The Journal of Experimental Biology 207, 2935-2946
Published by The Company of Biologists 2004
doi:10.1242/jeb.01105

Cytotoxicity of diatom-derived oxylipins in organisms belonging to different phyla

Sven Adolph[1], Stéphane Bach[2], Marc Blondel[2], Anne Cueff[2], Marjolaine Moreau[2], Georg Pohnert[1], Serge André Poulet[2,*], Thomas Wichard[1] and Alga Zuccaro[3]

[1]*Max-Planck Institute, Hans-Knöll-Str. 8, D-07745 Jena, Germany,* [2]*Station Biologique, CNRS, Mer et Santé (FRE 2775), INSU, UPMC, PO Box 74, 29682 Roscoff, France* and [3]*Institute of Microbiology, Technical University Braunschweig, Spielmannstrasse 7, D-38106 Braunschweig, Germany*

*Author for correspondence (e-mail: poulet@sb-roscoff.fr)

Accepted 20 May 2004

Summary

The cytotoxicity of several saturated and unsaturated marine diatom-derived aldehydes and an oxo-acid have been screened *in vitro* and *in vivo* against different organisms, such as bacteria, algae, fungi, echinoderms, molluscs and crustaceans. Conjugated unsaturated aldehydes like 2*E*,4*E*-decadienal, 2*E*,4*E*-octadienal, 5*E*,7*E*-9-oxo-nonadienoic acid and 2*E*-decenal were active against bacteria and fungi and showed weak algicidal activity. By contrast, the saturated aldehyde decanal and the non-conjugated aldehyde 4*Z*-decenal had either low or no significant biological activity. In assays with oyster haemocytes, 2*E*,4*E*-decadienal exhibited a dose-dependent inhibition of cytoskeleton organisation, rate of phagocytosis and oxidative burst and a dose-dependent promotion of apoptosis. A maternal diatom diet that was rich in unsaturated aldehydes induced arrest of cell division and apoptotic cell degradation in copepod embryos and larvae, respectively. This wide spectrum of physiological pathologies reflects the potent cell toxicity of diatom-derived oxylipins, in relation to their non-specific chemical reactivity towards nucleophilic biomolecules. The cytotoxic activity is conserved across six phyla, from bacteria to crustaceans. Deregulation of cell homeostasis is supposed to induce the elimination of damaged cells through apoptosis. However, efficient protection mechanisms possibly exist in unicellular organisms. Experiments with a genetically modified yeast species exhibiting elevated membrane and/or cell wall permeability suggest that this protection can be related to the inability of the oxylipin compounds to enter the cell.

Key words: diatom, oxylipin, cell toxicity, marine, non-marine organism, unsaturated aldehyde.

Introduction

In the late 1960s, researchers became aware that some aldehydic products of lipid peroxidation, such as hydroxyalkenals, trigger a number of biological effects (Schauenstein, 1967). The biosynthesis, chemical reactivity and biological effects of aldehydic oxylipins (metabolites derived by oxidative transformation of fatty acids) were originally described from terrestrial plants and animals. Activities described include antibiotic and fungicidal properties and, accordingly, some of these metabolites were assumed to be responsible for chemical defence against pathogens and herbivores (see review by Blée, 1998; Comporti, 1998). Hitherto, most investigations have been performed with the reactive fatty acid derived 4-hydroxy-2*Z*-nonenal. Under physiological conditions, this substance is highly reactive towards biomolecules bearing sulphydryl groups, such as glutathione, or coenzyme A. Amino acids, proteins or enzymes containing $–NH_2$ and/or –SH groups are also attacked (Schauenstein, 1967). The main reaction pathway is the Michael-addition of the unsaturated aldehyde with these nucleophilic groups. The resulting substituted hydroxynonenals are stable under physiological conditions (Esterbauer et al., 1975; Ferrali et al., 1980). Reactions are not specific and, consequently, the adduct formation induces adverse effects on a broad range of cell functions. The resulting effects include depletion of glutathione (Poot et al., 1987), the inhibition of DNA and protein synthesis (Poot et al., 1988) and induction of cell cycle arrest (Barrera et al., 1991; Wonisch et al., 1998). Fewer studies have been carried out with α,β,γ,δ-unsaturated aldehydes, which are also derived by oxidative transformations of polyunsaturated fatty acids. Like 4-hydroxy-2*E*-nonenal, these compounds are also potent Michael-acceptors with comparable reactivity towards biomolecules. Observed cellular activities inhibited by 2*E*,4*E*-decadienal, the best investigated member of this structure class, include reduced cell growth and induction of DNA fragmentation in human erythroleukaemia (HEL) cells

(Nappez et al., 1996) and the induction of apoptosis in copepod and sea urchin embryos (Romano et al., 2003).

α,β,γ,δ-unsaturated aldehydes are synthesised by numerous organisms including mammals and higher plants (Glasgow et al., 1986; Salch et al., 1995). Subsequently, this compound class was also detected in planktonic algae. Wendel and Jüttner (1996) first detected 2*E*,4*Z*-decadienal in the freshwater diatom *Melosira varians* (Bacillariophyceae). A series of molecules with this structural element was subsequently found in marine diatoms (Miralto et al., 1999a; Pohnert and Boland, 2002; d'Ippolito et al., 2002, 2003; Pohnert et al., 2002; Adolph et al., 2003), but not all diatoms have the capability for aldehyde production (Pohnert et al., 2002). More recently, other freshwater microalga belonging to the Chrysophytes and Synurophytes were added to the list of producers of unsaturated aldehydes (Watson and Satchwill, 2003). Several diatom species have developed the ability to synthesise these noxious compounds by lipoxygenase-mediated transformation of polyunsaturated fatty acids (Pohnert and Boland, 2002). The production of reactive oxylipins is initiated upon cell disruption such as would occur during mesozooplankton grazing (Pohnert, 2000). Aldehyde formation is initiated by the release of polyunsaturated fatty acids, which are further metabolized *via* a lipoxygenase/hydroperoxide lyase pathway (Pohnert, 2000, 2002; Pohnert and Boland, 2002). The deleterious effect of unsaturated aldehydes from diatoms on marine organisms was first identified in copepods (Miralto et al., 1999a). When ingested by spawning females, these aldehydes are responsible for a suite of physiological dysfunctions during egg development, hatching and morphogenesis in larvae (Miralto et al., 1999a; Romano et al., 2003; Poulet et al., 2003; Ianora et al., 2004). In fact, depending on the quantity of diatoms ingested by females (Chaudron et al., 1996), the number of normal embryos and offspring is low or survivors do not reach adulthood (Carotenuto et al., 2002; Poulet et al., 2003; Ianora et al., 2004). These molecules affect hatching and embryogenesis, two key reproductive processes in copepods, and have therefore been classified as antiproliferative oxylipins.

Following the work initiated by Poulet et al. (1994) and Miralto et al. (1999a), which focused on the diatom–copepod interactions, several other types of marine organisms have been recently tested. Up until now, the noxious effects of unsaturated aldehydes have been reported against a marine diatom (R. Casotti, S. Mazza, A. Ianora and A. Miralto, unpublished) and for several marine invertebrates, including ascidians (Tosti et al., 2003), tunicates (Miralto et al., 1999b), echinoderms (Caldwell et al., 2002; Adolph et al., 2003; Romano et al., 2003), polychaetes (Caldwell et al., 2002) and crustaceans (copepods – Romano et al., 2003; Poulet et al., 2003; *Artemia* – Caldwell et al., 2003). The majority of previous bioassays, conducted *in vitro* with eggs and embryos, were related to the noxious effects of 2*E*,4*E*-decadienal on the embryonic and larval development of these organisms. Although biological activity was observed in all cases, the inhibitory mechanism still remains unknown.

