

## COMMENT

**Are diatoms good or toxic for copepods?  
Reply to comment by Jónasdóttir et al.**Adrianna Ianora<sup>1,\*</sup>, Antonio Miralto<sup>1</sup>, Serge André Poulet<sup>2</sup><sup>1</sup>Stazione Zoologica 'A Dohrn', Villa Comunale, I-80121 Naples, Italy<sup>2</sup>Station Biologique de Roscoff, CNRS, Place Georges Teissier, F-29682 Roscoff, France

In a recent comment on copepod egg production and hatching success, Jónasdóttir et al. (1998) raised doubts about whether diatoms contain toxic compounds arresting copepod embryonic development. The authors maintain that reduced hatching rates are due to the absence of essential nutritional requisites in the diet rather than to the presence of toxic compounds affecting embryogenesis. Since reduced egg production and hatchability are reportedly well-established symptoms of essential fatty acid deficient diets in other marine organisms, including crustaceans, such a deficiency must also, therefore, be the likely cause of low hatching success in copepods. In our opinion, this deduction is questionable, because it has never really been demonstrated in copepods. Moreover, there is mounting evidence that diatoms possess anti-mitotic properties similar to the cytotoxic compounds isolated from numerous benthic marine algae. In this comment, we therefore present further evidence demonstrating that diatoms are indeed 'toxic' for the reproductive biology of copepods.

A considerable literature has developed on the marine natural products reported to have cytotoxic and antitumor activities (see Schmitz et al. 1993 for review). The majority of these have been isolated from algae, coelenterates, echinoderms and sponges, which accounted for 93% of the 5000 new metabolites reported from marine organisms in the last decade (Wright 1998). In addition to bioassays showing that these compounds inhibit development of fertilized sea urchin or starfish eggs, several have also been reported as toxic to a variety of cultured tumor lines (Ireland et al. 1993). These 'secondary metabolites' are often unique or novel and have potential applications as pharmaceuticals and in agriculture or aquaculture even though the biosynthetic pathways and ecological function of these compounds are still largely unknown (Paul & Fenical 1986, Hay & Fenical 1988). In Table 1, we give a few examples of the most studied metabolites isolated from benthic marine algae with known cytotoxic effects. Included in this list is also okadaic acid, isolated from marine dinoflagellates, which is a potent tumor promo-

tor through the inhibition of protein phosphatases 1 and 2A during the cell cycle (Gliksman et al. 1992). To date, this is the only known anti-mitotic compound isolated from marine phytoplankton. However, our recent studies have shown that the diatom *Thalassiosira rotula* can also inhibit cell cleavage in copepod, sea urchin and tunicate embryos whereas control dinoflagellate (*Prorocentrum minimum*) extracts, at the same concentrations, have no effect on cell division (Miralto et al. 1999).

Initial studies on diatom-copepod interactions had shown that egg viability decreased dramatically when female copepods were fed on a diatom diet (Ianora & Poulet 1993). The greater the number of cells ingested by females, the lower the hatching success, and vice versa (Chaudron et al. 1996). This inhibition was reversible when a diatom diet was substituted with a dinoflagellate diet (Laabir et al. 1995, Uye 1996). The same type of inhibition was also observed using 'extract' procedures whereby freshly spawned copepod eggs were exposed to increasing concentrations of extracts prepared from diatom and non-diatom (control) cells (Poulet et al. 1994, 1995, Ianora et al. 1996, Uye 1996, Ban et al. 1997, Lee et al. 1999).

This toxic effect is not due to anoxia, as suggested by Jónasdóttir & Kjørboe (1996) and Jónasdóttir et al. (1998). Jónasdóttir & Kjørboe (1996) conducted oxygen measurements in sealed bottles containing high concentrations of diatom extracts but did not design control experiments with non-diatom species. Since oxygen levels diminished with time, the authors concluded that low hatching rates were due to low oxygen rather than toxicity. However, Miralto et al. (1995) had already demonstrated that hatching failure was not due to anoxia. In containers with high concentrations of diatom (*Thalassiosira rotula*) and dinoflagellate (*Prorocentrum minimum*: as control) extracts ( $10^6$  to  $10^7$  cells  $\text{ml}^{-1}$ ), these authors showed that oxygen did not modify hatching rates in *Centropages typicus*. With or without bubbling air in containers, hatching success was 100% with dinoflagellate extracts as opposed to 0% with diatom extracts. Unfortunately, Jónasdóttir & Kjørboe (1996) and Jónasdóttir et al. (1998) continue

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Table 1. Cytotoxic compounds isolated from benthic marine algae which block cell cleavage in the sea urchin bioassay

