

Toxigenic effects of diatoms on grazers, phytoplankton and other microbes: a review

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Abstract Traditionally, diatoms have been regarded as providing the bulk of the food that sustains the marine food chain and important fisheries. However, this view was challenged almost two decades ago on the basis of laboratory and field studies showing that when copepods, the principal predators of diatoms, feed on certain diatom diets, they produce abnormal eggs that either fail to develop to hatching or hatch into malformed (i.e. teratogenic) nauplii that die soon afterwards. Over the years, many explanations have been advanced to explain the causes for reproductive failure in copepods and other marine and freshwater invertebrates including diatom toxicity, or nutritional deficiency and poor assimilation of essential compounds in the animal gut. Here we review the literature concerning the first possibility, that diatoms produce cytotoxic compounds responsible for growth inhibition and teratogenic activity, potentially sabotaging future generations of grazers by inducing poor recruitment. The cytotoxic compounds responsible for these effects are short chain polyunsaturated aldehydes (PUAs) and other oxygenated fatty acid degradation products such as hydroxides, oxo-acids, and epoxyalcohols (collectively termed oxylipins) that are cleaved from fatty acid precursors by enzymes activated within seconds after crushing of cells. Such toxins are suggested to have multiple simultaneous functions in that they not only deter herbivore feeding but some also act as allelopathic agents against other phytoplankton cells, thereby affecting the growth of competitors, and also signalling population-level cell death and termination of blooms, with possible consequences for food web

structure and community composition. Some oxylipins also play a role in driving marine bacterial community diversity, with neutral, positive or negative interactions depending on the species, thereby shaping the structure of bacterial communities during diatom blooms. Several reviews have already been published on diatom-grazer interactions so this paper does not attempt to provide a comprehensive overview, but rather to consider some of the more recent findings in this field. We also consider the role of diatom oxylipins in mediating physiological and ecological processes in the plankton and the multiple simultaneous functions of these secondary metabolites.

Keywords Diatoms · Copepods · Phytoplankton · Microbes · Reproduction · Teratogenic · Oxylipins · Polyunsaturated aldehydes

Introduction

Diatoms have traditionally been viewed as beneficial to the growth and survival of marine organisms, and to the transfer of organic material through the food chain to top consumers and important fisheries. As of 1993, however, there has been accumulating evidence that has progressively challenged the view that diatoms are good and harmless food items for copepods, the dominant constituent of the marine zooplankton. It has been shown that while diatoms may provide a source of energy for copepod larval growth, they often reduce fecundity and/or hatching success. These results constitute the paradox of diatom-copepod interactions in the pelagic food web (Ban et al. 1997). This biological model is new and has no other equivalent in marine plant-herbivore systems, since most of the known negative plant-animal interactions are generally related to

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repellent or poisoning processes, but never to reproductive failure.

In the course of these years, a series of basic characteristics of the inhibitory effect of diatoms on the reproductive biology of copepods have been demonstrated. Ianora and Poulet (1993) first showed that when the copepod *Temora stylifera* was fed on a mono-algal diet of the diatom *Thalassiosira rotula*, egg production remained high for more than 15 days but hatching success was seriously impaired compared to when animals were fed the dinoflagellate *Prorocentrum minimum*. These differences were not due to the biochemical composition of the two algae since both *T. rotula* and *P. minimum* contained high levels of proteins, vitamins and fatty acids essential for growth and development of copepods. Successively, Poulet et al. (1994) proposed that reduced hatching rates were due to the presence of unidentified anti-mitotic compounds blocking embryogenesis in the copepod *Calanus helgolandicus*. Depending on the age of the eggs prior to exposure to water-soluble extracts prepared from the diatom *Phaeodactylum tricornerutum*, cell division was blocked either before fusion of the male and female pronuclei, or during mitosis. Embryos underwent strikingly abnormal development, showing dispersed chromatin and asymmetrical division of blastomeres. None of these eggs developed to hatching. This was due to the de-polymerization of tubulin microfilaments, leading to cell blockage and the absence of spindle formation (Buttino et al. 1999).

Inhibition of hatching was shown to be diatom density-dependent (Chaudron et al. 1996): the greater the number of diatoms fed upon, the greater the inhibition, and vice versa. With decreasing diatom concentrations, deleterious effects on hatching diminished and these effects took longer to induce (Ban et al. 1997; Starr et al. 1999). The unknown compounds were shown to be produced by the diatom cells and not by bacteria associated with diatom cultures (Ianora et al. 1996). Axenic diatoms were even more potent than their non-axenic counterparts by inducing greater inhibition of hatching indicating that bacteria may play a role in mediating the production of toxic compounds. Diatoms not only interfered with egg maturation but also induced strong developmental aberrations in those nauplii that developed to hatching. Teratogenic (abnormal) nauplii showed a variety of birth defects such as asymmetrical bodies and malformed or reduced number of swimming and feeding appendages (Poulet et al. 1995; Uye 1996; Starr et al. 1999; Ianora et al. 2004). Such individuals usually died soon after birth because they were unable to swim or feed properly.

A major breakthrough in research on diatom-copepod interactions was the discovery of the first chemicals responsible for these effects. Miralto et al. (1999) identified 3 low molecular weight polyunsaturated aldehydes (PUAs)

from the diatom *Thalassiosira rotula* and showed that they arrested embryonic development of copepod and sea urchin embryos in a dose-dependent manner, and also had anti-proliferative and apoptotic effects on human carcinoma cells. Although all three PUAs were known from other sources, this was the first report of their presence in marine diatoms. The first two PUAs had already been isolated from the freshwater diatom *Melosira varians* by other researchers (Wendel and Jüttner 1996), but the biological activity of these compounds was not known at the time. Miralto et al. (1999) showed that diatoms negatively impacted copepod hatching success in the field, with only 12% of *Acartia clausi* eggs hatching during a bloom of the diatom *Skeletonema marinoi* (recently separated from *Skeletonema costatum*, Sarno et al. 2005) in the North Adriatic Sea, compared to 90% in post-bloom conditions.

Pohnert (2000) successively showed that when diatoms are damaged due to feeding, there is a rapid onset of PUA production seconds after cell disruption, similar to the wound reaction in higher plants. PUAs are cleaved from fatty acid precursors by enzymes activated within seconds of crushing of cells (reviewed by Pohnert 2005). This insidious mechanism, which does not deter the herbivore from feeding but impairs its recruitment, will restrain the cohort size of the next generation (Ianora et al. 2004). Hence, certain diatom diets can negatively impact both copepod egg hatching success (up to 100%) and larval development, with biomass build-up of blooms on the ocean floor that may have significant consequences for ocean ecology and biogeochemistry. Although there is currently no specific data on changes in feeding response in copepods, there is evidence for PUAs eliciting behavioural changes in other predators, such as freshwater crustacean grazers that are repelled by these compounds (Jüttner 2005) and freshwater gastropods and nematodes, that utilize these compounds for food and/or habitat finding (Fink 2007 and references therein).

This type of chemical defence, with the production of teratogenic compounds that induce abortions, birth defects, poor development and high offspring mortality, had never been demonstrated before for unicellular algae. By definition, teratogens are substances that induce congenital malformations in the offspring of organisms exposed to them during gestation. Teratogenesis is therefore the result of developmental toxicity which can ultimately give rise to embryo or fetal mortality (i.e. abortion). This latter property may partially explain why diatoms cause problems in hatcheries where they are still widely used in aquaculture due to their ease of mass cultivation (Ianora 2005). Although the effects of such toxins are less catastrophic than those inducing poisoning and death, they are nonetheless insidious occurring through abortions, birth defects and reduced larval survivorship. Such antiproliferative

compounds may discourage herbivory by sabotaging future generations of grazers, thereby allowing diatom blooms to persist when grazing pressure would normally have caused them to crash.

In terrestrial environments, there are many reports of teratogenic compounds produced by plants that interfere with the reproductive capacity of grazing animals, and which act as a form of population control. Some classic examples include caffeine, present in many plants processed for use as beverages and foods, and nicotine, the principal chemical present in tobacco. Nicotine has been shown to have possible teratogenic effects on fetal development in humans and it is now widely accepted that pregnant woman should avoid smoking during their pregnancy. The same compound has been shown to have strong effects also in range animals that ingest tobacco plants, with newborns showing limb deformities and palate closure defects associated with the high content of nicotine in their tissues (Schardein and York 1995). But nothing was known on the presence and effects of such compounds in the marine environment. The following sections will focus on some of the main features of this unique predator–prey relationship and discuss how chemical defence molecules such as reactive PUAs and other oxylipins from diatoms can help shape plant–animal interactions at sea. Several reviews have addressed the effects of diatoms on copepod reproduction (Paffenhöfer 2002; Paffenhöfer et al. 2005; Ianora et al. 2003) and the chemistry of aldehyde (PUA) production (Pohnert 2005) and the influence of diatom PUAs on invertebrate reproduction and development (Caldwell 2009) so in the following sections we consider only some of the recent advances in this field and not discussed in previous reviews. We also discuss other possible functions of diatom PUAs as allelopathic compounds and anti-bacterial agents, with potential consequences on bloom dynamics and phytoplankton and bacterial community structure.