The results presented here show that the unsaturated aldehyde 2*E*,4*Z*-decadienal, taken as a representative of the noxious oxylipins synthesised by marine diatoms, could trigger several categories of cell inhibition in a wide range of marine and non-marine organisms belonging to different phyla. Observations were compared with structurally related diatom-derived unsaturated aldehydes and the saturated aldehyde decanal, which lacks the reactive Michael-acceptor element and which exhibited low activity in sea urchin egg cleavage assays (Adolph et al., 2003). Our main objective was to explore which cellular pre-requirements have to be present for activity and what key physiological functions could be inhibited by unsaturated aldehydes, using a range of cellular models. This study suggests that diatom-derived oxylipins trigger *in vitro* the inhibition of different cell processes, among which apoptosis is probably the ultimate symptom. Findings are discussed in the context of selecting key organisms as future tools to permit the further elucidation of the inhibitory mechanism(s) involved at cellular and molecular levels occurring in marine organisms naturally exposed to toxic diatom diets.

Materials and methods

Test compounds

All tested unsaturated aldehydes were synthesized according to a published procedure (Adolph et al., 2003). The aldehydes were purified by column chromatography on Florisil® (Sigma, Deisenhofen, Germany) or SiO_2 (Macherey and Nagel, Düren, Germany) prior to the assays. The stability of the aldehydes was monitored by gas chromatography/mass spectrometry (GC/MS; Pohnert, 2000) after extraction from the medium. Solvents and the saturated aldehyde decanal, used as a control, were purchased from Sigma.

Sub-samples of stock solutions of both unsaturated and saturated aldehydes, diluted in dimethyl sulphoxide (DMSO; Sigma; 10 mg ml^{-1}), were used for the assays. Control experiments with DMSO and seawater were performed in parallel. 2*E*,4*E*-decadienal, 2*E*,4*E*-octadienal, 5*E*,7*E*-9-oxo-nonadienoic acid and 4*Z*-decenal were tested against *Bacillus megaterium*, *Escherichia coli*, *Microbotryum violaceum*, *Mycotypha microspora*, *Dendryphiella salina*, *Fusarium oxysporum*, *Asteromyces cruciatus* and *Chlorella fusca*. 2*E*,4*E*-decadienal, 2*E*-decenal, 4*Z*-decenal and decanal were tested against the fungus *Saccharomyces cerevisiae*. 2*E*,4*E*-decadienal and decanal were tested with the bacterium *Vibrio splendidus*, the sea urchin *Sphaerechinus granularis* and the oyster *Crassostrea gigas*. With the copepod *Calanus helgolandicus*, the combined effects of 2*E*,4*E*/*Z*,7*Z*-decatrienal, 2*E*,4*E*/*Z*-decadienal, 2*E*,4*E*/*Z*-octadienal, 2*E*,4*E*/*Z*,7-octatrienal, 2*E*,4*E*/*Z*-heptadienal and tridecanal were determined with the diatom *Thalassiosira rotula*, which produces these metabolites upon wounding (Pohnert et al., 2002), and were tested through feeding experiments. A wide concentration range ($0–2\times10^6$ cells $vial^{-1}$) of diatoms was used in the diets.

Collection and culture of biological samples

Bacteria, fungi and algae

Test organisms were the bacteria *Bacillus megaterium* de Bary (Gram positive), *Escherichia coli* (Migula) Castellani and Chambers (Gram negative) and *Vibrio splendidus* biovar I ATCC 33125 (Gram negative), the fungi *Microbotryum violaceum* (Pers.) Roussel (Ustomycetes), *Mycotypha microspora* Fenner (Zygomycetes) and *Fusarium oxysporum* Schltdl. (mitosporic fungi), the marine fungi *Dendryphiella salina* (Sutherland) Pugh and Nigot and *Asteromyces cruciatus* Moreau and Moreau *ex* Hennebert (Ascomycetes), the budding yeast *Saccharomyces cerevisiae* (Ascomycetes), and the alga *Chlorella fusca* Shih Krauss (Chlorophyceae).

Biological activity of aldehydes against microorganisms was tested in agar diffusion assays. 50 µl of 1 mg ml^{-1} solutions of the test compounds in DMSO/water (1:10) were pipetted onto sterile filter disks (Schleicher & Schuell, Dassel, Germany). These were placed onto an appropriate agar medium sprayed with a suspension of the test organisms: *Bacillus megaterium*, *Escherichia coli*, *Microbotryum violaceum*, *Mycotypha microspora*, *Fusarium oxysporum*, *Dendryphiella salina*, *Asteromyces cruciatus* and *Chlorella fusca*. DMSO was used as control. Growth media, preparation of test organism suspensions and incubation conditions are described elsewhere (Schulz et al., 1995). Agar diffusion assays were carried out according to Schulz et al. (1995). The radii of the resultant inhibition zones were measured from the edge of the filter disks and reported in millimetres.

Strains of the marine bacterium *Vibrio splendidus* were obtained from Institut Pasteur, Lille, France, kept on Marine agar 2216 (DIFCO, Franklin Lakes, NJ, USA) and grown in a liquid medium (Mueller-Hinton broth added with sodium chloride 2%) for 24 h at room temperature, diluted with sterile filtered seawater up to a cell density of 2×10^7 cells ml^{-1}. Inoculums were then spread on solid agar medium in Petri dishes (Mueller-Hinton agar added with sodium chloride 15 g ml^{-1}). Plate 1 received five sterile disks, 6 mm in diameter (Schleicher & Schuell): two antibiotic disks (Oxoid, Basingstoke, UK; 15 µg $disk^{-1}$ gentamycin; 30 µg $disk^{-1}$ chloramphenicol), one control disk (20 µl $disk^{-1}$ DMSO), one with 2*E*,4*E*-decadienal (6.6 µg $disk^{-1}$) and one with decanal (6.6 µg $disk^{-1}$). Plate 2 received six disks: one antibiotic disk (15 µg $disk^{-1}$ gentamycin), two with decanal (66 µg $disk^{-1}$ and 6.6 µg $disk^{-1}$) and three with 2*E*,4*E*-decadienal (33.3 µg $disk^{-1}$, 6.6 µg $disk^{-1}$ and 0.66 µg $disk^{-1}$). Plates were incubated in duplicate at room temperature for 24 h. The diameters of the resultant inhibition zones were measured and reported in millimetres. This antibiotic susceptibility test (Bauer et al., 1966) was used to establish the bacteriostatic activity of 2*E*,4*E*-decadienal on *V. splendidus* cultures, in comparison to decanal, DMSO as a negative control and antibiotics as positive controls.

Budding yeast

The budding yeast strains used in this study were as follows. Wild-type (WT) strain: *Mata*, *ade1-14*, *trp1-289*, *his3Δ200*, *ura3-52*, *leu2-3,112* (strong strain of 74-D694), described by Chernoff et al. (1995). STRg6 strain: *Mata*, *erg6:TP/*, *ade1-14*, *trp1-289*, *his3Δ200*, *ura3-52*, *leu2-3,112*, described by Bach et al. (2003). A sample of an overnight culture of either *erg6Δ* mutant (STRg6 strain) or the corresponding wild-type strain was spread on a Petri dish containing YPD (yeast extract peptone dextrose medium)-rich medium and small filters (Schleicher & Schuell) placed on the agar surface. Individual compounds were applied to each filter (20 µl of a 3 mmol l^{-1} solution in DMSO). DMSO, the compound vehicle, was used as a negative control.

