Antipredatory Defensive Role of Planktonic Marine Natural Products

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Contents

Abstract

This chapter deals with chemical ecology and the role of antipredatory metabolites in the plankton and how such plant–herbivore interactions structure communities in the pelagic zone of the oceans. The first section deals with antiproliferative compounds produced by unicellular diatoms that induce abortions or congenital defects in the offspring of zooplankton crustaceans such as copepods exposed to them during gestation. Such teratogenic compounds include numerous oxylipins, and polyunsaturated aldehydes (PUAs) in particular, that are very similar to those produced in higher terrestrial plants. 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

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herbivore attack (e.g., in tritrophic interactions) and as protective compounds (antibacterial, wound healing). The second section in this chapter deals with neurotoxic compounds (e.g., saxitoxins, brevetoxins, and others) produced mainly by dinoflagellates which induce severe pathologies (i.e., paralytic, neurotoxic, diarrhetic, and amnesic shellfish poisoning) in humans that consume shellfish containing high levels of these toxins and which are generally considered as feeding deterrents in the plankton. Strong physiological responses have also been reported in several copepod species after <24 h of feeding on these cells, such as elevated heart rates, regurgitation, loss of motor control, and twitching of the mouthparts, decreased feeding, decreased fecundity, delayed development, and direct mortality. Another important feeding deterrent in the plankton includes dimethylsulfoniopropionate (DMSP) found in numerous species of phytoplankton, but most prominently in the prymnesiophytes and dinoflagellates, which appears to act as a signal molecule or indicator of inferior prey rather than as a toxin. We discuss the environmental conditions which promote increased production of these metabolites and some of the classic bioassays to test their biological activity. We also discuss the multiple functions of antipredatory metabolites and the importance of chemical interactions in the plankton for shaping biodiversity and ecological functioning both at the community and cellular level.

13.1 Introduction

Plankton is the collective name for small or microscopic organisms that float or drift in large numbers, especially in the photic zone of the oceans. Plankton is of great importance because it serves as food for fish and larger organisms, and indirectly for humans whose fisheries depend on plankton. The plant-like component of the plankton is called phytoplankton and includes single-celled algal groups such as diatoms, dinoflagellates, coccolithophores, and other protists. The animal component is called zooplankton and is dominated mainly by crustaceans that subsist on phytoplankton and other small zooplankton. Copepods and other zooplankton crustaceans graze on large quantities of phytoplankton cells that must escape, deter, or tolerate herbivores in order to persist. 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. These chemicals are secondary metabolites or natural products that are not directly involved in primary metabolism. They differ from the more prevalent macromolecules, such as proteins and nucleic acids that make up the basic machinery of life. Often, secondary metabolites constitute a very small fraction of the total biomass of an organism, and it is not always clear what the biological function of these compounds actually is. In recent years, it has become increasingly apparent that these metabolites have important ecological functions and may at times contribute as much as primary metabolites to the survival of the producing organism. The science that considers chemical interactions between

organisms and their environment is termed chemical ecology, considered one of the fastest growing and rapidly evolving environmental subdisciplines.

Research on chemically mediated interactions in the marine environment has historically focused on predator–prey interactions in the benthos, and there is now a considerable amount known about feeding preferences and deterrent molecules in macroalgae, sponges, mollusks, and corals (see ▶ Chapter 12). Much less is known on the chemical ecology of planktonic marine organisms. We also know very little as to why zooplankton avoid certain compounds, and few studies have assessed what happens when secondary metabolites are consumed. Studies on chemical interactions in the plankton are still in their infancy, but there is an increased awareness that natural products play fundamental roles as defenses against predators, competitors, and pathogens, and therefore drive ecosystem functionality in the plankton. In this chapter, we focus on some of the better studied antipredatory defenses in the plankton, and more specifically on where the specific compounds have been identified and tested in pure or semipurified form in manipulative experiments. The chapter does not attempt to provide a comprehensive overview on this subject, but rather to illustrate some examples of the diversity and importance of chemically mediated interactions involving planktonic organisms (Fig. 13.1), and some of the methodologies that have developed in this field in recent years. In the following sections, we focus on compounds that induce reproductive failure in grazers and those that induce feeding deterrence and mortality (summarized in Table 13.1). We then discuss the environmental conditions which promote increased production of these metabolites and some of the classic bioassays to test the biological activity of phytoplankton natural products on predatory copepods.

13.2 Antiproliferative Compounds

13.2.1 Diatom Oxylipins

The strongest evidence that phytoplankton use chemical defense to deter planktonic grazers comes from the work initiated over a decade ago on diatom oxylipins. 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. However, 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 [12]. 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 repellent or poisoning processes, but never to reproductive failure.

In terrestrial environments, there are many reports of compounds produced by plants that interfere with the reproductive capacity of grazing animals, and which act as a form of population control. These compounds are referred to as teratogens or substances that induce abortions or congenital malformations in the offspring of

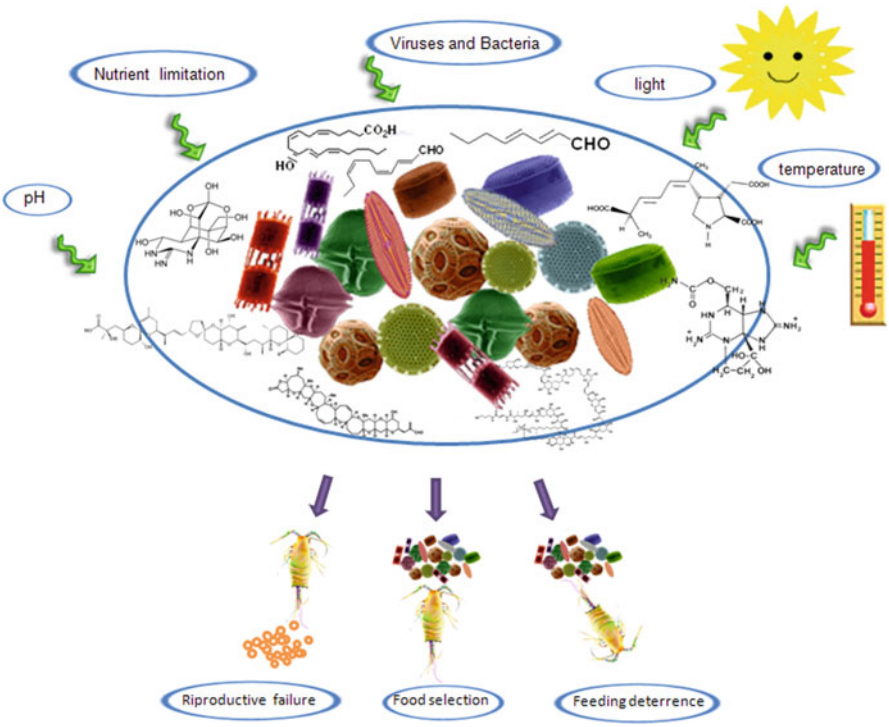

Fig. 13.1 Schematic representation of the environmental factors affecting the production of phytoplankton secondary metabolites (*green arrows*) and the effects of these metabolites on planktonic predatory copepods (*blue arrows*)

mothers exposed to them during gestation. Some classic examples of teratogens 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 [13].

Nothing was known on the presence and effects of such compounds in the marine environment until the discovery of diatom oxylipins, including polyunsaturated aldehydes (PUAs), produced by diatom cells from precursor membrane-bound lipids [5, 14–16]. Diatom PUAs were first isolated by Miralto et al. [1] who showed, in vitro, that they reduced copepod hatching success, cleavage of sea urchin embryos, and proliferation of human adenocarcinoma cells. The same authors showed that diatoms also modified copepod hatching success in the field in February 1997 and 1998 during two major diatom blooms in the North Adriatic

Table 13.1 Effects of secondary metabolites produced by phytoplankton on predatory copepods. Only characterized compounds known to be produced by microalgae and for which the specific toxicity has been determined are reported (modified from Ianora et al. [152])

Compounds	Producer	Target organism	Effect	Mode of action	References
PUAs					
Decatrienal	*Thalassiosira rotula* and other *Thalassiosira* species	Copepods	Reduced hatching success, abnormal nauplii, growth inhibition	Antimitotic, apoptosis	[1, 2]
Octatrienal, octadienal, heptadienal	*Thalassiosira rotula, Skeletonema marinoi*	Copepods	Reduced hatching success, abnormal nauplii, growth inhibition	Antimitotic, apoptosis	[3, 4]
Hydroxy- and epoxy-fatty acid pool	*Thalassiosira rotula*, *Skeletonema marinoi*, *Pseudo-nitzschia delicatissima*, *Chaetoceros affinis*, *Chaetoceros socialis*	Copepods	Reduced hatching success, abnormal nauplii, growth inhibition	Antimitotic apoptosis	[5, 6]
Fatty acid hydroperoxides	*Thalassiosira rotula*, *Skeletonema marinoi*, *Pseudo-nitzschia delicatissima*, *Chaetoceros affinis*, *Chaetoceros socialis*	Copepods	Reduced hatching success, abnormal nauplii, growth inhibition	Antimitotic apoptosis	[5, 6]
Oxoacids	*Pseudo-nitzschia delicatissima*	Copepods	Reduced hatching success, abnormal nauplii, growth inhibition	Antimitotic apoptosis	[7]
DMSP: acrylate, DMS	*Emiliana huxleyi*	Protistan, crustacean	Feeding deterrence, search behavior	Unknown	[8]
Apo-fucoxanthin	*Thalassiosira weissflogii*, *Phaeodactylum tricornutum*	Copepods	Feeding deterrence	Unknown	[9, 10]
PSP[a]	*Alexandrium* sp.	Copepods	Feeding deterrence	Unknown	[11]

[a]*PSP* saxitoxin (STX), neo-STX, gonyautoxin (GTX), C-toxins

Sea. Egg viability in these periods was only 12% and 24%, respectively, of the total number of eggs produced compared to 90% after the bloom in June indicating that copepod recruitment was higher during postbloom conditions and not during the bloom as previously assumed.

