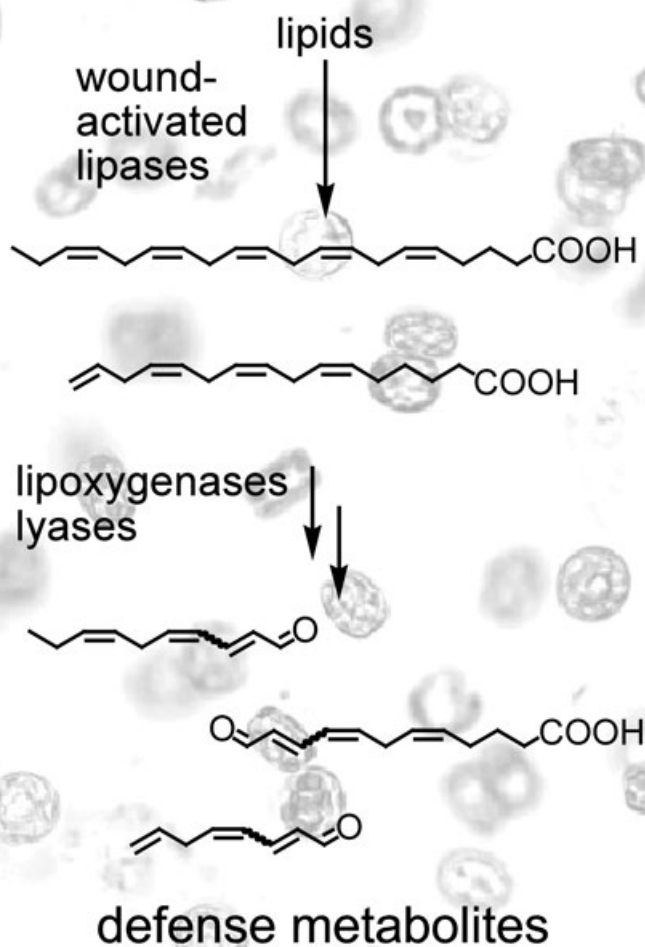
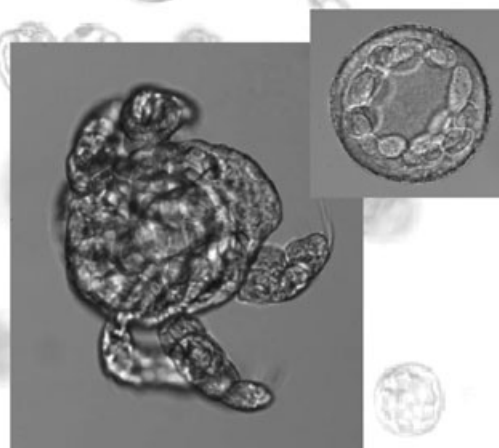
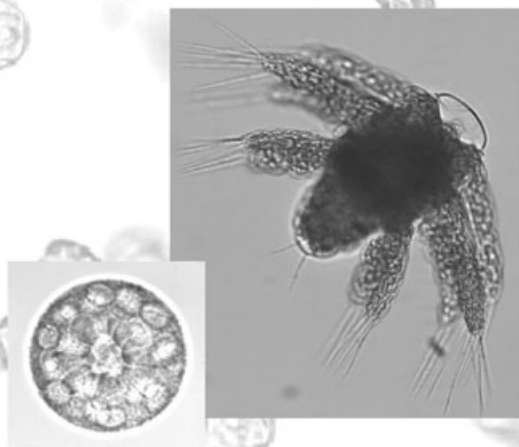


Defense of microalgae



normal herbivore
offspring

defense results in malformations

Diatom/Copepod Interactions in Plankton: The Indirect Chemical Defense of Unicellular Algae

Georg Pohnert^{*[a]}

Numerous coexisting species can be observed in the open oceans. This includes the complex community of the plankton, which comprises all free floating organisms in the sea. Traditionally, nutrient limitation, competition, predation, and abiotic factors have been assumed to shape the community structure in this environment. Only in recent years has the idea arisen that chemical signals and chemical defense can influence species interactions in the plankton as well. Key players at the base of the marine food web are diatoms (unicellular algae with silicified cell walls) and their main predators, the herbivorous copepods. It was assumed that diatoms represent a generally good food

source for the grazers but recent work indicates that some species use chemical defenses. Secondary metabolites, released by these algae immediately after wounding, are targeted not against the predators themselves but rather at interfering with their reproductive success. This strategy allows diatoms to reduce the grazer population, thereby influencing the marine food web. This review addresses the chemical ecology of the defensive oxylipins formed by diatoms and the question of how these metabolites can act in such a dilute environment. Aspects of biosynthesis, bioassays, and the possible implications of such a chemical defense for the plankton community structure are also discussed.

1. Introduction

Most of the surface of our planet is covered by water, and it should therefore come as no surprise that marine phototrophic organisms contribute substantially to global carbon fixation. Modern models predict that photosynthetic activity is divided into equal parts between marine and terrestrial ecosystems.^[1] Life in the seemingly homogeneous ocean water is multifaceted and ecological processes in the water are very complex. This might not be obvious because observation of life forms in the open water is challenging and the full diversity of this ecosystem is only revealed microscopically. In fact, the species diversity in seawater is extremely high. Contributing to this diversity are bacteria, viruses, small phototrophic algae (phytoplankton), and their predators. The observed richness in species has long puzzled ecologists. It was in 1961 that Hutchinson tried to explain what he called "the paradox of the plankton":

"The problem that I wish to discuss... is raised by the very paradoxical situation of the plankton, particularly the phytoplankton... How it is possible for a number of species to coexist in a relatively isotropic or unstructured environment, all competing for the same sorts of materials?"^[2]

The then-popular concept of competitive exclusion, predicting that two species that compete for exactly the same resources cannot stably coexist, did not provide a full explanation of this situation.^[2] The advantage of one species over the other will lead to the extinction of the second competitor in a homogeneous environment. In the ocean, the assemblage would consequently reduce to a population of a single species. Nevertheless, the complex situation in the plankton can be explained with competitive exclusion, if one assumes that the environment changes before the system reaches equilibrium.^[2] It has been observed that certain phytoplankton species dominate in specific seasons and so-called blooms (mass occurrences of a single dominant species) can persist for several days, weeks, or

even months. But if, for example, a diatom bloom dominates and consumes a limited nutritional factor such as silicate, which is used for cell-wall formation,^[3,4] this species will eventually encounter suboptimal conditions and competing species will arise. Ultimately, a final equilibrium is never reached in the plankton community and numerous species can coexist, even in the homogeneous water column.

Since these early concepts arose, numerous studies have been carried out to explain the interactions among plankton species.^[5–11] Different general factors have been identified which contribute to such spatial and temporal limitations. This includes, for example, allelopathic interactions among different algae,^[12,13] the above-mentioned limitation by resources, or a classic predator–prey relationship where the herbivores consume so many of the prey algae that the environment is depleted in this resource. Grazing by zooplankton is indeed a major factor that controls algal populations. This includes herbivores belonging to the microzooplankton (for example, dinoflagellates and ciliates) and mesozooplankton (for example, rotifers, herbivorous crustaceans, fish larvae, and jellyfish).^[14,15] These zooplankton species themselves form the food base for higher trophic levels, and carbon utilization by these grazers is thus a determining factor for the energy flow through the marine food web (Figure 1).^[16] Predator–prey interactions thus influence not only global carbon fixation by photosynthesis but also, directly, fisheries yields.