Bioassays with invertebrates

Echinoderm

Sea urchins, *Sphaerechinus granularis* (Lamarck), collected along the Brittany coast (France), were transported in seawater containers to the Marine Station within 3 h, where they were kept in running seawater. Male and female gametes were collected and fertilisation was conducted *in vitro*, following the protocol described by Meijer et al. (1991). Dense sample solutions (100 µl, with 15 000–20 000 embryos ml^{-1}) of newly fertilised eggs (5–9-min-old embryos) were placed in 900 µl filtered seawater in 5 ml culture wells (Nunc, Roskilde, Denmark) enriched with increasing concentrations of test aldehydes, in the range of 1–250 µmol l^{-1} (final concentration in well), at a constant temperature of 20°C. Observation of the proportions of first (two blastomeres) and second (four blastomeres) cell cleavages was performed for each compound 2–3 h later, in replicate samples of 100 embryos each, and compared with controls (embryos incubated in filtered seawater and with DMSO, 2.5% per volume seawater). This protocol was used to evaluate the effect of aldehydes on cell division during the early phase of embryonic development.

Mollusc

Oysters, *Crassostrea gigas* (Thumberg) (60–70 g wet mass each), purchased from an oyster farmer in the Bay of Morlaix (France), were maintained undisturbed for a 7-day acclimation period in tanks (50 oysters per tank) containing 110 litres of aerated and continuously flowing natural seawater (50 l h^{-1}) at 15–16°C in the laboratory. For all tests, individual oysters were taken from the tank prior to sampling haemolymph. The right side of the shell in each oyster was notched, allowing the sampling of blood in the adductor muscle using 2 ml syringes and 26 gauge × 1.3 cm needles. The rapidity of the procedure (1–2 min per sample) ensured that the effect of sampling on stress-induced catecholamine hormone release was minimal (Lacoste et al., 2001a). Haemolymph samples (0.5–1 ml) were pooled from 5–6 oysters in tubes kept on ice. Haemocyte concentration was determined with a haemocytometer and adjusted to 10^6 cells ml^{-1} by the addition of modified Hanks' balanced salt solution (MHBSS), consisting of HBSS adjusted to ambient seawater salinity (35 p.p.m.) and containing 2 g l^{-1} D-glucose (Anderson et al., 1994), in order to prevent cell aggregation. For each category of bioassays, new blood solution samples were prepared.

Four bioassay categories, with concentrations ranging between 2 and 50 μmol l^{-1}, were performed: (1) observation of cytoskeleton structure, (2) apoptosis induction, (3) phagocytosis and (4) haemocyte oxidative burst response. In assays related to cytoskeleton, apoptosis and phagocytosis, all blood samples enriched with 10 μl of DMSO (controls) or aldehydes were incubated in a humidified chamber in the dark at 20°C for 30 min in order to allow haemocytes to adhere on the plate and cell penetration of test compounds. Detailed protocols used with these assays are described by Panara et al. (1996) and by Lacoste et al. (2001a,b,c, 2002). These assays were based on the evaluation of the multiple responses of oyster haemocytes to aldehydes, in terms of proportions (%) of abnormal cytoskeleton, apoptotic and phagocytic cells, in comparison with controls incubated in DMSO and filtered seawater. The cytoskeleton was observed in rhodamine–phalloidin-stained oyster haemocytes (Sigma) (Panara et al., 1996). Cells entering apoptosis were revealed with fluorescein isothiocyanate (FITC)–Annexin V (Sigma) double labelling with propidium iodide (Sigma) (Bossy-Wetzel and Green, 2000; Lacoste et al., 2002). Phagocytosis was monitored with blood samples incubated on glass slides, following a protocol described by Lacoste et al. (2001b). At the end of the incubation period, 10 μl of 0.95 μm green fluorescent latex microspheres (Polysciences Europe, Epplheim, Germany) were added to the sample to obtain a ratio of 10 microspheres $cell^{-1}$. Haemocytes were further incubated for 30 min to allow phagocytosis to occur. In all assays, blood cell samples were fixed at the end of the incubation periods with 3.7% formaldehyde for 15 min and observed under an Olympus BX 61 epifluorescent microscope or under an IX Fluoview confocal microscope equipped with argon–krypton lasers. For chemiluminescence assays, a protocol described by Lacoste et al. (2001c) was utilised. Zymosan (Sigma) particles were used to stimulate the oyster haemocytes at a concentration of 1 mg ml^{-1}. The chemilumigenic probe used was luminol (Sigma; 10^{-4} mol l^{-1} final concentration) added to 1 ml cell suspensions containing 10^6 cells ml^{-1}. Baseline chemiluminescence was recorded 15 min before addition of Zymosan particles (80 particles $cell^{-1}$) and the luminescence response was recorded using a Lumat LB 9507 luminometer (EG&G Berthold, Pforzheim, Germany) every 3 min for 60 min. Chemiluminescence counts [relative light units per second (RLU s^{-1})] for each tested compound were plotted against time. All four series of assays were repeated three times each.

Crustacea

Copepods, *Calanus helgolandicus* (Claus), were collected offshore from Roscoff (France) and transported within 2 h to the Marine Station. Batches of 30 sexually mature females were sorted and acclimated individually in containers filled with 100 ml filtered seawater (0.22 μm) for 24 h at 17°C. At the end of this initial period, females were fed with a diatom culture in the exponential phase of growth (*Thalassiosira rotula*, strain CCMP 1647) known to produce several unsaturated aldehydes (Pohnert et al., 2002). The algal culture conditions were similar to those described by Pohnert et al. (2002). In each incubator, 20 ml of dense diatom cultures were added to 80 ml filtered seawater. After dilution of cultures, concentration of diatoms in diets ranged from 2×10^3 to 2×10^6 cells ml^{-1} in vials. Diet was renewed daily during an 8-day period. The concentrations of noxious aldehydes potentially available in the diets were measured (Pohnert et al., 2002; T. Wichard et al., unpublished) in a test *T. rotula* culture during the stationary phase of growth. This diatom can produce 2*E*,4*E*/*Z*-isomeric mixtures of 2,4,7*Z*-decatrienal and octadienal (not exceeding 2 fmol $cell^{-1}$ combined: this value was used to extrapolate potential oxylipin values in Fig. 8A) and minor amounts of 2,4-heptadienal, 2,4,7-octatrienal and 2*E*,4*E*-decadienal upon wounding. Feeding rates were not evaluated; however, ingestion of diatoms by *C. helgolandicus* females was estimated indirectly by counting faecal pellet production, which varied from 6 to 30 faeces $female^{-1}$ day^{-1}, increasing with cell density (Chaudron et al., 1996). The daily production and hatching success of eggs were monitored following a protocol described by Laabir et al. (1995). Eggs in control treatments were obtained following the same protocols, except that females were fed a culture (10^4 cells ml^{-1}) of the dinoflagellate *Prorocentrum minimum*, which is unable to synthesise the unsaturated aldehydes in question (Pohnert et al., 2002). This protocol was used to evaluate, *in vivo*, the effect of diatom-derived unsaturated aldehydes on cell division during embryogenesis in *C. helgolandicus* compared with dinoflagellate control.

Observations of normal and abnormal embryonic cell division were performed in samples stained with Hoechst 33342 (Sigma) specific for DNA, following the protocol described by Poulet et al. (1995). Samples were observed in fluorescent light with an Olympus microscope. Nauplius larvae, which hatched and survived the maternal-food effect during the incubation period with diatoms, were collected beyond incubation day 3–5. This is the minimum delay required to observe cell anomalies in *Calanus* embryos induced by toxic diatoms ingested by spawning females (Poulet et al., 1995). A double labelling method, FITC–Annexin V + propidium iodide, was used to diagnose cell degradation processes in N1 stage nauplii, following a protocol described by Poulet et al. (2003). Observation and estimate of the proportions (%) of nauplii (12–20 larvae per sample), intoxicated by maternal diatom diets and presenting apoptotic cell degradations, were compared with controls using the same confocal Olympus epifluorescent microscope.