Oxyplins, and PUAs in particular, have important biological and biochemical properties including the disruption of gametogenesis, gamete functionality,

fertilization, embryonic mitosis, and larval fitness and competence [17]. PUAs are also apoptogenic inducers through the activation of specific caspases that lead to the enzymatic breakdown of DNA [18, 19]. PUAs may be sequestered during oocyte development and be passed maternally to the embryo, or may act directly on embryos. By whichever route, the timing of reproduction in relation to toxic diatom abundance will have important consequences for invertebrate recruitment [20–22].

The PUA decadienal is the best-studied metabolite of this group and thus has become a model aldehyde for experimental studies on the effects of oxylipins on marine organisms. Numerous functions have been proposed for this highly reactive molecule, such as grazer defense [1, 23], allelopathy [24, 25], cell to cell signaling [26, 27], antibacterial activity [28], and bloom termination initiator [7, 29]. In terms of grazer defense, decadienal has been shown to negatively affect reproductive processes in planktonic copepods (reviewed by Ianora and Miralto [30]) and cladocerans [31], and benthic echinoderms [17, 18, 22], polychaetes [32], ascidians [33], mollusks [34], and benthic copepods [35] with widespread consequences on ecosystem functionality (Table 13.1).

The discovery of an enzyme cascade leading to the production of volatile biologically active oxylipins in marine phytoplankton is rather new [30] even though the oxidative cleavage of fatty acids to form similar defensive compounds is well known in higher terrestrial plants [36, 37] and freshwater microalgae [38]. Several papers have described the biochemical pathways leading to PUA production [6, 39]. Pohnert [40] described that the transformation of polyunsaturated fatty acids (PUFAs) such as C20 in the diatom *Thalassiosira 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 [40] 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 liberation of C20 EPA fatty acids by phospholipases to produce decadienal and decatrienal.

However, d'Ippolito et al. [15] described an alternative biochemical pathway from other complex lipids in the diatom *Skeletonema 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 [36]. 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. [41] 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 the diatom *S. marinoi*, C16 and C20 PUFAs serve as specific substrates for the production of 2,4-heptadienal and 2,4-octadienal.

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 [42], thereby avoiding intoxication to the diatom cells [24]. Fontana et al. [6] 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 *T. 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}) 40 min later. More recently, Romano et al. [22] have shown that decadienal can be detected spectrophotometrically for up to 14 days in closed culture containers.

A major advancement in the study of the chemistry of diatoms was the discovery that they produce cytotoxic compounds other than PUAs (Fig. 13.2). Wichard et al. [2] 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 do not produce these compounds. But, at about the same time, d'Ippolito et al. [43] showed that the diatom *T. rotula* had an oxidizing potential capable of converting PUFAs to a variety of other unprecedented oxylipins, as later confirmed by Fontana et al. [5]. Fontana and coworkers compared the effects of the well-known PUA-producing diatom *S. marinoi* [1, 23] 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 and oxylipins such as hydroxyacids (HEPEs) and epoxyalcohols (HepETEs), as well as highly reactive oxygen species (ROS) of low acute toxicity to adult copepods but which depressed the viability of copepod gametes and offspring. Two of these compounds, 15S-HEPE and threo-13,14-HepETE, have now also been reported by d'Ippolito et al. [7] in the non-PUA-producing pennate diatom *Pseudo-nitzschia delicatissima* which produces large amounts of these metabolites in the late exponential and stationary phase of growth. d'Ippolito et al. also reported the presence of another metabolite, 15-oxo-5Z,9E,11E,13E-pentadecatetraenoic acid, which was only present in small amounts in the late stationary phase of the culture, demonstrating a precise timing in the production of these compounds during various stages of the cell cycle. Pohnert et al. [44] had already suggested the presence of aldehydic 9-oxo-nonadienoic and 12-oxo-dodecatienoic acids (12-ODTE) in the non-PUA-producing diatom *Phaeodactylum tricornutum* on the basis of the detection of high amounts of the C11-hydrocarbon hormosirene and the C8-hydrocarbon fucoserratene after wounding. Indeed, as reported by Pohnert and Boland [45], these hydrocarbons are released together with the aldehydic acids 9-ONDE and 12-ODTE by

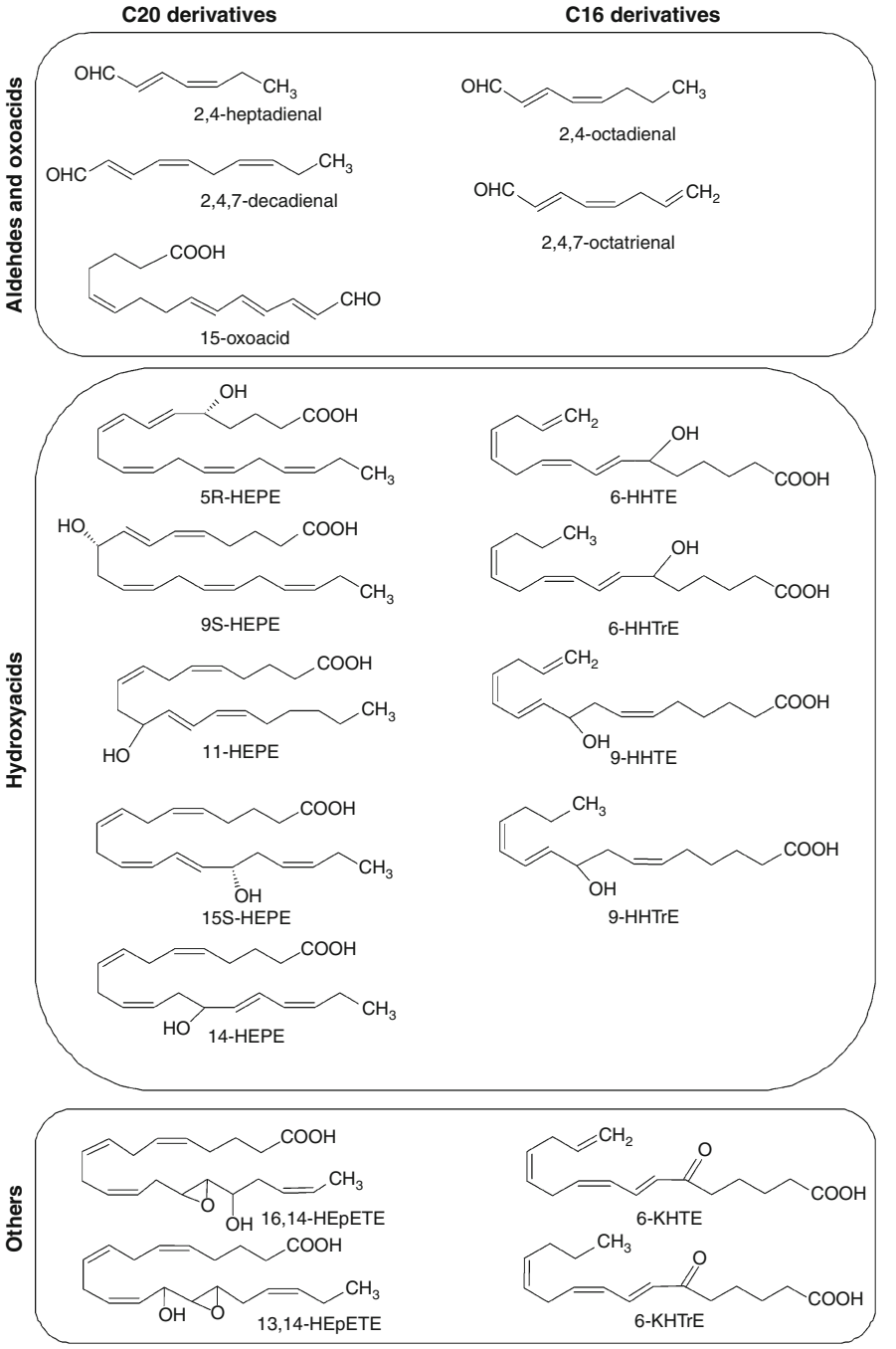

Fig. 13.2 (continued)

freshwater diatoms. These products are very similar to those produced by terrestrial plants [37], as shown in Fig. 13.3. A major difference, as in the case of PUAs, is in the precursor PUFAs, C16 and C20 fatty acids, used to synthesize these compounds in diatoms [39, 43] compared to the C18 fatty acids in terrestrial plants [36, 37]. Oxylipins formed in flowering plants include fatty acid hydroperoxides, hydroxyl- and keto-fatty acids, oxoacids, epoxyalcohols, divinyl ethers, PUAs, and the plant hormones 12-oxo-phytodienoic acid and jasmonic acid [46], 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), alarm signals against herbivore attack, 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 signaling and pheromone attraction between gametes of different sex [46].

