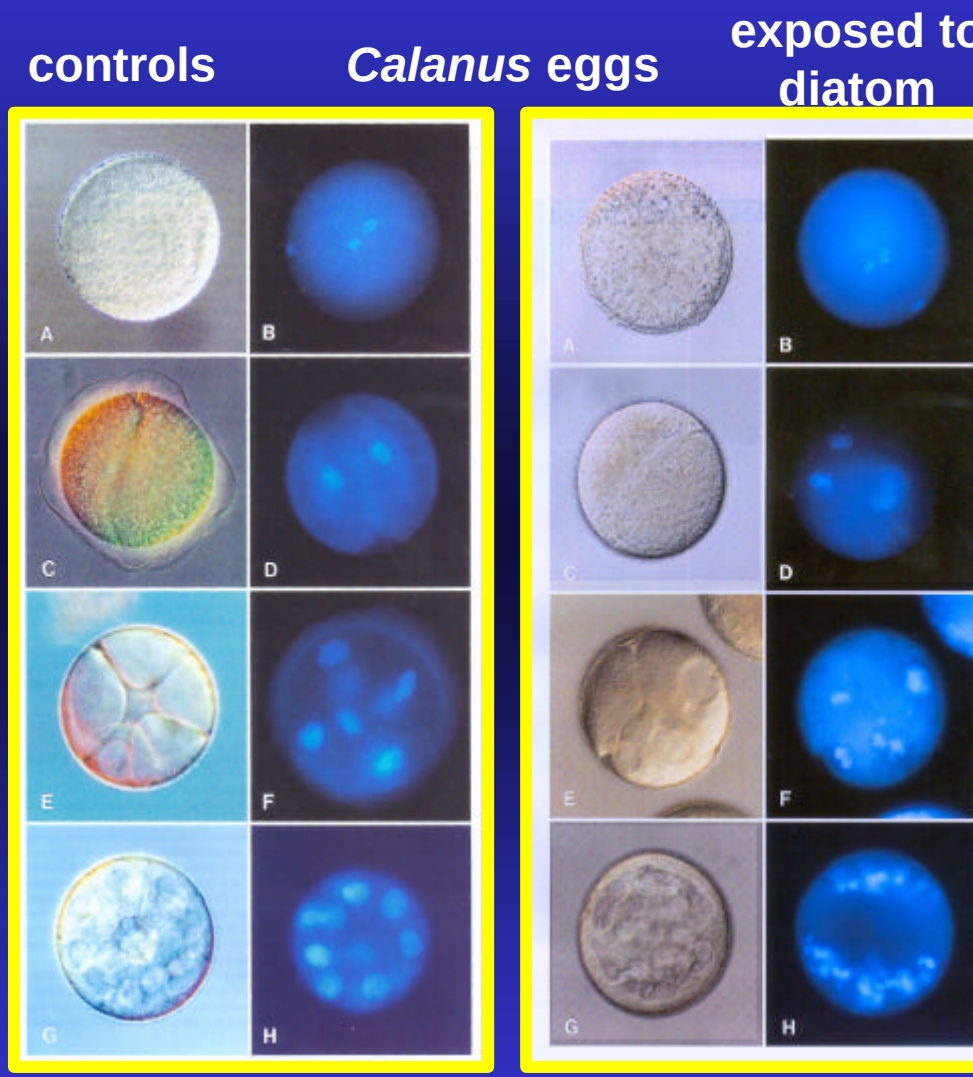
Centropages typicus fertilized and non-fertilized eggs