Traditionally, it was assumed that food availability and food quality are generally the limiting factors in phytoplankton/herbivore interactions. Indeed, the evidence that depletion of the

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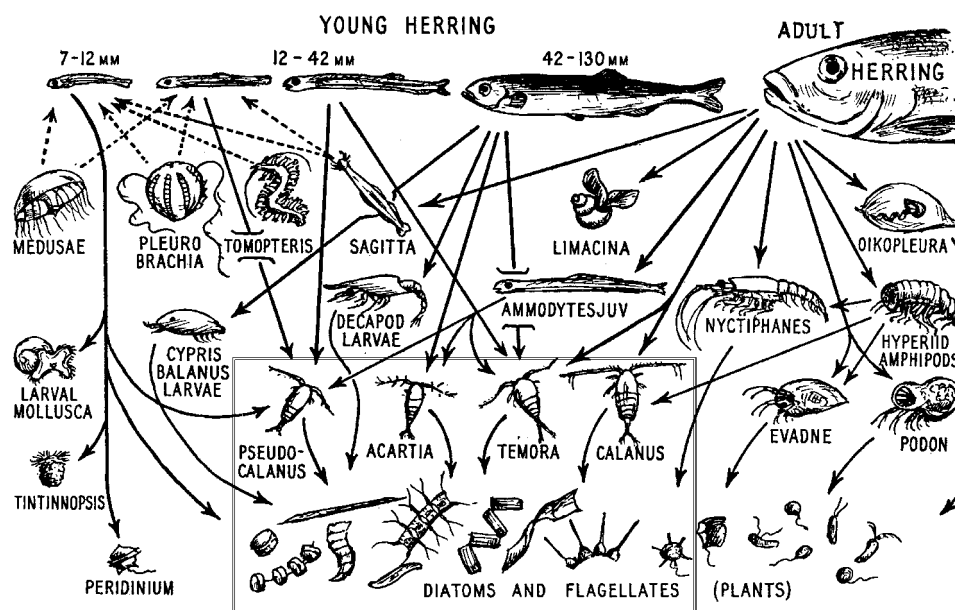


Figure 1. The classical view of the marine food web. The box highlights the groups of organisms discussed in this review. Reproduced from the work of Hardy^[16] with the kind permission of the Sir Alister Hardy foundation.

food containing certain nutrients or other essential components limits the population of herbivores is documented in numerous publications (see, for example, refs. [17–22]).

Only in recent years has resistance of phytoplankton to herbivores been discussed as an additional factor that may influence species interactions in the open water. Indeed, a “watery arms race”^[23] might be behind the success of certain species.^[9,24] This includes the direct mechanical defense exhibited in diatoms, which are protected by strong silicified cell walls,^[25] as well as chemical factors, such as antifeedants or toxins, which could provide additional benefits to the producing phytoplankton species.^[11,24,26–29] Chemical defense might be mediated by highly active metabolites, like the paralytic shellfish poisoning toxins from dinoflagellates that cause major losses year after year in fisheries and even result in some human fa-

talities. Remarkably, although most metabolites from these so-called red tides are strongly toxic to higher consumers, ecological roles providing direct benefits for the producers have rarely been discovered.^[28] But more subtle mechanisms of grazing regulation, such as the influence of feeding rates by dimethylsulfide or dimethylsulfoniopropionate, can also protect the producing algae.^[24,30–32] Ecological concepts, bioassay methods, and analytic chemistry are now advanced enough to allow the further exploration of chemical communication in open water.

The focus of this review will be the interactions of two key players in the plankton, namely, diatoms and copepods (Figure 1). Diatoms are unicellular algae, which are major contributors to phytoplankton biomass worldwide and are thus central in the global carbon cycle.^[33] These photosynthetic heterokonts have a fundamentally different evolutionary history from the higher plants that dominate photosynthesis on land. Diatoms are derived by a secondary endosymbiosis event whereby a nonphotosynthetic eukaryote acquired a chloroplast by engulfing a photosynthetic eukaryote. Data from the sequencing of the first diatom genome indicates that this photosynthetic organism was presumably a red alga. Accordingly, almost half of the diatom proteins have similar alignment scores to their closest homologues in plants, red algae, and animals.^[34] Copepods account for about 80% of the mesozooplankton biomass and are thus among the most dominant herbivores in the plankton. It used to be thought that diatoms are high-quality food for these herbivores.^[35,36] Only in recent years has evidence accumulated to suggest that this does not hold true in every case and that certain diatoms are chemically defended from their predators. Some reviews have been published that highlight aspects of these interactions covering the ecological perspective,^[37] the biosynthesis of algal oxylipins

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(metabolites derived from the oxidative transformation of fatty acids),^[38,39] or the distribution of volatile metabolites in the phytoplankton.^[40] Other reviews deal with general aspects of the chemical defense of phytoplankton (see, for example, refs. [24,26,28,29]). Here a comprehensive overview of the chemical ecology of oxylipins from diatoms is given. This article highlights the chemically mediated species interactions with a focus on the synergistic approaches from ecologists, chemists, and biochemists.

2. The Early Evidence of a Negative Influence of Diatoms on Copepod Reproduction

The hypothesis that diatoms are a good food source for copepods^[41] holds true when only the feeding behavior of these herbivores is observed. As early as 1935, Harvey et al. reported that the spring outburst of diatoms is controlled mainly by the grazing of herbivorous copepods.^[36] Copepods grow well on certain natural diatom-rich diets and can even be raised on monospecific diatom diets with no observable adverse effects. The classic pelagic food-web concept thus predicts a positive correlation among diatom-rich phytoplankton, copepod production, and fish abundance.^[42] This general concept does not hold when, besides copepod growth and survival, the reproductive success of the animals is taken into account. In the early 1990s, it was recognized that not all diatom species represent a good food source when copepod reproduction is considered. The observation that copepod egg production rates were low during certain diatom-rich situations suggests that these algae have an adverse effect. The cause for this phenomenon has, however, not yet been identified.^[43] In addition to egg production, hatching and successful larval development are also major fitness factors for herbivores that are strongly influenced by certain diatoms. When they fed the copepod *Temora stylifera* a diatom diet, Ianora and Poulet observed that the hatching success of the eggs produced was strongly decreased compared to success on a nondiatom diet.^[44]

An initial screening showed that the diatom food was obviously not diminished in essential nutrients, such as amino acids or fatty acids, and it was suggested that diatoms produce an inhibitory factor which blocks embryogenesis. This was manifested in experiments where copepod embryos incubated in diatom extracts showed abnormalities comparable to those embryos produced by females that fed on a diatom diet.^[45] This approach of incubating females with concentrated extracts or even purified metabolites does not, however, reflect the actual ecological situation. In the ocean, metabolites suffer significant dilution and would instead be administered through the food itself. Since an adverse effect of diatoms challenges the traditional pelagic food-web concept, several follow-up studies have been performed to test the true influence of diatoms or diatom metabolites on copepod reproduction. While some groups of researchers found no inhibitory effect at all or

a negative influence of diatoms that could not be explained by the presence of toxins,^[20,46,47] others observed antiproliferative activity.^[44,45,48–51] In an exceptional approach intended to sort out the discrepancies, 15 groups in 12 countries worldwide joined together to test combinations of different copepod species with 17 diatom diets.^[52] The effects of diatom diets that were found can be sorted into four major categories (Figure 2).

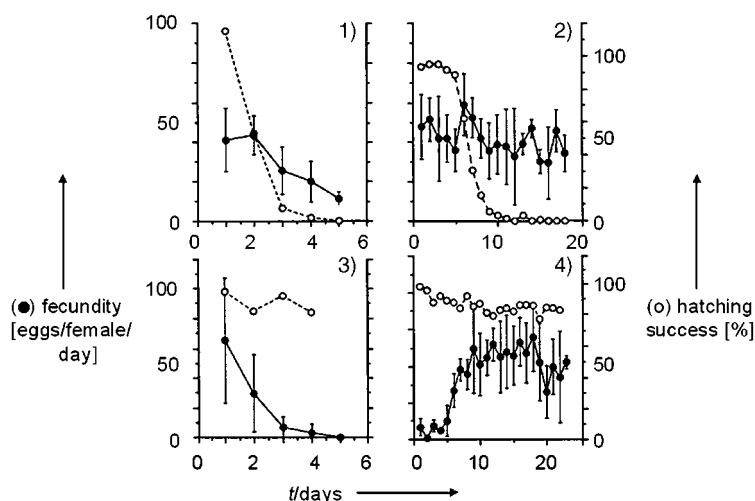


Figure 2. The effects of different diatom diets on the reproduction of *Calanus helgolandicus* can be grouped into four categories. Diatom diets can cause 1) reduced hatching success and reduced fecundity, 2) reduced hatching but normal fecundity, 3) normal hatching but reduced fecundity, or 4) no adverse effects at all. Reproduced from ref. [52] with the permission of Inter-Research.