Testing the biological activity of oxylipins other than PUAs is quite recent. Pohnert et al. [44] reported that the lethal dose to induce 50% mortality (DL-50) in sea urchins with 12-ODTE was reached at concentrations around 9–10 μM which was in the range observed for decadienal (7.3 μM). Fontana et al. [5] found that a pool of oxylipins, other than PUAs from the diatom *S. marinoi*, blocked hatching success of the copepod *Calanus helgolandicus* at about 70 μM compared to a pool of aldehydes from the same diatom (32 μM) with induction of apoptosis and malformations in hatched nauplii for both pools of compounds. More recently, Ianora et al. [47] tested the hydroxyacid 15S-HEPE on newly spawned eggs of the copepod *Temora stylifera*, showing that this metabolite blocked hatching success at about 94 μM compared to heptadienal and decadienal (45 and 32 μM, respectively) All three compounds induced apoptosis and malformations in hatched nauplii. Taken together, these results indicate that PUAs are not the only class of molecules inducing reproductive failure in copepods. 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 which grazers are exposed to during feeding. Oxylipins such as 9S-hydroxyhexadecatrienoic (9S-HHTrE) and 9S-hydroxyhexadecatetraenoic (9S-HHTE) have also been found at sea, during a late spring bloom in the North Adriatic Sea of the non-PUA-producing diatom

Fig. 13.2 Diatom oxylipins characterized to date deriving from the oxidation of C16 and C20 fatty acids. Information on diatom species producing these compounds is given in Ianora and Miralto [30]. 5R-HEPE = 5R-hydroxy-(6,8,11,14,17)-eicosapentaenoic acid; 9 S-HEPE = 9 S-hydroxy-(5,7,11,14,17)-eicosapentaenoic acid; 11-HEPE = 11-hydroxy-(5,8,11,15,17)-eicosapentaenoic acid; 15 S-HEPE = 15 S-hydroxy-(5,8,11,13,17)-eicosapentaenoic acid; 14-HEPE = 14-hydroxy-(5,8,11,15,17)-eicosapentaenoic acid; 6-HHTE = 6-hydroxyhexadeca-(7,9,12,15)-tetraenoic acid; 6-HHTrE = 6-hydroxyhexadeca-(7,9,12)-trienoic acid; 9-HHTE = 9-hydroxyhexadeca-(6,10,12,15)-tetraenoic acid; 9-HHTrE = 9-hydroxyhexadeca-(6,10,12)-trienoic acid; 13,14-HEpETE = 13-hydroxy-(14,15)-epoxy-5,8,11,17-eicosatetraenoic acid; 16,14-HEpETE = 16-hydroxy-(14,15)-epoxy-5,8,11,17-eicosatetraenoic acid; 6-KHTE = 6-ketohexadeca-(7,9,12,15)-tetraenoic acid; 6-KHTrE = 6-ketohexadeca-(7,9,12)-trienoic acid

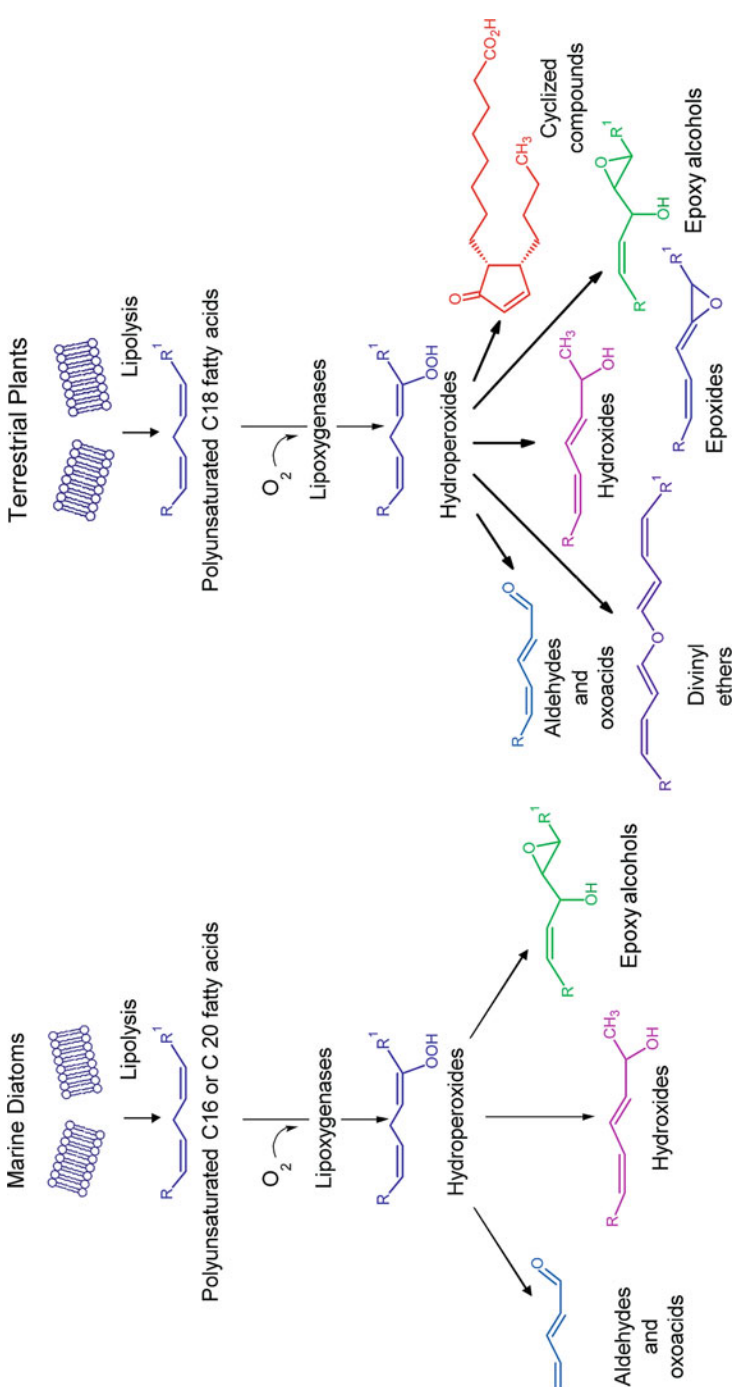

Fig. 13.3 Oxidative metabolism of fatty acids in (**a**) marine diatoms and (**b**) terrestrial plants. Starting mainly from eicosapentaenoic (C20) and exadecatrienoic (C16) fatty acids in diatoms or from octadeca(e)noic (C18) fatty acids in plants, both oxidative pathways involve oxygenation of these fatty acids by oxygen addition through lipoxygenase activity to a *cis,cis*-1,4-pentadiene moiety

Cerataulina pelagica, the likely cause for reduced egg production and hatching success in *Acartia clausi*, *Calanus helgolandicus*, and *Temora longicornis* [48].

Diatoms are not the only class of marine phytoplankton to produce oxylipins. The bloom-forming phytoplankter *Phaeocystis pouchetii* (Prymnesiophyceae) has been shown to produce and release decadienal [19] even though it remains unclear as to what amounts are produced. In freshwater environments, PUAs are commonly released by diatoms and chrysophytes (see [49] and references therein) through cell lysis, independently from grazing, conferring rancid smells to source drinking water [50]. In this case, PUAs may not only serve to deter grazers [51] but may also function as intra- or interspecific signals, for example, to modulate population density or to signal the presence of pathogens or predators [52]. Their widespread occurrence in marine and freshwater photoautotrophs may be an indication of the great ecological importance of these compounds.

An alternative hypothesis for the ecological function of PUAs by diatoms was recently developed by Jüttner ([49] and references therein). Upon cell damage, diatom-dominated biofilms release small quantities of PUAs that are efficient repellents for herbivorous zooplankton. These substances probably function as "warning cues," since upon cell damage, diatoms release high amounts of the polyunsaturated eicosapentaenoic acid (EPA) which is highly toxic for crustacean herbivores. Following cell wounding, a small fraction of the released EPA is further converted into PUAs which, in turn, can be detected by the grazers. Thus, grazers that initiate feeding on these diatoms cause the liberation of an activated warning cue that repels them, thereby protecting them from the toxic EPA and protecting the diatom from herbivores. A major challenge for the future will be to understand the ecological function of these metabolites and the physiological factors that lead to their production. We have just begun to understand the role of diatom oxylipins in the plankton, and there remains great scope for research into the effects of these toxins on gamete, embryonic, and larval development of herbivorous grazers.

13.2.2 Dinoflagellate Antiproliferative Compounds

Hatching failure in copepods can also depend on poor sperm quality. Interestingly, such effects have mainly been reported in copepods fed with dinoflagellate diets. For example, *Prorocentrum micans, Gymnodinium sanguineum* and *Gonyaulax polyedra* significantly modified spermatophore production and reduced the fertilization capacity of male sperm after 6–12 days of continuous feeding in the copepod *T. stylifera* [53]. A reduction in fertilization capacity was neither due to maternal effects nor to male age since hatching success returned to normal when males were substituted with freshly caught wild males or males conditioned with a good diet such as the dinoflagellate *Prorocentrum minimum* for the same length of time as couples fed with the poor diets. Microscope images of unhatched eggs colored with a nucleus-specific fluorescent dye showed that these eggs had not been fertilized.

Ianora et al. [54] later showed that extracts of a nontoxic strain of the dinoflagellate *Alexandrium tamarense* blocked fertilization but not cell divisions in sea

urchin embryos. Of the two organic extracts of *A. tamarense*, the one obtained in MeOH was the more active, and one of the fractions obtained by its separation on Sephadex LH-20 resin blocked fertilization of sea urchin oocytes at concentrations of 1 μg mL^{-1}. The PUA decadienal, used as a control, had little or no effect on fertilization success at any of the concentrations tested. By contrast, the *A. tamarense* active fraction had little or no effect on sea urchin first cell cleavage as opposed to decadienal, which blocked cell cleavage at concentrations of 1 μg mL^{-1}.

There are several examples of compounds isolated from marine dinoflagellates that block cell divisions in the classic sea urchin bioassay. These include goniodomin-A from *Goniodoma pseudogoniaulax* [55], amphidinolide-A from *Amphidinium* sp. [56], and okadaic acid and its derivative dinophysistoxin-1, which are common in some species of the genera *Prorocentrum* and *Dinophysis*, respectively [57]. Extracts of *A. tamarense* may also inhibit egg hatching and larval survival in the scallop *Chlamys farreri* [58]. In all of these studies, the molecules or extracts tested showed antimitotic activity with blockage of cell divisions in developing sea urchin embryos. In Ianora et al. [54], however, there was little or no adverse effect on cell cleavage, but very strong effects on fertilization success when oocytes were incubated for 30 min in *A. tamarense* extracts.

Clearly, these compounds were not feeding deterrents since both male and female *T. stylifera* copepods fed well on this *A. tamarense* strain, with fecal pellet production rates comparable to other dinoflagellate (*P. minimum*) diets [53]. Nonetheless, there were adverse effects on other life-history parameters such as fecundity, egg viability, and adult female survivorship.