from Ianora et al. 1992

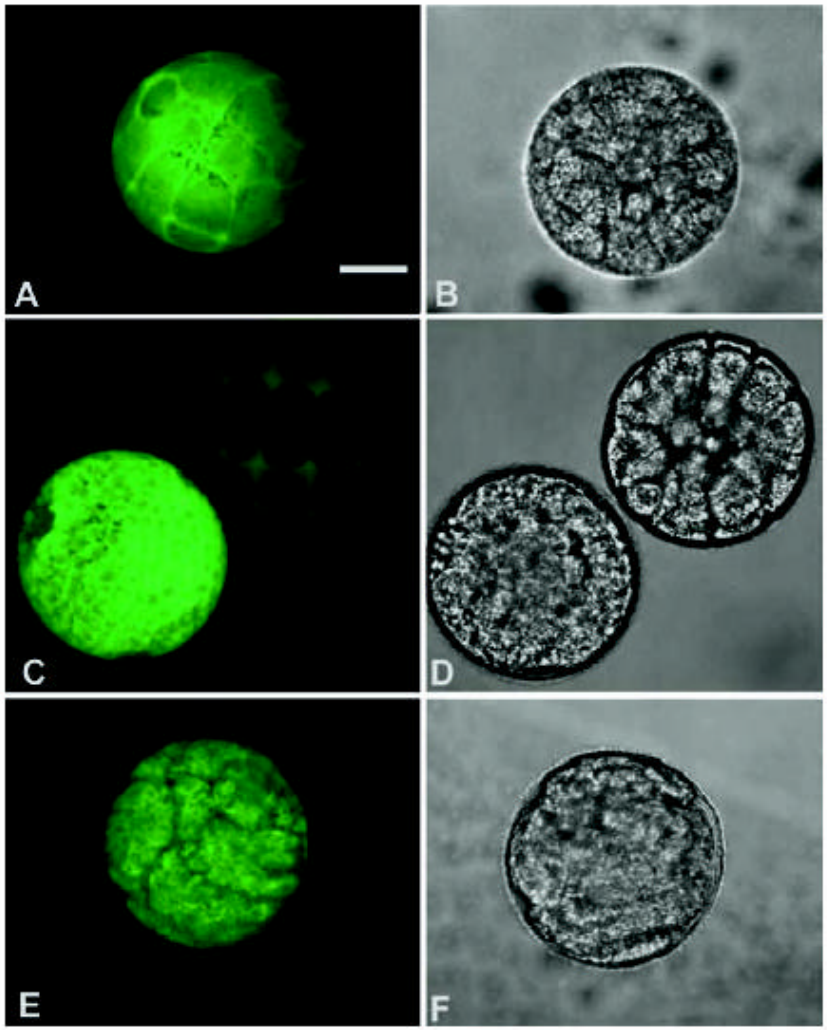
Calanus helgolandicus fed *Phaeodactylum tricornutum*





from Poulet et al. 1995

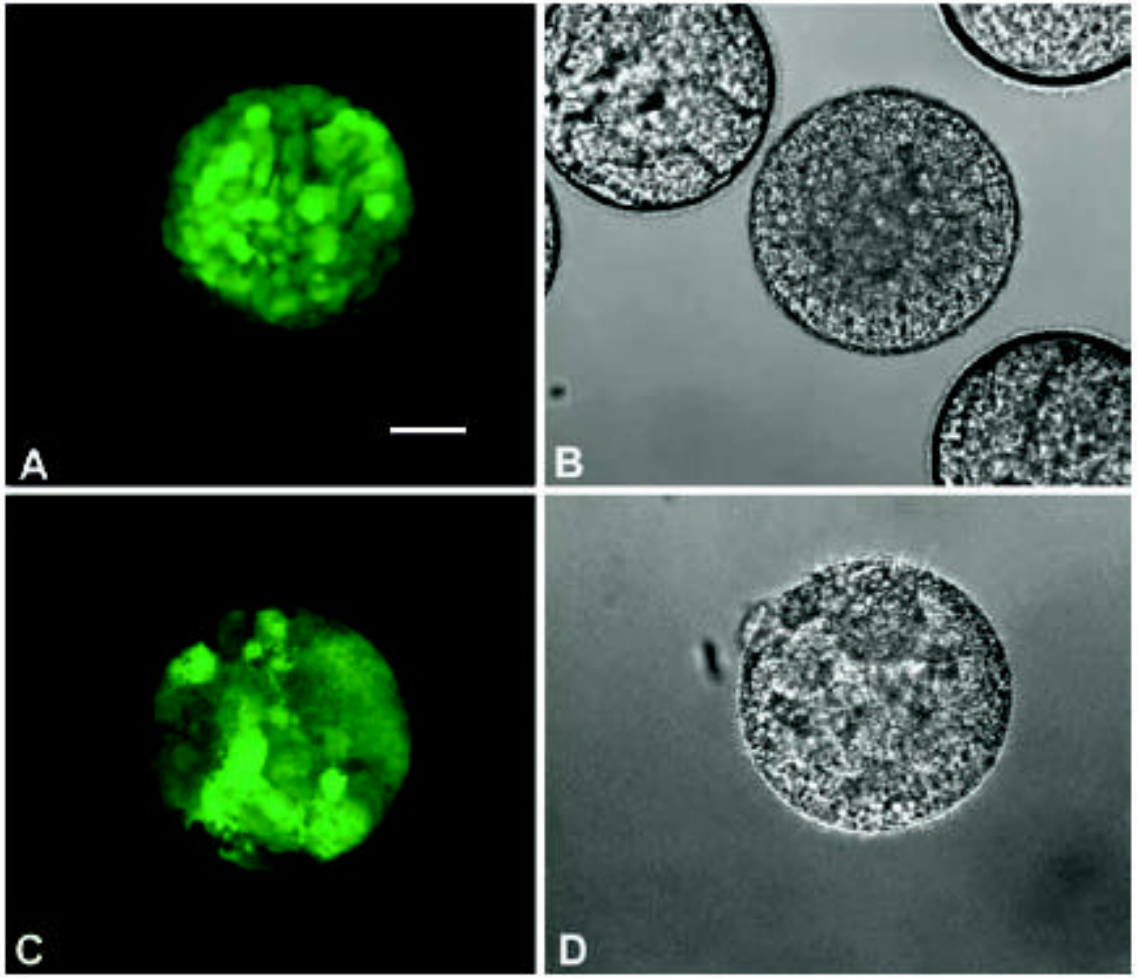
Fig. 1A–F *Calanus helgolandicus*. Viable embryos stained with fluorescein diacetate and observed with the confocal laser scanning microscope. **A** Fluorescent 3D image of a 64-cell-stage embryo. **B** Single focal plane of the same embryo in panel A observed in transmitted light. **C** 3D-reconstructed image of a fluorescent embryo at the gastrula stage (*left*), and a non-fluorescent 32-stage embryo (*right*). **D** The same embryos as in panel C observed in transmitted light on a single focal plane. **E** 3D reconstruction of a developed viable embryo before hatching. **F** The same embryo as in panel E observed in transmitted light on a single focal plane. *Scale bar*: 64.3 µm