In these standardized experiments, diatoms that lower copepod fecundity and hatching success were observed (category I), while other diatom diets caused the reduction of only one of these variables (categories II and III, respectively) or no adverse effect at all (category IV). This set of experiments clearly demonstrated negative effects of certain diatom species, but the factors involved remained elusive. Two major opinions surfaced about the reasons for these results, one favoring the existence of toxins from diatoms^[53] and the other favoring poor food quality of the monospecies laboratory diets, which would play no role under field conditions.^[46]

3. The Role of $\alpha,\beta,\gamma,\delta$ -Unsaturated Aldehydes in Diatom Defense

A major breakthrough for the understanding of chemically mediated diatom/copepod interactions was the finding that, under field conditions, the hatching success of wild copepods was strongly reduced when they fed on a diatom-dominated bloom. Even if egg production was high when diatoms dominated the phytoplankton, only 12% of the eggs hatched, in comparison to a 90% hatching rate after the bloom.^[54] A bioassay-guided structure elucidation pointed to the principle compounds that might underlie the reduced hatching success. Compared to other potent phytoplankton toxins,^[28,55] these

were the structurally remarkably simple aldehydes 2*E*,4*E*/*Z*-decadienal (in the following decadienal is used as shortened name) and 2*E*,4*E*/*Z*,7*Z*-decatrienal (decatrienal; Scheme 1).

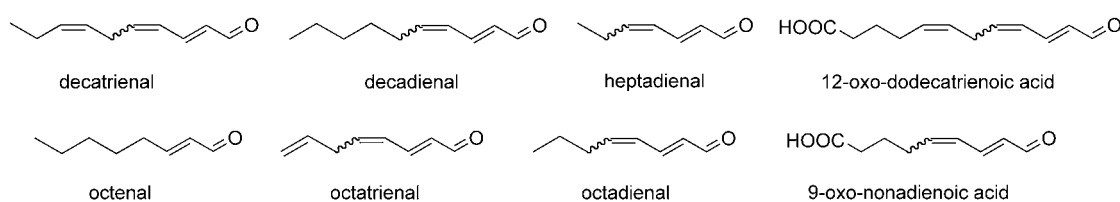
When added externally to newly spawned copepod eggs, these aldehydes reduced hatching success significantly at a concentration of $1\text{ }\mu\text{g mL}^{-1}$, and hatching was completely blocked with concentrations above $2\text{ }\mu\text{g mL}^{-1}$.^[54] These concentrations represent unrealistically high values with regard to the situation in the ocean, but the finding served as a stimulation to further unravel the role of these metabolites. In general, we know little about how consumers perceive secondary metabolites and have no direct means to apply ecologically realistic doses of defensive metabolites.^[56] Approaches using, for example, microencapsulated food or liposomes as toxin shuttles are currently being evaluated but have not been applied to studies of diatom oxylipins. External addition of chemicals, such as those performed with decadienal, can thus provide only indirect evidence pointing towards potential bioactivity.

The identified aldehydes have previously been detected in freshwater diatoms where they cooccur with homologues with shorter chain lengths.^[57] Although they are widely distributed in freshwater ecosystems,^[58,59] no function had been assigned to these metabolites up to that time. Tests with other marine organisms showed that the activity of the isolated aldehydes is not restricted to copepod egg hatching. Similar results were obtained when an easily accessible sea urchin egg cleavage assay was performed (Figure 3).^[60] These model animals, which can be stimulated to reliably produce high numbers of eggs, made it possible to study the effect at the cellular level. It could be shown that extracts of the diatom *Thalassiosira rotula* induce aberrations in embryonic tubulin organization and that microtubules were depolymerized. In extract-treated eggs, the chromatin appeared globular and compacted at the center of the cell.^[61] Examination of the influence of decadienal on key events of mitotic cell division revealed that this aldehyde inter-

feres with several viable cellular mechanisms. It affects the assembly of sperm aster, DNA synthesis, and mitotic events, probably through the inhibition of cyclin B/Cdk 1 kinase activity.^[62]

Detection of polyunsaturated aldehydes in diatoms

Once the C_{10} aldehydes were identified in a large-scale purification/structure-elucidation approach, subsequent studies focused on the investigation of volatiles from diatoms. The activity of the newly identified metabolites could then be evaluated with synthetic or commercially available material. Different approaches were used to analyze these volatiles. A sensitive protocol with the use of solid-phase microextraction (SPME) allows rapid screening of the production of volatiles from small culture samples. As no solvent extraction and derivatization are required, a first impression of the potential of phytoplankton to produce unsaturated aldehydes can easily be obtained. This method, however, does not allow samples to be stored or derivatized so structure elucidation is limited to metabolites that are available as reference compounds.^[63,64] Another approach, which requires more material but permits the purification and structure elucidation of newly identified metabolites, is based on a new Wittig derivatization protocol. The organic extract from diatoms is transformed by using a Wittig reagent that forms carboxyethylethylidene derivatives from the aldehydes. These can be further purified or analyzed directly by GC/MS.^[65] Both approaches have been independently applied to *T. rotula* cultures and have revealed that decadienal and decatrienal are not the only unsaturated aldehydes released; 2*E*,4*E*/*Z*-octadienal and 2*E*,4*E*/*Z*,7*Z*-octatrienal, as well as 2*E*,4*E*/*Z*-heptadienal, are also dominant polyunsaturated aldehydes (hereafter, PUA is used as an abbreviation for any conjugated polyunsaturated aldehyde) from certain isolates of this alga (Figure 4).^[63,65]



Scheme 1. $\alpha,\beta,\gamma,\delta$ -Unsaturated aldehydes from diatoms.

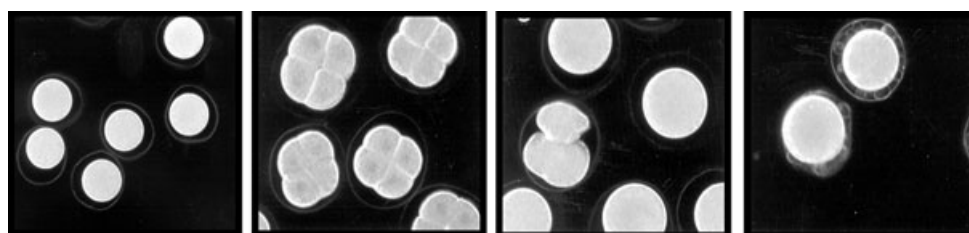


Figure 3. Inhibition of egg hatching in sea urchin egg cleavage assays. From left to right: fertilized eggs ($t=0$), fertilized eggs after the second cell cleavage, inhibition of hatching in the presence of 2,4-decadienal ($2\text{ }\mu\text{g mL}^{-1}$), and unspecific toxic effects caused by elevated concentrations of decanal. Reproduced with the kind permission of S. Poulet, Roscoff, France.