Why do microalgae produce such compounds if they do not serve to deter feeding in their predators? According to chemical defense theory, there should be a selective survival advantage for species that can "protect" themselves from grazers, allowing such species to grow explosively, because the growth of the populations of their predators has been suppressed. This could occur through the production of chemicals that not only deter feeding activity but also reduce population growth. For example, diatom oxylipins reduce egg production and/or hatching success, thereby sabotaging copepod population growth and, consequently, predator grazing impact. In the case of *A. tamarense*, the defense machinery is potentially even more effective, because female longevity is reduced as well. The chemical structures of these compounds remain unknown.

13.3 Feeding Deterrents

13.3.1 HAB Toxins

Phytoplankton are among the most diverse organisms in the oceans, and due to the complexity of the habitat in which they live, these unicellular eukaryotes have evolved some of the most unique metabolites ever isolated in nature. Some of these are potent neurotoxins that can make their way up the marine food chain and are responsible for massive fish kills, both wild and farmed, as well as the deaths of

many aquatic birds and mammals, including whales and sea lions [59, 60]. There are a wide range of organisms that produce toxic compounds that are often referred to as harmful algal bloom (HAB) toxins. These include flagellates especially dinoflagellates, cyanobacteria, and some species of diatoms, silicoflagellates, prymnesiophytes, raphidophytes, and other phytoplankton. It is estimated that of the 300 species reported to be HABs, only 2% (60–80 species) actually produce toxins. The other HABs cause mortality of marine organisms after reaching high concentrations that may cause damage due to clogging or oxygen depletion. A detailed description of the different types of HAB toxins is beyond the scope of this chapter. Here, we consider only some examples of toxins which potentially serve as antipredatory defense compounds in the plankton (Table 13.1, Fig. 13.4).

Interestingly, the neurotoxic compounds produced by dinoflagellates which induce severe pathologies (i.e., paralytic, neurotoxic, diarrhetic, and amnesic shellfish poisoning) in humans that consume shellfish containing high levels of these toxins may have little or no effect on their direct zooplankton grazers. Several studies have demonstrated that some copepods show little or no behavioral effects of consuming toxic dinoflagellates (e.g., [61, 62]). On the other hand, there are other studies showing that certain dinoflagellates induce strong physiological responses in copepods after $<$24 h of feeding on these cells, such as elevated heart rates, regurgitation, loss of motor control, and twitching of the mouthparts [63], decreased feeding [64], decreased fecundity [65], delayed development [66], and direct mortality [61]. Furthermore, the copepods *Calanus finmarchicus* [67], *Eurytemora herdmani*, and *Acartia tonsa* [68] have been shown to avoid feeding on paralytic shellfish toxin (PST)-containing prey when offered together with a nontoxic food option. Based on these findings and the negative effects that PST-containing phytoplankton have on some grazers, PSTs have been suggested to act as a protection against grazers [69], but direct demonstrations of paralytic shellfish toxins functioning as antigrazer defenses have not been demonstrated to date.

Most of these toxins are always present in the cells and are referred to as constitutive as compared to induced defenses that are produced only upon damage of the cells by predators (e.g., diatom oxylipins). If the primary function of these compounds is to protect the producers against grazers, the optimal defense theory predicts that cell-specific toxin content should correlate to the probability of attack [68]. Hence, their concentration in the cell should increase following contact with herbivore-specific chemicals [70]. This has, in fact, been shown for the marine copepod *A. tonsa* whereby waterborne chemicals produced by this copepod caused an increase in PST production in the harmful algal bloom-forming dinoflagellate *Alexandrium minutum* [11]. *A. minutum* contained up to 2.5 times more toxins than controls and was more resistant to further copepod grazing. Further investigations [71] showed that when *A. minutum* was grown under nitrate-rich conditions, but not in low-nitrate treatments, the presence of waterborne cues from grazers resulted in significantly increased cell-specific toxin content, implying that the magnitude of grazer-induced PST production is directly proportional to the degree of nitrogen availability. This response was also grazer-specific, with some species of copepods inducing a higher production of toxins (up to 20-fold increase in the presence of the

Fig. 13.4 (continued)

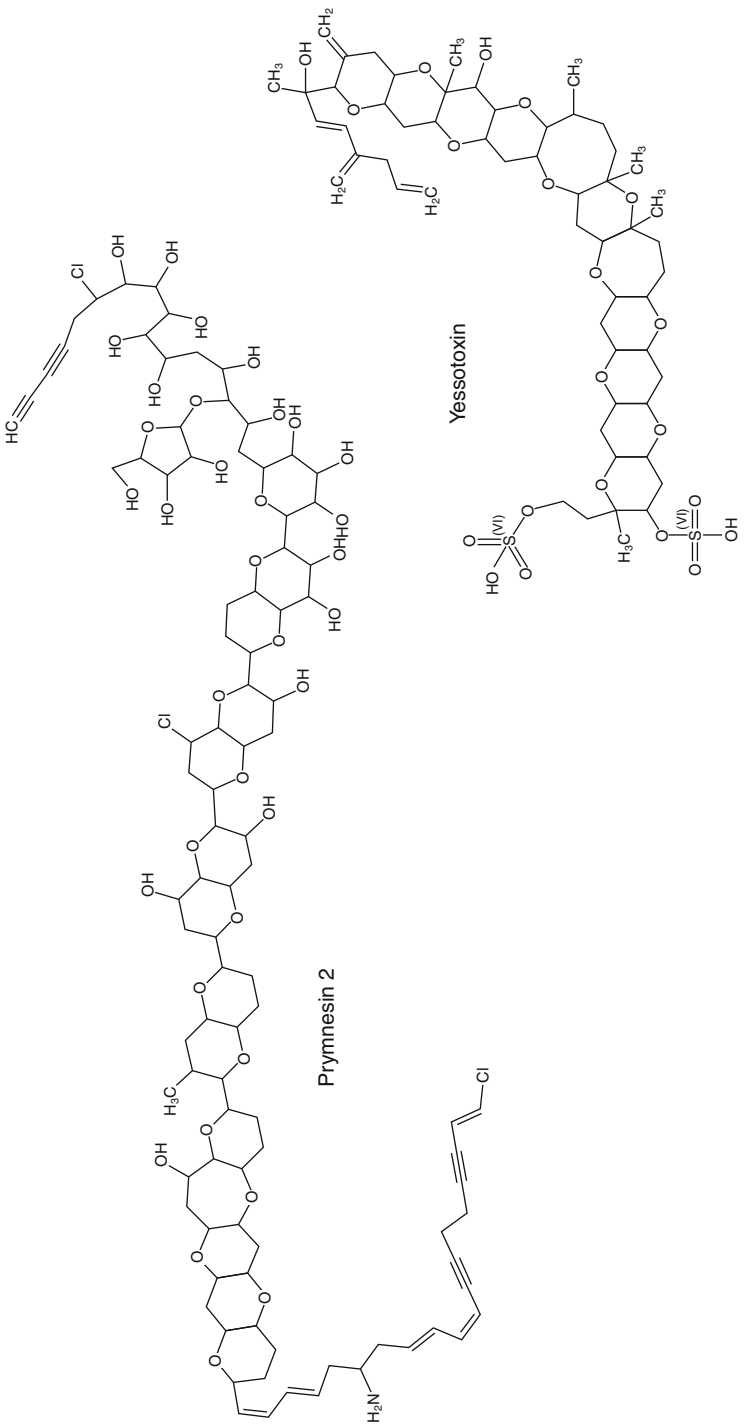

Fig. 13.4 Dinoflagellate and diatom neurotoxins suspected to have antifeeding and antireproductive activity on planktonic predatory copepods. Saxitoxin: $R^1 = H$, $R^2 = H$; Neosaxitoxin: $R^1 = OH$, $R^2 = H$; Gonyautoxin1: $R^1 = OH$, $R^2 = OSO_3$; Gonyautoxin2: $R^1 = H$, $R^2 = OSO_3^-$

copepod *Centropages typicus*) than others, which may induce no change at all (*Pseudocalanus* sp.). The ability of *A. minutum* to sense and respond to the presence of grazers by increased PST production, and the associated negative effect on grazers, is a strong evidence that these compounds are defensive metabolites, the purpose of which is not necessarily to intoxicate and kill the predator but to discourage further consumption.

The discovery and characterization of the molecules responsible for this biological activity are relatively recent (mainly 1980s). Okadaic acid was the first toxin isolated from a marine dinoflagellate even though it had previously been found in the sponge *Halichondria okadai*. The toxin was identified from a Tahitian strain of the dinoflagellate *Prorocentrum lima* and a derivative of this toxin, dinophysistoxin, was later isolated from temperate species of the dinoflagellate genus *Dinophysis*. Both okadaic acid and dinophysistoxin are associated with episodes of diarrhetic shellfish poisoning in humans (see [68, 72] for reviews on dinoflagellate toxins). Okadaic acid is a potent inhibitor of protein phosphorylase phosphatase 1 and 2A in the cytosol of mammalian cells that dephosphorylate serine and threonine. It is not only a potent tumor promoter but is also capable of reversing cell transformation in some oncogenes.

Other neurotoxins such as the brevetoxins are sodium channel activators causing repetitive depolarization of nerve membranes with an increase in the influx of sodium ions that ultimately deplete cellular reserves of acetylcholine at the synapses. The saxitoxins cause the opposite effect to the brevetoxins. They bind to the sodium channels and specifically block sodium permeability of the nerve membrane, ultimately causing paralysis and respiratory failure in humans. The differences in structure of the various saxitoxins alter the rates at which they bind to and depart from the binding site on the sodium channel. Yessotoxin, isolated from the dinoflagellate *Ptychodiscus brevis* (previously *Gymnodinium breve*), partially resembles the brevetoxins in structure and toxicity. Ciguatera poisoning produces various symptoms such as cardiovascular, gastrointestinal, sensory, and motor disturbances. No effective drug is currently known for therapy. The toxin is produced by a benthic dinoflagellate, *Gambierdiscus toxicus*, and is transmitted to fish along the marine food chain. *G. toxicus* also produces a more polar toxin, maitotoxin, which, together with ciguatoxin, is probably the most potent neurotoxin ever isolated from marine organisms.