Results

Bacteria, fungi and algae

In a first series of experiments, 2*E*,4*E*-decadienal (50 μg $disk^{-1}$) exhibited fungicidal activity against *Microbotryum violaceum* (>3 mm) and a limited inhibition (⩽3 mm) of the bacterium *Bacillus megaterium* and of the green alga *Chlorella fusca*. *Escherichia coli*, *Mycotypha microspora*, *Dendryphiella salina*, *Fusarium oxysporum* and *Asteromyces*

cruciatus were insensitive to this metabolite. Similar concentrations of 2*E*,4*E*-octadienal, 5*E*,7*E*-9-oxo-nonadienoic acid and 4*Z*-decenal exhibited no antiproliferative activity.

Results with the marine bacterium *Vibrio splendidus* are presented in Fig. 1. The diameter of the inhibition zone was high with antibiotics (15 μg disk^{-1} bis gentamycin, 21 mm; 30 μg disk^{-1} chloramphenicol, 36 mm) used as references. Growth inhibition was 14 mm with the 33.3 μg disk^{-1} 2*E*,4*E*-decadienal and lower (⩽9 mm) at concentrations of 6.6 μg disk^{-1} and 0.66 μg disk^{-1}. Elevated concentrations of decanal (66.6 μg disk^{-1}) resulted in slight growth inhibition. DMSO did not affect bacterial growth significantly. From these results and according to the chart of zone sizes (Bauer et al., 1966; ⩾12 mm), we concluded that *V. splendidus* was sensitive to 2*E*,4*E*-decadienal in a concentration range comparable with established antibiotics but insensitive to decanal.

Budding yeast

The wild-type (WT) *Saccharomyces cerevisiae* strain was found to be insensitive to 9.1 μg disk^{-1} 2*E*-decenal, 2*E*,4*E*-decadienal, 4*Z*-decenal and decanal, as indicated by the absence of a growth inhibition halo where these compounds were spotted (Fig. 2). Interestingly, a 9.1 μg disk^{-1} of the Michael-acceptors 2*E*-decenal and 2*E*,4*E*-decadienal was found to significantly inhibit cell growth of the STRg6 strain, whereas 4*Z*-decenal and decanal, the non-conjugated aldehydes of comparable chain length, did not result in growth inhibition compared with DMSO (Fig. 2). STRg6 strain lacks the *ERG6* gene. Such a deletion is known to increase cell permeability, probably due to an increase in cell wall and/or plasma membrane permeability (Blondel et al., 2000). Indeed, wild-type budding yeast cells are slightly permeable to a number of drugs, probably because, in addition to a plasma membrane, they also have a cell wall. Deletion of the *ERG6* gene, which is involved in ergosterol biosynthesis, is one of the genetic ways to increase cell permeability. The fact that *erg6Δ* cells were sensitive to 2*E*-decenal and 2*E*,4*E*-decadienal (as compared with WT cells) suggested that the cell wall and plasma membrane are involved in the resistance against these molecules and, furthermore, that, in order to be toxic, the inhibiting compounds must enter the cell or be in direct contact with the plasma membrane.

Echinoderm

Second embryonic cell cleavage in *Sphaerechinus granularis* occurred normally with DMSO in controls and elevated concentrations of decanal (>80 μmol l^{-1}). With <10 μmol l^{-1} 2*E*,4*E*-decadienal, cells did cleave normally. At 10 μmol l^{-1}, cell cleavage was blocked in >50% of embryos and reached 100% at concentrations above 20 μmol l^{-1} (Fig. 3A; Adolph et al., 2003). Observations of 2–3 h-old embryos revealed the normal four blastomeres in samples treated with DMSO, <80 μmol l^{-1} decanal or <10 μmol l^{-1}

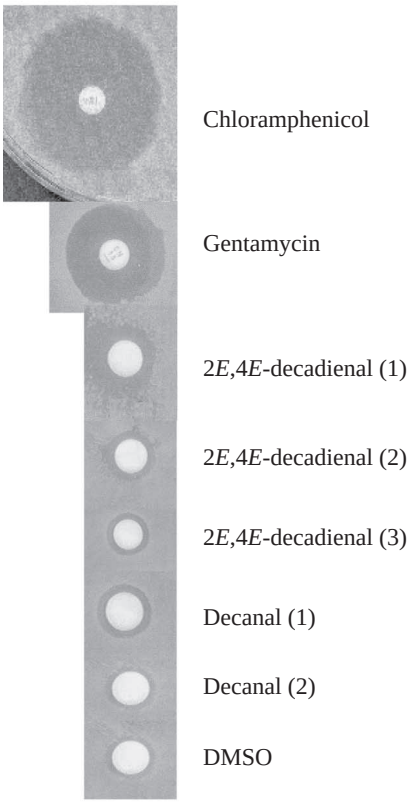


Fig. 1. *Vibrio splendidus* (bacterium). Effects of the aldehydes decanal (1=66 μg disk^{-1}, 2=6.6 μg disk^{-1}) and 2*E*,4*E*-decadienal (1=33.3 μg disk^{-1}, 2=6.6 μg disk^{-1}, 3=0.66 μg disk^{-1}) on cell proliferation, shown by the growth inhibition zone around the disk at different dilutions. Comparisons with DMSO and two antibiotics (15 μg disk^{-1} chloramphenicol and 30 μg disk^{-1} gentamycin) are shown.

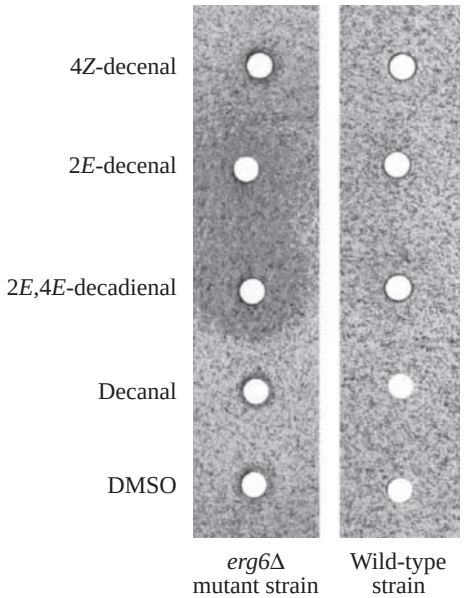


Fig. 2. *Saccharomyces cerevisiae* (fungus). *erg6Δ* cells are insensitive to decanal and 4*Z*-decenal but are highly sensitive to 2*E*,4*E*-decadienal and 2*E*-decenal, as indicated by the growth inhibition halo around the filter where these molecules were spotted. Concentration of each aldehyde tested was 9.1 μg disk^{-1}. *ERG6wt* cells (wild-type strain) are not sensitive to any of these molecules.