When FDA penetrates into viable cells, esterases produce free fluorescent fluorescein and cells appear fluorescent in green, whereas cells with an inactive metabolism are not fluorescent.

Buttino et al. Mar. Biol. 2004

Fig. 2A–D *Calanus helgolandicus*. Non-viable embryos stained with SYTOX green and observed with the confocal laser scanning microscope. **A** Fluorescent 3D image of non-viable morula embryo. **B** Transmitted light of the same field as in panel A; the darker embryo at the center of the field is positively stained with SYTOX green. **C** Fluorescent 3D image of an abnormal embryo with dispersed chromatin. **D** The same embryo as in panel C observed in transmitted light on a single focal plane. *Scale bar*: 58.5 µm



SYTOX green is a nucleic acid stain that enters only into cells with damaged plasma membranes, such as in dead cells, which then appear with green fluorescent nuclei.

Buttino et al. Mar. Biol. 2004

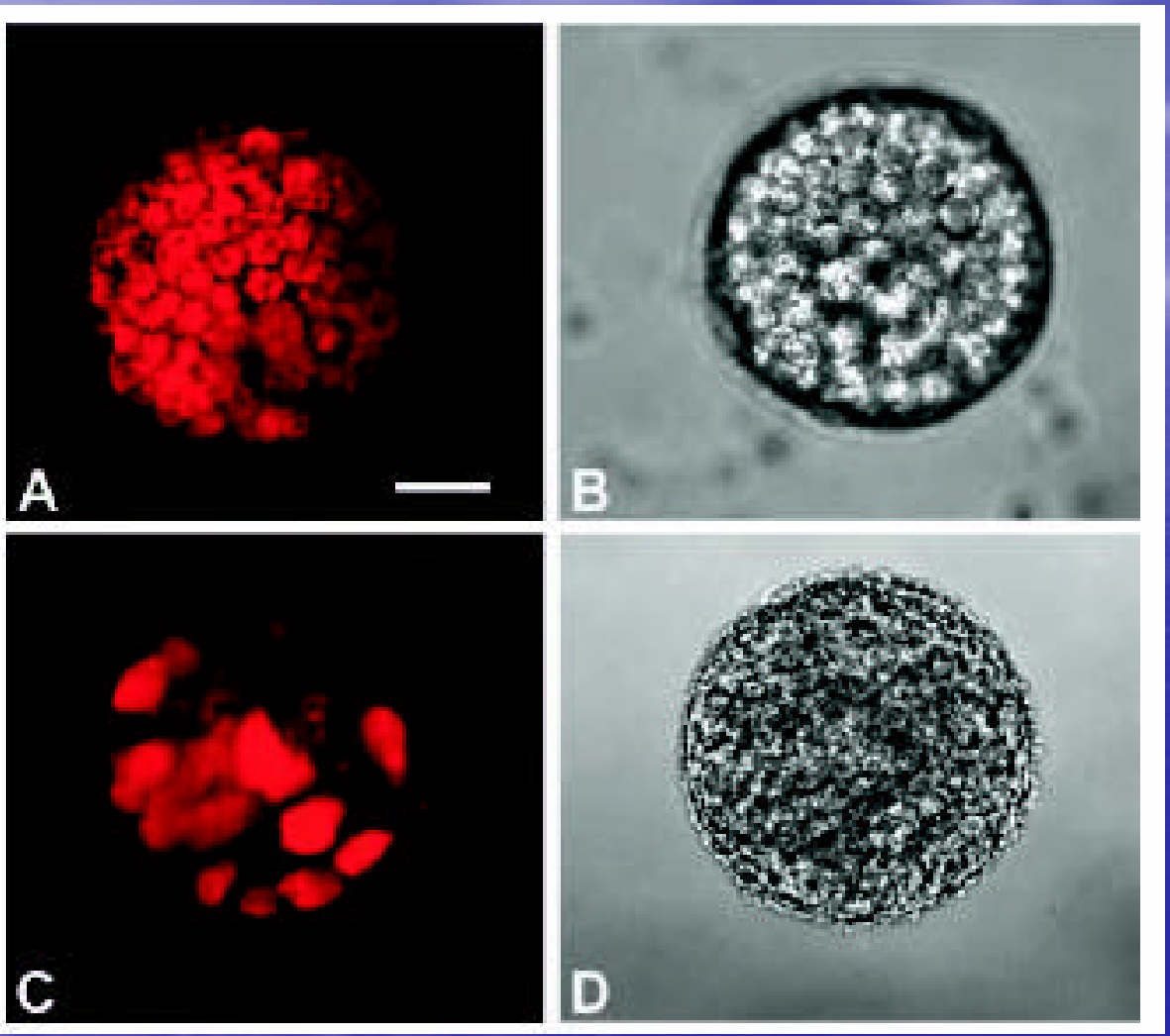


Fig. 3A–H *Calanus helgolandicus.* Non-viable embryos sta
with 7-aminoactinomicycin D (**A–D**) and auto-fluorescent
stained embryos (**E–H**), observed with the confocal laser scan
microscope. **A** 3D reconstruction of a fluorescent embryo at
morula stage. **B** The same embryo as in panel A observed
transmitted light on a single focal plane. **C** 3D reconstruction
fluorescent embryo; nuclei are asymmetrically distributed in
cytoplasm. **D** The same embryo as in panel C observed

Buttino et al. Mar. Biol. 200

7-AAD is a fluorescent DNA probe that is excluded from live cells; dead cells appear with red fluorescent nuclei.