There is high species-specific variability in the effects of these toxins on grazers, with effects ranging from severe physical incapacitation and death in some species [61] to no apparent physiological effects in others [73]. This variability may depend on the capability of some predators to detoxify or activate defense systems against these compounds. Recent studies [74] have shown that two days of feeding on a strong oxylipin-producing diatom (*Skeletonema marinoi*) is sufficient to inhibit a series of genes involved in aldehyde detoxification, apoptosis, cytoskeleton structure and stress response in the copepod *Calanus helgolandicus*. Of the 18 transcripts analyzed by RT-qPCR at least 50% were strongly downregulated (aldehyde dehydrogenase 9, 8 and 6, cellular apoptosis susceptibility and inhibitor of apoptosis IAP proteins, heat shock protein 40, alpha- and beta-tubulins) compared to animals fed

on a weak oxylipin-producing diet (*Chaetoceros socialis*) which showed no changes in gene expression profiles. These results provide molecular evidence of the toxic effects of strong oxylipin-producing diatoms on grazers showing that primary defense systems that should be activated to protect copepods against toxic algae can be inhibited. On the other hand other classical detoxification genes (glutathione S-transferase, superoxide dismutase, catalase, cytochrome P450) were not affected possibly due to short exposure times. At the same time, each herbivore species is likely to show unique features in detoxification/transport mechanisms as is the case for insect detoxification. Colin and Dam [62, 75] have shown that when two geographically distant populations of the copepod *Acartia hudsonica* were reared on the toxic dinoflagellate *Alexandrium fundyense*, the one that had not experienced recurrent blooms of the toxic algae had lower somatic growth, size at maturity, egg production, and survival, compared to the other population that showed no effects on these life-history parameters. Some copepod species also seem capable of concentrating toxins in their body tissues [76, 77], and such biotransformations typically yield the formation of more potent analogs of these toxins, as occurs in mollusks. Although there is no evidence that copepods are capable of long-term sequestration of such toxins, these predators provide not only a link for toxin flux in pelagic food webs but they may also act as a sink for toxins by metabolizing and removing them from the environment. Provided that they are not incapacitated by the toxin, in some cases, the accumulated toxin may even serve as a transient defense against more sensitive predators. Many benthic invertebrates are capable of sequestering compounds from the food they consume and using them as defensive molecules against predators [78]. There is no reason why this should not also occur in the plankton.

Some diatom species of the genus *Pseudo-nitzschia* produce the potent neurotoxin domoic acid which mimics the excitatory activity of the neurotransmitter L-glutamic acid, inducing destructive neuronal depolarization and successive degeneration of the hippocampus of the brain. In severe cases of this pathology, known as amnesic shellfish poisoning, victims show permanent loss of recent memory. Bioassays with pure compounds (<20 μM domoic acid) induced 100% mortality after 24 h in the copepod *Tigriopus californicus* [79]. However, domoic acid has not been shown to induce negative effects on planktonic organisms that consume *Pseudo-nitzschia* species [80]. Olson et al. [81] found that copepod grazing impacts on field populations of *Pseudo-nitzschia* sp. were negligible, but this lack of grazing was not attributed to domoic acid. In a similar field-based study, Olson et al. [82] found no correlation between low grazing rates and particulate or dissolved domoic acid concentrations in field samples. On the other hand, Bargu et al. [83] found that krill exposed to abnormally high concentrations of dissolved domoic acid fed significantly less on a nontoxic food source than krill unexposed to domoic acid. However, due to the high concentrations and exposure methods used by these authors, little can be concluded about the putative antipredatory role of this toxin. If domoic acid is not a feeding deterrent to zooplankton grazing, zooplankton may facilitate vectorial transmission of the toxin which could have serious impacts on the marine ecosystem as well as on

the marine economy and human health. The genome sequence for *Pseudo-nitzschia multiseries* which will soon be available, should provide more insights into domoic acid biosynthesis, allowing researchers to profile production capabilities across different species, to better characterize their responses to environmental triggers, and to examine the molecular interactions between toxin-producing cells and their predators.

Blooms of the haptophyte *Prymnesium parvum* are well-known nuisances in brackish waters around the world that are usually accompanied by massive fish kills due to the production of prymnesins which exhibit potent cytotoxic, hemolytic, neurotoxic, and ichthyotoxic effects. These secondary metabolites are especially damaging to gill-breathing organisms, and they are believed to interact directly with plasma membranes, compromising integrity by permitting ion leakage. Several factors appear to function in the activation and potency of prymnesins including salinity, pH, ion availability, and growth phase [84]. Prymnesins may function as defense compounds to prevent herbivory, and some investigations suggest that they have allelopathic roles. Exposure to *P. parvum* can cause inactivity in the copepods *Eurytemora affinis* and *Acartia bifflosa*, without the copepods actually consuming the toxic alga, resulting in reduced copepod reproductive success [85]. Sopanen et al. [86] found that cell-free filtrates of *P. parvum* also negatively impacted copepod survivorship. No studies are available testing the effects of pure compounds on grazers.

Karlodinium veneficum is a small athecate dinoflagellate commonly present in low levels in temperate, coastal waters. Occasionally, *K. veneficum* forms ichthyotoxic blooms due to the presence of cytotoxic, hemolytic compounds named karlotoxins. Exposure to a toxic strain of *K. veneficum* significantly lowered clearance and ingestion rates compared to a nontoxic strain and mixed diets dominated by the toxic strain, but did not induce mortality in the copepod *A. tonsa* [87]. These results support the hypothesis that karlotoxins in certain strains of *K. veneficum* deter grazing by potential predators and contribute to the formation and continuation of blooms. Another HAB dinoflagellate *Karenia brevis* produces brevetoxins, polyketide neurotoxins with acute toxic activity in waterborne or aerosol form against vertebrates [88]. However, according to Poulson et al. [89], negative effects of *K. brevis* on copepod egg production and survivability are not due to a chemical deterrent, but likely caused by the nutritional inadequacy of *K. brevis* as a food source. Again, there are no studies testing the effects of pure molecules on grazers.

There is also the possibility that compounds inducing negative effects on grazers are not only the PSP toxins produced by HABs but also other unknown allelopathic compounds that inhibit the growth, survival, and reproduction of competing algae [90, 91]. In *A. tamarense*, these compounds are presumably large molecules (>5 kDa), stable over broad temperature and pH ranges, and refractory to bacterial degradation [92]. *Alexandrium* allelochemicals have lytic activity targeting both competitors and grazers underlying the success of this species during blooms [93], suggesting that they are very important for structuring plankton communities.

13.3.2 DMSP and DMS

Dimethylsulfoniopropionate (DMSP) is found in numerous species of phytoplankton, but most prominently in the Prymnesiophyceae and Dinophyceae [94]. DMSP is cleaved during feeding by predators into the gas dimethyl sulfide (DMS) and acrylate which was originally hypothesized to act as a grazing deterrent (Table 13.1) via the accumulation of acrylate in grazer food vacuoles [95]. However, Strom et al. [96] showed that DMSP, not DMS or acrylate, acted as a chemical defense against protist grazers, causing a notable feeding rate reduction in several species.

DMSP released by phytoplankton into ambient seawater appears to act as a signal molecule or indicator of inferior prey rather than as a toxin. Fredrickson and Strom [97] have shown that adding DMSP (20 mM) to laboratory cultures of two ciliates (*Strombidinopsis acuminatum* and *Favella* sp.) and one dinoflagellate (*Noctiluca scintillans*) causes a 28–75% decrease in feeding rates and that these decreases were concentration-dependent with 20 nM as the lower threshold for an effect. Partial but not complete recovery of grazing rates occurred during long-term (24 h) exposure to dissolved DMSP, as long as concentrations remained above 12 mM.

There are other studies showing that DMSP-producing species of phytoplankton do not deter grazers, suggesting that some predators are less sensitive to or more capable of detoxifying this metabolite. The bloom-forming coccolithophore *Emiliania huxleyi* contains high intracellular levels of DMSP, yet some strains of *E. huxleyi* are a suitable food source for protist grazers [98–100]. Copepods also consume *E. huxleyi*, though not as a preferred food source [99]. Like *E. huxleyi*, the prymnesiophyte *Phaeocystis* is a suitable food source for some protist grazers even if it is a strong DMSP producer [101]. However, studies of copepod feeding upon *Phaeocystis* generally give varied results, many of which related to sizes of colonies, differences between copepod species and experimental techniques. Turner et al. [102] concluded that even though copepods may feed well upon *Phaeocystis*, resulting poor fecundity on this diet may inhibit copepod population increases during blooms, thereby contributing to the perpetuation of blooms. The high egg-hatching success on this diet argues against *Phaeocystis* containing chemical compounds that act as mitotic inhibitors reducing copepod egg viability, such as those found in diatoms and dinoflagellates.

Phaeocystis may also use morphological defenses to deter grazers. The life cycle of *Phaeocystis* includes solitary flagellated cells 3–8 μm in diameter, which are usually succeeded by blooms of colonial aggregations with hundreds of nonflagellated cells embedded in gelatinous colonies of up to 2 mm diameter ([102] and references therein). Gelatinous *Phaeocystis* colonies can form enormous nuisance blooms contributing to anoxia, beach fouling, and clogging of bivalve gills and fishing nets. Colony formation in *Phaeocystis* has been interpreted as a defense strategy against small grazers, because large colonies create a size-mismatch problem for small zooplankton ([70] references therein). *Phaeocystis* cells may therefore face a dilemma in a more complex food web context since colony formation will make the cells more vulnerable to larger grazers in the system

such as copepods. Long et al. [103] have shown that *Phaeocystis* colony formation was suppressed in the presence of a larger grazer (the copepod *A. tonsa*), but when small ciliate (*Euplotes* sp.) grazers attacked this alga, it shifted to the colonial form which was too large to be grazed by these ciliates. These results provide strong evidence that *Phaeocystis* cells not only sense that neighbors were being attacked but also that they "identified" the attacker and responded with opposing phenotypic shifts depending on the identity of the attacker [104]. The chemicals responsible for colony formation in this species have not yet been identified.