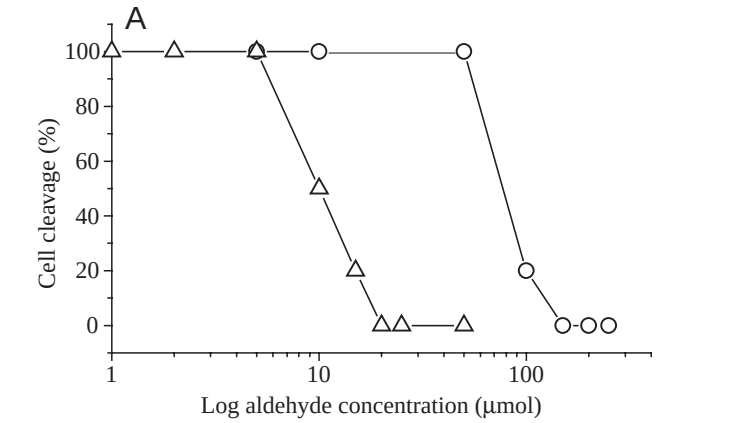


Fig. 3. *Sphaerechinus granularis* (echinoderm). (A) Dose-dependent effects of decanal (circles) and 2*E*,4*E*-decadienal (triangles) on cell division during the early embryogenic phase (four blastomeres) in 2.5 h-old embryos. Values are means of three replicate measurements. Standard error (<3% of the mean) is not shown. (B) Light microscope photographs of (1) normal divided embryos observed either in seawater controls or in 2*E*,4*E*-decadienal (<5 µmol l^{-1}) and decanal (<80 µmol l^{-1}) test solutions, (2) abnormal embryos presenting totally blocked or abnormal cell divisions in 2*E*,4*E*-decadienal (>10 µmol l^{-1}) and (3) blocked embryos presenting intoxication features with decanal (>80 µmol l^{-1}). Egg size: 95±6 µm. Scale bar applies to 1, 2 and 3.

2*E*,4*E*-decadienal (Fig. 3B1). Cell division was blocked in samples assayed with 2*E*,4*E*-decadienal at concentrations of >15 µmol l^{-1} (Fig. 3B2), whereas impairment of development by elevated concentrations of decanal (>80 µmol l^{-1}) was induced by a subsidiary toxic effect, as revealed by small spheres next to the egg membrane (Fig. 3B3).

Oyster

Results in Figs 4–7 show the multiple inhibitory effects of 2*E*,4*E*-decadienal (2–50 µmol l^{-1}) on the structure and key physiological functions of oyster haemocytes. The results are compared with the effects of decanal, DMSO and filtered seawater.

The haemocyte cytoskeleton was affected to different intensities by all treatments (Fig. 4A). The shapes of the cytoskeleton of disturbed and non-disturbed haemocytes are compared in Fig. 4B. Cytoskeleton was well extended in normal cells (Fig. 4A1) whereas it presented a compact, spherical shape in abnormal cells (Fig. 4A2). The background inhibition induced by the handling of cells in seawater and in DMSO was 27–30%. In the presence of decanal, 41–46% of haemocytes also presented abnormal spherical shapes. With added 2*E*,4*E*-decadienal, 43–59% of cells were affected. This effect was dose dependent and significantly higher than with decanal (Student's *t*-test, N=100, α=0.05). With both aldehydes, shape anomaly of the cytoskeleton was 12–28% above the DMSO control (significant Student's *t*-test, N_1, N_2 and N_3=100 cells each, α=0.05).

Apoptotic haemocytes, detected with the FITC–Annexin V + propidium iodide double labelling method, were observed in all treatments (Fig. 5). Pictures of normal (propidium positive, annexin negative; nucleus in red) and abnormal (propidium positive, annexin positive: nucleus in red and cell membrane in green) haemocytes are shown in Fig. 5B. Proportions of abnormal cells in seawater and DMSO controls were 32 and 39%, respectively. Proportions of apoptotic cells in samples treated with the respective aldehydes were dose dependent, ranging between 45 and 75%, which was significantly higher than in controls

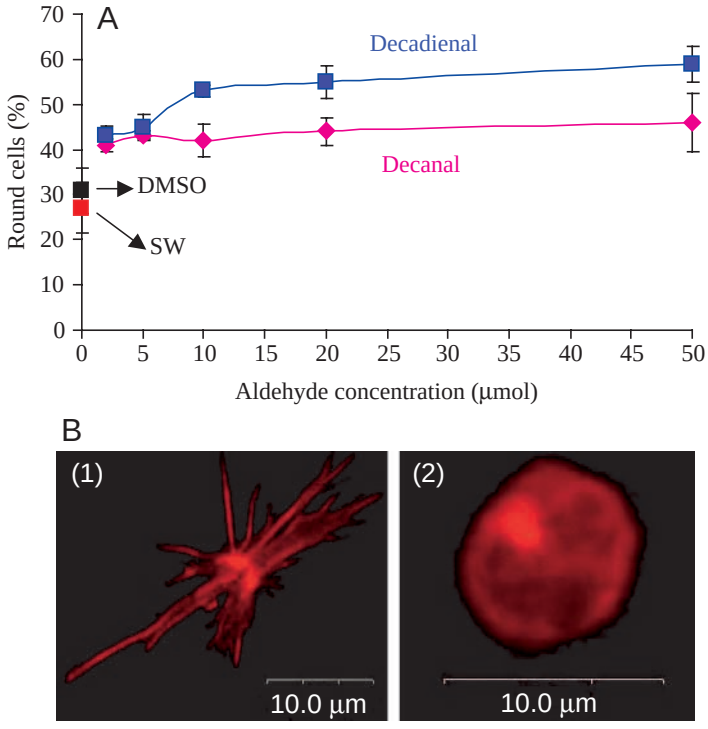


Fig. 4. *Crassostrea gigas* (mollusc). (A) Dose-dependent response of oyster haemocytes incubated in seawater (SW, controls), DMSO (solvent control), 2*E*,4*E*-decadienal and decanal. Values (mean ± S.D.) are the estimates of the proportions of abnormal, round cells reflecting the impact of these treatments on the cytoskeleton. (B) Fluorescent micrographs of normal (1) and abnormal (2) cytoskeleton revealed in rhodamine–phalloidin-stained cells.

(Fig. 5A; Student's *t*-test, N_1 and N_2=100 cells in each control and test sample, α=0.05). Highest proportions of apoptotic cells occurred in samples treated with 2*E*,4*E*-decadienal, which were 10–20% above decanal, 17–36% above DMSO and 25–45% above seawater backgrounds.

Results on the phagocytosis bioassays are shown in Fig. 6. With DMSO, inhibition of phagocytosis in haemocytes was low: ~3% above background level measured with seawater. With decanal, inhibition values of 2 and 9% (2 μmol l^{-1} and 50 μmol l^{-1} decanal) were not significantly different from controls. With 2*E*,4*E*-decadienal, inhibition was also dose dependent. Values, corresponding to 6 and 18% (2 μmol l^{-1} and 50 μmol l^{-1} decadienal), were significantly higher than those measured with both DMSO and decanal (Fig. 6A; Student's *t*-test, N_1 and N_2=100 cells in each control and test sample, α=0.05). Pictures of three optical sections of haemocytes observed with the confocal microscope, with (phagocytosis) and without (blocked phagocytosis) fluorescent green microspheres inside the cells (see green spot P in Fig. 6B1), reflecting active and non-active cells blocked by decadienal, respectively, are shown in Fig. 6B1,2.

Fig. 7 shows the chemiluminescence responses of oyster haemocytes, assayed with seawater, DMSO, decanal and 2*E*,4*E*-decadienal before and after addition of Zymosan. The background levels of the oxidative burst measured in assays were, on average, below 800 RLU s^{-1} in all samples before addition of Zymosan. The maximum oxidative-burst response of haemocytes, with peaks detected 15 min after addition of Zymosan, reflected the inhibition level of each chemical treatment. In seawater controls, the mean oxidative burst was 7000 RLU s^{-1}. With DMSO, it was slightly above 5000 RLU s^{-1}, corresponding to 21–24% inhibition compared with controls. With decanal and 2*E*,4*E*-decadienal, the dose-dependent responses were below 3200 RLU s^{-1}. At the same concentration (2 or 50 μmol l^{-1}), inhibition exerted by decadienal was significantly higher than that by decanal and chemiluminescence was 63–73% below values in seawater controls (Student's *t*-test, *N*=16, α=0.05).