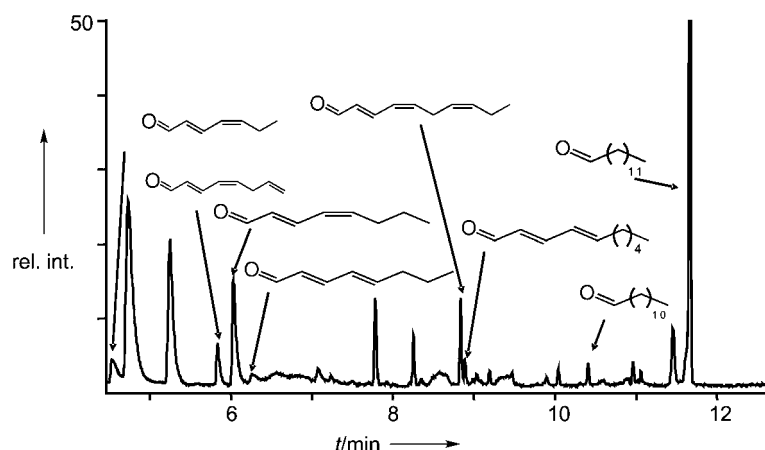


Figure 4. GC/MS trace of volatiles from *T. rotula*. Cells were wounded by sonication before solid-phase microextraction. The metabolites were identified by comparison with standards.

Other diatoms, such as *Skeletonema costatum* and *Skeletonema pseudocostatum*, lack the ability to form the C_{10} aldehydes but do release the unsaturated C_7 and C_8 aldehydes.^[63,66] Both analytical methods have been solely applied to diatom cultures but no information about the situation in the field has been available. This is due to the fact that aldehyde-producing phytoplankton are often present in low concentrations in the open oceans and laborious concentration steps would be required to obtain suitable samples. A pentafluorobenzoyloxime-based derivatization method is now available to overcome these limitations, thereby allowing investigation of field samples after a simple filtration protocol.^[67] Detection limits corresponding to less than 2000 cells of an aldehyde-producing species in 1 liter of seawater (which is well below the natural concentration of diatoms in the phytoplankton during bloom conditions) allow surveys to be made of field situations with varying plankton compositions.

Structure–activity relationship of diatom-derived aldehydes

Comparison of the major PUA isolated from *T. rotula* and *S. costatum* showed that both 2,4-octadienal and 2,4-decadienal exhibit antiproliferative activity and inhibit cleavage of sea urchin eggs.^[66] A synthetic approach that yields stereospecifically $\alpha,\beta,\gamma,\delta$ -unsaturated aldehydes gave enough pure material to perform a detailed structure–activity investigation.^[68] With sea urchin eggs as a model, activity was observed to increase with the increase in chain length from the C_7 to C_{10} PUA and even the acidic 12-oxo-dodeca-5Z,8Z,10E-trienoic acid, which has also been reported from diatoms,^[38,64] inhibited sea urchin embryonic development. Only aldehydes bearing a α,β - or $\alpha,\beta,\gamma,\delta$ -unsaturated structure element are active. Saturated and unsaturated aldehydes, which lack such a Michael acceptor system, exhibit no activity at all. Together with the observation that both 2E,4Z- and 2E,4E-dienals exhibit similar activity, it can be concluded that no specific receptor or enzymatic activity is addressed by the diatom-derived PUA but rather that unspecific interactions of the unsaturated aldehydes are responsible for

the activity.^[60,68] The only inactive $\alpha,\beta,\gamma,\delta$ -unsaturated aldehyde was 9-oxo-nona-5Z,7E-dienoic acid, which, presumably due to intermolecular interactions, was not inhibitory at all.^[68]

4. Wound-Activated Defense

Despite this progress in unraveling the defensive chemistry of diatoms, the community raised doubts about the significance of the laboratory experiments for the situation in the open ocean. One of the most severe criticisms was the high concentration of PUA or diatoms used in the assays. The total carbon equivalent of $1\ \mu\text{g mL}^{-1}$ of decadienal, which is the concentration required to reduce egg hatching of copepod or sea urchin eggs, exceeds the overall organic carbon content often found in open water. The possibility that the herbivores are being poisoned by toxins released into the open water can thus be excluded. Careful reinvestigation has shown that healthy diatom cultures do not in fact release the metabolites in question into the seawater. Even intact diatom cells do not contain these metabolites. Only after wounding of the unicellular algae can a rapid onset of aldehyde production be observed. Thus, for example, mechanical wounding of *T. rotula* in seawater triggers the release of aldehydes within seconds, and saturation levels in the range of 5 fmol of PUA per cell are reached within 2 minutes.^[64] A comparable dependence of PUA production on cellular disintegration or osmotic stress has also been shown with preparations of freshwater diatoms.^[57] This wound-activated chemical defense might be a way for the unicellular planktonic algae to overcome several limitations. On the one hand, it reduces the costs implicit in continuous toxin production and release. On the other hand, it minimizes the risk of self poisoning that might occur if defensive metabolites are stored in the cells. This risk is especially high in the case of the reactive PUA, which can form unspecific adducts to biopolymers such as DNA.^[69–71] A wound-activated defense mechanism thus permits investment in cellular resources during normal growth, and the production of defensive metabolites from these resources is only initiated upon demand. Wound-activated defense strategies are widely distributed among higher plants^[72] but are rare in plankton. In fact, only one other example has been reported to date. The coccolithophore *Emiliania huxleyi* also relies on an enzyme that acts upon wounding and transforms dimethylsulfoniopropionate (DMSP) to dimethylsulfide and acrylate. An *E. huxleyi* isolate with low DMSP-lyase activity is a preferred food source for a protozoan grazer if provided in a choice assay together with an isolate with a comparably high DMSP-lyase activity.^[30] However, the situation is complicated by recent findings demonstrating that both dimethylsulfide and acrylate might not act as a direct chemical defense but might instead indicate other adverse nutritional properties of the prey to the herbivore.^[31,32]

At first view, wound-activated defense strategies might not make evolutionary sense for unicellular algae that have no tissue to sacrifice and do not survive the ingestion process

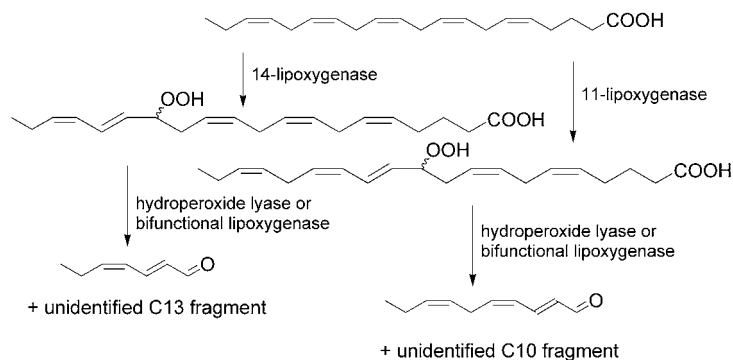
that triggers toxin production. However, it has to be taken into account that, during diatom blooms, algae proliferate predominantly asexually and thus the proportion of genetically identical algae can be high. The whole population could very well benefit from a reduction of the pool of grazers, even if certain individuals are sacrificed.^[24] Nevertheless, this concept lacks experimental verification since the molecular analysis of phytoplankton population structure is still in its infancy.^[73] Studies exist that question the uniclonal nature of phytoplankton blooms and suggest other mechanisms of selection,^[74,75] but as of yet this field has remained largely open to discussion. Work on the evolutionary role of activated defense in plankton thus still awaits confirmation.