Laboratory studies on diatom–copepod interactions

Several recent studies have focused on the effects of maternal diets on egg production, hatching success and later copepod growth and development (summarized in Table 1). Carotenuto et al. (2002) showed that *Temora stylifera* was unable to complete development from hatching to adulthood when larvae were reared on three different diatom species (*Thalassiosira rotula*, *Skeletonema costatum* and *Phaeodactylum tricornutum*) as opposed to three non-diatom (*Prorocentrum minimum*, *Isochrysis galbana* and *Oxyrrhis marina*) controls, with daily mortality rates as high as 20.3–35.5% day⁻¹. Survivorship of larvae to adulthood improved from 7 to 34% when larvae

were generated from females preconditioned with a non-diatom diet (*P. minimum*) for 24 h but, in any case, survivorship was much lower than with non-diatom diets (70–80% survivorship). Nauplii raised on diatom diets were normal whereas those generated when females fed on diatom diets for 7 days were strongly deformed demonstrating that maternal diets were responsible for the production of teratogenic nauplii. Such nauplii had already been observed in several laboratory studies (Poulet et al. 1995; Uye 1996; Starr et al. 1999), when newly spawned eggs were exposed to increasing concentrations of diatom extract, or had been recorded in the wild during natural diatom blooms (Ban et al. 2000).

Ianora et al. (2004) also explored the effect of maternal diatom diets on offspring fitness. They showed that when female *Calanus helgolandicus* were fed the diatom *Skeletonema marinoi*, development was arrested in all larvae in which both mothers and their larvae were fed the diatom diet. Mortality remained high even if larvae generated from females fed *S. marinoi* were switched to a diet of the dinoflagellate *Prorocentrum minimum*. On the other hand, when females were fed *P. minimum*, and the nauplii were then reared on *S. marinoi*, survivorship improved considerably, more so than when the offspring of females fed *S. marinoi* were raised on a diet of *P. minimum*, indicating that it was more important for mothers to receive the “good” food rather than their offspring. The unsaturated diatom aldehyde 2-trans-4-trans-decadienal (herewith referred to as decadienal) was shown to elicit this teratogenic effect. Ianora et al. (2004) used *P. minimum* cells loaded with a 1.5 µgml⁻¹ decadienal solution to deliver aldehydes to mothers for 3 days, and then placed females in containers with seawater and *S. marinoi* cells at natural concentrations and followed the development of the spawned nauplii. The offspring showed the same arrested development at the first copepodite stage as when both mothers and offspring were fed *S. marinoi*. The authors concluded that this insidious mechanism of induced defence by diatoms, which does not deter the herbivore from feeding but impairs its recruitment, will restrain the cohort size of the next generation of copepods.

Copepods have been shown to respond differently to the same diatom species. Ianora (2005) showed that the diatom *Thalassiosira rotula* induced different reproductive responses in the copepods *Acartia clausi*, *Centropages typicus*, *Calanus helgolandicus* and *Temora stylifera*. While *T. rotula* induced dramatic effects in *C. typicus*, the effect was somewhat reduced in *T. stylifera* and there was no effect in *A. clausi* and *C. helgolandicus*, denoting species-specific responses to the toxic metabolites in diatoms. Caldwell et al. (2004b) and Ianora (2005) suggested that some copepod species may have evolved counter-defences, such as detoxifying enzymes as aldehyde dehydrogenases

Table 1 Summary of the reproductive responses of copepods to different diatom diets

Diatom Species	Copepod Species	EPR	HS	AL	D	Authors
<i>Thalassiosira rotula</i>	<i>Temora stylifera</i>			–	–	Carotenuto et al. (2002)
<i>Skeletonema costatum</i>	<i>Temora stylifera</i>			–	–	Carotenuto et al. (2002)
<i>Phaeodactylum tricornutum</i>	<i>Temora stylifera</i>			–	–	Carotenuto et al. (2002)
<i>Thalassiosira rotula</i> strain TR1	<i>Calanus helgolandicus</i>	+	–			Pohnert et al. (2002)
<i>Thalassiosira rotula</i> strain TR2	<i>Calanus helgolandicus</i>	+	+			Pohnert et al. (2002)
<i>Skeletonema pseudocostatum</i>	<i>Calanus helgolandicus</i>	–	+			Pohnert et al. (2002)
<i>Thalassiosira rotula</i>	<i>Temora stylifera</i>	+	–			Ceballos and Ianora (2003)
<i>Thalassiosira weissflogii</i>	<i>Temora stylifera</i>	+	–			Ceballos and Ianora (2003)
<i>Phaeodactylum tricornutum</i>	<i>Temora stylifera</i>	–	–			Ceballos and Ianora (2003)
<i>Skeletonema costatum</i>	<i>Temora stylifera</i>	–	–			Ceballos and Ianora (2003)
<i>Skeletonema marinoi</i>	<i>Calanus helgolandicus</i>		–	–	–	Ianora et al. (2004)
<i>Thalassiosira rotula</i>	<i>Calanus helgolandicus</i>	+	–			Ianora (2005)
<i>Thalassiosira rotula</i>	<i>Temora stylifera</i>	+	–			Ianora (2005)
<i>Thalassiosira rotula</i>	<i>Acartia clausi</i>	+	–			Ianora (2005)
<i>Thalassiosira rotula</i>	<i>Centropages typicus</i>	–	–			Ianora (2005)
<i>Skeletonema marinoi</i>	<i>Calanus helgolandicus</i>	–	–			Ianora (2005)
<i>Lauderia annulata</i>	<i>Calanus helgolandicus</i>	–	–			Ianora (2005)
<i>Skeletonema costatum</i>	<i>Tisbe holothuriae</i>	+	+		+	Taylor et al. (2007)
<i>Skeletonema marinoi</i>	<i>Tisbe holothuriae</i>	+	+		+	Taylor et al. (2007)
<i>Navicula hansenii</i>	<i>Tisbe holothuriae</i>	+	+		+	Taylor et al. (2007)
<i>Melosira nummuloides</i>	<i>Tisbe holothuriae</i>	+	+		+	Taylor et al. (2007)
<i>Thalassiosira rotula</i>	<i>Calanus helgolandicus</i>	+	+	+		Poulet et al. (2007a)
<i>Chaetoceros calcitrans</i>	<i>Calanus helgolandicus</i>	–	+	+		Poulet et al. (2007a)
<i>Guinardia striata</i>	<i>Calanus helgolandicus</i>	–	+	+		Poulet et al. (2007a)
<i>Odontella regia</i>	<i>Calanus helgolandicus</i>	–	+	+		Poulet et al. (2007a)
<i>Rhizosolenia setigera</i>	<i>Calanus helgolandicus</i>	–	+	+		Poulet et al. (2007a)
<i>Stephanopyxis turris</i>	<i>Calanus helgolandicus</i>	–	+	+		Poulet et al. (2007a)
<i>Navicula sp.</i>	<i>Calanus helgolandicus</i>	+	–	–		Poulet et al. (2007a)
<i>Nitzschia sp.</i>	<i>Calanus helgolandicus</i>	+	–	–		Poulet et al. (2007a)
<i>Skeletonema costatum</i>	<i>Calanus helgolandicus</i>	+	–	–		Poulet et al. (2007a)
<i>Thalassiosira pseudonana</i>	<i>Calanus helgolandicus</i>	–	–	–		Poulet et al. (2007a)
<i>Guinardia delicatula</i>	<i>Calanus helgolandicus</i>	–	–	–		Poulet et al. (2007a)
<i>Skeletonema marinoi</i>	<i>Calanus helgolandicus</i>		–	–		Fontana et al. (2007b)
<i>Chaetoceros socialis</i>	<i>Calanus helgolandicus</i>		–	–		Fontana et al. (2007b)
<i>Chaetoceros affinis</i>	<i>Calanus helgolandicus</i>		–	–		Fontana et al. (2007b)
<i>Thalassiosira rotula</i>	<i>Temora longicornis</i>				+	Koski et al. (2008)
<i>Thalassiosira weissflogii</i>	<i>Temora longicornis</i>				+	Koski et al. (2008)
<i>Leptocylindricus danicus</i>	<i>Temora longicornis</i>				+	Koski et al. (2008)
<i>Skeletonema costatum</i>	<i>Temora longicornis</i>				+	Koski et al. (2008)
<i>Chaetoceros affinis</i>	<i>Temora longicornis</i>				–	Koski et al. (2008)
<i>Chaetoceros decipiens</i>	<i>Temora longicornis</i>				–	Koski et al. (2008)
<i>Chaetoceros socialis</i>	<i>Temora longicornis</i>				–	Koski et al. (2008)
<i>Thalassiosira rotula</i>	<i>Temora longicornis</i>				–	Koski et al. (2008)
<i>Thalassiosira pseudo nana</i>	<i>Temora longicornis</i>				–	Koski et al. (2008)
<i>Thalassiosira rotula</i>	<i>Temora longicornis</i>	+	–			Dutz et al. (2008)
<i>Thalassiosira weissflogii</i>	<i>Temora longicornis</i>	+	–			Dutz et al. (2008)
<i>Chaetoceros affinis</i>	<i>Temora longicornis</i>	+	–			Dutz et al. (2008)

Table 1 continued

Diatom Species	Copepod Species	EPR	HS	AL	D	Authors
<i>Leptocylindricus danicus</i>	<i>Temora longicornis</i>	–	–			Dutz et al. (2008)
<i>Skeletonema costatum</i>	<i>Temora longicornis</i>	–	–			Dutz et al. (2008)

Abbreviations are for egg production rates (EPR), hatching success (HS), abnormal larvae (AL) and development from nauplius stage 1 to adulthood (D) where (–) indicates adverse effects and (+) favorable effects of diets on these parameters

or glutathion reductases, thereby deleting the negative effects of diatom PUAs. This would explain past conflicting results in which diatoms at times did not reduce hatching success in copepods, as in the case of *Skeletonema costatum* which reduced hatching in *Calanus hegolandicus* (Ianora et al. 2003) but not in *C. finmarchicus* (Ban et al. 1997; Starr et al. 1999). These conflicting findings may also depend on strain-specific differences in PUA production within a given species. Pohnert et al. (2002) tested the effects of two strains of the diatom *Thalassiosira rotula* isolated off the coast of Naples, Italy (TR1), and California, USA (TR2), on fecundity and egg hatching rates of *C. helgolandicus* and showed that only the TR1 strain producing PUAs affected hatching success. They concluded that the capability to produce reactive PUAs was highly species and even isolate dependent.