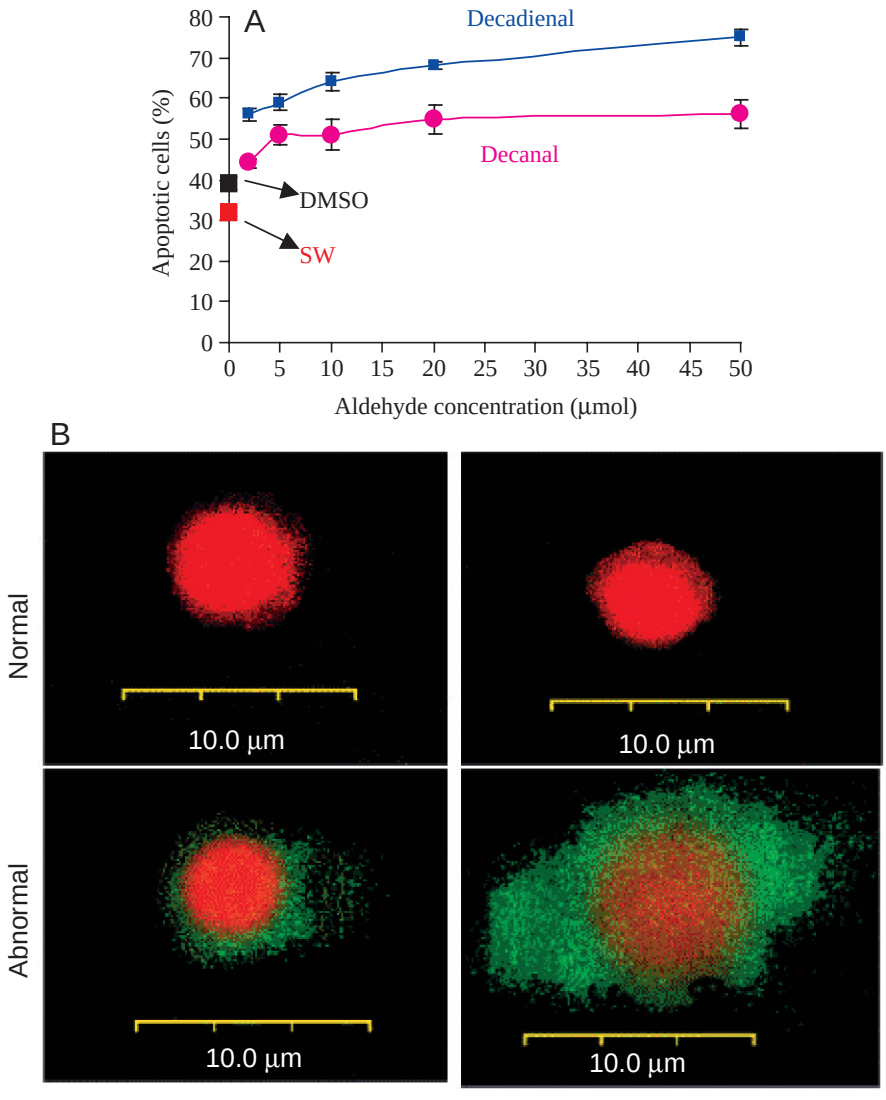


Fig. 5. *Crassostrea gigas* (mollusc). (A) Dose-dependent response of oyster haemocytes incubated in seawater (SW, controls), DMSO (solvent control), 2*E*,4*E*-decadienal and decanal. Values (mean ± S.D.) are the proportions of abnormal, apoptotic cell degradations reflecting the noxious impact of the treatments on the haemocytes. (B) Fluorescent micrographs of normal (1) and apoptotic (2) FITC–Annexin V-stained haemocytes.

Copepod

Results with *Calanus helgolandicus* are reported in Fig. 8. Eggs spawned by females fed the dinoflagellate *Prorocentrum minimum* (control diet; unsaturated-aldehyde free) underwent 100% normal cell division during embryonic development (Fig. 8A,B1). In these series, embryos collected during the 8-day incubation period hatched normally, giving rise to normal N1 stage larvae (Fig. 8C1,2). With females fed the diatom *Thalassiosira rotula*, the proportion of eggs presenting abnormal cell division increased beyond day 3 in relation to diatom concentrations in diets (Fig. 8A). *T. rotula* produces reactive aldehydes upon wounding. The potential availability of these unsaturated aldehydes *via* a natural diet was measured on one occasion (10^4 cells ml^{-1}) and extrapolated to the other diatom cell concentrations in vials (Fig. 8A; T. Wichard, S. Poulet and G. Pohnert, unpublished). In this case, the majority of embryos collected on day 4

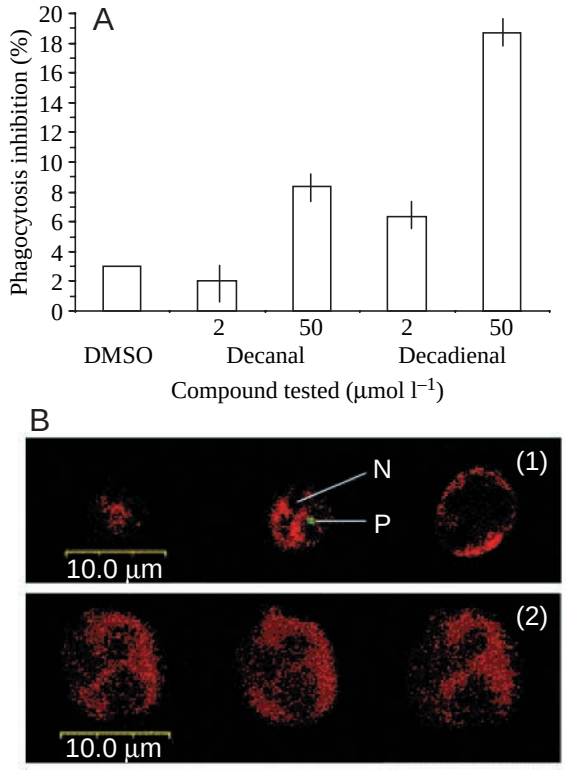


Fig. 6. *Crassostrea gigas* (mollusc). (A) Response of oyster haemocytes incubated in 2*E*,4*E*-decadienal or decanal (at concentrations of 2 and 50 µmol l^{-1}) and DMSO (solvent control). Values (mean ± S.E.M.) are given for three replicate tests, showing the proportions of blood cells presenting phagocytosis inhibition with each treatment. (B) Fluorescent confocal micrographs of three consecutive optical sections of normal (1) and inhibited (2) haemocytes, related to presence (P) or absence of fluorescent phagocytosed beads observed inside the cytoplasm. N, cell nucleus.

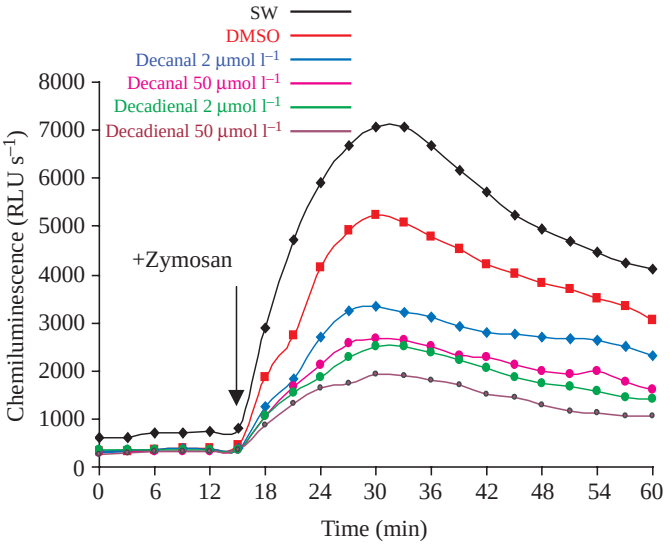


Fig. 7. *Crassostrea gigas* (mollusc). Concentration-dependent inhibition of the luminol-dependent chemiluminescence response of oyster haemocytes from a common pool of cells (10^6 cells ml^{-1}) by aldehydes (at concentrations of 2 and 50 µmol l^{-1}). For each treatment, values are the results of triplicate measurements of the chemiluminescence responses, within 15 min before and 45 min after addition of the stimulatory Zymosan particles (arrow).

showed abnormal cell divisions (Fig. 8B2,3). The few hatched larvae expressed apoptotic cell degradations inside their bodies (green spots), as revealed by the FITC–Annexin V double labelling method (Fig. 8C3).