5. Biosynthesis of Polyunsaturated Aldehydes

Lipoxygenase-mediated transformation of fatty acids

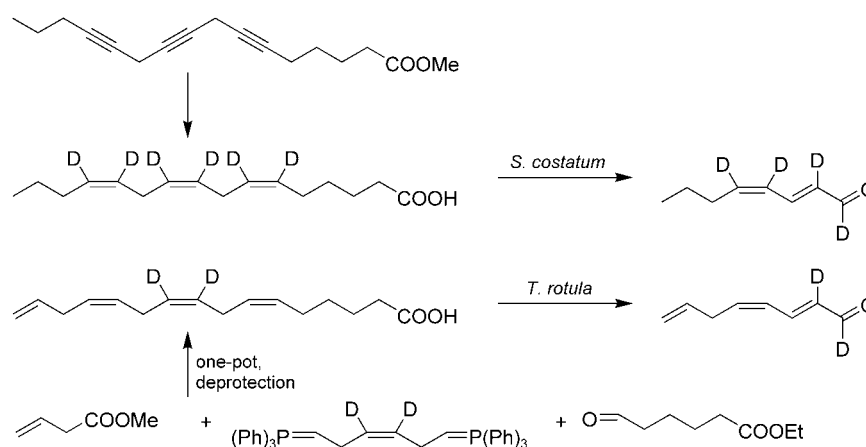
The unbranched structures of PUA suggest a common origin from polyunsaturated fatty acids. Throughout the plant, algal, and animal kingdoms, structurally related unsaturated aldehydes are known to be break-down products from the oxidative transformation of fatty acids.^[76–80] Higher plants use only the pool of C_{18} fatty acids that are transformed by lipoxygenases (LOX) to form intermediate hydroperoxides. These then serve as substrates for a family of hydroperoxide lyases (HPL) that release aldehydes with shorter chain lengths.^[81] In general, HPL-mediated reactions result in short-chain aldehydes such as (3Z)-hexenal, which is a component of the so-called green leafy volatiles, or (3Z)-nonenal with a cucumber-like odor. A characteristic structural feature of these HPL-derived products is that they contain only isolated double bonds and lack a Michael acceptor element. In plants, further transformations by 3Z:2E-enal isomerases result in α,β -conjugated aldehydes such as the phytohormone traumatic acid.^[81,82] Remarkably, diatoms do not follow plant-like pathways to generate PUA. Linolenic acid that could serve as a putative precursor for decatrienal is not accepted as a substrate for transformation with broken diatom cells or cell-free systems. By contrast, the C_{20} fatty acids eicosapentaenoic acid and arachidonic acid are incorporated with highly efficiency into decatrienal and decadienal, respectively. Experiments with deuterated arachidonic acid proved unambiguously that the entire C_{11} – C_{20} terminus of this fatty acid is incorporated into the reactive aldehydes (Scheme 2).^[64]

The shorter-chain PUA 2,4-heptadienal is derived from eicosa-



Scheme 2. Postulated biosynthetic pathways to the eicosanoid-derived C_7 and C_{10} PUA.^[63,65]

pentaenoic acid as well,^[83] but different precursors are required for the biosynthesis of the C_8 aldehydes. Judging from the position of the double bonds, 2,4-octadienal and 2,4,7-octatrienal cannot be derived from any of the C_{20} fatty acids found in diatoms. Nevertheless these metabolites are of fatty acid origin. Biosynthetic considerations point to unusual C_{16} fatty acids, first detected in fish oil^[84] but also common in diatoms,^[85,86] as candidate precursors for the C_8 aldehydes. The synthesis of labeled $\delta 6,9,12$ - $C_{16}:3$ based on coupling reactions of propargylic halides with terminal alkynes provided material with which to test this hypothesis. When applied to damaged *S. costatum* cells, the octadienal produced has a high degree of labeling.^[87] The short *bis*-Wittig based synthesis of labeled $\delta 6,9,12,15$ - $C_{16}:4$ and the application of the compound to *T. rotula* demonstrated the origin of 2,4,7-octatrienal from this tetraenoic fatty acid (Scheme 3).^[88] The incorporation patterns found in transformations of labeled precursors are in accordance with mechanisms involving LOX. The generated hydroperoxides may be cleaved by the action of the LOX itself or by additional HPL, but no further proof of this hypothesis is available. Neither the intermediate hydroperoxides nor the acidic cleavage products that should result as a second fragment from the hydroperoxide transformation have yet been detected.



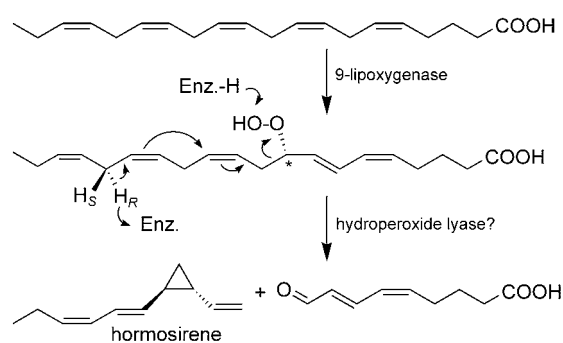
Scheme 3. Synthesis and transformation of C_{16} fatty acids from diatoms with unusual double-bond positions.^[85,86]

In contrast to this sparse knowledge about the mechanism for the production of volatile PUA, a detailed picture of the generation of acidic PUA from diatoms is available. This stems mainly from investigations of the biosynthesis of volatile hydrocarbons in the freshwater diatom *Gomphonema parvulum*. The production of cycloheptadiene C_{11} hydrocarbon ectocarpene was reported early on from this and other diatoms.^[57,58,89] This structure was later revised to the thermolabile divinylcyclopropane hormosirene (Scheme 4).^[90] Interestingly, these hydrocarbons were previously identified as brown algal pheromones with fatty acid origins.^[91] This motivated an in-depth investigation of their biosynthesis.^[38,91] Cell-free preparations of *G. parvulum* transformed the eicosanoids arachidonic acid and eicosapentaenoic acid efficiently into the cyclic C_{11} hydrocarbons. An acidic PUA, namely 9-oxo-nona-5Z,7E-dienoic acid, resulted as second fragment in these transformations.^[90] Trapping experiments revealed that this transformation involves a lipoxygenase-mediated oxygenation of the fatty acids, which are transformed into the (9S)-hydroperoxy intermediates. These are subsequently cleaved into the acidic PUA and the cyclic C_{11} hydrocarbons (Scheme 4). Metabolic studies with chiral deuterated fatty acids enabled an investigation of the mode of cyclization and the rate-determining steps in this transformation.^[92]

These biosynthetic studies have all been performed on crude cell preparations or cell-free systems and the involved enzymes have not been isolated. It is thus not known if the transformations are catalyzed by a multifunctional LOX or by both a LOX and an HPL.^[92]

The other example of well-investigated PUA production comes from the planktonic freshwater diatom *Asterionella formosa*. Upon wounding, this alga releases 1,3E,5Z octatriene (fucoserratene) as the major volatile compound.^[57,93] This metabolite, which is also known from the pheromone chemistry of brown algae, is derived from eicosapentaenoic acid as well.^[38] In this case, a 12-LOX with broad substrate tolerance^[94] forms an intermediate hydroperoxide, which is then cleaved to octatriene or corresponding higher saturated or unsaturated hydrocarbons and the PUA 12-oxo-dodeca-5Z,8Z,10E-trienoic acid (Scheme 5).^[64]

It is likely that the generation of volatile PUA by marine diatoms follows similar routes to those that have been found for the acidic PUA in freshwater species, but different general mechanisms have to be considered as well. In general, the transformation of fatty acid hydroperoxides to PUA can involve several different enzymatic activities. For example, at least three independent pathways from 12-hydroperoxyeicosatetraenoic acid to the

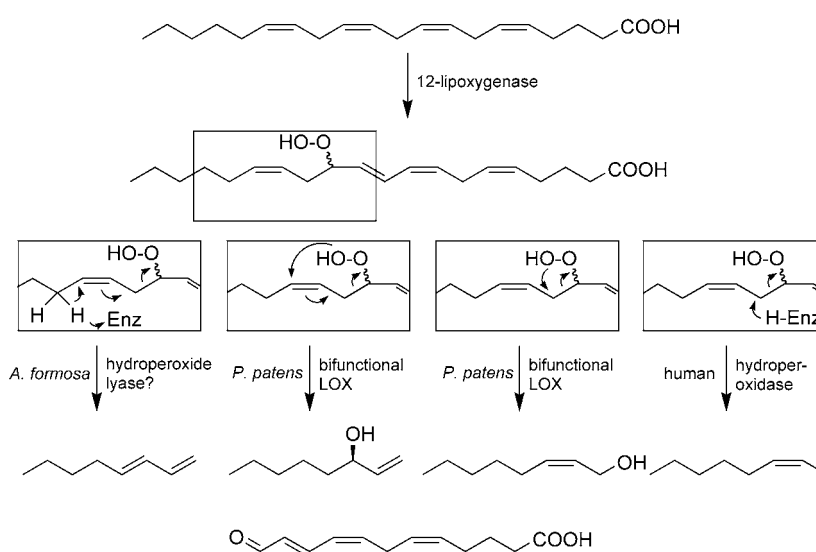


Scheme 4. Biosynthesis of hormosirene and the PUA 9-oxo-nona-5,7-dienoic acid.^[88]