Not all induced morphological changes involve colony formation. For example, Pondaven et al. [105] reported grazing-induced changes in cell wall silicification in a marine diatom. Grazing-induced silicification may increase the mechanical resistance of the diatom's frustule to copepod mandibules [106]. Pondaven et al. [105] compared changes in cell wall silicification of the marine diatom *Thalassiosira weissflogii* grown on three different preconditioned media which only differed by the fact that they had contained, either diatoms alone (control medium), nonfed copepods of the species *Calanus helgolandicus* (starved-copepods medium), or both diatoms and copepods ("diatoms and copepods" medium) before being used for culture experiments. Cells grown in preconditioned media that had contained both diatoms and herbivores were significantly more silicified than diatoms grown in media that contained diatoms alone or starved herbivores. These observations indicate that diatom cell wall silicification is not only a constitutive mechanical protection for the cell but also a phenotypically plastic trait modulated by grazing-related cues [70]. At present, it is still unknown which chemical compound(s) induces this defense.

DMS derived from the breakdown of DMSP also shows biological activity. A wide range of organisms seem to use DMS as a cue for food finding [107, 108]. It is possible that DMS odor plumes produced during zooplankton grazing are used by predators to detect, locate, and capture their prey. Plumes of DMS trigger a tail-flapping response in the copepod *Temora longicornis* which results in altered flow patterns and probably assists copepods in locating food [8]. The identification of the involved signal is important for general considerations about food finding by copepods. Since grazers are involved in the cell disintegration that triggers DMS production, this process could attract herbivores to patches with high food concentrations or assist predation on actively feeding herbivores. Carnivores can also benefit from the production of these infochemicals (information-conveying chemicals) which they sense and use to detect herbivores, thereby reducing the grazing pressure exerted by the herbivores on the plants [107]. Very little is known on such tritrophic (involving three trophic [feeding] levels) interactions in the plankton. A classic example of such complex feeding interactions in terrestrial ecosystems involves flies that sip nectar from flowers and are, in turn, eaten by spiders.

Despite the abundance and obvious presence in the plankton of these compounds, we still know little about the regulation of their production and their direct delivery to the interacting organisms. In phytoplankton cells, DMSP functions as a compatible solute, a cryoprotectant and, perhaps, an antioxidant [109–111]. Variation in phytoplankton intracellular DMSP concentration appears to facilitate

phytoplankton survival in a fluctuating environment. For example, increased DMSP production may occur under nutrient limitation [112] and when there is a decrease in temperature [113].

13.3.3 Apo-fucoxanthinoids

Another class of feeding deterrents in the plankton is the apo-fucoxanthinoids produced by the diatoms *Phaeodactylum tricornutum* and *Thalassiosira pseudonana* [9]. The compounds were identified as apo-10'-fucoxanthinal, apo-12'-fucoxanthinal, apo-12-fucoxanthinal, and apo-13'-fucoxanthinone. Feeding deterrent responses in the copepod *Tigriopus californicus* were observed at concentrations of 2–20 ppm which is about 1,000 times lower than the total apo-fucoxanthinoid concentration in *P. tricornutum* [10]. Mortality occurred at concentrations ranging from 36.8 to 76.7 ppm. Thus, these compounds are present in concentrations which may have ecological significance in the control of bloom formation and grazing, even if *P. tricornutum* and *T. pseudonana* are not major blooming species.

The activity of these molecules was tested by dissolving them into the water in which copepods had been incubated, and fecal pellet production was used as an indication of feeding activity. A decrease in fecal pellet production after 24 h was interpreted as a result of feeding deterrence compared to control containers that had not been inoculated with these compounds. A reduction in fecal pellet production as an indication of feeding deterrence has also been inferred in other studies on copepod feeding behavior, although it remains unclear if negative effects on copepod fitness are due to reduced ingestion of cells, toxins interfering with digestive processes, or enhanced energy expenditure due to detoxification.

13.4 Environmental Factors Affecting Toxin Production

In general, changes in toxin content are associated with disturbed (unbalanced) physiology with the up-shock or down-shock of cells exiting or entering stationary phases (see [114] and references therein). Table 13.2 illustrates the importance of up/down-shock events in affecting toxicity for some of the toxin-producing species discussed until now. Phosphorus limitation often seems to enhance cellular toxin content. N limitation does so to a lesser extent, or may in the cases of domoic acid and PSPs do the opposite and lead to a fall in toxin content. Si-limited growth of *Pseudo-nitzschia* promotes synthesis of domoic acid [115]. The same occurs for PUA production in the diatom *S. marinoi* with a 7.5-fold increase (27.5 fmol cell^{-1}) in Si-limited cells with respect to controls [116]. In most instances, toxin content is relatively low under nutrient-balanced conditions (N:P = 16:1). Physical factors can also affect toxin production. For example, low temperature decreased the growth rate of the PSP-producing dinoflagellates *Alexandrium catenella*,

Table 13.2 Toxic species and factors affecting toxin production

Toxic species	Factors increasing cell toxin	Toxin[a]	References
Toxins containing nitrogen			
Alexandrium catenella, *A. cohorticula*	Low temperature, low growth rate	PSP	[117]
A. fundyense	N- and P- limitation, temperature	PSP	[118–120]
A. minutum	P-limitation	PSP	[64]
A. tamarense	P-limitation, N-excess, organic matter	PSP	[117, 121–123]
Pseudo-nitzschia australi, P. delicatissima, P. multiseries, P. pungens, P. seriata	P-/Si-limitation, N excess, growth phase, high light, high pH, high or low temperature, organic matter, Fe-limitation or Cu-stress	DA	[114, 124, 125]
Toxins with little or no nitrogen			
Chrysochromulina leadbeateri, C. polylepis	P-limitation, salinity, high pH, N or P-limitation	ICHT	[117, 126, 127]
Dinophysis sp., D. acuminate, D. acuta	N-/or P-limitation, N-limitation; N-/P-sufficiency	OA, DTX, PTX	[117, 128]
Fibrocapsa japonica	Low salinity	PUFA	[129]
Gymnodinium catenatum	Low temperature, P-limitation	GYM	[117, 121, 130]
Heterosigma akashiwo	High temperature, Fe-limitation	ROS	[131]
Karenia brevis	Urea	Brevetoxin	[132]
Ostreopsis ovata	High temperature	Ostreocin	[133]
Prymnesium parvum, P. patelliferum	N-/or P-limitation	Prymnesin	[134, 135]
Prorocentrum lima	N-/or P-limitation, organic matter	OA, DTX	[117]
Pseudo-nitzschia delicatissima	Growth phase	15-HEPE, 15-oxoacid, 13,14- HepTE	[7]
Skeletonema marinoi	Growth phase, N- and P- limitation; Si- limitation	PUAs	[29, 115, 136]

Adapted from Graneli and Flynn [114]
GYM gymnodimine, *ICHT* ichthyotoxins, *DA* domoic acid, *OA* okadaic acid, *DTX* dinophysistoxin, *PTX* pectenotoxin, *15-HEPE* 15-hydroxyeicosapentaenoic acid, *13,14- HepTE* 13,14-hydroxy, epoxyeicosatetraenoic acid, *PUAs* polyunsaturated fatty aldehydes, *ROS* reactive oxygen species
[a]*PSP* saxitoxin (STX), neo-STX, gonyautoxin (GTX), C-toxins

A. cohorticula, and *Gymnodinium catenatum*, whereas it increased toxin content per cell. In contrast, a low growth rate due to light inhibition did not cause an increase in toxin content, compared to cells growing at optimum illumination. In other studies, light seems to be essential for toxin production (e.g., *Prymnesium parvum*) with higher irradiance augmenting toxin production, but once the toxins were extracellular, they were rapidly inactivated by exposure to both visible light and UV radiation [117]. Briefly, there is wide variation in toxin production not only

between algal groups but also species and strains in response to different environmental conditions, and this will affect toxin production levels at sea in different years with cascading effects on predators.

13.5 Laboratory Protocols to Assess the Effects of Phytoplankton Metabolites

Marine chemical ecology is a young science, and the methodologies to study the effects of natural products on marine organisms are subject to rapid change. During the last two decades, bioassays have progressed from those that detected bioactivity without much concern for ecological relevance, to ecologically relevant tests to investigate possible functions of secondary metabolites in the phytoplankton. The problem is that often the natural concentrations of a compound, and therefore the concentrations that should be tested, are not known. Most feeding deterrents have been isolated and identified by natural products chemists looking for unusual secondary metabolites. Rarely have chemical studies provided information on the yield of these compounds after extraction. Hence, it is difficult for ecologists conducting bioassay experiments to know the natural concentrations of these metabolites to be tested. To further complicate matters, there are geographical variations in the concentration of secondary metabolites and within-region variation in some cases. Notwithstanding, much progress has been made in recent years in designing ecologically relevant bioassays with natural concentrations of a compound in many feeding trials. Here, we give a few examples of bioassay methods currently used to study chemical interactions in the plankton especially those involving antipredation and antigrowth activities.

The antiproliferative activity of phytoplankton metabolites on plankton predators has been tested mainly on zooplankton copepods ([30] and references therein) and cladocerans [31]. The majority of these studies have tested the effects of aqueous solutions containing toxic compounds by incubating embryos, larvae, or adults and studying the effects on fertilization, embryogenesis, and postembryonic development. Although the ecological relevance of these assays is questionable, because animals usually come into contact with these metabolites only through direct grazing and possibly during lysis of cells during the decay of a bloom, these assays are very useful for a preliminary screening of bioactive compounds in phytoplankton. They are also very useful for bioassay-guided separation of bioactive compounds or for testing the effects of pure compounds.