The greater tolerance of some copepods to PUAs was also demonstrated by Taylor et al. (2007) who studied the response of the benthic harpacticoid copepod, *Tisbe holothuriae*, to PUA-producing diatom diets on reproductive success. The least tolerant life-stage was the nauplius followed by adult males, and then adult females. Short-term exposure to PUA-producing diatoms *S. marinoi* and *Melosira nummuloides* in maternal diets had no significant impact on reproductive success or on the survival and development of naupliar stages to adulthood indicating that *T. holothuriae* expresses a higher degree of tolerance to PUA-producing diatoms than many planktonic calanoids, possibly reflecting a degree of adaptation to higher stress levels associated with the benthos.

Another issue that has been addressed in recent years regards the biological activity of PUAs of different chain lengths (C7, C8, C10). Ceballos and Ianora (2003) conducted experiments testing the effects of these PUAs on egg hatching success showing that the longer the chain length of the PUAs, the stronger the biological activity of these molecules, as also confirmed by Adolph et al. (2003) using sea urchin eggs. Whereas total blockage of egg development in *T. stylifera* occurred at concentrations of $2 \mu\text{g ml}^{-1}$ of decadienal, the concentrations to induce blockage by octadienal and heptadienal were $\geq 2.5 \mu\text{g ml}^{-1}$. Similar findings were also found for microalgae (see sections on the effects of PUAs on diatoms and bacteria).

There have been several recent studies that failed to find a correlation between reduced hatching success and PUA production. Poulet et al. (2007a) studied the influence of diatoms on egg production and hatching success in *Calanus hegolandicus* fed 20 different algal diets in the laboratory. They reported that egg production was impaired by several diatom diets (*Chaetoceros calcitrans*, *Guinardia delicatula*, *G. striata*, *Odontella regia*, *Rhizosolenia setigera*, *Stephanopyxis turris*, *Thalassiosira pseudonana*) and mixed diatom assemblages collected in the field, as well as by the prymnesiophyte *Pavlova lutherii*. However, reproductive failure was not correlated to PUAs or any other chemical factors such as polyunsaturated fatty acids (PUFAs), including eicosapentaenoic acid (EPA) or docosahexaenoic acid (DHA) that are considered essential for copepod development (see Jónasdóttir et al. 2009 and references therein). Koski et al. (2008) also failed to find the reason behind the negative response to ingested diatom diets; neither toxicity nor nutritional quality (represented by C:N, PUFA and sterols) could directly explain the observed negative effects on growth of 4 out of 11 diatoms species tested. Similar results were obtained by Dutz et al. (2008) who investigated whether reduced reproductive success in *T. longicornis* fed diatom diets was related to nutritional imbalances with regard to essential PUFAs or to the production of toxic PUAs. Egg hatching success decreased after 4 days with all six diatom diets but there was no correlation to PUA production or nutritional content of the food. The authors suggested that the causes could have been due to the production of hitherto unknown antiproliferative compounds or to incomplete digestion following from the low gut passage time of diatoms. We discuss this possibility later in the text on the chemistry of diatoms.

Field studies on the negative impact of diatoms on copepod reproduction

Halsband-Lenk et al. (2005) found that hatching success in the copepod *Pseudocalanus newmani* dropped from 90–95 to 43% during a *Thalassiosira* spp. bloom in Dabob Bay, Washington USA, especially when the bloom consisted of

PUA-producing species (Horner et al. 2005). The same bloom also depressed hatching success in the copepod *Calanus pacificus*, but the effect was less dramatic (Pierson et al. 2005). In both studies, naupliar survival to the nauplius three stage was more severely affected by the *Thalassiosira* bloom than hatching success, and was as low as <1% in the case of *P. newmani*. Many of the nauplii looked abnormal and were hardly moving, developing to the nauplius two stage and then dying shortly afterwards. Leising et al. (2005a) showed that *C. pacificus* often rejected the most abundant phytoplankton species, particularly certain PUA-producing *Thalassiosira* species, explaining why this copepod species was less deleteriously affected by the bloom. On the other hand, *P. newmani* was a less selective feeder and often consumed toxic diatoms which induced lower hatching success and naupliar survival compared to *C. pacificus* (Leising et al. 2005b). These studies showed that the “diatom effect” operates well when all the prerequisites—(1) high concentrations of PUAs, (2) few prey alternatives, and (3) feeding of copepods on these algae—occurred at sea (Halsband-Lenk et al. 2005).

In another field study by Ask et al. (2006), hatching frequencies of *Eurytemora affinis* in the Baltic Sea between May and October 2003 were lowest during the spring diatom bloom. The reverse was true for clutch size, with the highest average egg number during the diatom bloom. In a separate laboratory experiment, nine different local clones of the diatom *S. marinoi* were used as food for adult *E. affinis* females, in order to screen for possible differences in toxicity. The resulting average copepod hatching frequency varied between 5 and 75% for the different clones, indicating that there can be large within-species variation in the toxic properties of diatoms.

Also during a bloom in the highly productive, coastal upwelling area off the coast of central Chile, Vargas et al. (2006) reported that ingestion of diatom cells induced a negative effect on egg hatching success, naupliar survival, and diatom ingestion. Most of the larvae at the nauplius one stage had asymmetrical bodies and malformed appendages and died within 24 h after hatching, whereas most of the others died at the nauplius two stage. During the spring/summer months, when the dominant blooming species were the PUA-producing species *Thalassiosira rotula* and *Skeletonema japonicum*, only 20–50% of the naupliar stage three individuals were viable.

Variations in egg production rates, hatching success, and production of abnormal larvae were also investigated in *Calanus chilensis* females sampled weekly, from late November to December 2004, at a station located in the same coastal zone off central Chile, at a time when diatom concentrations in the phytoplankton bloom were high (Poulet et al. 2007b). Weekly egg production rates did not

change significantly during this period and remained close to normal values (25–40 eggs female⁻¹day⁻¹), whereas hatching success was constantly low and high proportions of abnormal larvae were always observed. In parallel, feeding experiments showed that egg production rates were strongly depressed by artificially enriched diets, corresponding to natural diatom assemblages occurring in the field, while values for hatching success and abnormal larvae could not be improved. Hatching success and production of abnormal larvae did not improve when females were offered a favourable food such as the dinoflagellate *Prorocentrum minimum*.

Carotenuto et al. (2006) did not find a relation between diatom concentrations and copepod hatching success during a 1-year study in the Gulf of Naples, Italy, but did report low survivorship of hatched nauplii. They found that hatching success was generally >80% in the copepods *Temora stylifera* and *Centropages typicus*, but survivorship of the first naupliar stage was very low, with mean values of 12% in *T. stylifera* and 67% in *C. typicus*. Furthermore, in *T. stylifera*, on nearly 40% of the sampling dates not a single nauplius 1 survived to moult to the second larval stage, similar to the findings of Halsband-Lenk et al. (2005) and Poulet et al. (2007a). Laboratory experiments showed that maternal feeding on *Isochrysis galbana* or *Prorocentrum minimum* for 7 days did not enhance naupliar survival in either copepod species, indicating that the negative effects of maternal diets did not disappear after feeding on a high quality food. Carotenuto et al. (2006) explained the more attenuated effects of diatoms on copepod hatching success to the highly diversified and short lasting blooms in the Gulf of Naples compared to the intense, long lasting (3 months) and almost monospecific diatom blooms that characterize the Adriatic Sea (Miralto et al. 1999). Hence the “diatom effect” was diluted and more apparent after hatching, during the nauplius one stage. Lack of a correlation between diatom abundance and copepod hatching may also be due to species-specific or strain-specific differences in diatom PUA production (Wichard et al. 2005a), differing sensitivity of copepod species to diatom PUAs (Taylor et al. 2007), copepod feeding behavior and food selection, as well as intensity and duration of diatom blooms in the field. It follows, therefore, that the diatom effect (*sensu* Halsband-Lenk et al. 2005) does not operate well when at least two of the prerequisites—short-lasting blooms and many prey alternatives—occur.