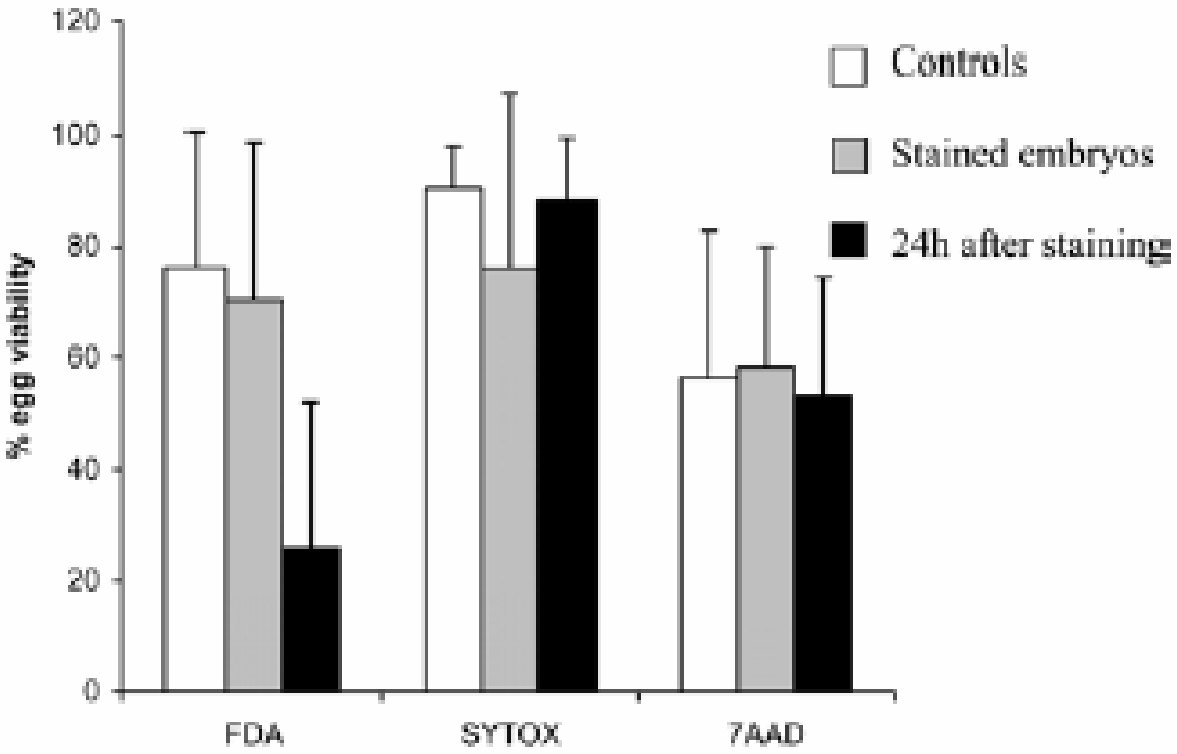
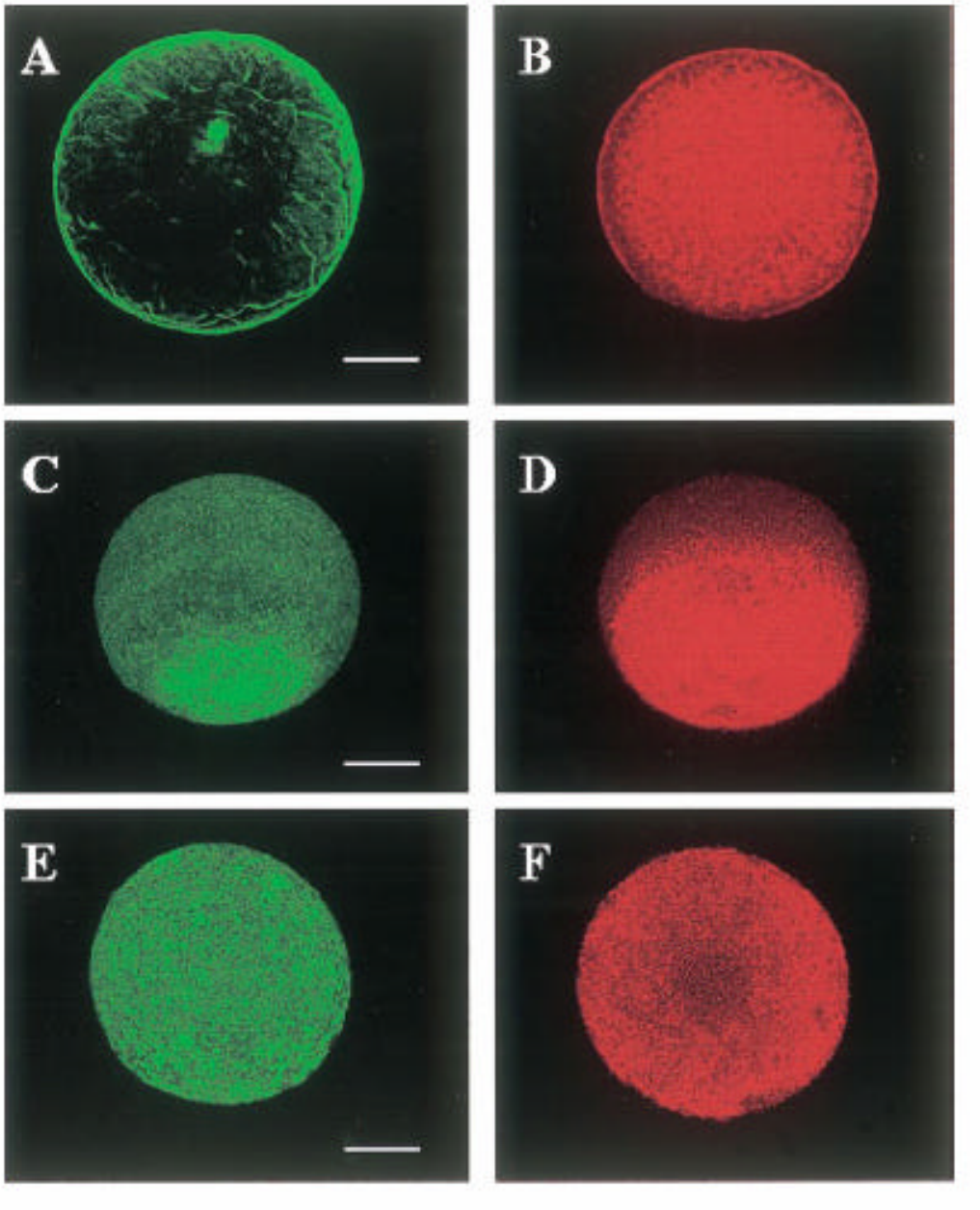


Fig. 4 *Calanus helgolandicus*. Comparison between the percentage of egg viability for unstained embryos (*controls*), FDA-fluorescent embryos, for SYTOX green– and 7-AAD–non-fluorescent embryos (*stained embryos*), and for embryos stained with the three probes and then allowed to hatch (*24 h after staining*) (mean ± SD)