PUA 12-oxo-dodeca-5Z,8Z,10E-trienoic acid are known. While diatoms cleave this hydroperoxide and release the hydrocarbon octa-1,3-diene, arachidonic acid stimulated human leucocytes presumably release oct-2-ene as a second product.^[80,95] A third independent pathway is reported from the moss *Physcomitrella patens*. There a LOX alone can account for the release of a mixture of the hydroxylated C_8 alcohols 1-oct-2-en-ol and 3-oct-1-enol as fragments, in addition to the 12-oxo acid.^[96] Another possible source for PUA is the direct transformation of lipids rather than free fatty acids. Lipid-body LOX^[97] could generate lipid hydroperoxides, which could be cleaved by HPL,^[98] but experiments investigating the regulation of the wound-activated defense exclude this pathway in diatoms.

Regulation of the wound-activated defense

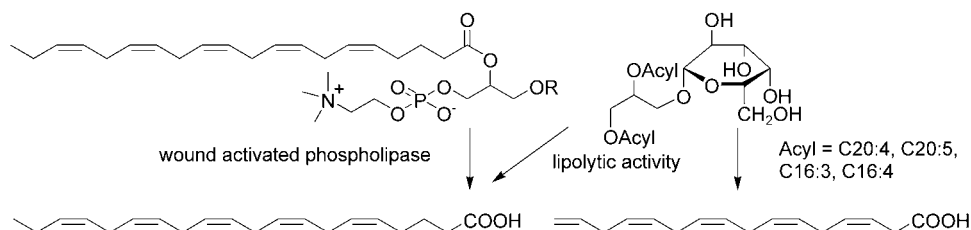
The question has arisen as to how diatoms regulate the fast production of the reactive PUA. PUA production starts within seconds after wounding in seawater, a fact that makes regulation through transcription and de novo protein biosynthesis of the required LOX highly unlikely.^[64] Since addition of eicosa-



Scheme 5. Different pathways leading to the formation of the PUA 12-oxo-dodeca-5,8,10-trienoic acid in diatoms (left),^[63] mosses (center),^[94] and humans (right).^[78,93]

pentaenoic acid or arachidonic acid to wounded *T. rotula* leads to the increased formation of decatrienal and decadienal, respectively, aldehyde production is apparently substrate limited. That only the free acids are transformed into the aldehydes was shown in further experiments with esterified fatty acids. Careful isolation of the fragile diatom cells followed by immediate deactivation of the enzymes with boiling water before extraction revealed that only free saturated fatty acids are present in intact *T. rotula* cells. These fatty acids cannot be transformed by LOX. Only upon wounding is a rapid onset of polyunsaturated fatty acid release observed (Figure 5).^[88,99]

Diatom phospholipids can play a role as a resource for PUA production since they are rich in polyunsaturated fatty



Scheme 6. Phospholipids and glycolipids (monogalactosyldiacylglycerol shown as a representative) serve diatoms as a resource for wound-activated defense.^[81,97]

phospholipids, thereby releasing the substrates for prestored LOX and HPL activity.

However, chloroplast-derived glycolipids can also provide free fatty acids for the generation of PUA upon wounding.^[83] In the diatom *S. costatum*, monogalactosyldiacylglycerol, which is especially rich in hexadecatrienoic acid and hexecatetraenoic acid, is transformed upon wounding by a galactolipid-hydrolyzing enzyme (Scheme 6). As

indicated above, these C₁₆ fatty acids are subsequently transformed to octadienal and octatrienal, respectively (Scheme 3). By contrast, the eicosapentaenoic acid derived heptadienal can be formed from both galactolipid- and phospholipid-bound precursors in *S. costatum*.^[83] Related principles of regulation are also known in wounded higher plants: in these, lipases release C₁₈ fatty acids, which are transformed to short-chain oxylipins, such as the green leafy volatiles.^[101]

The release of free fatty acids upon tissue disruption has also been observed in other diatoms.^[102,103] In freshwater biofilms, this response to tissue disruption correlates with a chemical defense, because the released eicosapentaenoic acid is toxic to the grazer *Thamnoce-*

phalus platyurus.^[102] This defense strategy seems not to be limited to diatoms since free polyunsaturated fatty acids have also been identified as haemolytic principles in the brevetoxin-producing raphidophyceae *Fibrocapsa japonica*.^[104]

The entire wound reaction of planktonic diatoms thus comprises at least two fast-acting enzymes that are active in sea water and preferably also in the nearly neutral environment of the copepod gut. This would allow the toxins to be targeted efficiently against herbivores and reduce dilution or self intoxication to a minimum. In any case, the production of PUA occurs close to the feeding instruments of the herbivores or in the herbivore gut. The use of phospho- and galactolipids as

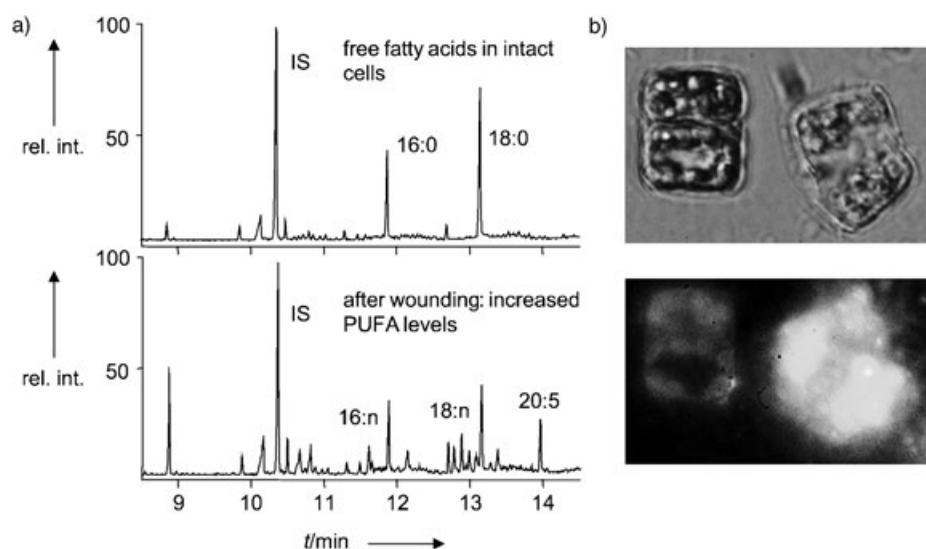


Figure 5. Wound-activated release of unsaturated fatty acids by *T. rotula*. a) Upper gas chromatogram: Predominantly free saturated fatty acids are found in intact algae. Lower gas chromatogram: After wounding, the release of free unsaturated fatty acids (PUFA) that serve as precursors for PUA production is observed. b) Upper micrograph: Intact and wounded diatom cell in the light microscope. Lower micrograph: Intact and wounded diatom cell in the epifluorescence microscope after staining with a phospholipase-sensitive fluorescent dye. Phospholipase activity can be detected in the extruding cytoplasm.^[99]

acids.^[100] After addition of an arachidonic acid rich phospholipid to wounded *T. rotula*, the arachidonic acid derived decadienal increased sharply in comparison to decatrienal, which is formed from the higher unsaturated eicosapentaenoic acid.^[99] The site specificity in transformations of fluorescently labeled phospholipids allowed the identification of phospholipase A₂, which regulates the release of the precursor fatty acids for PUA in *T. rotula* (Scheme 6). The phospholipase activity is found to be located in the extruding cytoplasm of wounded diatoms when labeled lipids are monitored with fluorescence spectroscopy. It can thus be assumed that cell disruption allows the lipase to come into contact with membrane phos-

direct precursors for a wound-activated chemical defense bears the additional advantage that, in times of low herbivore pressure, cellular resources can be invested in the formation of valuable lipids, which are essential during regular cell growth.