Irigoiien et al. (2002) in a worldwide survey across several coastal and oceanic regions, failed to find a link between diatom density and copepod hatching success and concluded that there is no ecologically relevant deleterious effect of field concentrations on in situ copepod egg hatching success even though their measurements of hatching success ranged from 20 to 100% indicating that

copepod reproduction was often compromised. Irigoien et al. (2005) successively also failed to find a relation between the proportion of *Skeletonema costatum* cells that dominated the major bloom in the Benguela upwelling system and hatching success in the copepods *Calanoides carinatus* and *Rhincalanus nasutus*. Koski (2007) also did not find a correlation between egg or naupliar production and diatom abundance of *Calanus finmarchicus* in three north Norwegian fjords during a spring bloom dominated by diatoms and the haptophyte *Phaeocystis pouchetii*, even though hatching success was slightly negatively correlated with diatom biomass. However, the overall high reproductive rates suggested that the main food items were not harmful for *C. finmarchicus* reproduction in the area, although direct chemical measurements were not conducted. Egg production rates were high, ranging from ca 40 up to 90 eggs $f^{-1} day^{-1}$, with a hatching success of 70–85%, and fast naupliar development were recorded through the first non-feeding stages. The majority of the copepod diet consisted of diatoms, mainly *Thalassiosira* spp. and *Chaetoceros* spp. More recently, Sommer (2009) found that a dominance of diatoms in the diet did not harm growth and reproduction in the copepod *Acartia tonsa* in mesocosms fertilized at different Si:N ratios promoting the growth of different phytoplankton assemblages. The author concluded that the frequently reported deleterious effect of diatoms on copepod reproduction may be unusual when copepods are confronted with a naturally diverse phytoplankton assemblage instead of clonal cultures in the laboratory.

Poulet et al. (2006) found that phytoplankton dominated by diatoms impaired *Calanus helgolandicus* egg production from April to November 2003 and from March to October 2004, at a station located in the English Channel off Roscoff, but there was no relationship between low fecundity and the production of diatom PUAs. They concluded that these effects were either due to limiting unidentified essential compounds not provided by diatoms, or to unknown diatom-derived toxins. In a successive paper, hatching success was examined as well, and the authors found that PUAs did not explain hatching failure which varied from 0 to 80% in that period (Wichard et al. 2008). Nutritional parameters of phytoplankton such as PUFAs, particulate organic carbon and nitrogen were measured as well, but these were also found to be unrelated to the frequently observed hatching failure of *C. helgolandicus* from the coastal waters off Roscoff. The lack of a correlation between hatching success and PUA production may in fact be due to the production by diatoms of other toxic metabolites as discussed in the following section.

Using a modeling approach Flynn and Irigoien (2009) questioned the importance of PUAs for copepod reproduction. They concluded that despite the potential impacts

of PUAs on copepods, the timing of events (matching of predators with bloom dynamics and age structure of copepods) and the behavior of other components of the system, such as microzooplankton or the noxious status of flagellates, have as much, if not more, capacity to affect diatom and copepod growth than does the liberation of PUAs upon copepod reproduction. These authors suggested that PUAs may not represent a defense developed against copepods as much as a defense against microzooplankton or as allelopaths against other phytoplankton since “slowly killing” a predator (copepods) responsible for consuming competitor primary producers (non-diatoms) and other predators (microzooplankton) would not be advantageous to the diatoms. Although this is an interesting hypothesis, to our knowledge there are as yet no studies that have examined the effects of PUAs on microzooplankton reproduction, even though there is a clear need for future research on the effects of these compounds on such predators.

Do diatoms produce cytotoxic compounds other than PUAs?

Although laboratory and field studies have unequivocally shown that high concentrations of diatoms often cause reproductive failure in copepods, the ecological significance and underlying mechanism for the “diatom effect” remain controversial. This controversy also stems from the fact that several studies have reported the absence of a correlation between diatom PUA production and copepod reduced hatching success (see previous sections), thereby placing in doubt the importance of these compounds in inducing poor reproductive performance. In this section we address this problem and discuss the chemistry of diatoms, also in the light of the recent discovery of new oxygenated fatty acid degradation products that, like PUAs, are produced from fatty acid precursors by enzymes activated within seconds after crushing of cells, and which cause the same biological effects on grazer reproductive success as the better-known diatom PUAs.

Methods for the detection and quantification of linear PUAs developed in recent years by d’Ippolito et al. (2002a) and Wichard et al. (2005a) have been fundamental for quantifying the production of these metabolites in diatoms. The first method is based on a procedure for the conversion of aldehydes into ethyl esters by the Wittig reaction with carboxylethylidene-triphenylphosphorane and the formation of stable carboxyethylidene derivatives that are easily analysed by GCMS, NMR and HPLC techniques. The second method is based on the treatment of diatom samples with O-(2,3,4,5,6-pentafluorobenzyl) hydroxylamine hydrochloride before wounding (sonication) of cells.

Using the first method, d'Ippolito et al. (2002b) described new linear PUAs in diatoms, other than the ones initially described by Miralto et al. (1999). In particular, two series of compounds were identified in the diatom *Skeletonema marinoi*: PUAs characterized by a conjugated polyunsaturated chain and aldehydes with a saturated or monounsaturated alkyl-1 tail. According to these authors, these compounds are probably derived by fatty acid oxidation, although two distinct mechanisms seem to be involved. Pentadecanal, 8-pentadecanal and tridecanal may be derived from the α -oxidation of palmitic acid (C16:0), palmitoleic acid (C16:1) and stearic acid (C18:0). Conversely, the PUAs octadienals and octatrienal are most likely formed by lipoxygenase-hydroperoxide-lyase-catalysed oxidation. Rapid transformation of fatty acids, particularly those of the eicosanoid series, had already been reported in other diatoms (Pohnert 2000).

Several papers have described the biochemical pathways leading to PUA production (see Pohnert 2005 for a review). Pohnert (2002) described that the transformation of polyunsaturated fatty acids (PUFAs) such as C₂₀ in *T. rotula* was initiated by phospholipases, in particular phospholipase A₂, that act immediately after cell damage. Liberated C₂₀ PUFAs such as eicosapentaenoic (EPA) and arachidonic acids are then further converted by lipoxygenases and lyases to PUAs. Pohnert (2002) suggested that in contrast to higher terrestrial plants that use lipases acting on galactolipids to release C₁₈ linoleic fatty acids for the production of aldehydes, diatoms rely on phospholipids and the transformation of C₂₀ EPA fatty acids by phospholipases to produce decadienal and decatrienal.

However, d'Ippolito et al. (2004) described another biochemical pathway from other complex lipids in *S. marinoi* which involves the hydrolysis of chloroplastic glycolipids and release of C₂₀ EPA and C₁₆ PUFAs such as hexadecatrienoic and hexadecadienoic acids. They showed that chloroplasts have a direct role in the production of PUAs in diatoms, similar to what occurs in higher terrestrial plants (Blée 1998). They proposed a biochemical pathway leading to the production of PUAs in diatoms whereby C₂₀ and C₁₆ PUFAs are liberated from chloroplast glycolipids and phospholipids by lipolytic acyl hydrolases. These PUFAs are then rapidly transformed into unstable hydroperoxides by either 9-lipoxygenase (LOX), 11-LOX or 14-LOX enzymes. Hydroperoxides are in turn rapidly transformed to PUAs such as 2,4 heptadienal, 2,4 octadienal and 2,4,7 decatrienal depending on the LOXs and other downstream enzymes present in the diatom cells.

The synthesis of PUAs begins immediately after cell wounding thus implying that the proteins responsible for the oxidative metabolism of C₁₆ and C₂₀ fatty acids are expressed constitutively in diatom cells. By studying the downstream enzymatic activity responsible for PUA

synthesis, d'Ippolito et al. (2006) have shown that lipoxygenase and lyase activities are both found in the microsomal fractions, the same fraction where the highest PUA production is found. The authors demonstrated that the diatom *T. rotula* possesses an enzymatic arsenal capable of transforming C₁₆ and C₂₀ PUFAs to 2,4 octadienal or 2,4,7 decatrienal whereas in *S. marinoi* C₁₆ and C₂₀ PUFAs serve as specific substrates for the production of 2,4 heptadienal and 2,4 octadienal. On the contrary, synthesis of decatrienal appears to depend mainly on C₂₀. Further studies are in progress to isolate and characterize the enzymes involved in this process (discussed in d'Ippolito et al. 2005). The ultimate goal is to define a useful molecular tool to easily evaluate the processes leading to the production of PUAs and/or other oxylipins.

A mechanism for the production of PUAs only after cell lysis has been proposed so that toxins are released directly into the body of grazers (Wichard et al. 2007) thereby avoiding intoxication to the diatom cells (Casotti et al. 2005). Fontana et al. (2007a) recently measured PUA production with time and showed that formation of PUAs begins soon after sonication of cells and increases steadily for several minutes thereafter, reaching concentrations of up to 50 fg cell⁻¹ in the diatom *Thalassiosira rotula*. When PUAs are removed by keeping the cells under vacuum for 15 min, production is reinitiated immediately, giving rise to even higher levels (up to 100 fg cell⁻¹). The results demonstrate that diatom enzymes are active for a substantial period in seawater (up to 40 min in the experiments by Fontana et al. 2007a) thereby enhancing local concentrations of these toxins either in the surrounding water or, if ingested, in the body of grazers.