Buttino et al. 2004

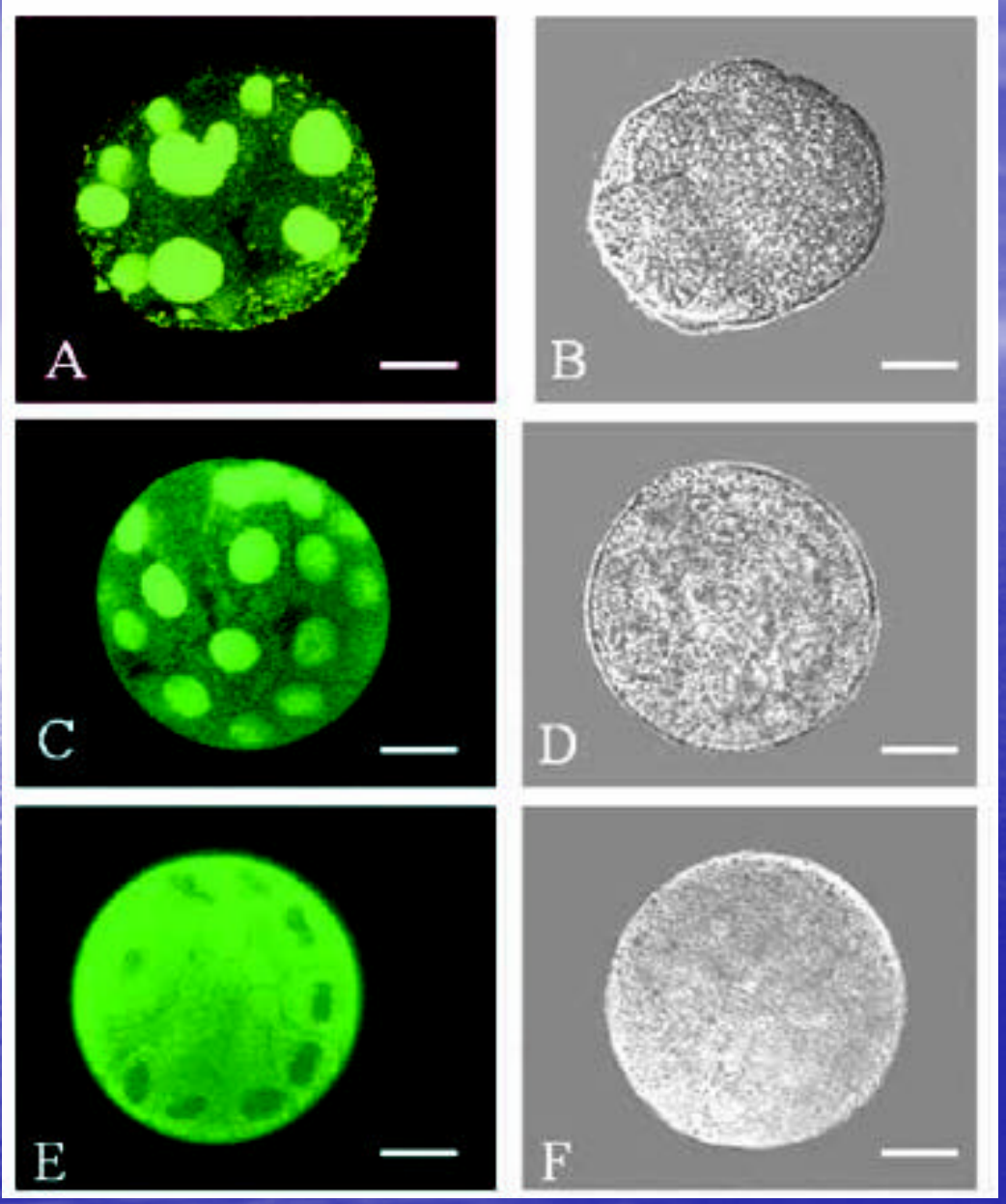


Alexa-Fluor 488 phalloidin stains filamentous actin green (left panel)

MitoTraker stains mitochondria red (right panel)

Fig. 3. Confocal laser scanning 3D reconstructions of *Cion intestinalis* embryos fixed after cortex contraction following fertiliza tion, dyed with the fluorochrome Alexa-Fluor 488 phalloidin to stai filamentous actin green (**left panels**) and MitoTraker to stai mitochondria red (**right panels**). **A**, **B**: Embryos incubated for 10 mi in 2 μg/ml of the diatom aldehyde DD before fertilization (bar = 37. μm). **C**, **D**: Control embryos (bar = 35.5 μm). **E**, **F**: Embryos incubate 10 min in 2 μg/ml cytochalasin B before fertilization (bar = 39.6 μm).