Discussion

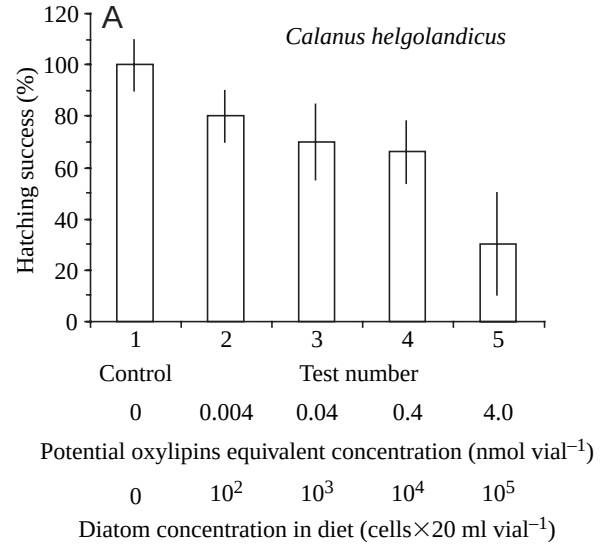
Results reported here show that diatom-derived α,β,γ,δ-unsaturated aldehydes induced, *in vitro* and *in vivo*, antiproliferative activity in a broad range of organisms. The saturated aldehyde decanal had no detectable or only minor inhibitory effects. According to Adolph et al. (2003), the antiproliferative activity of diatom-derived unsaturated aldehydes in sea urchin egg cleavage assays is related to the presence of a Michael-acceptor and not to a specific feature of 2*E*,4*E*- or 2*E*,4*Z*-decadienal. The minimum structural element required for activity is one double bond, conjugated to the aldehyde functionality. This pre-requirement for activity is also reflected in the assays, with the conjugated aldehydes tested being most active. Comparing the activity of different aldehydes in the yeast *erg6* mutant shows that the metabolites of similar chain length exhibited different activity (Fig. 2). The conjugated 2*E*,4*E*-decadienal and 2*E*-decenal did exhibit inhibitory activity, whereas the non-conjugated 4*Z*-decenal and decanal were not active. In this system, the same pre-requirement for activity is found as in sea urchin egg cleavage assays. In comparison with 2*E*,4*Z*-decadienal, the more polar conjugated aldehydes 2*E*,4*Z*-octadienal and the acidic 5*Z*,7*E*-9-oxo-nonadienoic acid were less active against several microorganisms (inhibition zones: 0 to <3 mm). This trend was also observed with sea urchin egg assays (Adolph et al., 2003). We thus conclude that the presence of a Michael-acceptor structural element with low polarity is required for maximum activity in a broad range of organisms. For this reason, we performed an in-depth comparison of 2*E*,4*E*-decadienal and the non-active decanal to elucidate the mode of action of the unsaturated diatom-derived aldehydes.

Depending on the organism, inhibitions triggered by α,β,γ,δ-unsaturated aldehydes concerned either cell proliferation, cell division, structure of cytoskeleton, phagocytosis, the inhibition of the induction of an oxidative burst, or apoptotic cell degradations (Figs 1–8). Previous results by Bisignano et al. (2001) have shown that unsaturated aldehydes have a broad antimicrobial spectrum. Our results suggest that these oxylipins can affect various key physiological cell processes in relation to their non-specific chemical affinities for biomolecules (Esterbauer et al., 1975; Spiteller, 2001; Luczaj and Skrzydlewska, 2003). They reveal, for the first time, that inhibition of cell proliferation by α,β,γ,δ-unsaturated aldehydes, determined in marine and non-marine organisms, is conserved from bacteria to crustaceans. The fact that 2*E*,4*E*-decadienal and

2*E*-decenal were active in both *erg6Δ* cells of *Saccharomyces cerevisiae* and other tested organisms (*Vibrio splendidus*, *Sphaerechinus granularis*, *Crassostrea gigas* and *Calanus helgolandicus*) suggests that, among the biochemical pathways targeted by the reactive aldehydes, at least those involved in the cell proliferation process might be evolutionarily conserved.

Among prokaryotes and eukaryotes tested in the present paper, the fungi *Dendryphiella salina*, *Fusarium oxysporum*, *Asteromyces cruciatus* and *Saccharomyces cerevisiae* (WT strain) and the bacteria *Bacillus megaterium* and *Escherichia coli* were not significantly affected by unsaturated nor saturated aldehydes under our experimental conditions. Cell proliferation was weakly inhibited by 2*E*,4*E*-decadienal in the terrestrial fungus *Mycotypha microspora* and the alga *Chlorella fusca* and strongly inhibited in the marine bacterium *Vibrio splendidus* and the genetically modified yeast *Saccharomyces cerevisiae* (strain STRg6) (Fig. 2). Comparison of results obtained for WT and STRg6 strains of the budding yeast, *Saccharomyces cerevisiae*, strongly suggests that in this eukaryotic species, the chemical resistance to α,β,γ,δ-unsaturated aldehydes is related to cell wall and/or plasma membrane impermeability. WT and STRg6 strains are only distinguished by the presence or absence of the *ERG6* gene, which results in increased cell permeability in the mutant (Blondel et al., 2000). This direct comparison suggests that the α,β,γ,δ-unsaturated aldehydes can cause unspecific damage when they reach the inside of the cells. The reduced cell permeability might thus be the reason for resistance in the insensitive organisms tested here.

Cells belonging to marine invertebrates in our assays all responded to unsaturated aldehydes. Many of these marine invertebrates rely on diatom diets and are therefore exposed to diatom-derived oxylipins. This includes crustaceans (copepods; Fig. 8), molluscs (oysters; Fig. 7), echinoderms