A major advancement in the study of the chemistry of diatoms was the discovery that they produced cytotoxic compounds other than PUAs. Wichard et al. (2005b) had shown that only 36% of the investigated 51 species released PUAs upon cell damage, with PUA concentrations ranging from 0.01 to 9.8 fmol cell⁻¹, depending on the species or strain, thereby suggesting that many diatoms did not produce these compounds, or produced very low amounts of these. But, at about the same time, d'Ippolito et al. (2005) showed that the diatom *Thalassiosira rotula* had an oxidizing potential capable of converting PUFAs to a variety of other unprecedented oxylipins, as later confirmed by Fontana et al. (2007b). Fontana et al. compared the effects of the well-known PUA-producing diatom *Skeletonema marinoi* (Miralto et al. 1999; Ianora et al. 2004) with two *Chaetoceros* (*C. similis* and *C. affinis*) species which did not produce PUAs, but which nonetheless impaired hatching success. They showed that when the *Chaetoceros* species were damaged, they produced fatty acid hydroperoxides (FAHs) and oxylipins such as hydroxyacids (HEPEs) and epoxyalcohols (HepETEs), as

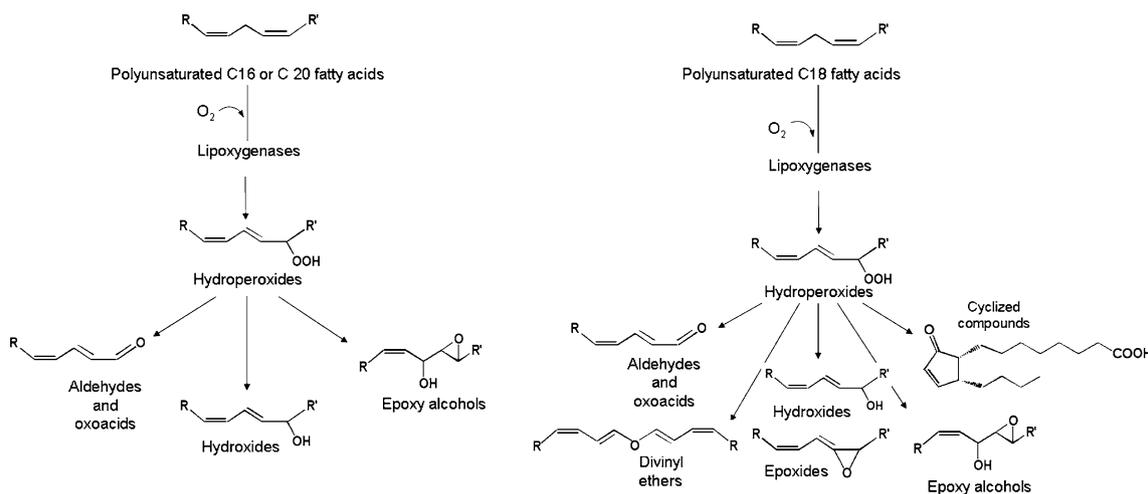


Fig. 1 Schematic representation of the oxidative metabolism of fatty acids in diatoms (modified from Fontana et al. 2007b) and terrestrial plants (modified from Blée 2002). Oxidation of C20 and C16 fatty acids in diatoms and C18 fatty acids in plants via lipoxygenase

well as highly reactive oxygen species (ROS) of low acute toxicity to adult copepods but which depressed the viability of copepod gametes and offspring. A schematic representation leading to the production of these new metabolites from PUFAs is given in Fig. 1 which also compares these oxylipins with those produced as a wound-activated defense mechanism in terrestrial plants.

These products are very similar suggesting that they are so fundamental for the survival of plant cells, that they have been conserved through evolution. A major difference is in the precursor PUFAs, C16 and C20 fatty acids, used to synthesize these compounds in diatoms (Pohnert 2005 and d'Ippolito et al. 2005) compared to the C18 fatty acids in terrestrial plants (Blée 1998, 2002). Oxylipins formed in flowering plants include fatty acid hydroperoxides, hydroxyl- and keto-fatty acids, oxo-acids, epoxyalcohols, divinyl ethers, PUAs, and the plant hormones 12-oxo-phytodienoic acid and jasmonic acid (Andreou et al. 2009), several of which have not yet been found in diatoms (e.g. jasmonic acid). Oxylipins are believed to play a pivotal role in plant defense because they act as chemical attractors (e.g. pheromones, pollinator attraction) or alarm signals against herbivore attack (e.g. in tritrophic interactions) and protective compounds (antibacterial, wound healing). Diatom oxylipins also show a high similarity to volatile organic carbons released from brown algae which are suggested to be involved in chemical signalling and pheromone attraction between gametes of different sex (Andreou et al. 2009 and references therein).

Several of these new compounds were also present in PUA producing species such as *Thalassiosira rotula* (d'Ippolito et al. 2005) and *S. marinoi* (Fontana et al. 2007b), indicating that some diatoms produce both PUAs

enzymes leads to the production of hydroperoxides and many final products such as aldehydes and oxo-acids, hydroxides and epoxyalcohols

and these other oxylipins, whereas other species produce only these new metabolites (Fig. 2). Two of these compounds, 15*S*-HEPE and *threo*-13,14-HepETE have now also been reported by d'Ippolito et al. (2009) in the non-PUA producing pennate diatom *Pseudo-nitzschia delicatissima*. Interestingly, d'Ippolito et al. also reported the presence of another metabolite, 15-oxo-5*Z*,9*E*,11*E*,13*E*-pentadecatetraenoic acid which was produced only in the late stationary phase of the culture. Pohnert (2002) had reported the presence of similar aldehydic 9-oxo-nonadienoic and 12-oxo-dodecatienoic acids in *Phaeodactylum tricornutum*, testing their activity on sea urchin embryos.

The impact of HEPES and HepETEs was tested on larval development in the copepod *Calanus helgolandicus* (Fontana et al. 2007b). Nauplii spawned by females reared with *C. affinis* showed incomplete development of swimming appendages with segments that differed from normal both in number and shape. Fluorescent images of the same specimen showed apoptotic regions corresponding to these morphological anomalies. These results demonstrated that PUAs were not the only class of molecules inducing malformations and apoptosis in copepods. We here confirm that diatoms producing these compounds induce reproductive failure in copepods (Fig. 3). Our new unpublished results show that a diet of *Pseudo-nitzschia delicatissima* induces low hatching success and apoptosis in the offspring of *Calanus helgolandicus* compared to control diets of the dinoflagellate *Prorocentrum minimum* which does not produce any of these oxylipins (Fontana et al. 2007b) and which does not induce apoptosis in copepod nauplii (Poulet et al. 2003). Although this is the only other report showing the effects of HEPES and HepETEs on grazer survival, these findings may shed light on past conflicting results

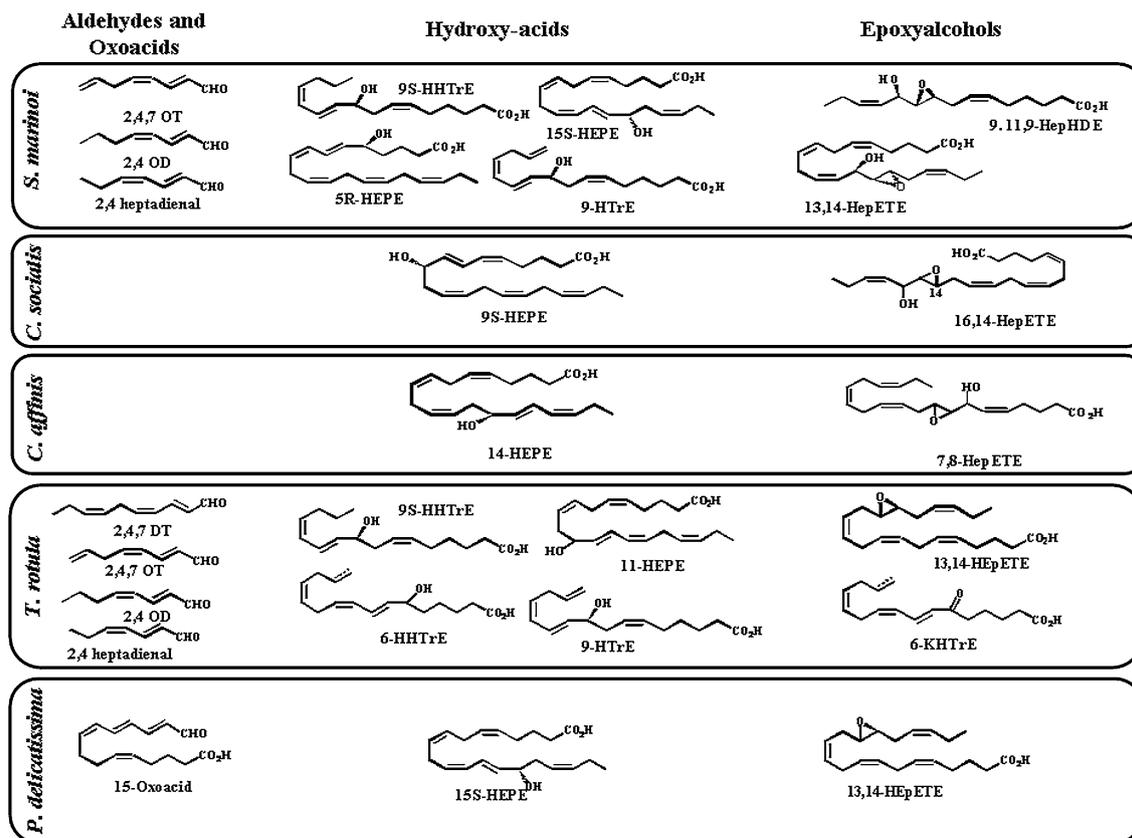


Fig. 2 Overview of oxylipins produced in the diatom species *Skeletonema marinoi*, *Chaetoceros socialis*, *C. affinis*, *Thalassiosira rotula* and *Pseudo-nitzschia delicatissima*, from Fontana et al. (2007a,

b), d'Ippolito et al. (2005, 2009). Production of these compounds is highly species- and strain-specific

where several authors were unable to find a relationship between PUA production and copepod reproductive failure (see previous sections). The “diatom effect” is therefore not due to a single class of compounds (i.e. PUAs), as previously believed, but rather to a blended mixture or bouquet of toxins to which grazers are exposed upon feeding on diatoms.