Algal source	Name of compound	Type of compound	References
<i>Stypodium zonale</i>	Stypoldione	Ortho-quinone	O'Brien et al. (1986)
<i>Cystoseira mediterranea</i>	Mediterraneols A–D	Diterpenoids	Francisco et al. (1986)
<i>Turbinaria ornata</i>	Turbinaric acid	Triterpenoid	Asari et al. (1989)
<i>Laurencia elata</i>	Elatol	Halogenated sesquiterpene alcohol	Sims et al. (1974)
<i>Bifurcaria galapagensis</i>	Bifurcarenone	Diterpenoid	Sun et al. (1980)
<i>Halimeda</i> spp.	Halimedatrial	Diterpenoid cyclopropane trialdehyde	Paul and Fenical (1984)
<i>Caulerpa taxifolia</i>	Caulerpenyne	Sesquiterpenoid	Paul & Fenical (1986)
<i>Udotea cyathiformis</i>	Udoteal	Diterpenoid diacetate aldehyde	Paul & Fenical (1986)
<i>Spatoglossum schmittii</i>	Spatol	Diterpenoid	Faulkner (1984)
<i>Prorocentrum concavum</i>	Okadaic acid	Polyether carboxylic acid	Dickey et al. (1990)

not to cite this study even though the results clearly show that hatching failure is not due to variations in oxygen levels during the incubation period, as further confirmed by Uye (1996) and Lee et al. (1999).

We now know the chemical nature of the toxic compounds that induce inhibition of cell division during copepod embryogenesis. They are 3 aldehydes of low molecular weight, a class of compounds that arrests cell division and induces apoptosis (i.e. programmed cell death) in cultured cell lines (Zimmerman et al. 1995). These aldehydes, recently isolated from *Thalassiosira rotula*, will be described elsewhere (Miralto et al. unpubl.). Here, we wish only to point out that several of the metabolites listed in Table 1 are aldehydes and that marine plants, which are abundant producers of polyunsaturated fatty acids such as 20- and 22-carbon  $\alpha$ -3 fatty acids (eicosapentaenoic and docosahexenoic acids), are known to synthesize various unsaturated aldehydes from these and other fatty acids via different oxidative pathways (Gerwick & Bernart 1993).

The mechanism through which aldehydes affect copepod reproduction is still unknown. Probably, inhibitory compounds are transferred to the gonads via feeding, followed by diffusion through the gut epithelium and accumulation in the developing oocytes during oogenesis. Preliminary studies with sea urchin eggs showed that these inhibitory compounds targeted tubulin microfilaments that were de-polymerized during cell cleavage (Buttino et al. 1999). Immunofluorescent dyes specific for DNA and  $\alpha$ -tubulin were used to stain the nuclei and microfilaments of sea urchin embryos at different times prior to the first mitotic division. Extracts of the diatom *Thalassiosira rotula* induced aberrations in tubulin organization similar to those reported for other toxicants, such as colchicine, classically defined as anti-mitotic drugs. In addition, recent studies using cultured cancer lines have demonstrated that extracts of the diatom *Skeletonema costatum* inhibit proliferation of human bronchopulmonary tumoral cells (Bergé et al. 1997). Cell growth is blocked in the G1 phase of the cell cycle and this growth arrest is irreversible.

The alternative explanation for reduced hatching success proposed by Jónasdóttir et al. (1998), that copepods exhaust their own storage of a limiting nutritional component essential for hatching and that depletion is faster when egg production is high, is unlikely. Presumably, if something is limiting in the food, the more an organism eats of this food, the more it can supplement the lack of an essential requisite in this food. In all our experiments, we fed copepods ad libitum so that food was not limiting. In addition, mixed food experiments such as those recommended but not conducted by Jónasdóttir et al. (1998) further confirm the idea that reduced hatchability is not due to the absence of any limiting nutrient. Experiments by Lee et al. (1999) on *Pseudocalanus newmani* showed that with mixed diets of the diatom *Chaetoceros gracilis* (CHA) and the Prymnesiophyceae *Pavlova* spp. (PAV) hatching success was lower than with PAV alone but higher than with CHA alone.

It is believed that several nutritional components are necessary for crustacean maturation and reproduction, such as long-chained polyunsaturated fatty acids and amino acids for the synthesis of yolk proteins and peptide hormones (Harrison 1990), but evidence that these compounds are lacking in the food grazed by copepods is still uncertain. Laabir et al. (1999) showed that hatching success was not due to the lack of any amino acids. With a diatom diet, there was a drastic increase in the amino acid pool in developing embryos notwithstanding reduced hatching rates. Jónasdóttir (1994) found no correlation between hatching success and the fatty acid content of the food, whereas Jónasdóttir & Kiørboe (1996) later found that hatching of *Acartia tonsa* eggs increased with increasing ratios of  $\alpha$ 3 to  $\alpha$ 6 and 22:6 $\alpha$ 3 to 20:5 $\alpha$ 3 fatty acids in the diet. However, such relationships in the ratio of these fatty acids were not observed by Lee et al. (1999). Clearly, further studies are necessary to assess the fatty acid requirements during copepod embryonic development, such as those specifically monitoring not only the composition of the food but also fatty acid levels in gravid females and newly spawned eggs.