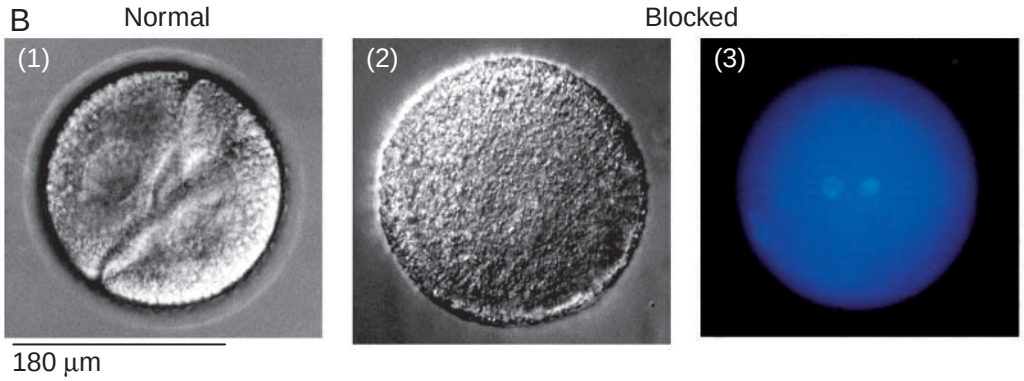


Fig. 8. *Calanus helgolandicus* (crustacean). (A) Post-ingestion effect of the noxious diatom *Thalassiosira rotula*, provided to spawning females. Dose-dependent values (mean ± S.D.) of the hatching success are the results of triplicate observations for each diet treatment in batch samples of 30–100 eggs each. The control diet (test number 1, with the dinoflagellate *Prorocentrum minimum*) does not contain any noxious unsaturated aldehydes. Values of the potential production of two dominant unsaturated aldehydes (2,4-octadienal and 2,4,7-decatrienal combined), measured as the mean aldehyde production (2 fmol cell^{-1}) of these major unsaturated aldehydes in a *T. rotula* initial culture (T. Wichard, S. Poulet and G. Pohnert, unpublished). Potential yields of these aldehydes are calculated in relation to the equivalent diatom cell concentrations in female diets (test numbers 2–5). (B) Micrographs of a normal embryo at the two-blastomere stage produced by females fed the dinoflagellate diet (1), blockage of cell division in abnormal embryos produced by females fed the diatom diet at >10^3 cells ml^{-1} (2), fluorescent micrograph of a similar abnormal embryo stained with Hoechst 33342, specific to DNA, showing two nuclei blocked in the egg matrix (3). (C) Fate of N1 stage naplius produced by females fed two different diets [1 and 2, dinoflagellate (10^4 cells ml^{-1}); 3, toxic diatom (10^4 cells ml^{-1})], sampled on day 5 during an 8-day incubation period. Light micrograph of a normal larva (1). Fluorescent confocal micrographs of normal (2) and apoptotic (arrow) (3) larvae double-stained with FITC–Annexin V + propidium iodide. Size of eggs= 172±4 µm; size of larvae=208±10 µm. Scale bars in B and C apply to 1, 2 and 3.

(sea urchins; Fig. 3) and annelids (Caldwell et al., 2002). Activity of 2*E*,4*E*-decadienal was reported earlier in both marine invertebrates (Tosti et al., 2003; Caldwell et al., 2002) and non-marine vertebrates, such as human cell lines (Nappez et al., 1996; Miralto et al., 1999a; Spiteller, 2001). That the observed effects are not only found *in vitro* is demonstrated by the female incubation experiments (Fig. 8) where the concentration-dependent effect of diatom diets on copepods is shown. The aldehyde-producing diatom *T. rotula*, as a diet, clearly reduced the hatching success of copepod eggs in a concentration-dependant way, compared with incubation experiments where *C. helgolandicus* was fed either *P. minimum* (Fig. 8A) or *T. rotula* (strain CCMP 1018), diets that do not produce any of the unsaturated aldehydes (Pohnert et al., 2002). These results indicate that unsaturated aldehydes, potentially available in diets and ingested by spawning females, are presumably responsible *in vivo* for mitotic cell dysfunction in embryos and apoptotic cell degradations in the newborn larvae, following a dose-dependent response.

Inhibition of cell division, apoptotic and necrotic cell degradations by 2*E*,4*E*-decadienal have already been reported in experiments achieved *in vitro* and *in vivo* (Caldwell et al., 2002; Romano et al., 2003; Poulet et al., 2003; Ianora et al., 2004). Recent results (Romano et al., 2003) have suggested that decadienal induces caspase-independent apoptosis in copepod embryos, whereas decadienal-mediated apoptosis in sea urchin embryos was caspase dependent. Once again, these results reflect the non-specific activity of decadienal, which has a potential reactivity against DNA, enzymes, peptides, neurotransmitters, hormones and other cell-signalling molecules involved in different key cell-signalling pathways. We suggest that once one of these key pathways and corresponding physiological cell functions are disturbed, damaged cells will enter apoptosis. In fact, present results (Figs 4–7), focused on the oyster haemocyte response, strengthen the hypothesis that decadienal non-specifically targets several biochemical pathways and thus can induce a range of cell disorders before cell death. Decadienal seems to be a strong inhibitor of several physiological functions in oyster cells, since the noxious effects of this aldehyde could be clearly distinguished from the toxic effects caused by decanal (Figs 4–7). This is also the case for sea urchin embryos (Fig. 3B). As shown in oyster blood cells (Fig. 4B), disturbance of the cytoskeleton in sea urchin embryos by toxic diatom extracts has been also reported by Buttino et al. (1999), who assumed that depolymerisation of microtubules was involved in the blockage of tubulin organization.

Significant progress has been recently achieved regarding the formation, reactivity and toxicity of aldehydes originating from diatoms (Pohnert, 2000; Pohnert and Boland, 2002; Adolph et al., 2003; d'Ippolito et al., 2002, 2003; Romano et al., 2003). Although apoptosis has been identified as one cause of cell death in copepods (Romano et al., 2003; Poulet et al., 2003), we suspect based on our results that it may not be the first nor the unique cell disorder triggered by these noxious compounds. In fact, previous reports with the Michael-acceptor 4-hydroxynonenal have shown that many other processes could be inhibited by this aldehyde before cells enter apoptosis (Brambilla et al., 1986; Comporti, 1998; Buszczak and Cooley, 2000; see review by Comporti, 1998). This view is also supported for the α,β,γ,δ-unsaturated aldehydes tested with oyster haemocytes (Figs 4–7). Together, our results have identified the wide spectrum of cell symptoms related to diatom-oxylipin toxicity. Despite our first steps in marine invertebrates, neither the molecular targets nor the inhibitory mechanisms are clearly identified. Clarifying these processes remains a challenge. Assuming that apoptotic and necrotic cell degradations observed in copepod embryos (Romano et al., 2003) and larvae (Fig. 8C3; Poulet et al., 2003) are probably the ultimate phases of cell disorders, we still do not know the timing and link between the cell symptoms, observed in the sea urchin, oyster and copepod samples in response to the unsaturated aldehydes, or their adducts. Clarifying the impact of aldehydes, in terms of cell location, adduct formation, molecular targets and timing between the cell symptoms, would greatly help our understanding of these puzzling antiproliferative principles produced by diatoms. In fact, new assays are requested to clarify how oxylipins deregulate cell homeostasis in marine invertebrates.

Although the antiproliferative activity of diatom-derived oxylipins was first identified in copepods (Miralto et al., 1999a), these crustaceans may not be the ideal tools for elucidating cell mechanisms involved in the inhibitory process. Several practical limitations are related to the low egg numbers and non-synchronous cell divisions in successive batches spawned by females. As shown by Wonisch et al. (1998), the yeast *Saccharomyces cerevisiae* could be one suitable tool for the elucidation of the mode of action of reactive oxylipins, because the genome has been sequenced and culturing of strain STRg6 is easy, which is not the case with copepods, sea urchins or oysters. The identified *erg6* mutant, which is susceptible to a broad range of diatom-derived oxylipins, can provide a useful model system for further studies on inhibitory mechanisms on the cellular level. Knowing that the antiproliferative model is conserved among different phyla, we recommend usage of this genetically modified yeast strain in future bioassays in order to further elucidate the molecular targets and cell mechanisms involved in numerous marine invertebrates naturally exposed to diatom-derived oxylipins.

Sailors from the Roscoff Marine Station are deeply thanked for collecting samples at sea. This work was partially funded by the European program EGIDE/PROCOPE, CNRS and DFG (G.P., T.W.). Prof. W. Boland is acknowledged for the support during the preparation of this work. We thank Ch. Cordevant, from Institut Pasteur, Lille, for providing the *V. splendidus* strain, and Dr P. Potin, from our Biological Station, for the use of his luminometer. Referees are deeply acknowledged for their helpful comments and suggestions.

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