For example, Miralto et al. [1] tested the effects of PUAs which they had isolated for the first time from marine diatoms by incubating *T. stylifera* copepods in 5-mL tissue culture wells containing PUAs (2-*trans*-4-*cis*-7-*cis*-decatrienal, 2-*trans*-4-*trans*-7-*cis*-decatrienal, and 2-*trans*-4-*trans*-decadienal) at concentrations ranging from 0.5 to 5 μg mL^{-1} (Fig. 13.5d). Fertilized eggs were freely spawned by females into containers, and the percentage of hatched eggs was determined 48 h later when

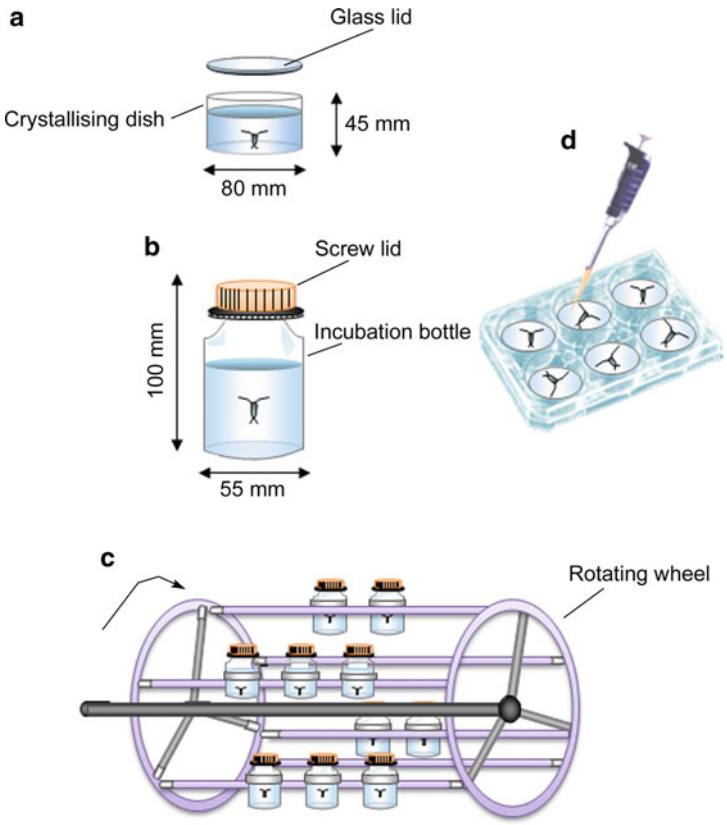

Fig. 13.5 Classic bioassays to test the biological activity of phytoplankton secondary metabolites on planktonic copepods. (**a**) 100-mL crystallizing dishes containing single females or male and female couples and natural food suspensions. (**b**): 1-L jars containing up to 20 copepods and food suspensions are placed on rotating wheel, as shown in (**c**). (**d**) 5-mL multitissue culture wells containing single copepods exposed to phytoplankton extracts or pure molecules. For explanation see text

control untreated eggs had hatched. Ianora et al. later used the same protocol to compare the effects of PUAs and 15S-HEPE on egg-hatching success of *T. stylifera*. Females were incubated individually in 5-mL tissue culture with known solutions of compounds and were removed when they had spawned to avoid cannibalization of eggs (Fig. 13.5d). Hatching was observed 48 h later.

More ecologically relevant studies on the antigrowth activity of phytoplankton metabolites have been conducted using grazing experiments whereby copepods are fed microalgae that are known to produce toxic metabolites (e.g., diatom oxylipins) for several days (usually 15 days). Using these long-term feeding assays, Ceballos and Ianora [138] followed the effects of diatoms that produced toxic metabolites on reproductive success of adult copepod females (egg production and hatching viability of eggs), demonstrating that the microalgae tested arrested cell divisions

and embryonic development compared to control microalgae that did not produce these compounds. Feeding assays consisted in incubating females individually in 100-mL crystallizing dishes filled with 0.45-μm filtered seawater and a known quantity of toxic-producing and nontoxin-producing microalgae, at saturating food concentrations equivalent to about 1 μg C mL^{-1} (Fig. 13.5a). Females were transferred to new containers with fresh algal suspensions daily for 15 days. A daily tally was kept of egg production and hatching success.

Feeding assays testing the effects of different algal diets have traditionally used larger experimental jars that are filled with food suspensions and then mounted on a rotating wheel (0.5 rpm), to keep the food suspended, in a controlled temperature room and on a fixed day–night light cycle (Fig. 13.5b). In this case, several copepods (5–10 in the case of 500 mL jars) are added to each of triplicate 500-mL experimental jars containing food suspension. A control jar with no copepods is treated in the same manner as the experimental jars, and an initial sample of food suspension is preserved at the beginning of each experiment. Experimental jars are filled and sealed, without air bubbles inside, and mounted on a rotating wheel (0.5 rpm), in a controlled temperature room at 20.5°C, on a 12:12 h L:D cycle for 24 h (Fig. 13.5c). After 24 h, copepods are removed from the experimental jars by pouring them into bowls to check for dead copepods. Food suspensions are then preserved with Lugol's solution, and phytoplankton cell numbers counted in Sedgwick-Rafter cells. At least 400 cells are counted in all cases to ensure ±10% precision. Ingestion rates are determined from differences in phytoplankton cell concentrations in initial, control, and triplicate experimental suspensions using the formulae of Frost [139]. Grazing rates for dead copepods are calculated assuming that they had fed for half of the experiment. Using these assays, Turner et al. [140] demonstrated that copepod egg-hatching success was strongly modified by some diatom diets, with hatching diminishing to 0% after 10 days of feeding on a diatom diet, and that this effect was diluted but not removed when the diatom was mixed with a dinoflagellate diet.

Bioassays have also been designed to test the antigrowth activity of metabolites on larval development to adulthood. Development assays consisted in incubating hatched copepod nauplii in experimental jars mounted on a rotating wheel with known quantities of algal food and following the development of nauplii to adulthood (Fig. 13.5b, c). Ianora et al. [23] sampled juveniles daily, counted and checked them under the microscope to determine the stage of development, and transferred them to new jars with fresh algal medium. A tally was kept of the most abundant larval stage and percentage of surviving individuals to calculate development and survival curves. Using this assay, Ianora et al. [23] showed that maternal diets affected offspring fitness. When mothers and their offspring were fed a diatom that produced unsaturated aldehydes, none of the larvae reached adulthood compared to control mothers and offspring that were fed a nondiatom diet. The effects on development were less severe when mothers received the control food and the offspring the treatment food.

A recent study has explored the possibility of using liposomes as a delivery system for copepods [141]. Giant liposomes were prepared and characterized in the

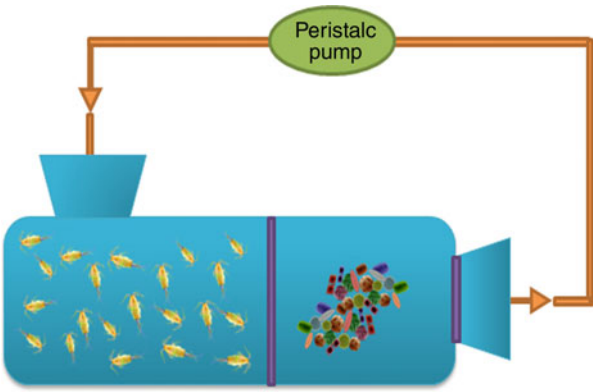

Fig. 13.6 Device to assess the effect of phytoplankton exudates on copepod activity. Phytoplankton and copepods are separated by a glass filter (see also Miralto et al. [143])

same size range of food ingested by copepods (mean diameter = 7 lm) and then encapsulated with the fluorescent dye fluorescein isothiocyanate (FITC) to verify copepod ingestion with fluorescence microscopy. Females of the calanoid copepod *T. stylifera* were fed with FITC-encapsulated liposomes alone or mixed with the dinoflagellate alga *P. 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 fecal 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 fecal 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. [142] used these giant liposomes to encapsulate decadienal in order to investigate the effect of PUAs on the reproductive biology of the copepods *T.* and *C. 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 that those used in classical feeding experiments (e.g., Ianora et al. [23]), demonstrating that liposomes were a useful tool to quantitatively analyze the impact of toxins on copepods. This type of technology for the delivery of toxins and drugs in feeding trials has considerable potential as a means of delivering a known quantity of toxin under seminatural conditions, allowing the toxins ingested to be determined.

An experimental flow-through chamber was designed by Miralto et al. [143] to verify if copepod fertility was modified due to the release of chemical cues by other conspecifics. The apparatus (Fig. 13.6) was divided into two chambers, each of which had a 50-mL volume capacity. The chambers were separated by a glass fiber filter (20 μm pore size), and a peristaltic pump ensured diffusion of 0.45-μm filtered SW water at a flow rate of 8 mL min^{-1}. A similar chamber could be used to test if copepod exudates induce an increased production of defensive metabolites in

phytoplankton, as described by Selander et al. [72] who observed a 2.5-fold increase in PST production in the dinoflagellate *A. minutum* when exposed to waterborne cues from copepods.

The application of "omics" technologies (genomics, transcriptomics, proteomics, metabolomics, etc.) to studies of biosynthesis and regulation of bioactive secondary metabolites, and the concomitant effects on plankton population dynamics is an emerging but rapidly advancing field. Complete genome sequences have been reported for two diatoms, *Thalassiosira pseudonana* [144] and *Phaeodactylum tricornutum* [145], but others will soon be available for representative species of major groups (e.g., heterotrophic and photosynthetic bacteria, prasinophytes, diatoms, *Emiliania huxleyi*, *Phaeocystis*, and copepods). With access to these tools, chemical ecologists have an unprecedented opportunity to learn more about the biochemistry and ecological functioning of pelagic systems more quickly and comprehensively than ever. This is manifested by altered expression of superoxide dismutase and metacaspases, key components of stress and death pathways. Transgenic approaches for manipulating genes in key signaling and stress-related pathways in phytoplankton may provide the opportunity to gain further insights into trophic-level interactions.