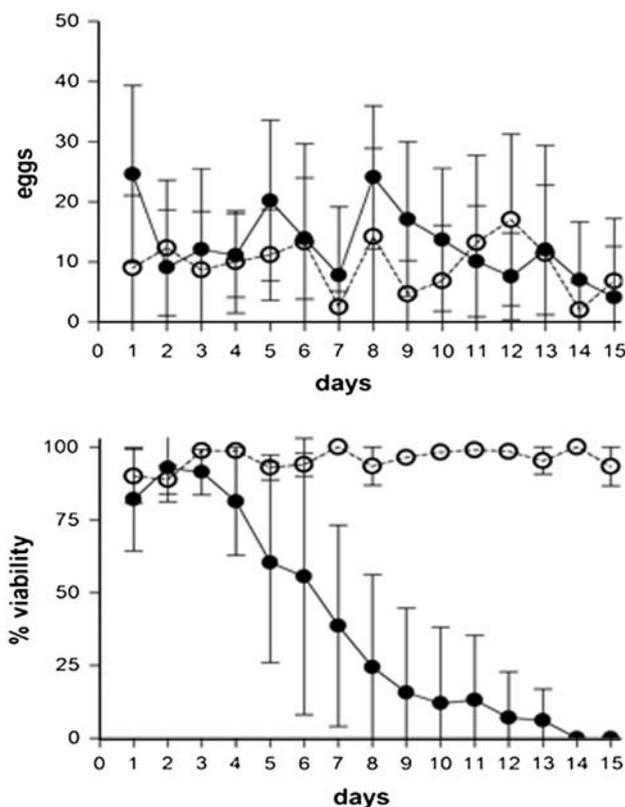
New oxylipins have also been found at sea, during a late spring bloom in the North Adriatic Sea of the non-PUA producing diatom *Cerataulina pelagica*, the likely cause for reduced egg production and hatching success in *Acartia clausi*, *Calanus helgolandicus* and *Temora longicornis* (Ianora et al. 2008). Hence, these compounds seem to be very diffuse among diatom species even if their exact function remains elusive. In any case, it seems evident that we have only begun to unravel the complex chemistry of diatoms. A major challenge for the future, therefore, will be to understand the ecological function of these metabolites and the biochemical pathways and physiological factors that lead to their production. By way of a coda, Cutignano et al. (2009) have now published methods for the detection of these new compounds which will certainly lead to a better understanding of their importance in diatoms.

New methods to study diatom-copepod interactions

In recent years there have been a series of methodological advancements in the study of diatom effects on copepod reproduction. New protocols for the rapid assessment of copepod hatching success have been developed to replace current laborious and time-consuming incubation methods. Three probes, fluorescein diacetate (FDA), SYTOX Green and 7-aminoactinomycin D (7-AAD), were shown to rapidly assess egg viability in the copepod *Calanus helgolandicus* (Buttino et al. 2004). Treatment with these stains did not interfere with hatching success. Embryos pre-treated with chitinase enzyme were stained with each of the probes and then observed with the confocal laser scanning microscope. The percentage of fluorescent-FDA embryos and non-fluorescent SYTOX Green and 7-AAD embryos were compared with the percentage of hatched unstained embryos and with the percentage of embryos that had been stained, washed, and allowed to hatch. Results showed that all three dyes accurately predicted embryo viability and could be used to rapidly predict egg hatching success.

Cell-specific probes have been used to detect apoptosis (cell death) in eggs and in nauplii spawned by copepods fed

Fig. 3 Unpublished results showing daily mean egg production rates (\pm SD) and percent egg viability for *Calanus helgolandicus* females ($n = 15$ replicates) maintained for 15 days on monoalgal diatom diets of the non-PUA producing pennate diatom *Pseudo-nitzschia delicatissima* (same clone as in d'Ippolito et al. 2009) (black circles) and control diets of the dinoflagellate *Prorocentrum minimum* (white circles). There were no statistical differences between the two diets in terms of egg production rates ($t_{28} = 1.77$; $P > 0.05$) but highly significant differences were found in terms of egg hatching success ($t_{28} = 6.25$; $P < 0.0001$). Experimental protocols to measure egg production and egg viability are the same as in Turner et al. (2001). Food concentrations tested were 10^4 cells ml^{-1} . Lower left panel shows a transmitted light image of a newly hatched nauplius generated from a mother fed on a diet of *P. delicatissima* which is TUNEL positive (green fluorescence, right panel) indicating apoptotic dead tissues. Nauplii from females fed *P. minimum* were non-fluorescent (not shown) as in Poulet et al. 2003



diatom diets. Romano et al. (2003) used deoxynucleotidyl-transferase-mediated dUTP nick-end labelling (TUNEL) and DNA fragmentation profiling (laddering) to show that the PUA decadienal induced apoptosis in copepod embryos spawned by *Calanus helgolandicus* females fed the diatom *Thalassiosira rotula* for 10–15 days, or when newly spawned eggs of this species were exposed for 1 h to $5 \mu\text{g ml}^{-1}$ decadienal. Poulet et al. (2003) used two double-labelling methods, TUNEL+propidium iodide and Annexin V-FITC+propidium iodide, to diagnose cell death in N1–N2 naupliar stages of the copepod *C. helgolandicus* fed

the diatom *T. rotula*, showing that apoptosis and necrosis occurred in 80–100% of the offspring, which sooner or later died. Such cell degradation processes and high mortality were not observed in nauplii produced by females fed the non-toxic dinoflagellate *Prorocentrum minimum*. Ianora et al. (2004) also used the TUNEL assay and propidium iodide to show that *C. helgolandicus* abnormal nauplii generated from mothers fed the diatom *Skeletonema marinoi* were apoptotic, and that apoptosis and teratogenesis of nauplii increased the longer the females fed on the diatom diet. Fontana et al. (2007b) used the TUNEL dye to reveal

apoptotic cells and tissues in newborn nauplii generated from *C. helgolandicus* and *Temora stylifera* copepods fed the non-PUA producing diatoms *Chaetoceros affinis* and *C. socialis*. They showed that apoptosis was due to a blended mixture of oxylipins other than PUAs, such as HEPEs and HepETEs, as well as massive concentrations of FAHs and ROS, explaining the strong negative effects on early development of copepod nauplii.

A recent study has explored the possibility of using liposomes as a delivery system for copepods (Buttino et al. 2006). Giant liposomes were prepared and characterised in the same size range of food ingested by copepods (mean diameter = 7 μm) and then encapsulated with the fluorescent dye fluorescein isothiocyanate (FITC) to verify copepod ingestion with the confocal laser-scanning microscope. Females of the calanoid copepod *Temora stylifera* were fed with FITC-encapsulated liposomes alone or mixed with the dinoflagellate alga *Prorocentrum minimum*. Control copepods were incubated with the *P. minimum* diet alone. When liposomes were supplied together with the algal diet, egg production rate, egg-hatching success, and faecal pellet production were as high as those observed for the control diet. On the contrary, egg production and hatching success was very low with a diet of liposomes alone and faecal pellet production was similar to that recorded in starved females. This suggests that liposomes alone did not add any nutritive value to the diet, making them a good candidate as inert carriers to study the nutrient requirements or biological activity of different compounds.

In another study, Buttino et al. (2008) used these giant liposomes to encapsulate decadienal in order to investigate the effect of PUAs on the reproductive biology of the copepods *Temora stylifera* and *Calanus helgolandicus*. After 10 days of feeding, liposomes reduced egg hatching success and female survival with a concomitant appearance of apoptosis in both copepod embryos and female tissues. Concentrations of decadienal inducing blockage were one order of magnitude lower than those used in classical feeding experiments (e.g. Ianora et al. 2004) demonstrating that liposomes were a useful tool to quantitatively analyze the impact of toxins on copepods.

Effects of diatoms on other invertebrates

Broadcast spawning benthic invertebrates have been shown to differ widely in their susceptibility to the PUA decadienal (Caldwell et al. 2002) with polychaetes being more vulnerable than echinoderms. For example, exposure of gametes to decadienal inhibited fertilization success which declined to 50% of control values at concentrations of 1.55 $\mu\text{g ml}^{-1}$ in *Arenicola marina*, 3.98 $\mu\text{g ml}^{-1}$ in *Nereis*

virens, 7.94 $\mu\text{g ml}^{-1}$ in *Psammechinus miliaris* and 10 $\mu\text{g ml}^{-1}$ in *Asterias rubens* (Caldwell et al. 2004a). The cause of reduced fertilization success was shown to depend on sperm motility, as demonstrated by pre-incubation of sperm in decadienal which caused a pronounced dose-dependent decline in sperm migration rates. Lewis et al. (2004) and Caldwell et al. (2005) studied the effect of diatom PUAs on larval fitness and used random deviations from perfect bilateral symmetry to analyze fitness in the polychaete *N. virens* and the sea urchin *P. miliaris* exposed to decadienal. They showed that the degree and frequency of asymmetrical (teratogenic) development increased with increasing decadienal concentrations, and that there was a clear stage-specific effect, with earlier larval stages being the most affected.