Even in the fish and crustacean literature, there is only limited evidence that essential fatty acid (EFA) deficiency can modify hatching rates. Watanabe (1982) reported that several fish species had lower egg production, proportion of eyed eggs and reduced hatchability but only after they were fed EFA-deficient diets for several weeks. And Harrison (1990) discussed the general importance of EFAs in crustacean reproduction and development without presenting evidence that EFA-deficient diets blocked hatching success. In any case, the problem of reduced hatchability with diatoms is not related to the absence of specific EFAs since *Thalassiosira rotula* contains high levels of 20:5 $\alpha$ 3 and 22:6 $\alpha$ 3 fatty acids, which comprise 30.5% and 5.4%, respectively, of the total fatty acid content in this species (Kattner unpubl.).

Jónasdóttir et al. (1998) have suggested that our extract experiments are a 'procedure no more realistic than suffocating humans in chocolate syrup and then concluding that chocolate is toxic' and that 'such procedures should be discontinued'. Apparently they are not aware that many biologically active compounds have been screened using similar procedures (e.g. Schmitz et al. 1993). To further demonstrate that extract experiments are indeed a robust bioassay, we have tested the effect of one of the pure compounds (i.e. aldehydes) extracted from the diatom *Thalassiosira rotula* or purchased from SIGMA on the hatchability of *Temora stylifera* eggs. Details of the extraction protocol are described elsewhere (Miralto et al. unpubl.). Copepod couples were placed in crystallizing dishes containing 100 ml of 0.45  $\mu$ m filtered seawater with  $3 \times 10^3$  cells  $\text{ml}^{-1}$  of the diet *P. minimum*, enriched with aldehydes at a concentration of 1  $\mu\text{g ml}^{-1}$ . Control couples were fed the same concentration of dinoflagellate cells without the addition of aldehydes. Each day, couples were transferred to new containers with fresh media and the same concentration of aldehydes. Hatching success was determined 48 h after egg laying. Experiments were run for 5 consecutive days. The results show that hatching success immediately dropped to <40% within 24 h with the addition of aldehydes as opposed to control couples (Fig. 1). Females continued to lay eggs and to swim and feed normally in the presence of aldehydes, showing that these inhibitory compounds were not noxious to the adults. However, they were very toxic for eggs which rapidly degenerated with time. In other words, diatom extracts acted as the 'birth control pill' for female copepods that continued to lay eggs, few of which were viable. In summary, both the discovery of aldehydes in a marine diatom and their highly significant inhibitory effects (Fig. 1) lend unequivocal support to our toxicity hypothesis.

Jónasdóttir et al. (1998) conclude that if the toxicity hypothesis were true, an entirely new interpretation of

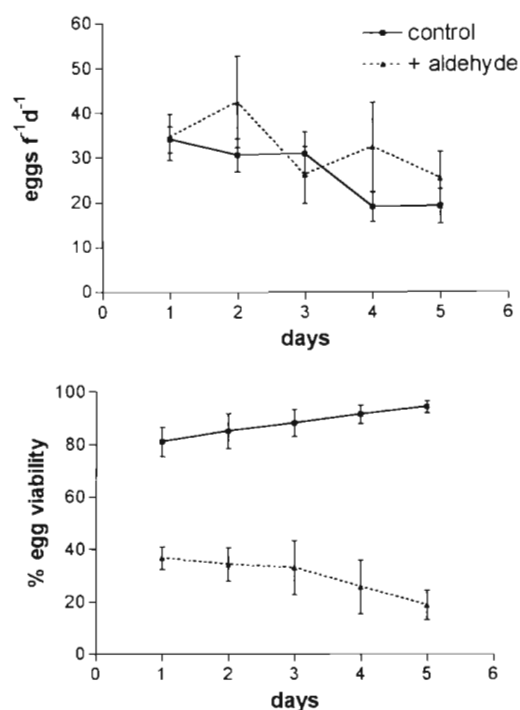


Fig. 1. Daily egg production and % egg viability for *Temora stylifera* couples maintained on a diet of the dinoflagellate *Prorocentrum minimum* (control couples: continuous line). Experimental couples were fed the same diet at the same concentrations but with the addition of 1  $\mu\text{g ml}^{-1}$  of aldehydes extracted from the diatom *Thalassiosira rotula* (dashed line)

copepod feeding ecology would be required. We agree. The mounting evidence of the negative impact of diatoms on the reproductive biology of copepods (Poulet et al. 1995, Ban et al. 1997, Lee et al. 1999) clearly indicates that it is time to revisit the classic view on diatom-copepod interactions (Riley 1947). As suggested for the secondary metabolites isolated from several benthic marine algae (Paul & Fenical 1986), toxic compounds may discourage herbivory or play a role in algal-algal or algal-bacterial interactions. Whatever their ecological role in the marine plankton, our understanding of diatom-copepod chemical interactions will advance only through well designed field observations and laboratory experiments testing the effects of phytoplankton metabolites on copepod feeding and reproductive behavior. In the future, it would be more useful, therefore, for those that challenge our hypothesis to present data showing that diatoms are non-toxic rather than opening academic discussions against this view. As Huntley et al. (1986) once pointed out, the compounds that make a food 'bad' or 'toxic' are specific metabolites other than nutritious substances such as proteins, lipids and carbohydrates. It is time to look for such specific molecules and to understand their role in the pelagic realm.



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