6. Role of PUA in the Regulation of Algal/Herbivore Interactions

Since the first discovery of the role of unsaturated aldehydes in reducing the hatching success of copepod eggs, many studies have been conducted to investigate the effect of these reactive metabolites in more detail. This has included investigation of the possible ecological scenario in which these aldehydes are able to act efficiently, but searches for new target organisms and other modes of PUA action have also been conducted.

Maternal PUA-rich diets can induce teratogenic effects in offspring

One important explanation of how unicellular algae could benefit from a wound-activated defense was made by Ianora et al.^[105] The question of whether or not diatom chemical defense acts in a regulatory manner on the annual growth cycles in nutrient-rich aquatic environments motivated a series of experiments in which the reproduction of copepods was monitored from egg to adult organism. It is often observed that diatom blooms, which occur early in the year, suffer only low grazing pressure.^[106,107] This has been attributed to the inability of overwintering copepod populations to track these blooms since their life cycles, which involve 11 larval stages, take weeks or months to complete.^[41] An alternative explanation for this poor usage of the spring diatom bloom is the chemical defense of these algae against the common overwintering copepod *C. helgolandicus*. When nauplii (freshly hatched offspring) were collected from wild *C. helgolandicus* females during a spring diatom bloom and then raised on a diatom diet, the larvae did not survive to adulthood. Control groups, raised on a neutral dinoflagellate diet that did not produce any PUA, showed higher survival (Figure 6a).

Model experiments were conducted in the laboratory, where similar patterns could be observed. When females were raised on a diatom diet the survival and development of their offspring was significantly impaired. The low survival levels were due to teratogenic birth defects^[49] that were manifested in malformations of the hatched nauplii (Figure 7).^[105] The observed malformations were further investigated by double labeling methods, which showed that aberrations from diatom-rich diets were caused by induced apoptosis and necrosis.^[108,109] Most interestingly, in these examples the fitness of the offspring is influenced by the maternal diet, as evidenced by incubations in which both mothers and nauplii were raised on four different nutritional regimes. When both females and offspring were raised on dinoflagellate diets the survival rate was high, whereas 100% mortality was observed when the diatom *S. costatum* was offered as a diet. Percentage of survival was intermediate for the cases where mothers were fed the

diatom and the offspring the dinoflagellate or vice versa (Figure 6b,c). That these effects can indeed be attributed to the action of PUA was shown in experiments in which the neutral dinoflagellate diet was treated with decadienal before being fed to copepod females and offspring. There, in a concentration-dependent manner, the pattern of reduced survival resembled that observed on the diatom diet (Figure 6d,e). Parallel monitoring of the decadienal content in this artificial diet revealed that the content of PUA administered through decadienal-treated cells was similar to that reached on an *S. costatum* rich diet. Even if this evidence points strongly towards the function of PUA, more efficient ways to deliver toxins in plankton will have to be developed in the future for a final proof. *P. minimum* is a poor shuttle for PUA, in that it carries only a fraction of the externally added aldehydes to the consumers, while the major part of this aldehyde remained in the water during the assay. Thus, interference of 2,4-decadienal by passive absorption, rather than from its presence in the diet, cannot be excluded.

Taken together, these findings provide a plausible mechanism for the apparent poor timing between bloom development and the late arrival of the bulk of the overwintering copepod stock. Since the recruitment of herbivores is impaired during the diatom-rich spring blooms, the cohort size of the early-rising overwinterers, and therewith the grazing pressure on the spring diatom bloom, is kept low.^[105]

7. Effect of PUA on Other Marine Organisms

Since diatoms are consumed not only by herbivorous copepods but by other marine animals as well, the question arose as to whether or not the deleterious effects of PUA are restricted to copepods. Earlier, the effect of the compounds on sea urchin embryos as model organisms was demonstrated (see above). Diatom extracts as well as decadienal were also assayed on polychaete and sea star embryos.^[110] Both crude diatom extracts and the synthetic decadienal were found to inhibit fertilization, embryogenesis, and hatching success. The polychaete embryos were very sensitive and even concentrations of 125 ng mL⁻¹ of decadienal reduced hatching success significantly. Consistent with the concept of wound-activated defense, intact diatom cells had no effect on these parameters.^[111] These results were discussed in the context of the knowledge that the assayed sensitive polychaetes *Arenicola marina* and *Nereis virens* breed during a food-limited period. Such behavior contradicts the general view that breeding occurs preferentially under optimal conditions.^[111] This behavior might be explained by the occurrence of diatom-derived developmental inhibitors during optimal breeding conditions. Rather than evolving detoxification mechanisms, these organisms might have shifted their breeding patterns in order to minimize the adverse effects of diatom-derived PUA.^[110]

Other processes involved in reproduction can also be inhibited by decadienal. When broadcast-spawning echinoderm sperm were incubated with decadienal for a short time, fertilization success was markedly reduced. This was attributed to