Tosti et al. Molecular Reproduction And development 2003

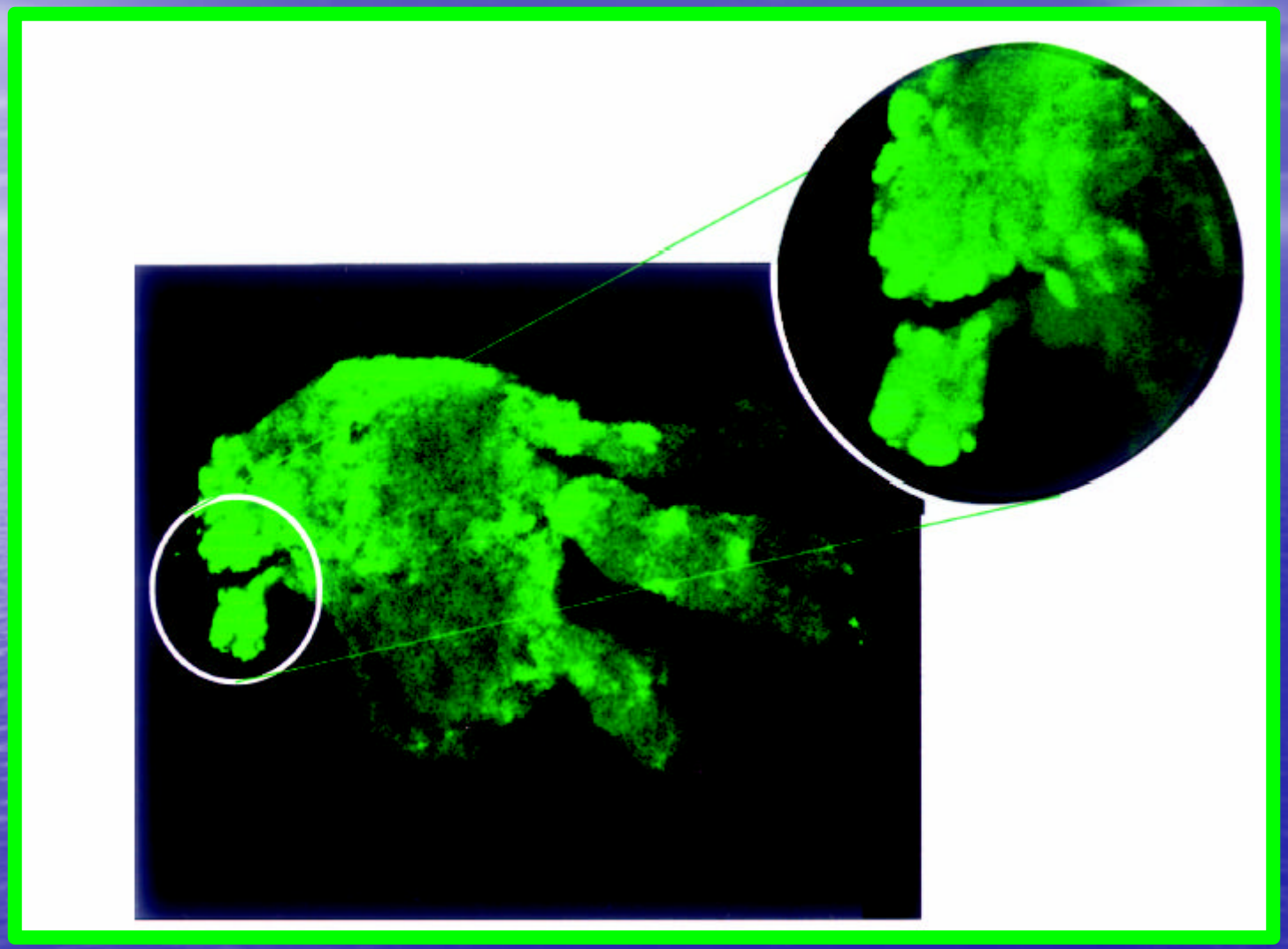


Calanus helgolandicus embryos stained with TUNEL dye to detect apoptosis (cell death)

Fig. 1. *Calanus helgolandicus* embryos after TUNEL staining and observed by confocal laser scanning microscopy in fluorescence (A,C,E,G) and transmitted (B,D,F,H) light. (A) Fluorescent, three-dimensional image of an embryo produced by females fed for 10 days with the diatom *Thalassiosira rotula*. Nuclei are positively stained (green) by the TUNEL. Bar, 39.7 μm. (B) The same embryo as in A observed in transmitted light. Bar, 42.1 μm. (C) Fluorescent three-dimensional image of a *C. helgolandicus* embryo produced by female fed non-diatom *Prorocentrum minimun* (PRO) algae for 24 h then incubated for 1 h in 5 μg ml^{-1} DD. Nuclei (green) are positively stained by TUNEL. Bar, 40 μm. (D) Embryo in C observed in transmitted light. (E) Three-dimensional image of the embryo produced by female fed 24 PRO. Nuclei are not stained in green and appear as black shadows. Bar, 42.2 μm. (F) The same embryo as in E observed in transmitted light. Bar, 40.3 μm. (G) Three-dimensional fluorescent image of a TUNEL-positive control embryo obtained by female fed for 24 h with PRO and incubated in DNase to simulate apoptosis. Nuclei are stained in green as in A and C. Bar, 34.6 μm. (H) The same embryo as in G observed in transmitted light. Bar, 34.6 μm.