The effect of decadienal on fertilization processes was also studied by Tosti et al. (2003) who showed that decadienal was able to selectively inhibit sperm-activated ion channels in ascidian *Ciona intestinalis* oocytes. In particular, decadienal acted as a specific fertilization channel inhibitor, altering actin filaments and mitochondrial migration after contraction, leading to a disturbance in cleavage formation that may be responsible for teratogenic development of embryos. This was the first report of a compound (decadienal) that specifically blocked fertilization channels in animals without altering the internal release of calcium stores generating contraction of oocytes.

Romano et al. (2003) tested the effect of decadienal as an apoptogenic inducer in sea urchin embryos, using TUNEL, DNA fragmentation profiling (laddering) and an assay for caspase-3 activity. Decadienal induced apoptosis and also activated a caspase-3-like protease. The saturated aldehyde decanal induced apoptosis at a higher concentration and after a longer incubation period than decadienal, indicating that a,b-unsaturation of the molecule, coupled with the aldehyde group, was responsible for the greater biological activity of decadienal.

The cytotoxicity of several saturated and unsaturated PUAs and an oxo-acid have been screened in vitro and in vivo on other organisms such as bacteria, algae, fungi, echinoderms, molluscs and crustaceans (Adolph et al. 2004). Decadienal, octadienal, and 9-oxo-nonadienoic acid were active against bacteria and fungi and showed weak algicidal activity. By contrast, the saturated aldehyde decanal had either low or no significant biological activity. In assays with oyster haemocytes, decadienal exhibited a dose-dependent inhibition of cytoskeleton organization, rate of phagocytosis and oxidative burst and a dose-dependent promotion of apoptosis. This wide spectrum of physiological pathologies reflects the potent cell toxicity of diatom-derived PUAs, in relation to their non-specific chemical reactivity towards nucleophilic biomolecules.

Adolph et al. (2004) pointed out that this cytotoxic activity was conserved across six phyla, from bacteria to crustaceans.

Caldwell et al. (2003) studied the effects of water soluble algal extracts, the aldehydes decadienal, decanal, undecanal and the fatty acid eicosapentaenoic acid (EPA) on the hatching success and larval mortality of the brine shrimp *Artemia salina*. Both crude cellular extracts of the diatoms *Skeletonema costatum* and *Nitzschia commutata* and decadienal were found to inhibit hatching success of *A. salina* cysts in a dose-dependent manner. Decadienal also significantly affected larval mortality rates in 24 and 72 h exposure incubations. By contrast, the two saturated aldehydes and EPA showed a limited toxic effect on hatching success and naupliar mortality trials.

The acute toxicities of five PUAs to the rotifer *Brachionus plicatilis* and nauplii of the brine shrimp *Artemia salina* was also studied by Taylor et al. (2005), who examined the effect of a representative of these aldehydes in the presence of sub-lethal levels of heavy metals. *B. plicatilis* generally showed greater sensitivity to PUAs than *A. salina*. Of the aldehydes tested, decadienal was the most toxic to both species having 24 h LD₅₀ values of 7 and 20 μM for *B. plicatilis* and *A. salina*, respectively. The presence of 1 μM of copper sulphate in solutions of decadienal resulted in the reduction of the 24 h LD₅₀ of decadienal by approximately a third for both species. One μM of copper chloride in solutions of decadienal reduced the 24 h LD₅₀ of decadienal to *A. salina* nauplii by $\sim 11\%$ and 1 μM zinc sulphate caused a reduction of only 3%. Pre-exposure of the organisms to 1 μM copper sulphate had no significant impact on their subsequent mortality in decadienal.

Carotenuto et al. (2005) have shown that in the freshwater cladoceran *Daphnia pulex* egg hatching success was reduced with diets of the decadienal-producing diatom species *Fragilaria* sp. and in vitro tests indicated a dose-dependent inhibition for this molecule. Arrested egg development and egg abortion had already been observed in field populations of *Daphnia* and had been linked to food composition but not to particular species of diatoms (Boersma and Vijverberg 1995). *Daphnia* and other freshwater pelagic grazers such as the copepods *Cyclops* and *Eudiaptomus* have been shown to perceive decadienal and other PUAs which cause a repellent action that reduces swimming activity in assay vials suggesting that aldehydes modify behavioural responses of potential grazers (Jüttner 2005). This author suggested that PUAs may act as warning signals for the toxic EPA that is formed in high concentrations in cells upon wounding and which is retained in the diatom cells.

PUAs are also produced by benthic diatoms such as *Cocconeis scutellum* and it has been suggested that this or

other compounds may be responsible for the early apoptosis of the male gonad of the protandric shrimp *Hippolyte inermis* (Zupo et al. 2007). This benthic shrimp is characterized by a peculiar mechanism of sex reversal influenced by diatom foods. The appearance of primary females in spring is due to an apoptotic early disruption of the androgenic gland and of the male gonad, triggered by still unknown compounds present in diatoms of the genus *Cocconeis*. Zupo et al. (2007) experimentally administered specific planktonic diatoms, their extracts and specific compounds (decadienal) known to induce apoptosis in planktonic copepods, to *H. inermis* postlarvae, to check whether the apoptotic effect was due to PUAs, but they found only negligible effects on the sex ratios of cultured shrimps indicating that compounds other than PUAs were responsible for sex reversal.

Effects of PUAs on diatoms

Casotti et al. (2005) were the first to show that PUAs are also toxic to diatom cells themselves. Decadienal inhibited the growth of *Thalassiosira weissflogii* in a dose- and time-dependent manner, with irreversible effects after 24 h exposure. EC₅₀ for growth ranged between 0.1 and 0.2 $\mu\text{g ml}^{-1}$ of decadienal on *T. weissflogii* growing at continuous or alternate light (12:12 = D:L), and at different cell concentrations (10 or 20 thousand cell/ml, exponential phase of growth). The effect was both concentration-dependent and time dependent, and treated cells appeared granulated and TUNEL-positive, suggesting the induction of an active process of cell death, closely resembling apoptosis. Casotti et al. (2005) proposed a possible non-toxic role of PUAs to signal changes in biomass, structure and dynamics of algal communities suggesting an active endogenous control of the population by these molecules when environmental factors become unfavourable for growth. Hence PUAs may not only act as defensive molecules against grazers but act as signal molecules that determine cell fate and death of diatoms.

PUAs also act as allelochemicals by suppressing the growth of other phytoplankton, with smaller species such as the prymnesiophyte *Isochrysis galbana* that are more sensitive to these compounds compared to larger species like the chlorophyte *Tetraselmis suecica* and the diatom *Skeletonema marinoi* (Ribalet et al. 2007a). Based on published reports, these authors estimated that the release of PUAs from each diatom cell was 46.9, 4.7 and 0.5 $\mu\text{mol PUA l}^{-1}$ at a distance of 1, 10 and 100 μm from the cell surface which is well within the significant range for affecting growth and performance of surrounding organisms.

PUA production has been shown to increase depending on the age of the culture, from 1.2 fmol cell⁻¹ in the

exponential phase to $4.2 \text{ fmol cell}^{-1}$ in the stationary phase of the diatom *Skeletonema marinoi* (Ribalet et al. 2007b). PUA production was enhanced under nutrient limitation, and N- and P-limited cells produced 10.7 and 4.6 times more PUAs in the stationary phase, respectively, suggesting a direct link between toxin production, physiological conditions of the cells, and nutrient stress. These authors suggested that under natural conditions nutrient stress could act as an upstream signal for bloom termination by switching the cell response towards cell death. Ribalet et al. (2009) have now measured silica content, as an indication of cell wall thickness, and PUA content in *S. marinoi* grown under Si-limitation in continuous cultures. A 7.5-fold increase in PUA production was observed ($27.5 \text{ fmol cell}^{-1}$) in Si-limited cells with respect to the controls, while Si content decreased by 50% suggesting that high PUA levels produced under Si-limitation, when cells have a thinner cell wall, may denote a compensatory alternation of mechanical and chemical defense mechanisms in diatoms. Interestingly, there seems to be a synchronized release of PUAs from intact cells immediately before the declining phase of growth, supporting the idea that PUAs may indeed play a role as infochemicals in mediating diatom bloom dynamics (Vidoudez and Pohnert 2008).

Production of PUAs seems to vary considerably between strains and even within the same strain. Taylor et al. (2009) studied the production of PUAs in nine different *S. marinoi* strains isolated at three different times of the year (spring, summer, and autumn) in relation to the predominant conditions at the time of isolation from Gullmar Fjord, Skagerrak. During the initial stages of growth, PUA production potential was highest in summer strains whereas spring strains showed a strong capacity for increased PUA production potential in later stage cultures with diminishing nutrient levels, reaching amounts similar to those observed in summer strains. In contrast, PUA production potentials of summer and autumn strains did not change significantly from the original values. Grazing pressure was negligible during the spring bloom and was much greater during summer and autumn. Hence PUA production potentials of *S. marinoi* appear to reflect the ecological conditions at the time of isolation with higher production potentials in strains isolated when conditions were likely to be less beneficial for survival of the species.