Given the massive genome size and complexity characteristic of marine dinoflagellates, whole genome sequencing remains impractical. A functional genomics approach, involving generation of a cDNA library and sequencing of expressed sequence tags (ESTs), has proven useful in the search for putative biosynthetic genes for secondary metabolites, stress response genes, and those involved in growth regulation leading to bloom formation [146]. Particularly, when coupled with DNA microarrays, molecular approaches have the potential to reveal novel pathways that are up- or downregulated during certain stress situations and might reveal the biochemical and chemical basis for some observed species interactions that cannot be explained to date. One detailed study of intrapopulation diversity within the toxigenic dinoflagellate *A. tamarense* [147] revealed that the abundant genetic diversity, determined by two independent molecular markers, was not directly correlated with the phenotypic variation in toxin spectrum and intracellular concentration.

Another powerful postgenomics tool, advanced metabolite profiling, also termed "metabolomics," allows mapping of the chemical diversity and dynamic range of bioactive secondary metabolites of potential importance in ecological interactions. Thus, the response of phytoplankton cultures or communities to competing organisms, predators, and viruses can be monitored with limited bias toward a certain compound class. Statistical evaluation of the data provides insight into the metabolic changes within the cells as well as into the released metabolites that might represent the chemical language of the cells [148]. It is likely that knowledge of the complex patterns of chemicals released by microalgae and other plankton species will result in a paradigm shift revealing new mechanisms for processes such as community function, food location, or complex defensive and allelopathic interactions. The marriage of metabolomics and metagenomics has the potential to be a particularly powerful

combination in shedding light on planktonic and microbial interactions in marine ecosystems [149].

Another important tool in plankton chemical ecology studies will be the adoption of model organisms that will provide insight into the functioning of other organisms. This approach is widely used in the biomedical field to explore potential causes and treatments for human disease and is based on the concept of conservation of biochemical pathways and genetic material over the course of evolution. Models are chosen on the basis that they are suitable for experimental manipulation, have characteristics such as a short life cycle, are amenable for maintenance and breeding in the laboratory, and have nonspecialist living requirements. In terrestrial chemical ecology, the introduction of *Arabidopsis thaliana* as the first fully sequenced plant proved to be highly successful for the elucidation of rather general plant defensive pathways. Such an approach could also be useful in the plankton and would allow for the elucidation of fundamental biochemical mechanisms such as regulatory and metabolic pathways in producer organisms as well as the identification of the mechanisms of action in target organisms. Ideally, the models selected in plankton ecological studies would also be supported by genomic and postgenomic resources, databases, and infrastructure.

13.6 Concluding Remarks

In comparison to the large number of studies on the antipredatory compounds produced by terrestrial plants, those regarding marine phytoplankton are underrepresented. Most studies on the secondary metabolites of these microalgae have focused on bioactivity and structural elucidation, with a view toward potential pharmaceuticals or other commercial purposes. Nevertheless, the environmental and human health consequences of HABs have provided great impetus to conduct ecologically relevant studies on microalgae in the last decade (reviewed by Poulson et al. [89] and Sieg et al. [150]).

Marine chemical ecology is a young science that requires the collaborative effort of biologists, ecologists, and chemists. Identifying the compounds responsible for mediating feeding, reproduction, and behavioral interactions is only the first step in understanding the ecological relevance of a compound. These effects then need to be translated from laboratory assays to their natural context in order to provide the ultimate test and major challenge for field ecologists. An increased understanding of chemical defenses will be achieved when we know how ecologically realistic doses of these metabolites affect growth, reproduction, and survivorship of consumers. In the long run, such studies will lead to a better understanding of how these compounds can help regulate ecosystem functionality by underpinning the chemical and molecular processes that are crucial for the fitness and survival of the producing organisms.

A major challenge for the future will be to understand the multiple simultaneous functions of many of these secondary metabolites. For example, diatom PUAs may have an antipredatory function [14] and also act as allelopathic agents [135]. Furthermore, PUAs can affect growth of some bacterial strains [28] and possibly also

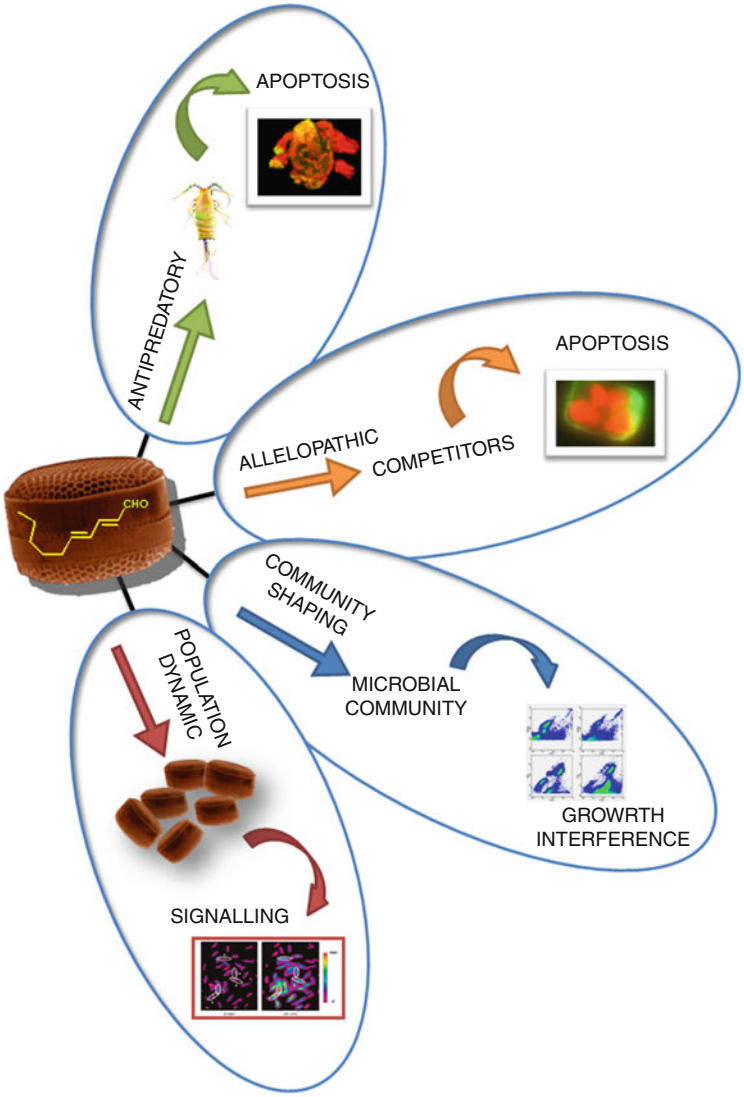

Fig. 13.7 Multiple effects of diatom polyunsaturated aldehydes (PUAs): From the *top*, *Antiproliferative (teratogenic) effect on copepods*. Abnormal *Calanus helgolandicus* copepod nauplius hatched from a mother fed with the PUA-producing diatom *Skeletonema marinoi*. *Yellow* parts indicate TUNEL-positive apoptotic tissues (from Ianora et al. [23]). *Allelopathic effect* on phytoplankton species in culture, *Skeletonema marinoi*, cell after incubation with decadienal. *Red* fluorescence indicates annexin positivity (apoptosis) (from Casotti et al. [24]). *Growth interference* of picoplankton. Flow cytograms represent a natural seawater sample from the Adriatic Sea; *left panels* are controls, *right panels* are picophytoplankton (*top*) and heterotrophic bacteria (*bottom*), after 24 h inoculation with a mixture of octadienal and heptadienal [28]). *Signaling effect*. Intracellular transmission of decadienal-derived signal induces NO in neighboring (from Vardi et al. [26]) influencing population dynamic

have a stress-signaling function [17], with potential consequences for food web structure and community composition (Fig. 13.7). Thus, the same secondary metabolites may act to deter different groups of organisms by different modes of action.

The chemical multifunctionality of secondary metabolies is well documented in higher terrestrial plants. For example, the anthraquinone emodin, identified in 17 plant families distributed worldwide, has numerous biological activities, some of which exhibit a wide spectrum of ecological impacts by mediating biotic or abiotic interactions of plants with their environment. In vegetative organs emodin may help protect plants against herbivores, pathogens, competitors and extrinsic abiotic factors (e.g., high light intensities). In unripe fruit pulp, emodin may facilitate seed dispersal by protecting the immature fruit against predispersal seed predation whereas in ripe pulp it may deter frugivores and thus reduce the chances that seeds will be defecated beneath the parent plant [151]. Natural selection should favor secondary metabolites with multiple functions because they protect the plants against a variety of unpredictable biotic and abiotic environments. Such metabolites also enhance plant defenses by using different molecular targets of specific enemies through a variety of mechanisms of action. As discussed in a recent paper [152] herbivores also vary profoundly in their tolerance to secondary metabolites, and future research should be directed to better understanding detoxification processes, or lack of such processes, in the plankton. The complexity of planktonic food webs allows a multitude of pathways for infochemicals that need to be better understood. The role of parasites and viruses, both in introducing toxins into the plankton, as well as removing them through detoxification or species-specific host–pathogen interactions is poorly known. Resulting trophic cascades may have important repercussions in plankton community structure with implications for higher trophic levels including important fisheries.

We have only barely begun to understand the importance of chemical interactions in the plankton and their role in shaping biodiversity and ecological functioning both at a community and cellular level. An increased understanding of the natural function of these compounds may lead to new strategies for the correct management and protection of these potentially important natural resources for the future and help find new biotechnological applications for these products in our day-to-day lives. Antifeedants have already been used to derive anticancer, antiviral, and antiaging products; toxins have been used to develop pain killers; and functional products have been obtained from the materials used by sessile organisms to colonize free surfaces. Knowledge of ecophysiological interactions may serve as a platform to facilitate the search for new biotechnological candidates, as well as to optimize culture conditions and achieve production of biomass for industrial applications.

13.7 Study Questions

1. What types of antipredator defensive strategies occur in the plankton and do these differ from those in benthic habitats?

2. How do antipredator defensive strategies in marine organisms compare to those in terrestrial habitats?
3. What is the function of oxylipins in diatoms?
4. Are dinoflagellate neurotoxins defensive metabolites against predators?
5. Are some classes of molecules more common as defensive metabolites than others?
6. How do toxin production levels vary in relation to environmental changes?

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