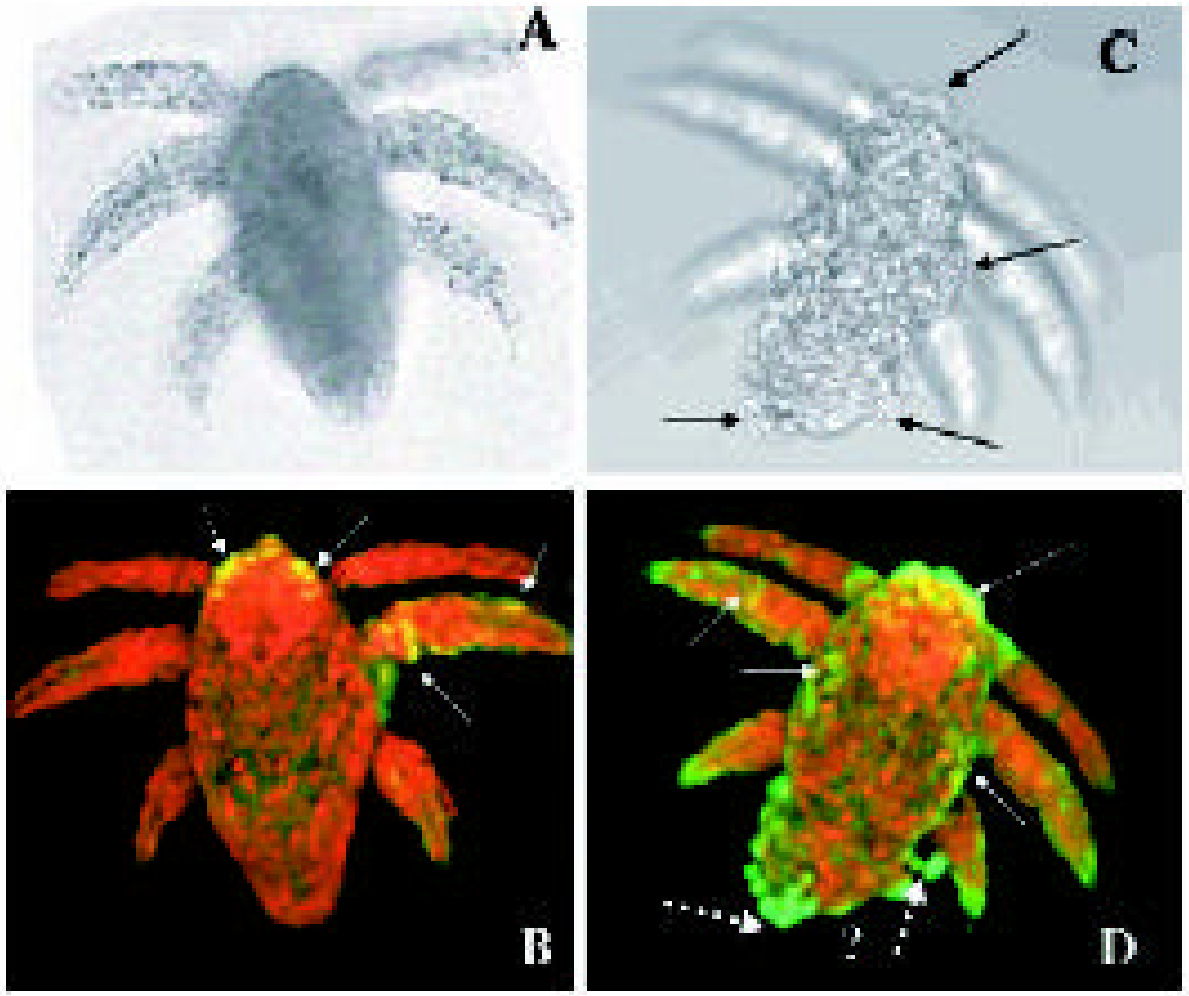
Romano et al. J Exp Biol 2003

TUNEL dye used to detect cell death in newly spawned embyros



Ianora et al. Phycologia 2003

Embryos stained with TUNEL (green) + Propidium iodide (red)



Poulet et al. Mar. Biol. 2003

MARBEF Advanced Course on

Chemical ecology and bioassay methods

Tjärnö Marine Biological Laboratory
Sweden, 9-14 September 2006

Course Coordinators

Adrianna Ianora
Henrik Pavia

Scientific Board

Jens Harder
Raffaella Casotti
Adrianna Ianora
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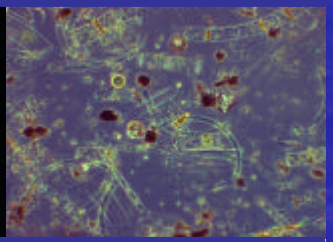
TOPICS: Chemical Ecology, Marine Natural Products, Ecotoxicology, Allelochemicals, Bioassays of natural compounds, Chemical defense, Deterrency, Trophic Interactions, Methods





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