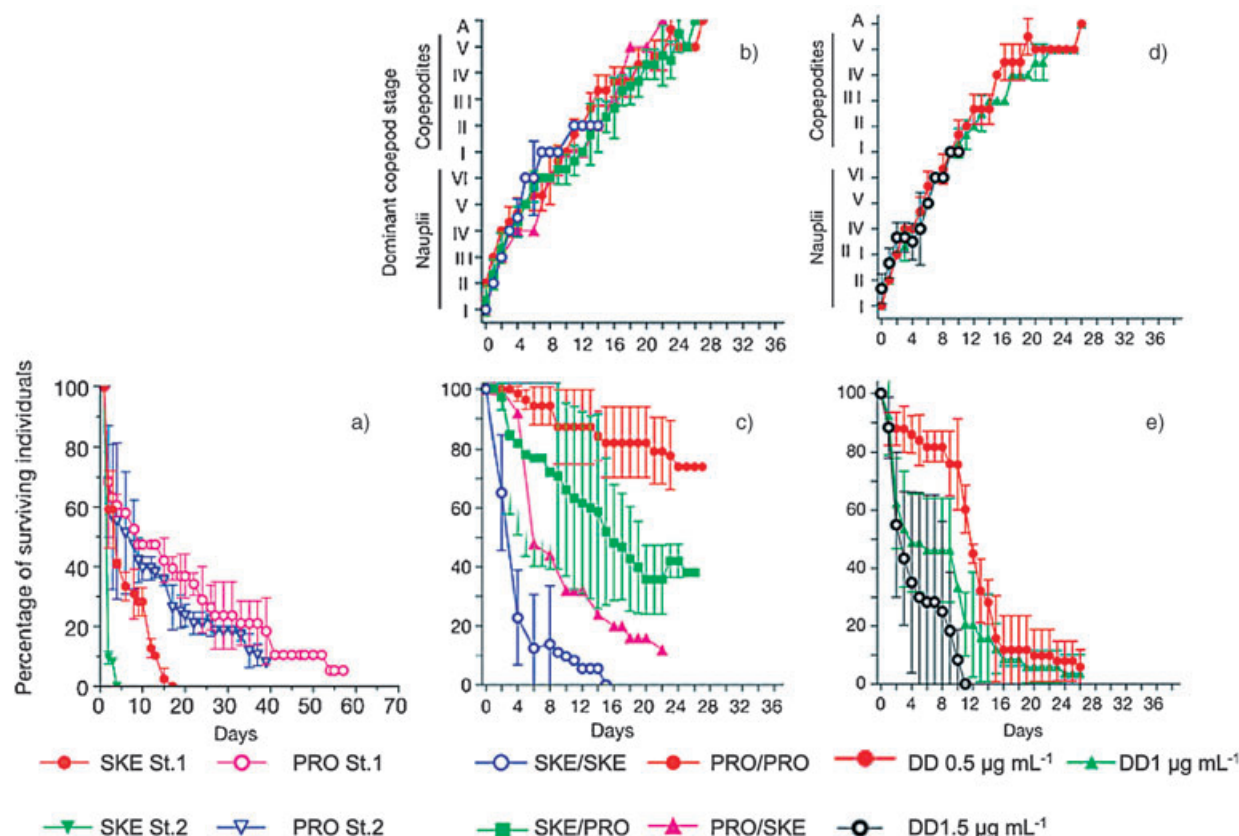


Figure 6. Effects of combinations of maternal and neonate diets on the development rates (top) and percentage survival (below) of *C. helgolandicus* larvae. a) Offspring of females caught during a spring diatom bloom at two different field stations (St.1 and St.2) suffer birth defects and low survival rates if raised on a diatom diet consisting of *S. costatum* (SKE), the offspring of the same females fed the control dinoflagellate *Prorocentrum minimum* (PRO) showed higher survival. b), c) When females and their larvae were fed the diatom SKE (SKE/SKE), survival was low, while a dinoflagellate diet of mother and offspring resulted in high survival (PRO/PRO). SKE/PRO indicates that females were fed SKE and their progeny were fed PRO; the opposite is true for PRO/SKE. In both cases, survival was intermediate. e) Similar effects to those caused by an SKE diet can be observed with a PRO diet that delivers externally applied decadienal. Reproduced from ref. [103] with the permission of the Nature Publishing Group.

the fact that decadienal limits the number of motile sperms in a concentration-dependent manner.^[112]

In a broader screen for cytotoxicity of PUA against different organisms, the unspecific reactivity of these metabolites was reflected in cytotoxicity conserved across six phyla, including two domains of life. PUA activities included inhibition of cell proliferation, cell division, phagocytosis, the induction of an oxidative burst, and the induction of apoptosis.^[60] This broad spectrum of activity is presumably also responsible for the activity of decadienal against human cancer cell lines.^[54] Experiments with yeast cells showed that a prerequisite for activity is cell-wall permeability. While a wild-type yeast strain proved to be highly resistant to decadienal, a mutant with increased cell-wall permeability did not survive decadienal treatments.^[60] Recently the active principle of the marine haptophyte *Phaeocystis pouchetii*, which produces compounds that adversely effect numerous marine organisms, was identified as decadienal as well, a result that demonstrates once more that nondiatom marine phytoplankton are also able to produce PUA.^[113] Although these metabolites have been discussed in the context of diatom defense, it is most likely that the same or related defensive principles are wide-spread in plankton.

8. Perspectives

The adverse effects of diatoms on copepod reproduction are quite convincing in the laboratory, but no general toxic effects can be observed throughout global ecosystems.^[114] A very controversial discussion has arisen in which “the oceanographic community has been reluctant to accept a possible toxic effect of diatoms, because in field studies per capita egg production generally increases during the spring diatom bloom.”^[47] To underline this argument, data sets have been published showing that, in a number of cases, diatom-rich diets in the field are not correlated with low hatching success.^[114] In addition, some of the bioassays used to demonstrate toxic effects of diatom metabolites were also considered as inappropriate for distinguishing between poor food quality and true toxicity.^[46] From another group, mechanistic investigations were performed to “present further evidence demonstrating that diatoms are indeed “toxic” for the reproductive biology of copepods”^[53] and that the deleterious effects of PUA can be found in a number of model systems. Most of the studies that led to these general opinions were based on experiments that focused only on partial aspects of the diatom copepod interac-

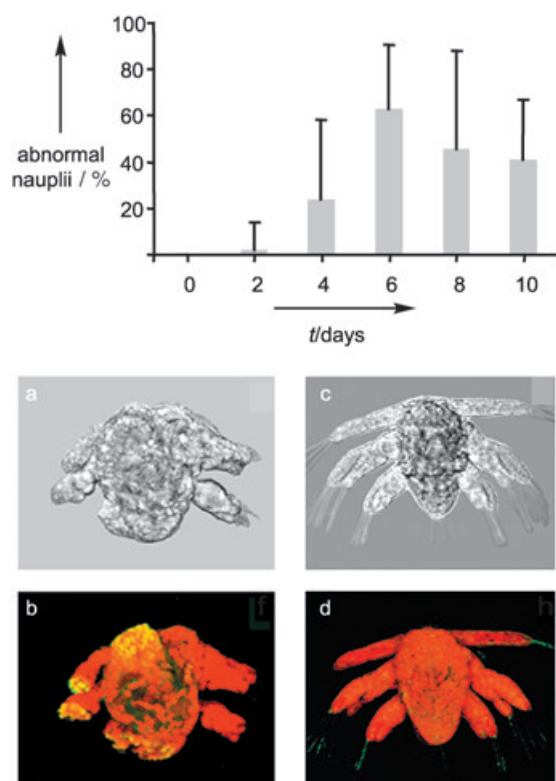


Figure 7. Effects of diet on *C. helgolandicus* offspring fitness. Top) After feeding on *S. costatum*, up to 65% of the hatched nauplii were abnormal, while nauplii developed normally on the control dinoflagellate *P. minimum*. After nine days on the diatom-rich diet, nauplii had deformed limbs (a; light microscope) that were positive (yellow) for apoptosis-specific TUNEL staining (b; epifluorescence microscope). Nauplii generated from females fed the control PRO diet appeared normal (c, d). Modified from ref. [103] with the permission of the Nature Publishing Group.

tions. On the one hand, data sets used to argue against the effect of PUA did not include monitoring of the PUA content in field samples. On the other hand, laboratory experiments did not distinguish between PUA producers and nonproducers and often did not include starvation controls, thus wrongly suggesting a general adverse effect of diatoms. Only in recent years have analytical methods become available to monitor PUA production of phytoplankton samples on a regular basis, and the first studies are underway to correlate these data series with the in situ reproductive success of copepods. Apparently not every diatom-rich bloom in the ocean is correlated with PUA production and only about a third of approximately 70 randomly selected diatom cultures were able to produce these aldehydes.^[115] Moreover, PUA production is not only species- but also strain-dependent, since various isolates of *T. rotula* differed in their ability to form these antiproliferative aldehydes. Comparison of two *T. rotula* isolates from a diatom culture collection revealed that, although genetically closely related and morphologically nearly identical, these isolates differed completely in their ability to produce PUA. While the total amount of aldehydes reached about 5 fmol cell⁻¹ in one isolate, the other did not produce any detectable aldehydes in SPME analysis. These two isolates allowed comparative bioas-

says to be performed, the results of which strongly supported the hypothesis that unsaturated aldehydes reduce the hatching success of copepods. While the PUA-producing strain reduced the hatching success significantly, the nonproducer represented a high-quality food, and copepod eggs from females fed the nonproducing strain showed comparable hatching success with eggs from females raised on a control diet.^[63]

Based on this new correlative evidence, and also on more elaborate modeling,^[9] the idea of a defense mechanism in a few selected species might become more acceptable. In the future, it will be a major task to truly understand what role the oxylipin-based defense of diatoms plays in global plankton dynamics. As outlined in a recent workshop contribution by 37 authors, all aspects of the interactions, including food quality, toxic properties, and also possible detoxification strategies, will have to be considered in an interdisciplinary way.^[116] To understand plankton dynamics, not only will the classical approaches be important but also the new tools offered by genetic analysis of bloom-forming diatoms^[75,117] and by genetic engineering.^[118,119] Paired with the data from the first diatom genome,^[34] these tools will open new perspectives for an in-depth investigation of interactions in open water.

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Keywords: aldehydes • biosynthesis • chemical ecology • defense mechanisms • plankton

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