Decadial has been shown to trigger the generation of nitric oxide (NO) which results in cell death (Vardi et al. 2006). Pre-treatment of cells with sublethal doses of decadial induces resistance to subsequent lethal doses demonstrating the existence of a sophisticated stress surveillance system in diatoms which allows diatom cells to sense local PUA concentrations and integrate this information in a temporal context. According to these authors,

when stress conditions are aggravated during a bloom, PUA concentrations could exceed a certain threshold and act as a diffusible bloom-termination signal triggering population-level cell death. Vardi et al. (2008) have now used a functional genomics approach to monitor the stress response of the diatom *Phaeodactylum tricornutum* exposed to decadial. They have shown that the gene responsible for NO generation, PtNOA, was upregulated in response to decadial and that overexpressing cell lines were hypersensitive to sublethal levels of this aldehyde, manifested by altered expression of superoxide dismutase and metacaspases, key components of stress and death pathways. These authors have reported NOA-like sequences in diverse oceanic regions, suggesting that a novel NO-based system operates in diatoms and may be widespread in phytoplankton, providing a biological context for NO in the upper ocean. Vardi et al. (2008) conclude that future studies using transgenic approaches for manipulating genes in key signaling and stress-related pathways in diatoms may provide the opportunity to gain further insights into the role of these compounds in controlling algal population dynamics and other trophic-level interactions in the aquatic environment.

Diatoms are not the only class of marine phytoplankton to produce PUAs (Hansen et al. 2004). A recent study by these authors showed that the bloom-forming phytoplankter *Phaeocystis pouchetii* (Prymnesiophyceae) from the coastal waters of northern Norway produced and released decadial. However, no significant adverse effects of *P. pouchetii* on diatom presence were observed in the field. *P. pouchetii* and *S. costatum* were frequently co-occurring species, and since they are both known producers of PUAs it remains unknown if the presence of decadial released from *P. pouchetii* induced by heavy grazing might influence the growth of other phytoplankton species (Hansen and Eilertsen 2007).

In freshwater environments, PUAs are commonly released by diatoms and chrysophytes (see Jüttner 2005 and references therein) through cell lysis, independently from grazing, conferring rancid smells to source drinking water (Watson and Satchwill 2003). In this case, PUAs may not only serve to deter grazers (Fink 2007) but may also function as intra or interspecific signals, for example, to modulate population density or to signal the presence of pathogens or predators (Watson 2003). Their wide spread occurrence in marine and freshwater photoautotrophs may be an indication of the great ecological importance of these compounds. It is interesting to note that similar compounds are also produced by terrestrial plants to signal an attack by herbivores to conspecifics which respond by up-regulating defensive genes against predators (e.g. Arimura et al. 2000).

Effect of PUAs on marine bacteria

Diatoms have long been known to have inhibitory or stimulatory effects on pathogenic bacteria (e.g. Bell et al. 1974). Recent evidence indicates that diatom PUAs block the growth of some pathogenic non-marine bacteria (Adolph et al. 2004) and have synergistic antibacterial effects when different aldehydes are employed in combination (Bisignano et al. 2001). There is also evidence that diatom PUAs can affect the growth of some marine bacteria but not others (Ribalet et al. 2008), with a concentration-dependent growth reduction for 19 of 33 bacterial strains tested at concentrations ranging from 3 to 145 $\mu\text{mol l}^{-1}$. This is one to two orders of magnitude higher than concentrations affecting growth of phytoplankton cells (Casotti et al. 2005; Ribalet et al. 2007b) suggesting that bacteria are much more resistant to PUAs than other microbes. Other bacterial strains were unaffected by even very high PUA concentrations, and two strains even showed growth stimulation in the presence of PUAs. Interestingly, most strains isolated during a bloom of the PUA producing diatom *Skeletonema marinoi* showed resistance to PUAs which could explain why some bacteria can thrive in close contact with diatoms. Therefore, these compounds may play a significant role in shaping the structure of bacterial communities during diatom blooms (Ribalet et al. 2007b). This may be even more important during the final stages of blooms, when senescence and nutrient limitation increase the potential production and release of PUAs.

Concluding remarks

Herbivory is very intense in the plankton. Copepods and other planktonic crustaceans are predominantly herbivorous, grazing on large quantities of phytoplankton cells. For example, copepods dominate the zooplankton in samples from most sea areas, representing from 55 to 95% of the total number of zooplankton organisms caught in plankton hauls. They are considered the most numerous multi-cellular organisms on earth, outnumbering the insects in terms of abundance if not in terms of biodiversity (Schminke 2007). Herbivory is therefore an important pressure for the evolution of defensive compounds in marine phytoplankton, as for terrestrial higher plants, and for shaping prey–predator relationships in the pelagic environment. Studies on chemical interactions in the plankton are still in their infancy but there is an increased awareness that such products may play fundamental roles as defenses against predators, competitors and pathogens, and therefore drive ecosystem functionality. However, the organism has to pay a price for this ecological advantage.

The chemical pathways that generate marine natural products are often complex and significant amounts of metabolic energy are expended to generate their production that could otherwise have been directed to growth or reproduction (Ianora et al. 2006). The high energetic cost of producing and maintaining potentially toxic compounds must therefore be compensated for by a defensive benefit to the producing organism.

In the case of diatoms, however, the compounds are not constitutively present in the cells but PUAs and other oxylipins are only produced when the cell is damaged as would occur during grazing (Pohnert 2000) or cell lysis (Vidoudez and Pohnert 2008). Thus, the cost for their production is expected to be lower than for other microalgal toxins which are always present in the cell, such as the saxitoxins, gonytoxins and other chemically complex neurotoxic compounds produced by dinoflagellates. Diatom defense relies on primary metabolites such as storage lipids, which are transformed by lipase and lipoxygenase enzymes after wounding or ingestion. The cost of defense would therefore be negligible and the evolution of such defenses could thus be driven by the need for processes involved in primary metabolism (Pohnert et al. 2007) together with the need for feeding pressure reduction.

Due to the teratogenic nature of diatoms PUAs and other oxylipins, the mechanism of chemical defense functions by mainly reducing the grazing effects of subsequent generations of copepods. Hence, these compounds differ from those that act as feeding deterrents, the purpose of which is not to intoxicate the predator but discourage further consumption, or those that lead to physical incapacitation such as paralysis and death of the predator. Cembella (2003) termed such stealth compounds of low acute toxicity to adult predators that lead to post-digestive reduction in fecundity or depressed viability the “kill-the-children” selection, even though recent evidence suggests that these compounds compromise adult fitness as well (Buttino et al. 2008). It simply takes longer for these effects to manifest themselves in adults compared to larval stages, as had already been observed by Taylor et al. (2007). Hence, feeding deterrence would not protect the individual ingested cells but the community as a whole and the defense compounds would not target the predator but mainly its offspring. In the end, grazing pressure would be reduced allowing blooms to persist when grazing pressure would otherwise have caused them to crash.

What advantages would chemical defenses confer to single cell-organisms such as diatoms? In asexually reproducing organisms the sum total of all the cells comprises the clone and thus the “individual” is a group of cells. Elimination of the individual does not destroy the clone so there is ample scope for evolution of chemical defenses in phytoplankton as also for copepods to sample

their food, learn which cells to avoid and eventually develop antidotes against toxins (Smetacek, personal observation). Copepods feed selectively on individual particles which are handled, prior to ingestion or rejection, with sensory mouth parts. So there is no reason why chemical warfare with its accompanying arms race, equivalent to that in all other biomes, should not also be raging in the plankton (Smetacek 2002). There is in fact recent evidence that copepods are able to discriminate between virtually identical cells of the same diatom species but with different biochemical properties resulting in variable PUA production (Broms et al. 2009) indicating that copepods actively select less deleterious cells with the result that egg hatching success increases. Similar suggestions were made by Leising et al. (2005a, 2005b), proposing that selection against specific diatoms in a field study from Dabob Bay, U.S.A. was most likely based on active particle rejection as a response to chemical properties of the cell surfaces of diatoms since other, morphologically similar cells, were not rejected. There is therefore a benefit for the algae to invest in oxylipin production since they are less attractive to the grazers.

It is interesting to speculate on the multiple simultaneous functions of diatom oxylipins which not only deter herbivore feeding but also act as allelopathic agents against other phytoplankton cells, affecting the growth of competitors with possible consequences on food web structure and community composition. Since PUAs are mainly produced during the stationary stage, when they can be naturally lysed from the cell, it has been suggested that these molecules are also implicated in signalling population-level cell death and termination of the bloom (e.g. Casotti et al. 2005). Wink and Schimmer (1999) explained the evolution of such compounds in terms of nature's tendency 'to catch as many flies with one clap as possible'. Thus, the same molecules may act to deter different groups of organisms by different modes of action and also act as signals mediating other plankton interactions. Wink (1999) claims that multiple functions of secondary metabolites in higher terrestrial plants are common and do not contradict their main role for chemical defense and signaling. Furthermore, he argues that natural selection will favor those metabolites that possess multiple functions. Future ecological studies of plankton secondary metabolites will therefore have to consider such multiple functions and the multiple pathways by which metabolites mediate chemical interactions among organisms and how these in turn are mediated by biotic and abiotic environmental factors.

The chemistry of diatoms is much more complex than we originally conceived when we isolated the first molecules from diatoms a decade ago (Miralto et al. 1999). The diversity of molecules at the species and strain-specific level suggests a communication function similar to what

occurs in higher terrestrial plants (Blée 2002). The chemical complexity of diatoms may therefore have been driven by the necessity to avoid co-evolution by predators to detoxify these molecules. Further studies on such detoxifying mechanisms may in fact shed light on diatom-copepod interactions and why some diatoms seem to be consumed more by some predators than others. These complex interactions recall the aphorism "one man's meat is another man's poison" and suggest that co-evolution between diatoms and copepods is also based on a chemical arms race.

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